



ARTICLE

Overexpression of the Sweet Potato ORANGE (*IbOr*) Gene Enhances Water Deficit Tolerance in *Arabidopsis thaliana*

Ertuğrul Kutlu^{1,2} , Erkan Anıl Aydın^{1,2} , Büşranur Demir^{1,2} , Ece Nisa Birinci^{1,2} ,
Melih Aktürk¹ , Sang-Soo Kwak³ and Aykut Sağlam^{1,*}

¹Department of Molecular Biology and Genetics, Faculty of Science, Karadeniz Technical University, Trabzon, Türkiye

²Graduate School of Natural and Applied Science, Karadeniz Technical University, Trabzon, Türkiye

³Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB),
Daejeon, Republic of Korea

*Corresponding Author: Aykut Sağlam. Email: saglama@ktu.edu.tr

Received: 04 August 2026; Accepted: 30 September 2026; Published: 19 October 2026

ABSTRACT: Water-deficit stress is a major environmental constraint that limits plant growth and productivity, highlighting the importance of identifying genetic factors that enhance stress tolerance. To evaluate the response of *Arabidopsis thaliana* plants overexpressing the *Ipomoea batatas* ORANGE gene (*IbOr*) under water-deficit and osmotic stress, empty vector (EV) controls and *IbOr*-OX lines were examined using two complementary stress systems: hydroponic 10% (w/v) polyethylene glycol 6000 (PEG-6000)-induced water-deficit stress and *in vitro* 200 mM mannitol-induced osmotic stress. Under PEG-6000-induced water-deficit stress, *IbOr*-OX lines exhibited less pronounced morphological damage, including leaf wilting, chlorosis, and desiccation, than EV plants. PEG-6000-induced water-deficit stress decreased fresh weight and relative water content (RWC) in all lines, although these reductions were less pronounced in *IbOr*-OX44. Furthermore, under PEG-6000-induced water-deficit stress, the *IbOr*-OX lines maintained higher total chlorophyll and carotenoid levels than EV plants. Under PEG-6000-induced water-deficit stress, H₂O₂ and malondialdehyde (MDA) levels were significantly lower in the *IbOr*-OX lines than in EV plants. This pattern was accompanied by line-dependent changes in antioxidant enzyme activities, with particularly strong induction of catalase (CAT) in *IbOr*-OX44 and guaiacol peroxidase (POD) in *IbOr*-OX34. To further characterize molecular and metabolic responses associated with *IbOr* overexpression, soluble sugar accumulation and the transcriptional profiles of nine carotenoid- and ABA-related genes (*GGPS1*, *ABI2*, *AAO3*, *NCED3*, *ZEP*, *ABA2*, *PSY*, *ABF3*, and *RD20*) were examined under mannitol-induced osmotic stress. Mannitol-induced osmotic stress markedly increased soluble sugar accumulation in all lines, with the highest level observed in *IbOr*-OX34. Mannitol-induced osmotic stress also produced line-dependent changes in the expression of carotenoid biosynthesis- and ABA signaling-related genes, including *GGPS1*, *PSY*, *ABI2*, and *ABF3*. Together, these findings suggest that *IbOr* overexpression is associated with enhanced soluble sugar accumulation, altered ROS homeostasis, and changes in ABA-related biosynthetic and signaling responses that may contribute to stress tolerance in *A. thaliana*.

KEYWORDS: Absciscic acid signaling; carotenoid biosynthesis; *IbOr*; reactive oxygen species; water deficit tolerance

1 Introduction

Water deficit has detrimental effects on plants' developmental processes, survival, biomass production, and yield. A major biochemical consequence of water-deficit stress is the increased production of reactive oxygen species (ROS), including singlet oxygen, superoxide anion, and hydrogen peroxide due to the

reduced rate of photosynthesis [1]. These reactive oxygen molecules are normal products of cellular metabolism and also function as important signaling components in plant stress responses. Recent evidence further emphasizes that ROS and reactive nitrogen species (RNS) are generated and tightly regulated across multiple cellular compartments, including chloroplasts, mitochondria, and peroxisomes, to maintain a balance between stress signaling and the prevention of cellular damage [2]. However, excessive ROS accumulation can promote lipid peroxidation, protein degradation, and DNA damage. To cope with stress conditions, plants must regulate ROS levels and limit their excessive accumulation during water-deficit stress. Plants have several antioxidants to regulate and detoxify reactive oxygen species for survival and to manage water-deficit stress. The plant antioxidant defense system is generally categorized into non-enzymatic and enzymatic components. Non-enzymatic antioxidants include ascorbic acid (AA), tocopherols (vitamin E), carotenoids, glutathione and phenolic compounds. Superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT) are enzymatic antioxidants. These antioxidant components also differ in their cellular localization and biochemical functions [3].

Carotenoids are pigments present in plants that help them to withstand abiotic stress [4]. Phytoene synthase (PSY) is a pivotal enzyme in carotenoid biosynthesis. The introduction of the *DcPSY2* gene from *Daucus carota* L. into *Nicotiana tabacum* plants resulted in increased tolerance to salt stress [5]. In addition to PSY, geranylgeranyl pyrophosphate synthase (GGPS)—which supplies the essential 20-carbon precursor geranylgeranyl diphosphate (GGPP)—serves as another critical metabolic node for enhancing environmental stress tolerance. For instance, transgenic overexpression of the *IbGGPS* gene from sweetpotato (*Ipomoea batatas* L.) in *A. thaliana* led to a substantial increase in total carotenoid levels and significantly improved tolerance to osmotic stress [6]. Similarly, the heterologous expression of *GbGGPPS1* from *Ginkgo biloba* in *Arabidopsis* boosted chlorophyll content and enhanced seed germination rates under high-salinity conditions [7]. Carotenoids can also act as antioxidants during stress. The application of salt or the manipulation of carotenoid levels can assist plants in their ability to cope with stress [8]. Carotenoids enhance desiccation tolerance in *Daucus carota* taproots by removing harmful substances, stimulating lutein production, and removing excess light [9]. The synthesis of epoxy carotenoids can also enhance plants' ability to withstand water deficits. The 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene, which is present in numerous species, facilitates the production of ABA, which enables plants to cope with stress [10]. However, basal transcript abundance is not the only factor. The sequestration and accumulation of carotenoids within specialized plastid structures under stress remains a primary limiting factor, which has drawn intense research focus toward the orange (*Or*) gene. A useful approach for increasing carotenoid levels in food crops may boost sink capacity and biosynthesis pathway catalytic activity. For example, an orange (*Or*) gene was isolated from an orange cauliflower mutant (*Brassica oleracea* var. botrytis) that has a high level of β -carotene accumulation in the curd and stem tissues. This is due to the differentiation of non-colored plastids into chromoplasts that accumulate carotenoids [11]. Transgenic potato tubers expressing the *Or* gene accumulated increased levels of carotenoids and had continuously increased β -carotene contents during long-term cold storage [12]. In addition, transgenic sweetpotato calli that express *IbOr* showed an increase in antioxidant activity and salt stress tolerance, as well as an increase in levels of β -carotene and total carotenoids [13]. The *Arabidopsis thaliana Or* gene has been reported to positively regulate responses to water-deficit stress. Shan et al. [14] reported that the *AtOr* overexpression lines showed increased accumulation of carotenoids and abscisic acid, decreased stomatal aperture, and modified expression of water-deficit stress-related genes, conferring enhanced water-deficit stress tolerance. Ali et al. [15] demonstrated that *Or* might improve tolerance to water-deficit stress via proline biosynthesis.

Kang et al. [16] reported that potato *Or* overexpression in *A. thaliana* lines had comparatively low H₂O₂ and MDA contents under water deficit and salt stress as compared to the wild type (WT). The increased activation of ROS scavenging and membrane stability might be attributed to an elevation of carotenoid content. The authors also suggested that *Or* genes may be involved in the enhancement of stress tolerance and the accumulation of carotenoids. Given the established role of ORANGE proteins in carotenoid metabolism, changes in carotenoid status associated with *IbOr* overexpression could potentially influence stress responses through interconnected effects on antioxidant protection, ABA-related regulation, and osmotic adjustment. Carotenoid derivatives, in addition to direct antioxidant scavenging, are important precursors for the biosynthesis of abscisic acid (ABA), a master signaling phytohormone that controls stomatal closure and stress-responsive gene expression under water deficit. The transformation of epoxy-carotenoids to ABA intermediates is governed by highly coordinated enzymatic steps involving zeaxanthin epoxidase (*ZEP*), 9-cis-epoxycarotenoid dioxygenase3 (*NCED3*), ABA deficient 2 (*ABA2*) and abscisic aldehyde oxidase 3 (*AAO3*). Downstream signaling nodes such as the negative regulator ABA Insensitive 2 (*ABI2*), the transcription factor abscisic acid responsive elements-binding factor 3 (*ABF3*) and responsive markers such as RESPONSIVE TO DEHYDRATION 20 (*RD20*) determine the effectiveness of the plant's survival strategy. Simultaneously, osmotic adjustments through the accumulation of compatible solutes provide critical mechanical protection for the maintenance of cell turgor, in conjunction with hormonal control. To date, while the role of *Or* genes in carotenoid accumulation is well documented, their potential regulatory influence on the interplay among soluble sugar accumulation, carotenoid metabolism, and core ABA biosynthetic and signaling pathways under water-deficit conditions remains to be fully elucidated.

Previous studies have shown that ORANGE (*Or*) genes from different plant species can enhance tolerance to abiotic stresses, including water-deficit and osmotic stress, when heterologously expressed in *Arabidopsis thaliana*. However, to our knowledge, whether the sweet potato *IbOr* gene produces a comparable stress-tolerance phenotype in *A. thaliana* and whether this response is accompanied by changes in carotenoid content, oxidative stress responses, osmotic adjustment, and ABA-related transcriptional regulation have not been comprehensively evaluated. We hypothesized that *IbOr* overexpression would enhance stress tolerance by influencing carotenoid content, oxidative stress responses, osmotic adjustment, and ABA-related transcriptional regulation. Accordingly, this study aimed to evaluate the physiological and biochemical responses of *IbOr*-overexpressing lines under PEG-6000-induced water-deficit stress and to characterize soluble sugar accumulation, ROS distribution, and the expression of carotenoid- and ABA-related genes under mannitol-induced osmotic stress.

2 Material and Methods

2.1 Plant Material and Growth Conditions

The biological material utilized in this research comprised transgenic *Arabidopsis thaliana* lines harboring either an empty vector control construct (EV/At) or the sweetpotato-derived *Ipomoea batatas* (L.) Lam. cv. *Sinzami* gene (*IbOr*-OX/At), originally characterized by Park et al. [17]. Seeds were kindly provided through donation by Prof. Sang-Soo KWAK from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon). Prior to germination, seeds underwent a 2-day stratification period at 4°C [18]. Surface sterilization protocol involved exposure to 70% (v/v) ethanol for 5 min, followed by treatment with a 30% (v/v) commercial sodium hypochlorite solution for an identical duration of 5 min, concluding with three consecutive rinses in sterile double-distilled water. Unless stated otherwise, plants were cultivated within a regulated environmental growth chamber (Mikrotest, MIT-400, Türkiye) maintained at 23 ± 2°C alongside

a relative humidity range of 50–55%, under a 16-h light/8-h dark photoperiod regime and a photon flux density set to $150 \mu\text{mol m}^{-2} \text{s}^{-1}$.

2.1.1 Hydroponic PEG-Induced Water-Deficit Stress

Surface-sterilized seeds were established in a hydroponic setup utilizing rock wool as mechanical support nourished with standard Hoagland medium. Seventeen days post-germination, uniform plantlets were segregated into control and experimental cohorts. Water-stress conditions were initiated by supplying the treatment group with Hoagland solution supplemented with 10% (w/v) polyethylene glycol 6000 (PEG-6000) [19], whereas control specimens continued receiving standard Hoagland nutrient solution. This PEG-6000 stress application lasted for 4 consecutive days. Visual phenotypic alterations—such as leaf senescence, chlorosis, and turgor loss—were continuously tracked throughout the experimental window. Harvesting of 21-day-old whole plants was subsequently executed to evaluate biomass metrics (fresh and dry weights), relative water content (RWC), photosynthetic pigment levels, proline accumulation, hydrogen peroxide (H_2O_2) concentrations, malondialdehyde (MDA) content, and specific antioxidant enzyme activities.

2.1.2 In Vitro Mannitol-Induced Osmotic Stress

To evaluate the performance of *IbOr*-expressing lines under an alternative osmotic constraint, a separate *in vitro* mannitol assay was conducted using EV and *IbOr-OX* seedlings. Sterilized seeds were seeded onto half-strength Murashige and Skoog (1/2 MS) solid medium containing 1% (w/v) sucrose and 0.5% (w/v) phytagel. After 14 days of uniform growth, healthy seedlings were transferred onto fresh 1/2 MS medium enriched with 200 mM mannitol [20] to provoke osmotic stress, while control seedlings were transferred onto mannitol-free 1/2 MS medium. Growth conditions were kept identical for another 5 days. Plantlets were then harvested to quantify total soluble carbohydrate reserves, perform histochemical reactive oxygen species (ROS) localization assays, and execute quantitative real-time PCR (qPCR) transcript analyses.

Both the hydroponic PEG-6000 and *in vitro* mannitol procedures served as complementary yet experimentally independent osmotic stress paradigms. PEG-driven hydroponics targeted physiological and biochemical profiling, whereas mannitol-supplemented *in vitro* plates were dedicated to soluble carbohydrate quantification, ROS histochemistry, and gene transcription profiling. Consequently, data derived from these two systems were evaluated strictly within their distinct methodological frameworks rather than as interchangeable equivalents.

2.2 Determination of Plant Fresh and Dry Weight

To calculate biomass parameters, five biological replicates were randomly harvested from each respective control and PEG-treated lot. Rosette fresh biomass was weighed immediately post-harvest. Samples were subsequently dehydrated in a forced-air oven at 65°C for 48 h to determine dry mass. All weighing procedures were executed utilizing a high-precision analytical balance (ABJ-NM/ABS-N, KERN & Sohn GmbH, Germany).

2.3 Relative Water Content

Relative water content (RWC) was quantified independently from bulk biomass determinations. Three biological replicates ($n = 3$) were tested per treatment, utilizing one fully expanded, visually healthy rosette leaf per independent plant, ensuring leaves were analyzed individually rather than pooled. Fresh weight (FW) was documented immediately upon excision. Leaves were then submerged in deionized water and stored in

darkness at 4°C for 24 h. Following full rehydration, superficial moisture was gently blotted away using filter paper to record turgid weight (TW). Samples were subsequently oven-dried at 65°C for 48 h to acquire dry weight (DW). RWC percentages were computed following the formula established by Castillo [21]:

$$RWC (\%) = \frac{FW - DW}{TW - DW} \times 100$$

2.4 Chlorophyll and Carotenoid Content

Photosynthetic pigments (chlorophylls and carotenoids) were quantified spectrophotometrically following the protocols of Arnon [22]. Fresh leaf tissue (0.1 g) was ground thoroughly in 1.8 mL of 80% (v/v) aqueous acetone using a bead mill homogenizer (Bioprep-6, Allsheng, China). Homogenates were centrifuged at 5000× g for 5 min, and optical densities of the resulting supernatants were recorded at wavelengths of 663, 645, and 450 nm via a UV-Vis spectrophotometer (Multiskan SkyHigh, Thermo Fisher Scientific, USA). Pigment concentrations were calculated using standard equations:

$$\text{Chlorophyll } a \text{ (mg L}^{-1}\text{)} = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$\text{Chlorophyll } b \text{ (mg L}^{-1}\text{)} = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$\text{Total Chlorophyll (mg L}^{-1}\text{)} = 20.2 \times A_{645} + 8.02 \times A_{663}$$

$$\text{Total Carotenoids (mg L}^{-1}\text{)} = 4.07 \times A_{450} - (0.0435 \times \text{Chl } a + 0.3367 \times \text{Chl } b)$$

To scale pigment values relative to tissue fresh weight ($\mu\text{g g}^{-1}$ FW), the following conversion was applied:

$$\text{Pigment Content (}\mu\text{g g}^{-1}\text{ FW)} = \frac{C \times V}{W}$$

where C represents extract pigment concentration ($\mu\text{g mL}^{-1}$), V denotes final extract volume (mL), and W indicates tissue fresh weight (g).

2.5 Proline Content

Proline accumulation was measured according to Bates et al. [23]. Fresh leaf material (0.1 g) was homogenized in 3% aqueous sulfosalicylic acid and centrifuged at 5000× g for 5 min. A 2 mL aliquot of the supernatant was mixed with 1 mL of glacial acetic acid and 1 mL of freshly prepared acid-ninhydrin solution. The reaction mixture was heated in a boiling water bath at 100°C for 1 h, after which the reaction was abruptly halted by transferring tubes to an ice bath. Following cooling, 3 mL of toluene was added, and the mixture was vigorously vortexed. The organic phase absorbance was measured at 520 nm. Proline quantities were deduced from an L-proline standard calibration curve (0–50 $\mu\text{g mL}^{-1}$) and reported as $\mu\text{g g}^{-1}$ fresh weight (FW).

2.6 Determination of Soluble Sugar Content

Total soluble carbohydrates were estimated spectrophotometrically following Dubois et al. [24]. Fresh leaf samples (0.2 g) were homogenized in 1.8 mL of 70% (v/v) ethanol. Extracts were incubated at 80°C for 3 min, allowed to cool to ambient temperature, and subsequently centrifuged at 10,000× g for 5 min. For

colorimetric evaluation, 100 μL of supernatant was diluted with 900 μL of ultrapure water. Then, 1 mL of 5% (w/v) phenol and 5 mL of concentrated 96% sulfuric acid were introduced, and the solution was vortexed thoroughly. Absorbance readings were taken at 490 nm. Soluble sugar concentrations were quantified against a glucose standard curve (0–100 $\mu\text{g mL}^{-1}$) and expressed as mg g^{-1} fresh weight (FW).

2.7 Hydrogen Peroxide Content

Endogenous hydrogen peroxide (H_2O_2) levels were quantified following Velikova et al. [25]. Fresh leaf tissue (0.1 g) was homogenized in 1.8 mL of 0.1% (w/v) trichloroacetic acid (TCA) via bead homogenization. Homogenates were centrifuged at $15,000\times g$ for 15 min at 4°C . A 1 mL volume of the clear supernatant was combined with 1 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). Following a 10-min dark incubation period, absorbance was read at 390 nm using a spectrophotometer (Multiskan SkyHigh, Thermo Fisher Scientific, USA). H_2O_2 levels were calculated from a standard reference curve of exogenous H_2O_2 (0–100 μM) and expressed as $\mu\text{mol g}^{-1}$ fresh weight.

2.8 Malondialdehyde Determination

Membrane lipid peroxidation was evaluated by quantifying malondialdehyde (MDA) accumulation as outlined by Heath and Packer [26]. Fresh leaf samples (0.1 g) were homogenized in 1.8 mL of 0.1% (w/v) TCA using a bead mill homogenizer and centrifuged at $15,000\times g$ for 5 min. A 1 mL aliquot of supernatant was mixed with 4 mL of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was incubated in a boiling water bath at $95\text{--}100^\circ\text{C}$ for 30 min, then promptly cooled in an ice bath. Following centrifugation at $10,000\times g$ for 5 min, absorbance was measured at 532 and 600 nm. Non-specific turbidity at 600 nm was deducted from the 532 nm reading. MDA concentration was derived utilizing an extinction coefficient of $155\text{ mM}^{-1}\text{cm}^{-1}$ and expressed as nmol g^{-1} fresh weight.

2.9 Histochemical Staining of ROS

In situ visualization of H_2O_2 distribution in *Arabidopsis thaliana* leaves was conducted via 3,3'-diaminobenzidine (DAB) staining based on a modified protocol from Thordal-Christensen et al. [27]. Rosette leaves were harvested from 19-day-old seedlings grown under control conditions (MS medium) or exposed to 200 mM mannitol-induced osmotic stress for 5 days. Freshly detached leaves were immersed in a 1 mg mL^{-1} DAB solution (pH 3.8) and incubated in darkness at room temperature for 8 h to facilitate the polymerization reaction between DAB and endogenous H_2O_2 . Post-staining, photosynthetic pigments were cleared by boiling the tissues in 96% (v/v) ethanol for 10–15 min. De-colored leaves were subsequently photographed using a high-resolution digital camera to record reddish-brown DAB polymerization spots.

Superoxide radical ($\text{O}_2^{\bullet-}$) localization was visualized using nitroblue tetrazolium (NBT) staining according to a modified method by Beauchamp and Fridovich [28]. Rosette leaves collected from 19-day-old control and stressed EV and *IbOr*-overexpressing lines were submerged in 0.1% (w/v) NBT dissolved in 50 mM potassium phosphate buffer (pH 7.8) and incubated under ambient light at room temperature for 6 h. Upon formation of dark blue-purple formazan precipitates, the reaction was stopped, and chlorophyll was removed by boiling in 96% (v/v) ethanol for 15 min. Cleared leaves were preserved in 70% (v/v) glycerol prior to digital camera documentation.

2.10 Determination of Antioxidant Enzyme Activities

For enzyme extraction, 0.1 g of fresh leaf tissue was pulverized in liquid nitrogen and homogenized in an extraction buffer composed of 50 mM K_2HPO_4 , 1 mM EDTA, and 1% (w/v) PVPP (pH 7.0). Extracts

were centrifuged at $14,000\times g$ for 20 min at 4°C , and the supernatant served as the crude enzyme source. All preparation steps were maintained at $0\text{--}4^{\circ}\text{C}$ to prevent protein degradation. Soluble protein content was quantified at 25°C via the Bradford assay [29] employing bovine serum albumin as a standard, and enzymatic specific activities were reported as U mg^{-1} protein.

2.10.1 Guaiacol Peroxidase (POD) Activity

Guaiacol peroxidase (POD; EC 1.11.1.7) activity was assayed according to Urbanek et al. [30]. The 2 mL reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 5 mM guaiacol, 15 mM H_2O_2 , and 50 μL of enzyme extract. Kinetic absorbance changes at 470 nm were recorded for 1 min. POD activity was calculated utilizing an extinction coefficient of $26.6 \text{ mM}^{-1}\text{cm}^{-1}$.

2.10.2 Catalase (CAT) Activity

Catalase (CAT; EC 1.11.1.6) activity was monitored following Aebi [31]. The 1 mL assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2 , and 100 μL of enzyme extract. The drop in absorbance at 240 nm was monitored for 3 min. CAT activity was evaluated using an extinction coefficient of $39.4 \text{ mM}^{-1}\text{cm}^{-1}$ for H_2O_2 consumption.

2.10.3 Ascorbate Peroxidase (APX) Activity

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano and Asada [32] by tracking absorbance reduction at 290 nm. The 1 mL reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.0), 250 μM sodium ascorbate (ASC), 5 mM H_2O_2 , and 100 μL of enzyme extract. APX activity was determined using an extinction coefficient of $2.8 \text{ mM}^{-1}\text{cm}^{-1}$ for ascorbate.

2.10.4 Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed following Beauchamp and Fridovich [28]. The 1 mL reaction solution consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM NBT, and 50 μL of enzyme extract. Photochemical reactions were initiated by adding 2 μM riboflavin, and mixtures were exposed to white fluorescent light ($375 \mu\text{mol m}^{-2} \text{ s}^{-1}$) for 10 min. Absorbance was subsequently measured at 560 nm.

2.11 Determination of Gene Expression Levels

2.11.1 RNA Isolation and cDNA Synthesis

Total RNA was extracted from 0.1 g of finely ground leaf tissue using TRIzol Reagent (Nucleogene, NGE023) following a modified Chomczynski [33] extraction protocol. RNA purity and concentration were assessed via NanoDrop 2000 spectrophotometry. For first-strand cDNA synthesis, exactly 2000 ng of total RNA template was reverse-transcribed utilizing the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's protocol.

2.11.2 Quantitative Real-Time PCR (qPCR) Measurement

Gene transcript profiling was conducted to analyze key genes associated with carotenoid biosynthesis, abscisic acid (ABA) biosynthesis, ABA signal transduction pathways, and ABA-mediated stress responses. Target genes evaluated included *AtGGPS1*, *AtPSY*, *AtZEP*, *AtNCED3*, *AtABA2*, *AtAAO3*, *AtABI2*, *AtRD20*, and *AtABF3*, with *Actin 2* (*AtACT2*) serving as the internal reference standard. Gene-specific primer sequences used for qPCR analysis are listed in Table 1.

Real-time quantitative PCR (RT-qPCR) reactions were carried out on a CFX96 Real-Time PCR System (Bio-Rad, USA) utilizing iTaq Universal SYBR Green Supermix (Bio-Rad). Reactions were set up in a 20 μ L total volume containing 10 μ L of 2 $\times$ SYBR Green Supermix, 2 μ L of cDNA template, 0.6 μ L of each gene-specific forward and reverse primer (10 μ M), and nuclease-free PCR-grade water. Thermal cycling conditions involved an initial denaturation step at 95°C for 3 min, followed by 39 amplification cycles consisting of denaturation at 95°C for 5 s and combined annealing/extension at 60°C for 30 s. Fluorescence emission signals were captured at the end of every cycle. Amplification specificity was confirmed through subsequent melting curve analysis across 65–95°C. All reactions were performed in triplicate technical replicates alongside no-template controls (NTC) for each target. Gene expression normalization was executed against *AtACT2*, and relative expression fold changes were determined via the $2^{-\Delta\Delta C_q}$ algorithm utilizing Bio-Rad CFX Maestro software, taking the control group mean ΔC_q as the baseline calibrator (1.0) [34].

Table 1: Specific primer sequences used for quantitative real-time PCR (qPCR) analysis.

Target Gene	NCBI Accession No.	Primer Sequence (5' $\rightarrow$ 3')
<i>AtAAO3</i>	NM_128273.3	Forward primer 5' CGCTGCTTTCTTGGCGCTAGTG 3' Reverse primer 5' GCCCTGATCGAATGATCCCCGC 3'
<i>AtZEP</i>	NM_180954.3	Forward primer 5' TACTCGACTTGTTGCATGCG 3' Reverse primer 5' TAACACGCCCTTTACCCCAA 3'
<i>AtABA2</i>	NM_104113.5	Forward primer 5' TGTGTTTCCAAGCATGCTG 3' Reverse primer 5' AGCGAGTTTAGTTGCAACCG 3'
<i>AtNCED3</i>	NM_112304.3	Forward primer 5' ACGCCGTCAAATTCGAACAC 3' Reverse primer 5' AAAACCGGTCGACCCAATTG 3'
<i>AtGGPS1</i>	NM_119845.3	Forward primer 5' GCCAATCTGAACCATCCTCT 3' Reverse primer 5' ACGGAGAGGAACAGCTGAAT 3'
<i>AtPSY</i>	NM_121729.3	Forward primer 5' ATCGATGCGGTGAAGTTTGC 3' Reverse primer 5' AAATCGCCTTTTCGCCTTTCG 3'
<i>AtABI2</i>	NM_125087.3	Forward primer 5' ACTCTAGGGCGGTTTTGTGT 3' Reverse primer 5' TTCTATCCTCGCCGCTTCAT 3'
<i>AtRD20</i>	NM_128898.4	Forward primer 5' TTCGACCAAAACGACGATGG 3' Reverse primer 5' AGTGTAACGTAGCTGAACGC 3'
<i>AtABF3</i>	NM_001342246.1	Forward primer 5' CACAGATGGTGCAGCAGCCACA 3' Reverse primer 5' TGGGAAAAGGCCTCTCCTGCGT 3'
<i>AtACT2</i>	NM_112764.4	Forward primer 5' TGCTGTTGACTACGAGCAGG 3' Reverse primer 5' CGAGGGCTGGAACAAGACTT 3'

2.12 Statistical Analysis

All biological experiments were conducted using three independent biological replicates, and subsequent assays were performed in triplicate technical replicates to guarantee data reproducibility. Statistical evaluation was performed through analysis of variance (ANOVA). Significant variance among control and transgenic lines (*IbOr-OX34* and *IbOr-OX44*) under both normal and stress environments was determined via Tukey's honestly significant difference (HSD) *post-hoc* test. All computational analyses were carried out using SPSS software (Version 23.0 for Windows; IBM Corp., Armonk, NY, USA). Differences were accepted as statistically significant at a threshold of $p < 0.05$. Statistically significant groupings are designated by distinct lowercase letters over histogram bars, and values are reported as means accompanied by standard deviation (SD).

2.13 Accession Numbers

Sequence data for the genes analyzed in this study can be found in the EMBL/GenBank/TAIR data libraries under the following accession numbers: *AtAAO3* (NM_128273.3), *AtZEP* (NM_180954.3), *AtABA2* (NM_104113.5), *AtNCED3* (NM_112304.3), *AtGGPS1* (NM_119845.3), *AtPSY* (NM_121729.3), *AtABI2* (NM_125087.3), *AtRD20* (NM_128898.4), *AtABF3* (NM_001342246.1), and *AtACT2* (NM_112764.4) and the sweetpotato ORANGE gene *IbOr* (HQ828087).

3 Results

3.1 Fresh and Dry Weights

Under control conditions, no obvious phenotypic differences were observed among EV, IbOr-OX34, and IbOr-OX44 plants. Following PEG-6000-induced water-deficit stress, EV plants showed more pronounced leaf wilting, chlorosis, and desiccation than the two IbOr-overexpressing lines (Fig. 1A). Under mannitol-induced osmotic stress, EV seedlings also exhibited more evident growth retardation and leaf chlorosis, whereas IbOr-OX34 and IbOr-OX44 retained greener rosette leaves and showed less visible growth inhibition (Fig. 1B).

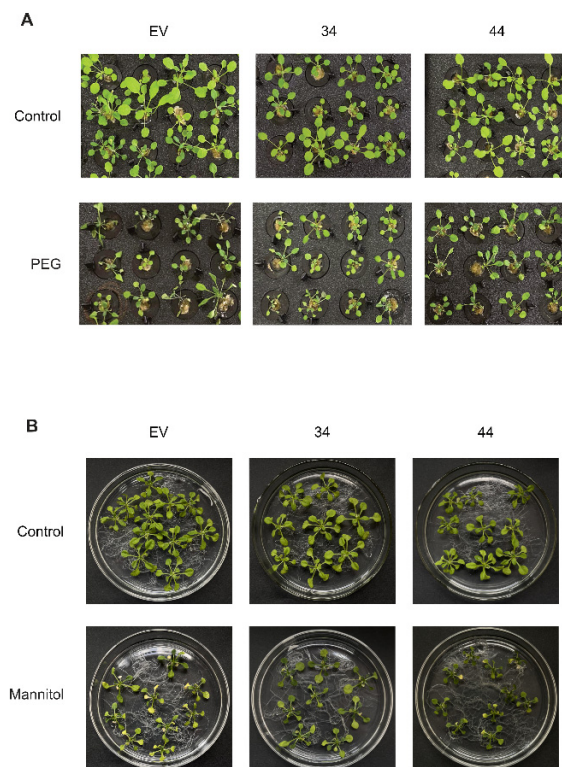


Figure 1: Phenotypic responses of *Arabidopsis thaliana* control (EV/At) and *IbOr*-overexpressing lines (IbOr-OX34 and IbOr-OX44) under PEG-6000-induced water-deficit stress and mannitol-induced osmotic stress. (A) Hydroponic PEG-6000-induced water-deficit stress. Seventeen-day-old seedlings were treated with 10% (w/v) PEG-6000 for 4 days in an Araponics system. (B) *In vitro* mannitol-induced osmotic stress. Fourteen-day-old seedlings were transferred to 1/2 MS medium with or without 200 mM mannitol for 5 days. Representative images show phenotypic differences among EV/At and the two independent transgenic lines.

Under control conditions, the fresh weight of EV plants was significantly higher than those of both IbOr-OX34 and IbOr-OX44 plants (Fig. 2A). PEG-6000-induced water-deficit stress significantly reduced

fresh weight in all lines. Under PEG treatment, IbOr-OX44 plants maintained significantly higher fresh weight than both EV and IbOr-OX34 plants, whereas EV and IbOr-OX34 did not differ significantly from each other. Relative to their respective controls, fresh weight decreased by approximately 43.3%, 25.7%, and 12.2% in EV, IbOr-OX34, and IbOr-OX44 plants, respectively.

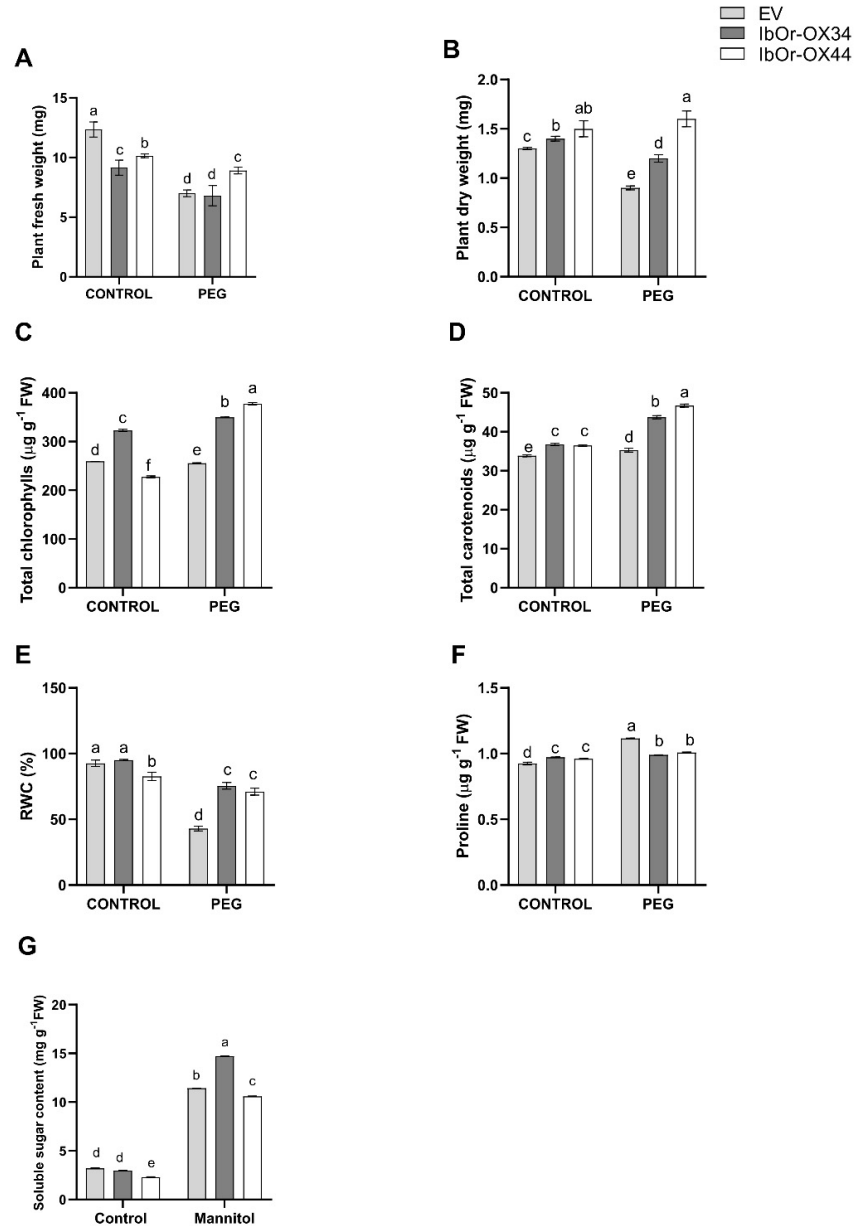


Figure 2: Physiological and biochemical parameters of control (EV) and *IbOr*-overexpressing (*IbOr*-OX34 and *IbOr*-OX44) *Arabidopsis thaliana* lines under two distinct stress systems. (A) Plant fresh weight, (B) plant dry weight, (C) total chlorophyll content, (D) total carotenoid content, (E) relative water content (RWC), and (F) proline content of 21-day-old plants subjected to hydroponic 10% (w/v) PEG-6000-induced water-deficit stress for 4 days. (G) Soluble sugar content of 19-day-old seedlings grown *in vitro* on 1/2 MS medium with or without 200 mM mannitol for 5 days. Data are presented as mean $\pm$ SD ($n = 3$ independent biological replicates). Different letters above the bars indicate statistically significant differences among groups according to ANOVA followed by Tukey's *post-hoc* test ($p < 0.05$).

Under control conditions, the dry weights of both *IbOr-OX34* and *IbOr-OX44* plants were significantly higher than that of EV plants (Fig. 2B). Following PEG-6000 treatment, *IbOr-OX44* plants exhibited the highest dry weight, followed by *IbOr-OX34* and EV plants, with significant differences among the three groups. Relative to their respective controls, dry weight decreased by approximately 30.8% in EV plants and 14.3% in *IbOr-OX34* plants. In contrast, although the mean dry weight of *IbOr-OX44* plants increased slightly by approximately 6.7% under PEG treatment, this difference was not statistically significant.

3.2 Chlorophyll and Carotenoid Content

PEG-6000-induced water-deficit stress significantly altered total chlorophyll and carotenoid contents in the examined lines (Fig. 2C,D). Relative to their respective controls, total chlorophyll content decreased slightly by approximately 1.6% in EV plants, whereas it increased by approximately 8.4% and 66.0% in *IbOr-OX34* and *IbOr-OX44* plants, respectively. Under PEG treatment, *IbOr-OX44* exhibited the highest total chlorophyll content, followed by *IbOr-OX34* and EV, with significant differences among all three lines. Total carotenoid content also increased significantly following PEG-6000 treatment, with increases of approximately 4.2%, 18.9%, and 28.0% in EV, *IbOr-OX34*, and *IbOr-OX44* plants, respectively, compared with their corresponding controls. Under PEG treatment, *IbOr-OX44* showed the highest carotenoid content, followed by *IbOr-OX34* and EV, with significant differences among all three lines.

3.3 Relative Water Content

PEG-6000-induced water-deficit stress significantly reduced relative water content (RWC) in all lines (Fig. 2E). Relative to their respective controls, RWC decreased by approximately 53.6%, 20.6%, and 14.1% in EV, *IbOr-OX34*, and *IbOr-OX44* plants, respectively. Under PEG treatment, both *IbOr-OX34* and *IbOr-OX44* maintained significantly higher RWC than EV plants, whereas no significant difference was observed between the two overexpressing lines.

3.4 Proline Content

PEG-6000-induced water-deficit stress increased proline content in all lines, although the magnitude of the response differed among genotypes (Fig. 2F). Relative to their respective controls, proline content increased by approximately 20.8%, 1.9%, and 5.0% in EV, *IbOr-OX34*, and *IbOr-OX44* plants, respectively. Under PEG treatment, EV plants exhibited significantly higher proline content than both *IbOr-OX34* and *IbOr-OX44* plants, whereas no significant difference was observed between the two overexpressing lines.

3.5 Soluble Sugar Content

Mannitol-induced osmotic stress markedly increased total soluble sugar content in all lines (Fig. 2G). Relative to their respective controls, soluble sugar content increased approximately 3.58-, 4.95-, and 4.63-fold in EV, *IbOr-OX34*, and *IbOr-OX44* plants, respectively. Under mannitol treatment, *IbOr-OX34* exhibited the highest soluble sugar content, followed by EV and *IbOr-OX44*, with significant differences among all three lines.

3.6 Lipid Peroxidation and Hydrogen Peroxide Content

PEG-6000-induced water-deficit stress significantly increased MDA content in all lines (Fig. 3A). Relative to their respective controls, MDA content increased approximately 11.32-, 3.90-, and 8.03-fold in EV, *IbOr-OX34*, and *IbOr-OX44* plants, respectively. Under PEG treatment, EV plants exhibited the highest MDA content, followed by *IbOr-OX44* and *IbOr-OX34*, with significant differences among all three lines.

H₂O₂ content showed a distinct genotype-dependent response to PEG-6000 treatment (Fig. 3B). Relative to their respective controls, H₂O₂ content increased approximately 9.96-fold in EV plants, whereas it decreased by approximately 12.4% and 28.6% in *IbOr*-OX34 and *IbOr*-OX44 plants, respectively. Under PEG treatment, EV plants exhibited the highest H₂O₂ content, followed by *IbOr*-OX44 and *IbOr*-OX34, with significant differences among all three lines.

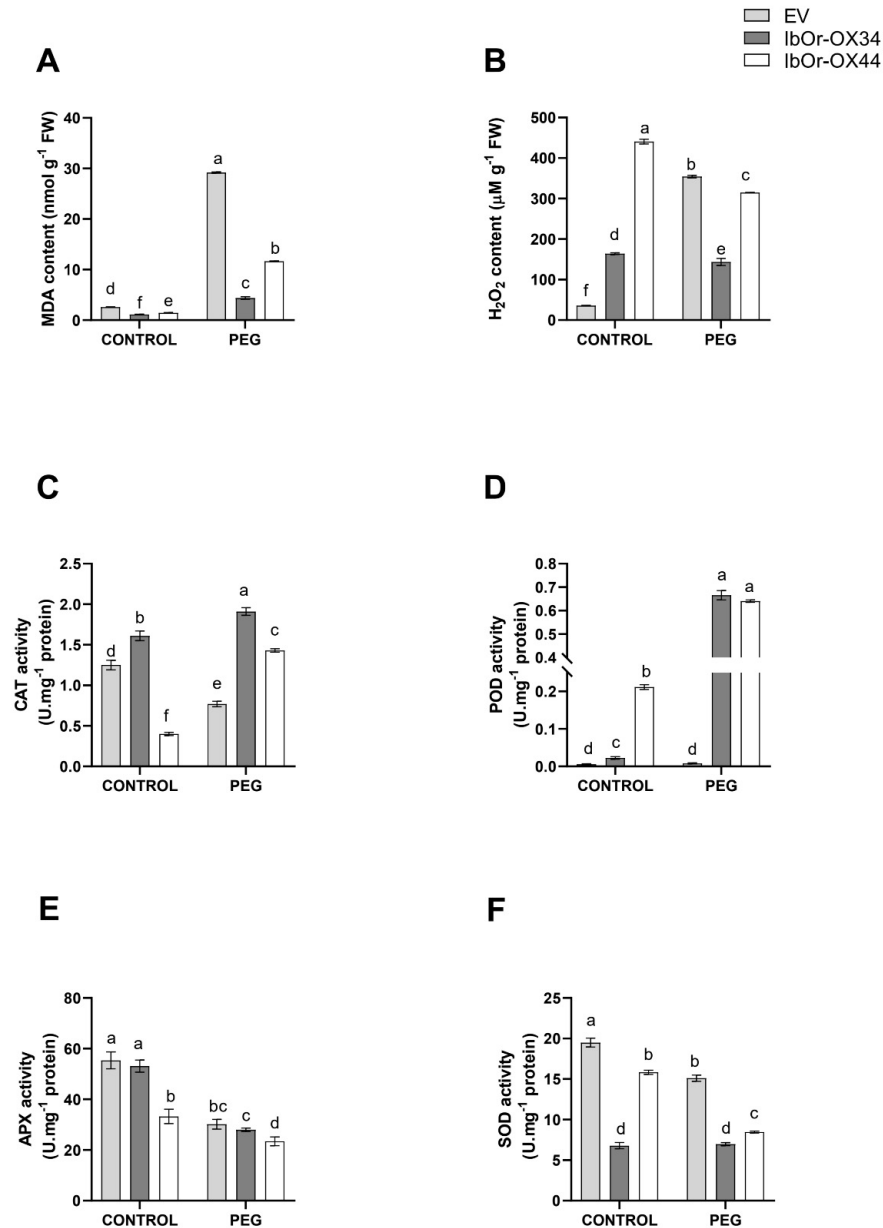


Figure 3: Oxidative stress markers and antioxidant enzyme activities of control (EV) and *IbOr*-overexpressing (*IbOr*-OX34 and *IbOr*-OX44) *Arabidopsis thaliana* lines under PEG-6000-induced water-deficit stress. (A) Malondialdehyde (MDA) content, (B) hydrogen peroxide (H₂O₂) content, (C) catalase (CAT) activity, (D) guaiacol peroxidase (POD) activity, (E) ascorbate peroxidase (APX) activity, and (F) superoxide dismutase (SOD) activity of 21-day-old plants subjected to hydroponic 10% (w/v) PEG-6000 treatment for 4 days. Data are presented as mean ± SD (*n* = 3 independent biological replicates). Different letters above the bars indicate statistically significant differences among groups according to ANOVA followed by Tukey's *post-hoc* test (*p* < 0.05).

3.7 Antioxidant Enzyme Activities

CAT activity showed a genotype-dependent response to PEG-6000-induced water-deficit stress (Fig. 3C). Relative to their respective controls, CAT activity decreased by approximately 38.4% in EV plants, whereas it increased by approximately 18.6% and 3.58-fold in *IbOr*-OX34 and *IbOr*-OX44 plants, respectively. Under PEG treatment, *IbOr*-OX34 exhibited the highest CAT activity, followed by *IbOr*-OX44 and EV, with significant differences among all three lines.

POD activity responded strongly to PEG-6000 treatment in the two *IbOr*-overexpressing lines (Fig. 3D). Compared with their respective controls, POD activity increased approximately 29.50-fold in *IbOr*-OX34 and 3.03-fold in *IbOr*-OX44 plants. Although the mean POD activity of EV plants increased by approximately 46.1%, this difference was not statistically significant. Under PEG treatment, POD activities of *IbOr*-OX34 and *IbOr*-OX44 were significantly higher than that of EV plants, whereas no significant difference was observed between the two overexpressing lines.

In contrast, APX activity decreased following PEG-6000 treatment in all lines (Fig. 3E). Relative to their respective controls, APX activity decreased by approximately 45.5%, 47.3%, and 29.5% in EV, *IbOr*-OX34, and *IbOr*-OX44 plants, respectively. Under PEG treatment, EV and *IbOr*-OX34 plants did not differ significantly in APX activity, whereas *IbOr*-OX44 exhibited the lowest activity.

SOD activity also showed line-dependent responses to PEG-6000 treatment (Fig. 3F). Relative to their respective controls, SOD activity decreased by approximately 22.5% in EV plants and 46.6% in *IbOr*-OX44 plants. In *IbOr*-OX34, SOD activity remained statistically unchanged between control and PEG-treated conditions despite a slight numerical increase of approximately 2.6%. Under PEG treatment, EV plants exhibited the highest SOD activity, followed by *IbOr*-OX44 and *IbOr*-OX34, with significant differences among the three lines.

3.8 Histochemical Detection of ROS

Histochemical DAB staining was used to visualize the spatial accumulation of H_2O_2 in leaves under control and mannitol-induced osmotic stress conditions (Fig. 4A). Under control conditions (MS medium), all tested lines (EV, *IbOr*-OX34, and *IbOr*-OX44) exhibited minimal background staining. Following mannitol-induced osmotic stress, EV leaves showed extensive dark-brown DAB precipitates, indicating greater H_2O_2 accumulation. In contrast, both *IbOr*-overexpressing lines exhibited visibly weaker and more restricted DAB staining than EV plants. Among the transgenic lines, *IbOr*-OX44 showed the least intense brown coloration and a more localized staining pattern.

Histochemical NBT staining was used to visualize the spatial accumulation of superoxide radicals ($O_2^{\cdot-}$) in rosette leaves under control and mannitol-induced osmotic stress conditions (Fig. 4B). Under control conditions, EV, *IbOr*-OX34, and *IbOr*-OX44 leaves exhibited relatively limited and localized NBT staining. Following mannitol-induced osmotic stress, EV leaves showed extensive dark blue/purple formazan deposition across the leaf lamina, consistent with increased superoxide accumulation. *IbOr*-OX44 displayed visibly weaker and more restricted staining under the same conditions. In *IbOr*-OX34 leaves, the staining pattern was more heterogeneous and was accompanied by noticeable background coloration, making a direct visual comparison of staining intensity less straightforward. Overall, the NBT staining patterns revealed line-dependent differences in the spatial distribution of superoxide under mannitol-induced osmotic stress.

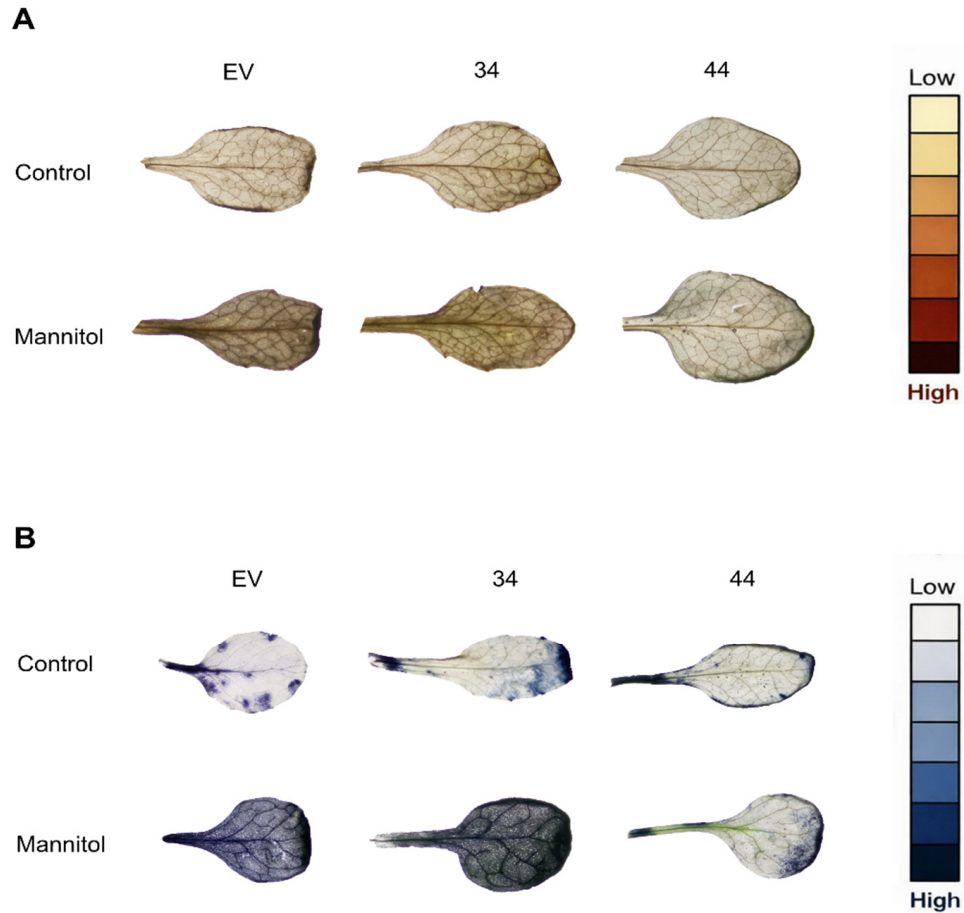


Figure 4: Histochemical detection of reactive oxygen species (ROS) in EV and IbOr-overexpressing (IbOr-OX34 and IbOr-OX44) *Arabidopsis thaliana* leaves under control and mannitol-induced osmotic stress conditions. (A) DAB (3,3'-diaminobenzidine) staining showing H₂O₂ accumulation as brown precipitates. (B) NBT (nitroblue tetrazolium) staining showing superoxide radical (O₂^{•-}) accumulation as dark blue/purple precipitates. Leaves were obtained from 19-day-old seedlings grown *in vitro* with or without 200 mM mannitol for 5 days. Color scales on the right are provided as visual guides to indicate increasing staining intensity from low to high and do not represent quantitative measurements.

3.9 Transcriptional Regulation of Upstream Carotenoid Biosynthetic Genes

Under control conditions, *AtGGPS1* transcript levels were relatively low in all lines (Fig. 5A). Mannitol-induced osmotic stress increased *AtGGPS1* expression in all genotypes. Under mannitol treatment, IbOr-OX44 exhibited the highest *AtGGPS1* transcript level at approximately 4.0-fold, whereas EV and IbOr-OX34 showed similar expression levels of approximately 2.6-fold and did not differ significantly from each other.

For *AtPSY*, transcript levels were also relatively low under control conditions (Fig. 5B). Mannitol-induced osmotic stress increased *AtPSY* expression in all three lines. Under mannitol treatment, IbOr-OX34 exhibited the highest transcript level at approximately 4.0-fold, followed by EV at approximately 3.4-fold and IbOr-OX44 at approximately 3.0-fold, with significant differences among all three lines.

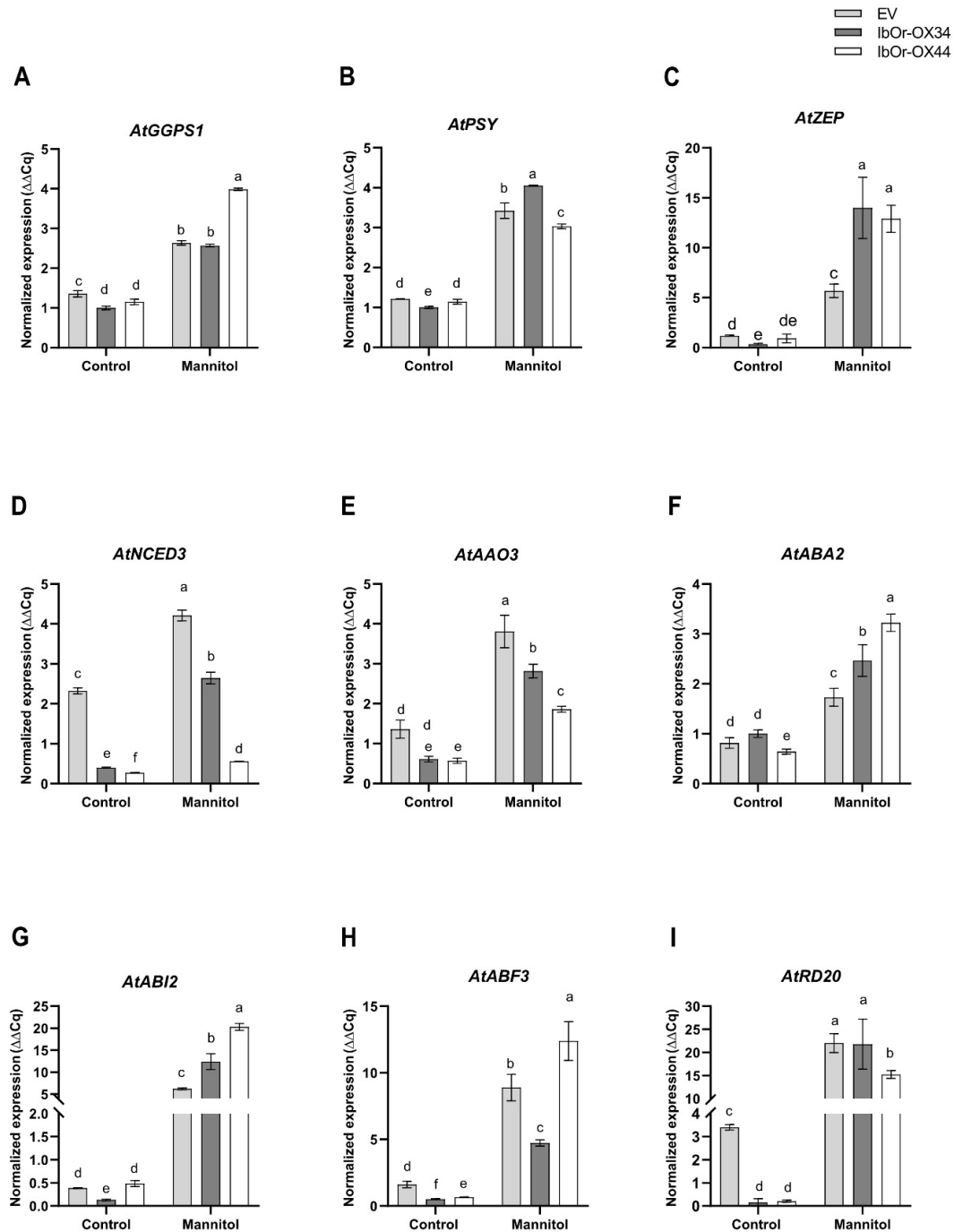


Figure 5: Relative expression levels of carotenoid/ABA biosynthesis- and ABA signaling-related genes in EV and IbOr-overexpressing (IbOr-OX34 and IbOr-OX44) *Arabidopsis thaliana* lines under control and mannitol-induced osmotic stress conditions. Quantitative real-time PCR (qPCR) analysis of (A) *AtGGPS1*, (B) *AtPSY*, (C) *AtZEP*, (D) *AtNCED3*, (E) *AtAAO3*, (F) *AtABA2*, (G) *AtABI2*, (H) *AtABF3*, and (I) *AtRD20* in 19-day-old seedlings grown *in vitro* on 1/2 MS medium with or without 200 mM mannitol for 5 days. Target gene expression was normalized to the internal reference gene *AtACT2*. Data are presented as mean $\pm$ SD ($n = 3$ independent biological replicates). Different letters above bars indicate statistically significant differences among groups according to ANOVA followed by Tukey's *post-hoc* test ($p < 0.05$).

3.10 Transcriptional Regulation of Downstream ABA Biosynthetic Genes

Under control conditions, *AtZEP* transcript levels were relatively low in all lines (Fig. 5C). Mannitol-induced osmotic stress significantly increased *AtZEP* expression. Under mannitol treatment, IbOr-OX34 and IbOr-OX44 exhibited the highest relative transcript levels at approximately 14- and 13-fold, respectively, with no significant difference between the two lines. Both overexpressing lines showed significantly higher *AtZEP* transcript levels than EV plants, which exhibited an approximately 5.5-fold relative expression level.

AtNCED3 and *AtAAO3* showed distinct line-dependent expression patterns under mannitol-induced osmotic stress (Fig. 5D,E). For *AtNCED3*, EV plants exhibited the highest relative transcript level at approximately 4.2-fold, followed by IbOr-OX34 at approximately 2.6-fold. In contrast, *AtNCED3* transcript abundance remained low in IbOr-OX44 under mannitol treatment at approximately 0.6-fold. For *AtAAO3*, EV plants also exhibited the highest relative transcript level at approximately 3.8-fold, followed by IbOr-OX34 at approximately 2.8-fold and IbOr-OX44 at approximately 1.8-fold, with significant differences among the stressed groups.

For *AtABA2*, transcript levels were relatively low under control conditions (Fig. 5F). Mannitol-induced osmotic stress increased *AtABA2* expression in a line-dependent manner. Under mannitol treatment, IbOr-OX44 exhibited the highest relative transcript level at approximately 3.2-fold, followed by IbOr-OX34 at approximately 2.5-fold and EV at approximately 1.7-fold, with significant differences among all three lines.

3.11 Transcriptional Regulation of ABA Signaling Core and Downstream Markers

Mannitol-induced osmotic stress increased *AtABI2* transcript levels in all genotypes compared with control conditions (Fig. 5G). Under mannitol treatment, IbOr-OX44 exhibited the highest relative transcript level at approximately 20-fold, followed by IbOr-OX34 at approximately 12.5-fold and EV at approximately 6-fold, with significant differences among all three lines.

Under control conditions, *AtABF3* transcript levels were relatively low in all genotypes (Fig. 5H). Following mannitol-induced osmotic stress, *AtABF3* transcript levels increased in all lines. Under mannitol treatment, IbOr-OX44 exhibited the highest relative transcript level at approximately 12.5-fold, followed by EV at approximately 8.8-fold and IbOr-OX34 at approximately 4.7-fold.

For *AtRD20*, transcript levels were low under control conditions and increased following mannitol-induced osmotic stress (Fig. 5I). Under mannitol treatment, EV and IbOr-OX34 exhibited relative transcript levels of approximately 22- and 23-fold, respectively, with no significant difference between the two lines. IbOr-OX44 exhibited a lower relative transcript level of approximately 14-fold and was significantly different from EV and IbOr-OX34.

4 Discussion

The present study examined water-deficit stress tolerance in transgenic *Arabidopsis thaliana* overexpressing the sweet potato *ORANGE* gene (*IbOr*). Plants can mitigate water deficit by altering their physiological, biochemical, molecular, and morphological characteristics, along with modifying gene expression. Plant responses to water-deficit stress are complex and involve both adaptive and detrimental physiological changes. The capacity of a plant to endure water deficit conditions is determined by various factors, including the species and cultivar, the duration and severity of the water deficit, environmental characteristics, variations in water content, and the plant's growth dynamics [35]. Water-deficit stress primarily results in a reduction of both fresh and dry weights in *A. thaliana* and other plant species [36,37]. Significant water deprivation resulting from water-deficit stress may restrict the buildup of dry matter by as much as 50%. Despite these severe reductions in biomass, plants can still exhibit water deficit toler-

ance by triggering the aforementioned physiological and molecular adaptations. Maintenance of tissue water status and cellular turgor is an important component of plant tolerance to water-deficit stress. The higher RWC maintained by the *IbOr*-overexpressing lines under PEG-6000-induced water-deficit stress suggests improved preservation of plant water status. This was accompanied by better maintenance of dry biomass, particularly in *IbOr*-OX44, in which dry weight did not differ significantly from the corresponding control. Together, these findings indicate improved maintenance of water status and biomass under PEG-6000-induced water-deficit stress.

Osmotic adjustment is an important component of plant responses to water-deficit stress and involves the accumulation of compatible solutes, including proline, soluble sugars, glycine betaine, and polyols [38,39]. These compounds contribute to the maintenance of cellular osmotic potential and can also protect proteins, membranes, and other macromolecular structures during stress. Among these osmolytes, proline is widely associated with plant responses to water-deficit and salinity stress and may contribute not only to osmotic adjustment but also to the stabilization of cellular components and stress-related signaling [40]. In the present study, proline content was evaluated under PEG-6000-induced water-deficit stress. PEG treatment increased proline content in all lines, but the magnitude of this response was substantially greater in EV plants than in the two *IbOr*-overexpressing lines. This genotype-dependent pattern indicates that the enhanced stress tolerance observed in the *IbOr*-OX lines was not accompanied by a correspondingly strong increase in proline accumulation. However, because proline content alone does not provide information on its biosynthetic flux or turnover, the lower stress-induced accumulation observed in the transgenic lines should not be interpreted as evidence of reduced proline biosynthesis or a specific metabolic reallocation.

Soluble sugar accumulation was examined in a separate experimental system using mannitol-induced osmotic stress. Soluble sugars are important compatible solutes that contribute to osmotic adjustment and the stabilization of cellular structures during stress [38,39]. In the present study, mannitol treatment markedly increased soluble sugar content in all genotypes, with the highest absolute level observed in the *IbOr*-OX34 line. Relative to their corresponding controls, both *IbOr*-overexpressing lines also showed pronounced increases in soluble sugar accumulation. Because proline and soluble sugars were quantified under different experimental stress systems, however, the present data do not allow a direct comparison of their relative contributions or provide evidence for a metabolic shift from proline-based to carbohydrate-based osmoprotection. Rather, these findings indicate distinct osmolyte responses of the *IbOr*-overexpressing lines under the two experimental stress conditions.

The photosynthetic apparatus represents one of the major targets of water-deficit-induced physiological impairment, and alterations in chlorophyll content are commonly associated with stress-related damage to photosynthetic tissues [41,42]. In the present study, PEG-6000-induced water-deficit stress decreased chlorophyll content in EV plants, whereas the *IbOr*-overexpressing lines maintained higher chlorophyll levels under stress. This contrasting response was consistent with the more pronounced chlorosis observed in EV plants. Together with the higher RWC and better maintenance of biomass in the *IbOr*-overexpressing lines, the preservation of chlorophyll content suggests that *IbOr* overexpression was associated with reduced physiological impairment under PEG-6000-induced water-deficit stress.

This phenotypic preservation may be related, at least in part, to the established biochemical function of ORANGE proteins. Previous studies have shown that ORANGE proteins can interact with and post-transcriptionally stabilize phytoene synthase (PSY), a key rate-limiting enzyme in the carotenoid biosynthetic pathway [17,43]. Consistent with this established role, total carotenoid content increased more strongly in the *IbOr*-overexpressing lines than in EV plants under PEG-6000-induced water-deficit stress. Importantly, carotenoids are not merely accessory pigments but also contribute to non-enzymatic antioxidant and

photoprotective processes. They can quench triplet chlorophyll and singlet oxygen generated under excess excitation, thereby limiting photo-oxidative damage [4]. Previous ORANGE gene manipulation studies have similarly demonstrated enhanced carotenoid accumulation [43], and increased carotenoid levels following IbOr overexpression have been reported in transgenic potato, alfalfa, sweet potato, and maize [44]. Therefore, the higher carotenoid status observed in the IbOr-OX lines may have contributed to the reduced oxidative damage observed under PEG-6000-induced water-deficit stress.

In the present study, PEG-6000-induced water-deficit stress resulted in marked oxidative stress in EV plants, as indicated by pronounced increases in H_2O_2 and MDA levels, consistent with enhanced lipid peroxidation and oxidative membrane damage. Intriguingly, under control conditions, the IbOr-OX lines—particularly IbOr-OX44—maintained higher baseline H_2O_2 levels than EV plants. Because ROS can function not only as potentially damaging molecules but also as important signaling components in plant stress responses [45], the elevated basal H_2O_2 level may contribute to a form of stress preparedness or priming. However, the present data do not allow this possibility to be established directly. Notably, the carotenoid- and ABA-related genes examined in the unstressed IbOr-OX44 line, including *AtGGPS1*, *AtPSY*, *AtZEP*, and *AtABF3*, remained at relatively low baseline expression levels and showed no clear constitutive induction. This observation does not support a simple constitutive transcriptional priming mechanism involving these genes. If a priming-related response is involved, it could potentially operate through post-transcriptional or enzymatic regulation, although such mechanisms were not directly assessed in the present study. Alternatively, the elevated basal H_2O_2 level observed in IbOr-OX44 may reflect a line-specific effect associated with the T-DNA insertion site. Random integration of the transgene could potentially influence local chromatin organization or endogenous regulatory networks and thereby alter basal ROS status independently of the primary effects of *IbOr* overexpression. Thus, the distinct basal H_2O_2 phenotype of IbOr-OX44 should be interpreted cautiously, and evaluation of multiple independent transgenic lines remains important for distinguishing gene-associated responses from insertion-specific effects.

In line with these observations, the IbOr-overexpressing lines maintained substantially lower H_2O_2 and MDA levels than the stressed EV plants under PEG-6000-induced water-deficit stress. This response was accompanied by marked line-dependent changes in antioxidant enzyme activities, particularly the strong induction of guaiacol peroxidase (POD) in IbOr-OX34 and catalase (CAT) in IbOr-OX44. Comparable enhancements in antioxidant enzyme responses have been reported in transgenic *Arabidopsis* lines overexpressing the *Malus baccata* *ICE1* gene [46] or the *Solanum tuberosum* *ERECTA* gene [47], where enhanced antioxidant capacity was associated with reduced oxidative damage. Despite decreases in APX activity and unchanged or decreased SOD activity under stress, the pronounced line-specific induction of POD and CAT may have contributed to the lower H_2O_2 and MDA accumulation observed in the IbOr-overexpressing lines. Such line-dependent antioxidant responses are consistent with the central role of regulated ROS production and scavenging in plant responses to abiotic stress [48]. These findings suggest that *IbOr* overexpression is associated with a distinct antioxidant enzyme response that may contribute to limiting oxidative damage under PEG-6000-induced water-deficit stress.

The spatial distribution and scavenging of reactive oxygen species (ROS) are important components of plant responses to abiotic stress. In the present study, histochemical DAB and NBT staining provided qualitative information on the accumulation patterns of H_2O_2 and superoxide radicals ($O_2^{\bullet-}$), respectively, under mannitol-induced osmotic stress (Fig. 4). EV leaves exhibited extensive DAB staining following mannitol treatment, whereas both IbOr-overexpressing lines showed more restricted DAB staining, particularly IbOr-OX44. NBT staining also revealed line-dependent differences in superoxide distribution, with IbOr-OX44 displaying a visibly more restricted staining pattern than EV plants. The staining pattern of

IbOr-OX34 was more heterogeneous and therefore did not permit a straightforward visual comparison of staining intensity. These qualitative observations suggest that IbOr overexpression may be associated with altered ROS accumulation patterns under mannitol-induced osmotic stress; however, the histochemical staining itself was not quantitatively analyzed.

Plant responses to water-deficit stress involve coordinated transcriptional regulation of multiple stress-responsive gene networks [49]. In the present study, analysis of transcript profiles under 200 mM mannitol-induced osmotic stress revealed distinct line-dependent responses at the carotenoid-ABA interface. *AtNCED3*, a key regulatory gene in ABA biosynthesis [50,51], was strongly induced in stressed EV plants but remained comparatively low in IbOr-OX44, despite the induction of other ABA-biosynthetic genes. Previous studies have shown that enhanced NCED activity can increase ABA accumulation and influence drought-related responses [52], highlighting the potential biological relevance of the divergent *AtNCED3* expression observed here. However, because ABA content was not measured in the present study, differences in *AtNCED3* transcript abundance cannot be directly translated into differences in ABA accumulation. *AtAAO3* also showed a distinct expression pattern among the lines, further indicating that ABA-related transcriptional responses were not uniformly coordinated across pathway components. *AtABI2*, which encodes a clade A protein phosphatase 2C (PP2C) and functions as a negative regulator of ABA signaling [53–55], was more strongly induced in the IbOr-overexpressing lines than in EV plants under mannitol-induced osmotic stress. This expression pattern is consistent with the possibility of enhanced negative-feedback regulation within ABA signaling. However, because ABA content, ABI2 protein abundance, and PP2C activity were not measured, the observed transcriptional response does not establish a direct effect on ABA accumulation or ABA signaling activity. Therefore, these findings support an association between *IbOr* overexpression and altered ABA-related transcriptional regulation rather than demonstrating ABA homeostasis directly.

Carotenoid biosynthesis-related genes also exhibited pronounced transcriptional responses under mannitol-induced osmotic stress. In particular, *AtGGPS1*, *AtPSY*, and *AtZEP* showed induction under mannitol-induced osmotic stress, with distinct expression patterns among EV and the IbOr-overexpressing lines. These genes participate in key steps of carotenoid and xanthophyll biosynthesis [43,56], and their expression patterns are consistent with altered transcriptional regulation of the carotenoid biosynthetic pathway associated with *IbOr* overexpression. However, because enzyme abundance, pathway intermediates, and metabolic flux were not directly measured, these transcriptional changes cannot be interpreted as direct evidence of increased carotenoid or xanthophyll flux under mannitol treatment. Downstream stress-responsive genes also showed distinct expression patterns. *AtABF3*, a well-established component of ABA-responsive stress signaling [57,58], showed strong line-dependent induction, particularly in IbOr-OX44, while *AtRD20* was induced in all stressed groups. Together, these expression patterns indicate that *IbOr* overexpression is associated with changes in carotenoid biosynthesis-related and ABA-responsive gene expression under mannitol-induced osmotic stress, although the causal relationships among these responses remain to be established.

It should also be noted that the physiological and biochemical responses were primarily evaluated under PEG-6000-induced water-deficit stress, whereas the transcriptional analyses were performed under mannitol-induced osmotic stress. Therefore, these datasets are interpreted as complementary responses to two distinct stress systems rather than as direct evidence that the transcriptional changes observed under mannitol mechanistically caused the physiological responses measured under PEG treatment. In addition, the qPCR analyses were performed using bulk leaf tissue and therefore represent average transcript responses across different leaf cell types. Future tissue- or cell-type-resolved analyses will be valuable for

determining whether the observed carotenoid- and ABA-related transcriptional responses are preferentially associated with specific cell types, such as guard cells, vascular tissues, or mesophyll cells.

In conclusion, this study demonstrates that overexpression of the sweet potato ORANGE gene (*IbOr*) enhances tolerance of *Arabidopsis thaliana* to PEG-6000-induced water-deficit stress. The *IbOr*-OX lines generally maintained higher relative water content, biomass, chlorophyll, and carotenoid levels and exhibited lower oxidative damage than EV plants, together with line-dependent changes in antioxidant enzyme activities. In the independent mannitol-induced osmotic stress system, *IbOr* overexpression was associated with pronounced soluble sugar accumulation, altered ROS distribution, and distinct transcriptional responses of carotenoid biosynthesis- and ABA-related genes. In particular, the expression patterns of *AtNCED3*, *AtAAO3*, *AtABI2*, *AtABF3*, and other stress-responsive genes suggest that *IbOr* overexpression influences ABA-related transcriptional regulation; however, ABA content and signaling activity were not directly measured, and therefore a specific ABA-mediated mechanism cannot be established from the present data. Overall, these findings identify *IbOr* as a promising candidate for further investigation of water-deficit tolerance, although validation in crop species and under soil-based and field-relevant drought conditions will be necessary before broader agricultural applications can be inferred.

Acknowledgement: Not applicable.

Funding Statement: This research was supported by the Scientific Research Projects Coordination Unit of Karadeniz Technical University under Project Number FBA-2025-16239. This study was also supported by the Scientific and Technological Research Council of Türkiye (TÜBİTAK 2209-A Program) under Project Number 1919B012112821.

Author Contributions: The authors confirm contribution to the paper as follows: conceptualization, Ertuğrul Kutlu and Aykut Sağlam; methodology, Ertuğrul Kutlu and Aykut Sağlam; validation, Ertuğrul Kutlu, Erkan Anıl Aydın and Aykut Sağlam; formal analysis, Aykut Sağlam; investigation, Ertuğrul Kutlu, Erkan Anıl Aydın, Büşranur Demir, Ece Nisa Birinci and Melih Aktürk; resources, Aykut Sağlam; data curation, Erkan Anıl Aydın and Aykut Sağlam; writing—original draft preparation, Erkan Anıl Aydın and Aykut Sağlam; writing—review and editing, Erkan Anıl Aydın, Sang-Soo Kwak and Aykut Sağlam; visualization, Erkan Anıl Aydın and Aykut Sağlam; supervision, Aykut Sağlam; project administration, Aykut Sağlam; funding acquisition, Aykut Sağlam. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The authors confirm that the data supporting the findings of this study are available within the article. Additional raw datasets generated and analyzed during the current study are available from the corresponding author, Aykut Sağlam, upon reasonable request.

Ethics Approval: Not applicable. This study does not contain any studies with human participants or animal subjects performed by any of the authors.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Samanta S, Seth CS, Roychoudhury A. The molecular paradigm of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with different phytohormone signaling pathways during drought stress in plants. *Plant Physiol Biochem.* 2024;206:108259. [[CrossRef](#)].
2. Ali M, Kaderbek T, Khan MA, Skalicky M, Brestic M, Elsabagh M, et al. Biosynthesis and multifaceted roles of reactive species in plant defense mechanisms during environmental cues. *Plant Stress.* 2025;18:101102. [[CrossRef](#)].
3. Guo W, Xing Y, Luo X, Li F, Ren M, Liang Y. Reactive oxygen species: a crosslink between plant and human eukaryotic cell systems. *Int J Mol Sci.* 2023;24(17):13052. [[CrossRef](#)].

4. Vieira EA, Gaspar M, Caldeira CF, Munné-Bosch S, Braga MR. Desiccation tolerance in the resurrection plant *Barbarea graminifolia* involves changes in redox metabolism and carotenoid oxidation. *Front Plant Sci.* 2024;15:1344820. [[CrossRef](#)].
5. Acevedo O, Contreras RA, Stange C. The carrot phytoene synthase 2 (*DcPSY2*) promotes salt stress tolerance through a positive regulation of abscisic acid and abiotic-related genes in *Nicotiana tabacum*. *Plants.* 2023;12(10):1925. [[CrossRef](#)].
6. Chen W, He S, Liu D, Patil GB, Zhai H, Wang F, et al. A sweetpotato geranylgeranyl pyrophosphate synthase gene, *IbGGPS*, increases carotenoid content and enhances osmotic stress tolerance in *Arabidopsis thaliana*. *PLoS One.* 2015;10(9):e0137623. [[CrossRef](#)].
7. Wang Q, Zhao X, Jiang Y, Wang L. Isolation, expression and functional analysis of the *GbGGPPS1* gene in *Ginkgo biloba*. *Fruit Res.* 2023;3(1):44. [[CrossRef](#)].
8. Ni K, Lu X, Li S, Li F, Zhang Y, Cui R, et al. *GhLCYE-3* characterized as a lycopene cyclase gene responding to drought stress in cotton. *Comput Struct Biotechnol J.* 2024;23:384–95. [[CrossRef](#)].
9. Zhang RR, Wang YH, Li T, Tan GF, Tao JP, Su XJ, et al. Effects of simulated drought stress on carotenoid contents and expression of related genes in carrot taproots. *Protoplasma.* 2021;258(2):379–90. [[CrossRef](#)].
10. Ni K, Dai M, Lu X, Zhang Y, Fan Y, Xu N, et al. Pretreatment of NaCl enhances the drought resistance of cotton by regulating the biosynthesis of carotenoids and abscisic acid. *Front Environ Sci.* 2022;10:998141. [[CrossRef](#)].
11. Shi L, Chang L, Yu Y, Zhang D, Zhao X, Wang W, et al. Recent advancements and biotechnological implications of carotenoid metabolism of *Brassica*. *Plants.* 2023;12(5):1117. [[CrossRef](#)].
12. Lopez AB, van Eck J, Conlin BJ, Paolillo DJ, O'Neill J, Li L. Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *J Exp Bot.* 2008;59(2):213–23. [[CrossRef](#)].
13. Kim SH, Ahn YO, Ahn MJ, Jeong JC, Lee HS, Kwak SS. Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures. *Plant Physiol Biochem.* 2013;70:445–54. [[CrossRef](#)].
14. Shan YJ, Li D, Cao JJ, Zhang L, Han LQ, Zhang MP, et al. Over-expression of *Arabidopsis ORANGE* gene enhances drought stress tolerance through ABA-dependent pathway in *Arabidopsis thaliana*. *Plant Growth Regul.* 2022;96(1):91–101. [[CrossRef](#)].
15. Ali F, Wang Q, Fazal A, Wang LJ, Song S, Kong MJ, et al. The DnaJ-like zinc finger protein *ORANGE* promotes proline biosynthesis in drought-stressed *Arabidopsis* seedlings. *Int J Mol Sci.* 2022;23(7):3907. [[CrossRef](#)].
16. Kang L, Zhang C, Liu J, Ye M, Zhang L, Chen F, et al. Overexpression of potato *ORANGE* (*StOR*) and *StOR* mutant in *Arabidopsis* confers increased carotenoid accumulation and tolerance to abiotic stress. *Plant Physiol Biochem.* 2023;201:107809. [[CrossRef](#)].
17. Park S, Kim HS, Jung YJ, Kim SH, Ji CY, Wang Z, et al. Orange protein has a role in phytoene synthase stabilization in sweetpotato. *Sci Rep.* 2016;6:33563. [[CrossRef](#)].
18. Tocquin P, Corbesier L, Havelange A, Pielain A, Kurtem E, Bernier G, et al. A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of *Arabidopsis thaliana*. *BMC Plant Biol.* 2003;3:2. [[CrossRef](#)].
19. Chen X, Favero BT, Liu F, Lütken H. Overexpression of *Rhizobium rhizogenes* A4-rolB enhances osmotic stress resistance in *Arabidopsis*. *BMC Plant Biol.* 2025;25(1):711. [[CrossRef](#)].
20. Pegler JL, Oultram JMJ, Grof CPL, Eamens AL. *DRB1*, *DRB2* and *DRB4* are required for an appropriate miRNA-mediated molecular response to osmotic stress in *Arabidopsis thaliana*. *Int J Mol Sci.* 2024;25(23):12562. [[CrossRef](#)].
21. Castillo FJ. Antioxidative protection in the inducible CAM plant *Sedum album* L. following the imposition of severe water stress and recovery. *Oecologia.* 1996;107(4):469–77. [[CrossRef](#)].
22. Arnon DI. Copper enzymes in isolated chloroplasts. polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 1949;24(1):1–15. [[CrossRef](#)].
23. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. *Plant Soil.* 1973;39(1):205–7. [[CrossRef](#)].
24. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem.* 1956;28(3):350–6. [[CrossRef](#)].
25. Velikova V, Yordanov I, Edreva A. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. *Plant Sci.* 2000;151(1):59–66. [[CrossRef](#)].

26. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys*. 1968;125(1):189–98. [[CrossRef](#)].
27. Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB. Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. *Plant J*. 1997;11(6):1187–94. [[CrossRef](#)].
28. Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem*. 1971;44(1):276–87. [[CrossRef](#)].
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–54. [[CrossRef](#)].
30. Urbanek H, Kuzniak-Gebarowska E. Elicitation of defence responses in bean leaves by *Botrytis cinerea* polygalacturonase. *Acta Physiol Plant*. 1991;13(1):43–50.
31. Aebi H. Catalase *in vitro*. *Methods Enzymol*. 1984;105:121–6. [[CrossRef](#)].
32. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol*. 1981;22(5):867–80. [[CrossRef](#)].
33. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*. 1993;15(3):532–7.
34. Bookout AL, Mangelsdorf DJ. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nucl Recept Signal*. 2003;1:e012. [[CrossRef](#)].
35. Panda D, Mishra SS, Behera PK. Drought tolerance in rice: focus on recent mechanisms and approaches. *Rice Sci*. 2021;28(2):119–32. [[CrossRef](#)].
36. Hummel I, Pantin F, Sulpice R, Piques M, Rolland G, Dauzat M, et al. *Arabidopsis* plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme, and gene expression analysis. *Plant Physiol*. 2010;154(1):357–72. [[CrossRef](#)].
37. Zia R, Nawaz MS, Siddique MJ, Hakim S, Imran A. Plant survival under drought stress: implications, adaptive responses, and integrated rhizosphere management strategy for stress mitigation. *Microbiol Res*. 2021;242:126626. [[CrossRef](#)].
38. Sezgin A, Altuntaş C, Sağlam A, Terzi R, Demiralay M, Kadioğlu A. Absciscic acid cross-talking with hydrogen peroxide and osmolyte compounds may regulate the leaf rolling mechanism under drought. *Acta Physiol Plant*. 2018;40(8):141. [[CrossRef](#)].
39. Roeder AHK, Shi Y, Yang S, Abbas M, Sasidharan R, Yanovsky MJ, et al. Translational insights into abiotic interactions: from *Arabidopsis* to crop plants. *Plant Cell*. 2025;37(7):koaf140. [[CrossRef](#)].
40. Spormann S, Nadais P, Sousa F, Pinto M, Martins M, Sousa B, et al. Accumulation of proline in plants under contaminated soils-are we on the same page? *Antioxidants*. 2023;12(3):666. [[CrossRef](#)].
41. Lawlor DW, Cornic G. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant Cell Environ*. 2002;25(2):275–94. [[CrossRef](#)].
42. Chaves MM, Flexas J, Pinheiro C. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann Bot*. 2009;103(4):551–60. [[CrossRef](#)].
43. Osorio CE. The role of *Orange* gene in carotenoid accumulation: manipulating chromoplasts toward a colored future. *Front Plant Sci*. 2019;10:1235. [[CrossRef](#)].
44. Tran TL, Ho TH, Nguyen DT. Overexpression of the IbOr gene from sweet potato (*Ipomea batatas* ‘Hoang Long’) in maize increases total carotenoid and β-carotene contents. *Turk J Biol*. 2017;41(6):1003–10. [[CrossRef](#)].
45. Sood M. Reactive oxygen species (ROS): plant perspectives on oxidative signalling and biotic stress response. *Discover Plants*. 2025;2(1):187. [[CrossRef](#)].
46. Duan Y, Han J, Guo B, Zhao W, Zhou S, Zhou C, et al. MbICE1 confers drought and cold tolerance through up-regulating antioxidant capacity and stress-resistant genes in *Arabidopsis thaliana*. *Int J Mol Sci*. 2022;23(24):16072. [[CrossRef](#)].
47. Liu X, Yang W, Zhang L, Nie F, Gong L, Zhang H. Overexpression of StERECTA enhances drought tolerance in *Arabidopsis thaliana*. *J Plant Physiol*. 2024;303:154353. [[CrossRef](#)].
48. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol*. 2004;55:373–99. [[CrossRef](#)].

49. Shinozaki K, Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. *J Exp Bot.* 2006;58(2):221–7. [[CrossRef](#)].
50. Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, et al. Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *Plant J.* 2001;27(4):325–33. [[CrossRef](#)].
51. Seo M, Koshihara T. Complex regulation of ABA biosynthesis in plants. *Trends Plant Sci.* 2002;7(1):41–8. [[CrossRef](#)].
52. Qin X, Zeevaert JA. Overexpression of a 9-*cis*-epoxycarotenoid dioxygenase gene in *Nicotiana glauca* increases abscisic acid and phaseic acid levels and enhances drought tolerance. *Plant Physiol.* 2002;128(2):544–51. [[CrossRef](#)].
53. Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J. The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant J.* 2001;25(3):295–303. [[CrossRef](#)].
54. Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, et al. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science.* 2009;324(5930):1064–8. [[CrossRef](#)].
55. Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, et al. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science.* 2009;324(5930):1068–71. [[CrossRef](#)].
56. Ruiz-Sola MÁ, Rodríguez-Concepción M. Carotenoid biosynthesis in *Arabidopsis*: a colorful pathway. *Arab Book.* 2012;10:e0158. [[CrossRef](#)].
57. Kang JY, Choi HI, Im MY, Kim SY. *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell.* 2002;14(2):343–57. [[CrossRef](#)].
58. Yoshida T, Mogami J, Yamaguchi-Shinozaki K. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Curr Opin Plant Biol.* 2014;21:133–9. [[CrossRef](#)].