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An animal-derived protein hydrolysate alleviates osmotic stress-induced oxidative damage in tomato by activating ascorbate and glutathione cycle

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Abstract

Background Protein hydrolysates, as a major group of biostimulants, have gained increasing attention because of their remarkable efficacy in enhancing plant stress resistance. However, the mechanism underlying protein hydrolysate-mediated tolerance to osmotic stress remains poorly understood. In this study, the alterations in oxidative damage and the ascorbate and glutathione (AsA-GSH) cycle were analyzed to investigate the potential effects of protein hydrolysates derived from pig blood (PP) against PEG-induced osmotic stress in tomato.

Results Osmotic stress triggers excessive accumulation of hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot-}$), leading to increased malondialdehyde (MDA), electrolyte leakage, and Evans blue uptake, which subsequently reduced photosynthetic efficiency, shoot fresh weight, and relative growth rate in tomato plants. Exogenous application of PP effectively alleviated the stress-induced negative effects by increasing the levels of AsA and GSH and ratios of AsA/DHA and GSH/GSSG. This protective effect was primarily attributed to the enhanced activity and gene expression of key AsA-GSH cycle enzymes, including glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX), γ -glutamylcysteine synthetase (γ -ECS), glutathione peroxidase (GPX), and glutathione S-transferase (GST). In addition, treatment with L-buthionine-sulfoximine (BSO), a GSH biosynthesis inhibitor, significantly reduced AsA and GSH levels and aggravated oxidative damage and growth inhibition, while PP application partially reversed the above negative effects caused by BSO addition. Meanwhile, PP application increased the proline accumulation by enhancing the activities of Δ^1 -pyrroline-5-carboxylate synthase (P5CS) and ornithine- δ -aminotransferase (OAT), while reducing the activity of proline dehydrogenase (ProDH).

Conclusion Exogenous PP confers tolerance to PEG-induced osmotic stress in tomato plants by activating the AsA-GSH cycle and enhancing cellular redox homeostasis.

Keywords Osmotic stress, Oxidative stress, Protein hydrolysates, Tomato, Ascorbate-glutathione cycle

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Introduction

Tomato (*Solanum lycopersicum* L.), one of the globally cultivated vegetables, is prized for its nutritional value and rich secondary metabolites. However, its agricultural productivity is severely constrained by abiotic stresses, especially osmotic stress induced by drought, which largely limits plant growth and fruit yield [1]. Osmotic stress disrupts cellular water homeostasis, compromises water-use efficiency, and induces overproduction of reactive oxygen species (ROS) [2]. The resulting oxidative damage affects essential biomolecules, such as membrane lipids, proteins, and nucleic acids, leading to lipid peroxidation and structural deterioration of cellular membranes, ultimately impairing photosynthesis [3]. To mitigate ROS-induced oxidative stress, plants have evolved a sophisticated antioxidant defense system comprising both enzymatic and non-enzymatic systems distributed across various subcellular compartments [4, 5].

The ascorbate and glutathione (AsA-GSH) cycle is a ubiquitous antioxidant mechanism localized in key subcellular compartments, including the cytosol, chloroplasts, mitochondria, and peroxisomes [6, 7]. This cycle involves non-enzymatic AsA and GSH and essential metabolic enzymes, including glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and ascorbate peroxidase (APX), which synergistically regulate cellular redox homeostasis and ROS scavenging. AsA serves as a primary electron donor in H_2O_2 detoxification, whereas GSH maintains redox buffering capacity across various metabolic and stress-responsive pathways. In addition, glutathione peroxidase (GPX) and glutathione S-transferase (GST) utilize GSH as a substrate for ROS elimination and xenobiotic detoxification, respectively [3, 7]. Emerging evidence has highlighted the pivotal role of the AsA-GSH cycle in increasing the tolerance to drought-induced osmotic stress by mitigating ROS and maintaining redox balance. Under drought stress, transcriptional and post-translational upregulation of AsA-GSH cycle enzymes has been consistently documented in *Dendranthema grandiflorum*, *Triticum aestivum*, and maize, etc [8–10]. Notably, the enhanced activities of APX, DHAR, and GR under stress conditions are associated with an increased GSH/GSSG ratio and improved ROS scavenging efficiency [11]. In addition, exogenous GSH has been widely reported to enhance drought and salinity tolerance by stimulating antioxidant defense systems and sustaining cellular redox homeostasis [12]. Collectively, these observations highlight the AsA-GSH cycle as an evolutionarily conserved mechanism for abiotic stress resilience, wherein its enzymatic and non-enzymatic constituents effectively strengthen the ROS detoxification and redox homeostasis.

Biostimulants are environmentally sustainable natural or synthetic substances that, even at low doses, activate intrinsic physiological and biochemical processes to enhance plant growth, nutrient use efficiency, crop yield and quality, and tolerance to abiotic stresses [13]. Protein hydrolysates (PHs), a prominent class of natural biostimulants, are rich in amino acids and bioactive peptides. They are primarily produced through chemical and enzymatic hydrolysis of low-cost, eco-friendly substrates such as crop residues and animal by-products [14]. Several studies have indicated that PHs significantly promote plant growth and improve the tolerance to osmotic stress induced by drought. For example, the vegetal proteins from *Malvaceae* effectively increased tomato resistance to water stress by regulating key antioxidant compounds, including phenolic compounds [15]. Similarly, plant-derived PHs also positively influenced antioxidant levels and enzymatic activity in tomato leaves, thereby improving tomato performance under water-deficient conditions [16]. Compared with plant-derived PHs, animal-derived PHs often contain higher levels of bioactive peptides and amino acids and are produced from abundant and low-value animal by-products, making them the potentially cost-effective option within a circular-economy framework [14, 17, 18]. In addition, our previous studies also found that seed priming with PHs derived from animal by-products substantially decreased ROS production and subsequent oxidative damage by improving non-enzymatic antioxidants, including AsA and GSH [19]. Collectively, these results highlight the considerable potential of protein hydrolysates as a feasible strategy for improving plant osmotic stress tolerance by enhancing the antioxidant system. However, the influence of protein hydrolysates on the AsA-GSH cycle and their exact mechanism by which they improve osmotic stress resistance in plants remains unclear.

In present study, we first explored the protective effects of foliar application of protein hydrolysate derived from pig blood (PP) on tomato plants subjected to osmotic stress. Subsequently, we focused on analyzing the components, enzymatic activities, and gene expression profiles of the AsA-GSH cycle, along with proline content and the activities of its biosynthetic enzymes. Moreover, the L-buthionine-sulfoximine (BSO), an inhibitor of GSH biosynthesis, was applied to determine whether the protective effects of PP under osmotic stress are regulated through GSH metabolism. The results may demonstrate that the application of protein hydrolysates confers osmotic stress tolerance in tomato plants by coordinately regulating the AsA-GSH cycle and thereby enhancing redox homeostasis.

Materials and methods

Plant material and experimental design

The tomato seeds (Dong-1) were commercially sourced from Shouguang, China. The protein hydrolysate derived from pig blood (PP) was supplied by Win Plus Biotech Co., Ltd., with its peptide composition and free amino acid levels presented in Table S1 and Table S2, respectively. The PP was produced from pig blood via controlled enzymatic hydrolysis followed by spray-drying, and the resulting powder was stored in airtight and light-proof containers. Tomato seeds were surface-sterilized with 10% (v/v) sodium hypochlorite solution for 5 min, followed by three thorough rinses with distilled water. The seeds were subsequently soaked in distilled water under dark conditions at 24 °C for one day. Then, seeds were transferred to a sterile growth substrate comprising peat, vermiculite, and perlite (3:1:1, v/v/v). Seedlings were cultivated in growth chambers under the following conditions: 16/8 h light/dark photoperiod with photosynthetic photon flux density of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and day/night temperatures of 25/16°C. At the 10-day growth stage, uniform seedlings were transplanted into the containers with 1 L of modified standard Hoagland nutrient solution, with five seedlings per pot. The nutrient solution was replenished every 5 days.

Nine days after transplantation, tomato seedlings were subjected to different experimental treatments. Osmotic stress was induced by supplementing the Hoagland nutrient solution with 10% (w/v) polyethylene glycol 6000 (PEG-6000), a high-molecular-weight osmotic agent that reduces water potential, thereby imposing osmotic stress that partially mimics drought-induced water deficit. Based on the results of our previous experiments [20], the 2 g L⁻¹ PP produced the greatest improvement in growth performance under osmotic stress, and therefore this concentration of PP was applied in this experiment. L-buthionine-sulfoximine (BSO, a glutathione synthesis inhibitor) was applied at 100 μM according to the prior experiment. Experiment 1 comprised four treatments: CK (nutrient solution and foliar application of double-distilled water), PP (nutrient solution and foliar application of PP), PEG (nutrient solution with 10% PEG-6000 and foliar application of double-distilled water), and PEG + PP (nutrient solution with 10% PEG-6000 and foliar application of PP). Experiment 2 included eight treatments: CK, BSO (Hoagland nutrient solution and foliar application of BSO), PP, PP + BSO (foliar application of BSO on the basis of PP treatment), PEG, PEG + PP, PEG + BSO (foliar application of BSO on the basis of PEG treatment), and PEG + BSO+PP (foliar application of BSO and PP on the basis of PEG treatment). In both experiments, the double-distilled water, PP, and BSO were sprayed daily for three days, with six replicates per group. Leaf samples were collected after 3 days of treatment, then

flash-frozen in liquid nitrogen, and stored at -80 °C until subsequent analyses.

Relative growth rate

The relative growth rate was evaluated using the following equation: relative growth rate (g day^{-1}) = $(W_2 - W_1)/(T_2 - T_1)$, where W_1 and W_2 were the dry weights at the initial (T_1) and final (T_2) time points, respectively.

Determination of photosynthetic parameters

The net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and water use efficiency (WUE) of the fully expanded tomato leaves were measured using a portable photosynthesis system (CIRAS-3, Hansatech Ltd., King's Lynn, UK) between 10:00 and 12:00 in the artificial climate chamber.

Determination of ROS content

The concentrations of H₂O₂ in tomato leaves were analyzed following the previous method with minor modifications [21]. Briefly, leaf tissue extracts were mixed with a solution containing 20% (v/v) H₂SO₄ and 0.1% (v/v) TiCl₄. After centrifugation, the absorbance of the supernatant was determined at 410 nm via a UV-visible spectrophotometer (X-5, Shanghai Metash Instruments Co., Ltd, China), and quantified by a standard curve of H₂O₂. The O₂⁻ concentration was analyzed spectrophotometrically following the protocol of Elstner and Heupel with modifications [22]. Fresh leaf tissue was extracted using 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 10,000 g for 10 min at 4 °C. Then, the supernatant was combined with the reaction mixture containing 65 mM phosphate buffer (pH 7.8), 10 mM hydroxylamine hydrochloride, 17 mM sulfanilamide, and 7 mM α -naphthylamine. After incubation, the absorbance at 530 nm was determined using a UV-visible spectrophotometer. The O₂⁻ concentration was measured using a sodium nitrite-based standard curve.

Determination of lipid peroxidation, Evans blue uptake, and electrolyte leakage

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) levels following the thiobarbituric acid (TBA) method with modifications [23]. Briefly, tomato leaf tissues were homogenized in 1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 g for 10 min at 4 °C. Subsequently, the supernatant was mixed with 20% (w/v) TCA containing 0.5% (w/v) TBA. The mixture was incubated in a water bath at 95 °C for 30 min. After centrifugation, the absorbance of the supernatant was determined at 532 nm and 600 nm.

Plasma membrane integrity was evaluated through Evans blue dye uptake assay [23]. Leaf samples were immersed in 0.25% (w/v) Evans blue solution for 10 min,

followed by three washes with deionized water to remove unbound dye. The stained tissues were then incubated in N, N-dimethylformamide for 1 h at 25 °C. The absorbance of the extracted dye was measured at 600 nm using a spectrophotometer.

Electrolyte leakage (EL) was determined by measuring electrical conductivity with minor modifications [24]. The relative EL was measured following the formula: $EL (\%) = [(EC_1 - EC_0)/(EC_2 - EC_0)] \times 100$, where EC_0 represents the initial conductivity of the solution, EC_1 represents the conductivity after incubation at room temperature, and EC_2 indicates the total conductivity after boiling the samples.

Determination of the ascorbate, glutathione, and proline contents

The concentrations of AsA and dehydroascorbate (DHA) were quantified using a colorimetric assay [25]. In this assay, AsA reduces ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), and then forms a pink-colored complex with α, α' -bipyridyl under acidic conditions. The absorbance of this complex was determined at 525 nm via a spectrophotometer. Total ascorbate (AsA + DHA) was measured after the reaction of DHA to AsA with dithiothreitol (DTT). DHA content was calculated by subtracting AsA from the total AsA.

GSH and oxidized glutathione (GSSG) levels were measured according to the previous method [26]. The GSH was reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and then its absorbance at 412 nm was determined. The oxidized glutathione (GSSG) was quantified by pretreatment with 2% (v/v) 2-vinylpyridine for 1 h at 25 °C prior to GSH analysis.

Proline content was determined via the acid ninhydrin method [27]. Briefly, fresh leaf tissues were homogenized in 3% (w/v) sulfosalicylic acid and centrifuged at 10,000 g for 10 min at 4 °C. The extracts were reacted with the ninhydrin reagent and quantified spectrophotometrically at 520 nm based on a standard curve of L-proline.

Enzyme activity assay

Plant tissues were homogenized in cold 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). The mixtures were centrifuged at 15,000 g for 15 min at 4 °C, and the supernatants were used for enzymatic assays.

APX activity was determined spectrophotometrically by measuring the reduction of absorbance at 290 nm [28]. DHAR and MDHAR activities were assayed by measuring the increase of reaction mixture at 265 nm and 340 nm, respectively [28]. GR activity was quantified based on the oxidation rate of NADPH at 340 nm [29]. Additionally, γ -ECS activity was assessed according to a previously described method [30]. GST activity was measured

as described by Cobbett et al. [31]. GPX activity was measured following the method of Drotar et al. [32]. The activity of Δ 1-pyrroline-5-carboxylate synthase (P5CS) was determined following the protocol of Song et al. [33]. The activity of ornithine- δ -aminotransferase (OAT) was determined via the method of Kim et al. [34]. Proline dehydrogenase (ProDH) activity was measured following the method described previously [35]. Enzyme activities were expressed as units per gram of fresh weight ($U\ g^{-1}\ FW$).

Total RNA isolation and qRT-PCR analysis

Total RNA was extracted from tomato leaves by a plant RNA purification kit (Sangon Biotech Co., Ltd., Shanghai, China) following the manufacturer's instructions. First-strand cDNA synthesis was conducted via HiScript II Q RT SuperMix (Vazyme, Nanjing, China). Quantitative real-time PCR (qRT-PCR) analysis was performed on an ABI QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA) by the ChamQ™ SYBR Color qPCR Master Mix (Vazyme, Nanjing, China). The gene-specific primers of APX, GR, MDHAR, DHAR, γ -ECS, GPX, and GST were presented in Table S3, and the β -actin gene was used as an internal reference. Three independent biological replicates were analyzed, each with three technical replicates. Relative gene expression levels were measured via the $2^{-\Delta\Delta C_t}$ method [36].

Statistical analysis

Data are shown the means $\pm$ standard deviation of at least three biological replicates. Statistical analyses were performed by one-way analysis of variance (ANOVA) using GraphPad Prism (version 9.5.1, GraphPad) for comparisons among treatment combinations and not for testing factorial interaction effects. Statistical comparisons among different treatments were carried out through Tukey's multiple comparison test at $P < 0.05$.

Results

Growth performance, ROS level, oxidative damage, and photosynthesis-related parameters

Compared to the control, osmotic stress significantly reduced shoot fresh weight by 22.3%, but PP application increased shoot fresh weight by 16.1% compared with PEG treatment alone (Fig. 1A). The similar significant difference of phenotypic presentation of tomato growth was also observed after different treatments (Fig. 1I). Accordingly, PP application also significantly reduced PEG-induced MDA accumulation, resulting in a 40.0% decrease in MDA content compared with PEG treatment alone (Fig. 1B). Compared with control, PEG treatment significantly increased the contents of $O_2^{\cdot-}$ and H_2O_2 by 166.7% and 173.1%, respectively, whereas PP application significantly reduced their contents by 26.8%

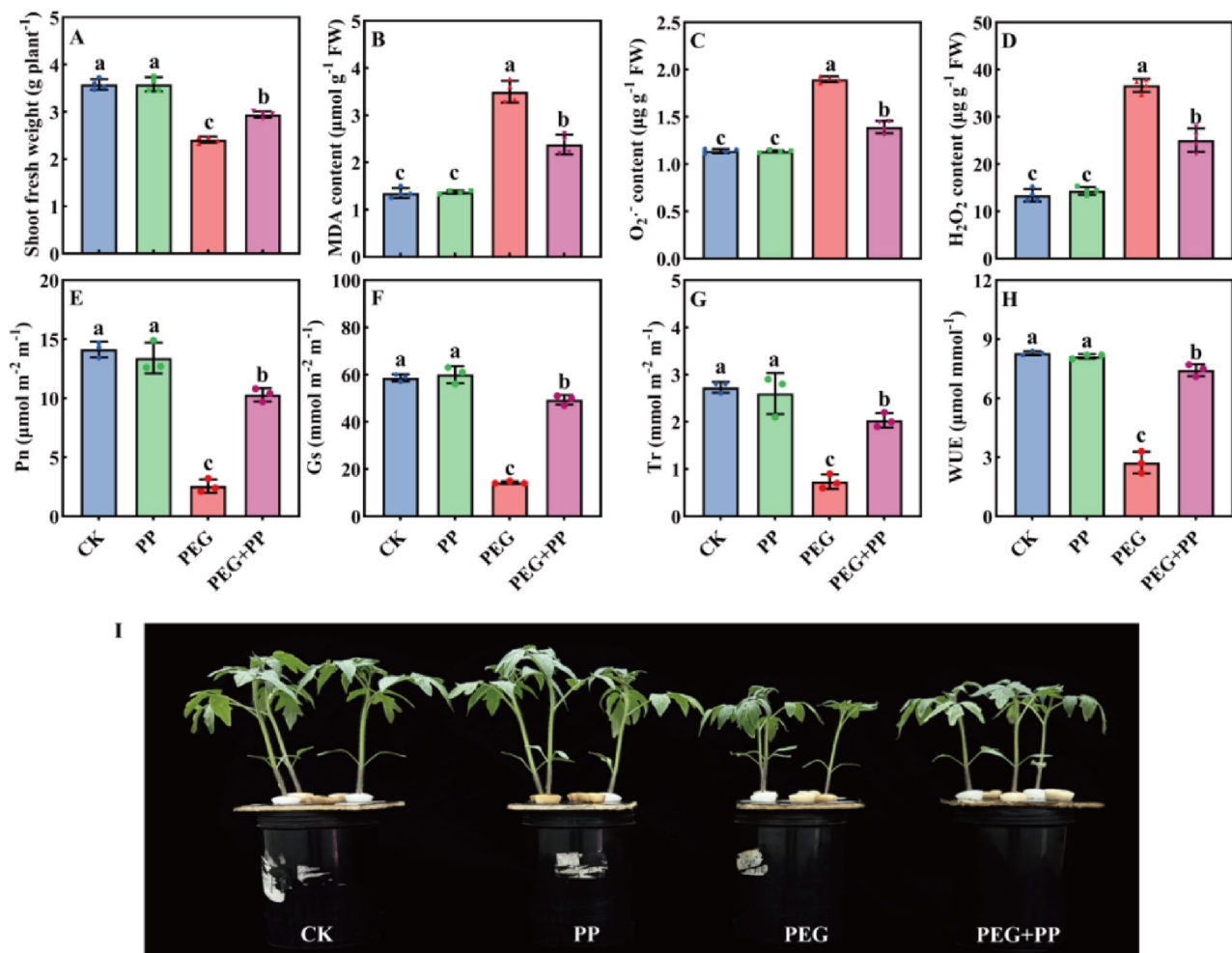


Fig. 1 Shoot fresh weight (A), MDA content (B), O₂⁻ content (C), H₂O₂ content (D), net photosynthetic rate (E), stomatal conductance (F), transpiration rate (G), water use efficiency (H), and (I) growth performance in tomato plants after 2 g L⁻¹ PP treatment under PEG-induced osmotic stress for 3 days. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$)

and 31.6%, respectively, compared with PEG treatment alone (Fig. 1C and D). Consistent with these changes in biomass and ROS, PEG-induced osmotic stress severely impaired the photosynthesis-related parameters (Fig. 1E–H). Compared with CK treatment, PEG treatment resulted in decreases of Pn, Gs, Tr and WUE by 81.8%, 75.6%, 73.3% and 67.1%, respectively. In contrast, the additional application of PP significantly alleviated these reductions, increasing Pn, Gs, Tr, and WUE by 300.8%, 244.2%, 178.1%, and 175.2%, respectively, under PEG treatment.

Ascorbate, glutathione, and redox status in tomato leaves

AsA and GSH contents were relatively constant over 3 days under the control treatment. In contrast, AsA and GSH levels in PEG-treated tomato plants significantly increased by 40.8% and 23.6%, respectively, on the first day, as their contents increased gradually with the enhancement of the duration of osmotic stress, and

the greatest relative increase occurred on the third day (Fig. 2A and B). Compared with PEG treatment alone, PP application further significantly increased AsA and GSH contents by 20.5% and 10.5%, respectively (Fig. 2C and F). Concurrently, PP application markedly decreased the levels of DHA and GSSG by 22.7% and 29.3%, respectively (Fig. 2D and G). As a result, the AsA/DHA and GSH/GSSG ratios were significantly elevated by 75.7% and 49.7%, respectively, after PP application under osmotic stress conditions (Fig. 2E and H), indicating that PP effectively enhances redox homeostasis by maintaining a more reduced intracellular environment during PEG-induced osmotic stress.

Enzyme activities and gene expression in the ascorbate-glutathione cycle

The effects of PP application on the activities of key enzymes related to the AsA-GSH cycle and their gene expressions in tomato seedlings were shown in Fig. 3.

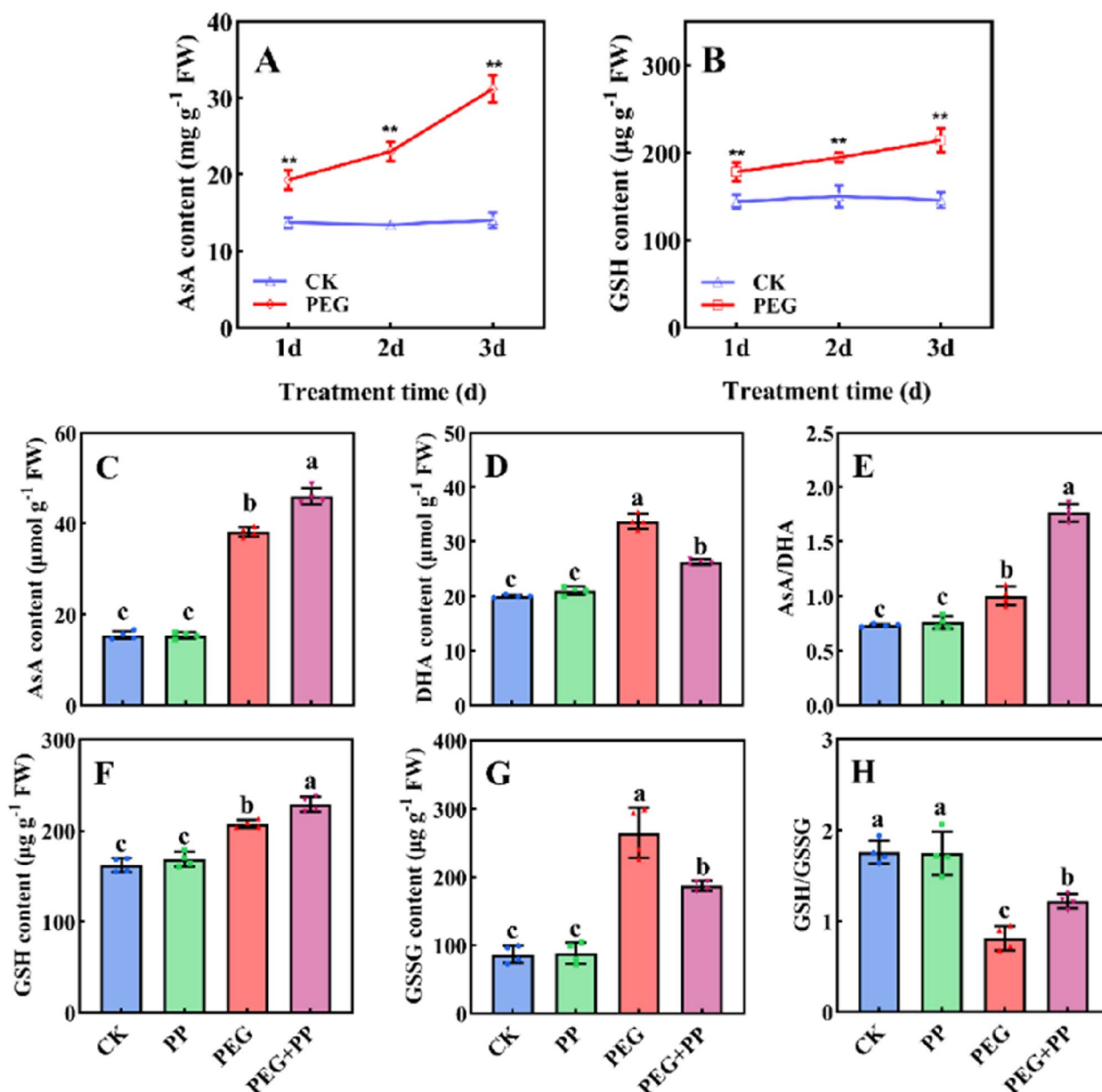


Fig. 2 Changes in AsA (A) and GSH (B) contents in PEG-treated tomato seedlings and the contents of AsA (C), DHA (D), AsA/DHA (E), GSH (F), GSSG (G), and GSH/GSSG (H) in tomato seedlings after 2 g L⁻¹ PP treatment under PEG-induced osmotic stress for 3 days. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$). ** indicates significant difference between CK and PEG treatments at $P < 0.01$

Compared with the control treatment, the activities of APX, MDHAR, DHAR, and GR were shown to increase significantly by 81.6%, 53.8%, 32.6%, and 53.2%, respectively, under PEG treatment. The PP application further enhanced the activities of APX, MDHAR, DHAR, and GR by 33.6%, 41.5%, 33.0%, and 34.2%, respectively, under osmotic stress. The similar trends in gene expression of APX, MDHAR, DHAR, and GR were also found, with a significant increase of 1.56, 1.42, 1.73, and 1.44-fold, respectively, after PP treatment compared with PEG treatment alone.

Enzyme activities and gene expression in glutathione biosynthesis

The activities and relative gene expression levels of key enzymes related to GSH metabolism were analyzed (Fig. 4). Compared with the control treatment, PEG-induced osmotic stress significantly increased the activities of γ -ECS, GPX, and GST by 61.3%, 54.5%, and 12.1%, respectively. Under osmotic stress, PP application further increased the activities of γ -ECS, GPX, and GST by 20.7%, 35.1%, and 31.0%, respectively, compared with PEG treatment alone. In addition, the PP application

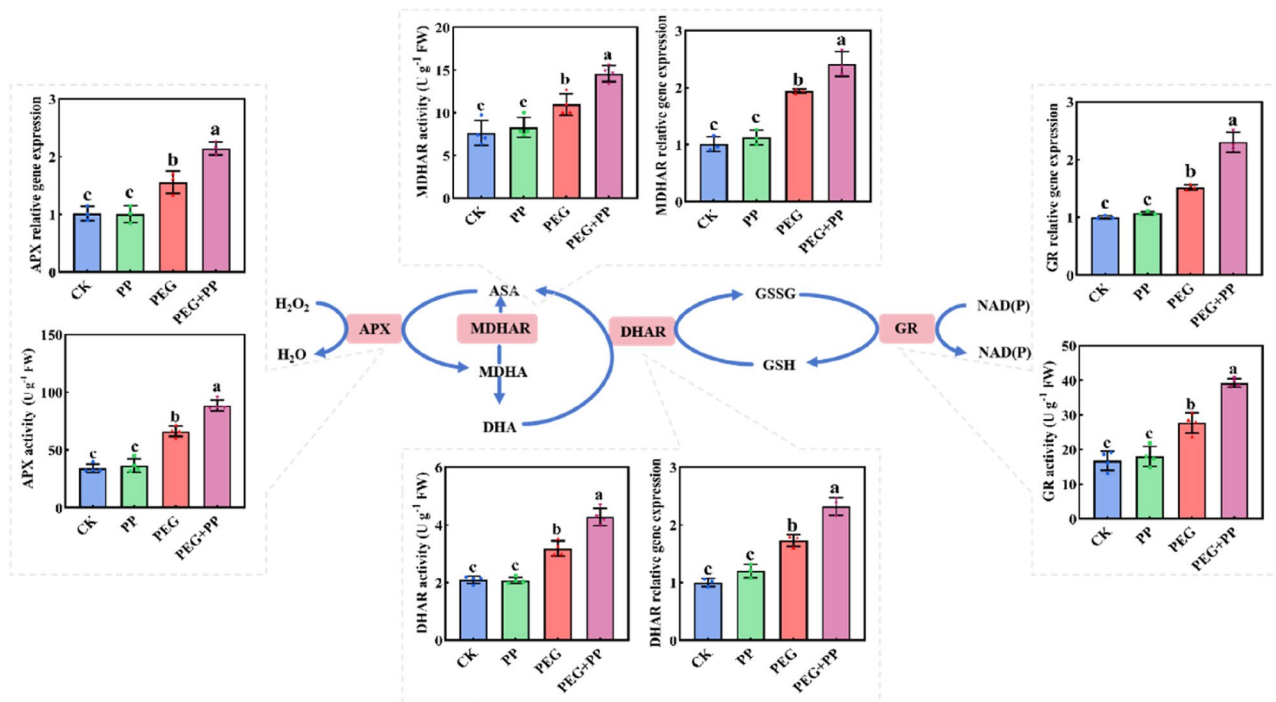


Fig. 3 The activities and gene expression levels of APX, MDHAR, DHAR, and GR in tomato plants after 2 g L⁻¹ PP treatment under PEG-induced osmotic stress for 3 days. Data are means $\pm$ SD. Different letters indicate significant differences ($P < 0.05$)

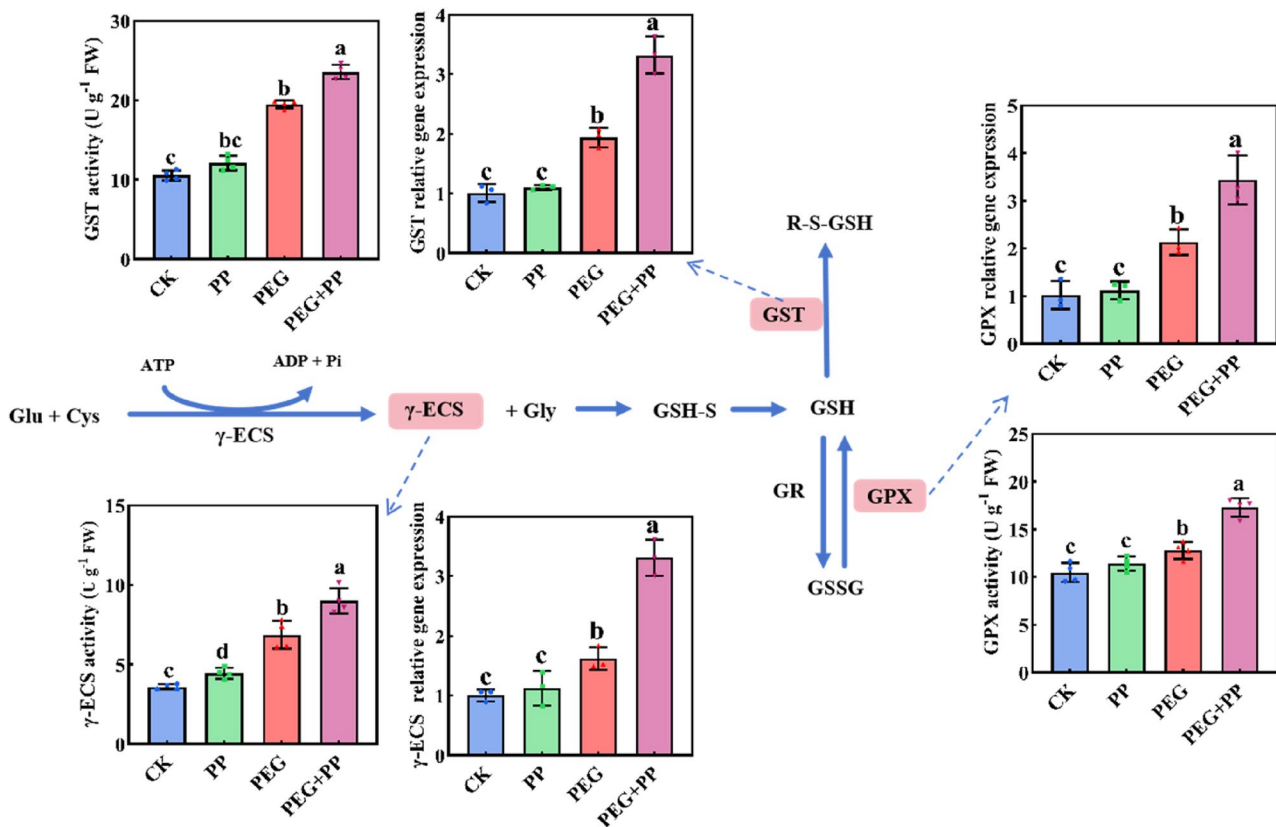


Fig. 4 Activities and gene expressions of GPX, GST, and γ -ECS related to glutathione metabolism in tomato plants after 2 g L⁻¹ PP treatment under PEG-induced osmotic stress for 3 days. Data are means $\pm$ SD. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA)

significantly increased the gene expressions of γ -ECS, GPX, and GST by 2.04, 1.71, and 1.61-fold, respectively, compared with PEG treatment alone.

Growth rate, ROS level, and oxidative damages after BSO treatment

PEG-induced osmotic stress significantly suppressed the relative growth rate of tomato plants, and the application of GSH synthesis inhibitor BSO further decreased the relative growth rate of tomato plants (Fig. 5A). In addition, compared with PEG+PP treatment, the BSO application markedly reduced the relative growth rate under osmotic stress conditions. Similarly, the PP application significantly reduced electrolyte leakage, Evans blue uptake, and MDA content by 18.6%, 27.1%, and 15.9%, respectively, whereas above parameters were largely increased after BSO application under osmotic stress (Fig. 5B–D). In terms of ROS, no significant differences in H_2O_2 and $O_2^{\cdot-}$ contents were found between

the application of PP and BSO under control conditions (Fig. 5E and F). However, compared with PEG treatment alone, PEG+BSO treatment significantly increased H_2O_2 and $O_2^{\cdot-}$ levels by 14.1% and 24.4%, respectively. Conversely, PEG+BSO+PP treatment significantly reduced the H_2O_2 and $O_2^{\cdot-}$ levels compared with PEG+BSO treatment.

Ascorbate and glutathione content after BSO treatment

Under control conditions, no significant differences in AsA and GSH contents were observed between PP and BSO treatments, whereas PEG-induced osmotic stress significantly increased AsA and GSH levels compared with the control. Although BSO treatment alone did not affect AsA and GSH contents under control conditions, it significantly reduced their contents under PEG-induced osmotic stress. Furthermore, PEG+BSO+PP treatment significantly increased the AsA and GSH contents, compared with PEG+BSO treatment (Fig. 6A). The

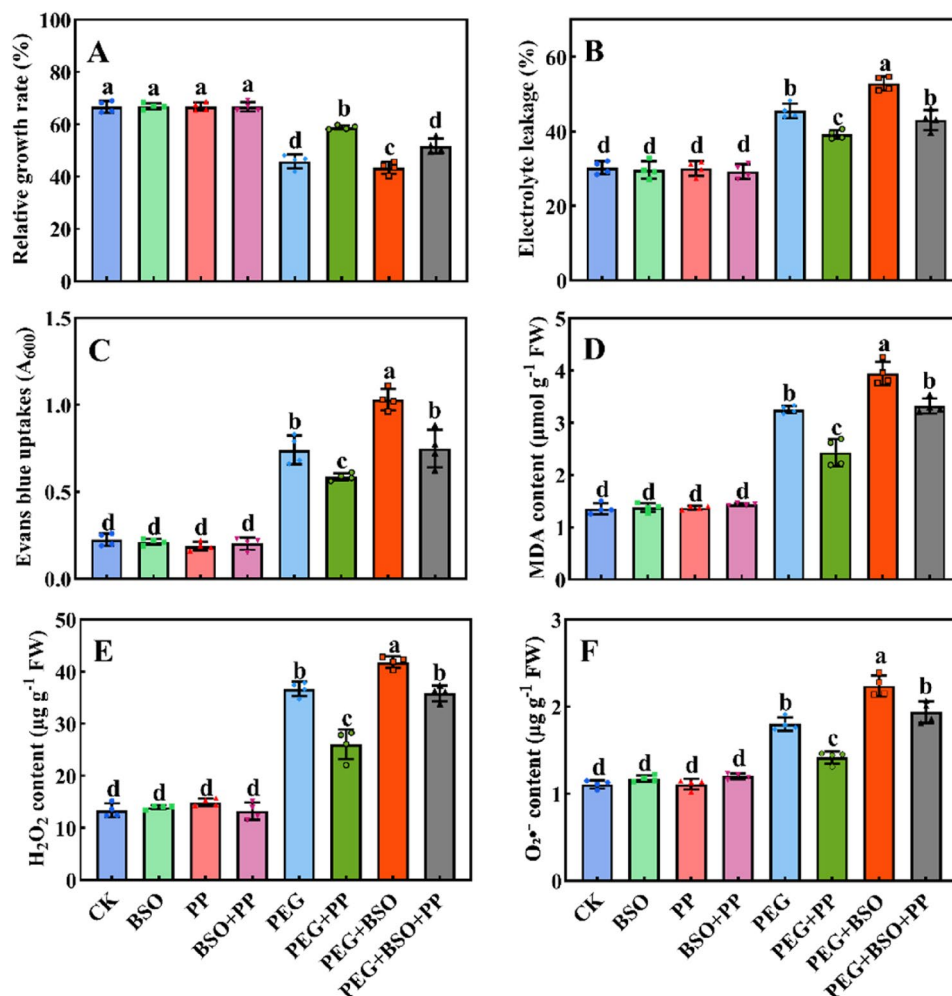


Fig. 5 Relative growth rate (A), Electrolyte leakage (B), Evans blue uptake (C), MDA content (D), H_2O_2 content (E), and $O_2^{\cdot-}$ content (F) in tomato plants after 2 g L⁻¹ PP and BSO treatment under PEG-induced osmotic stress for 3 days. Data are means $\pm$ SD. Different letters indicate significant differences ($P < 0.05$)

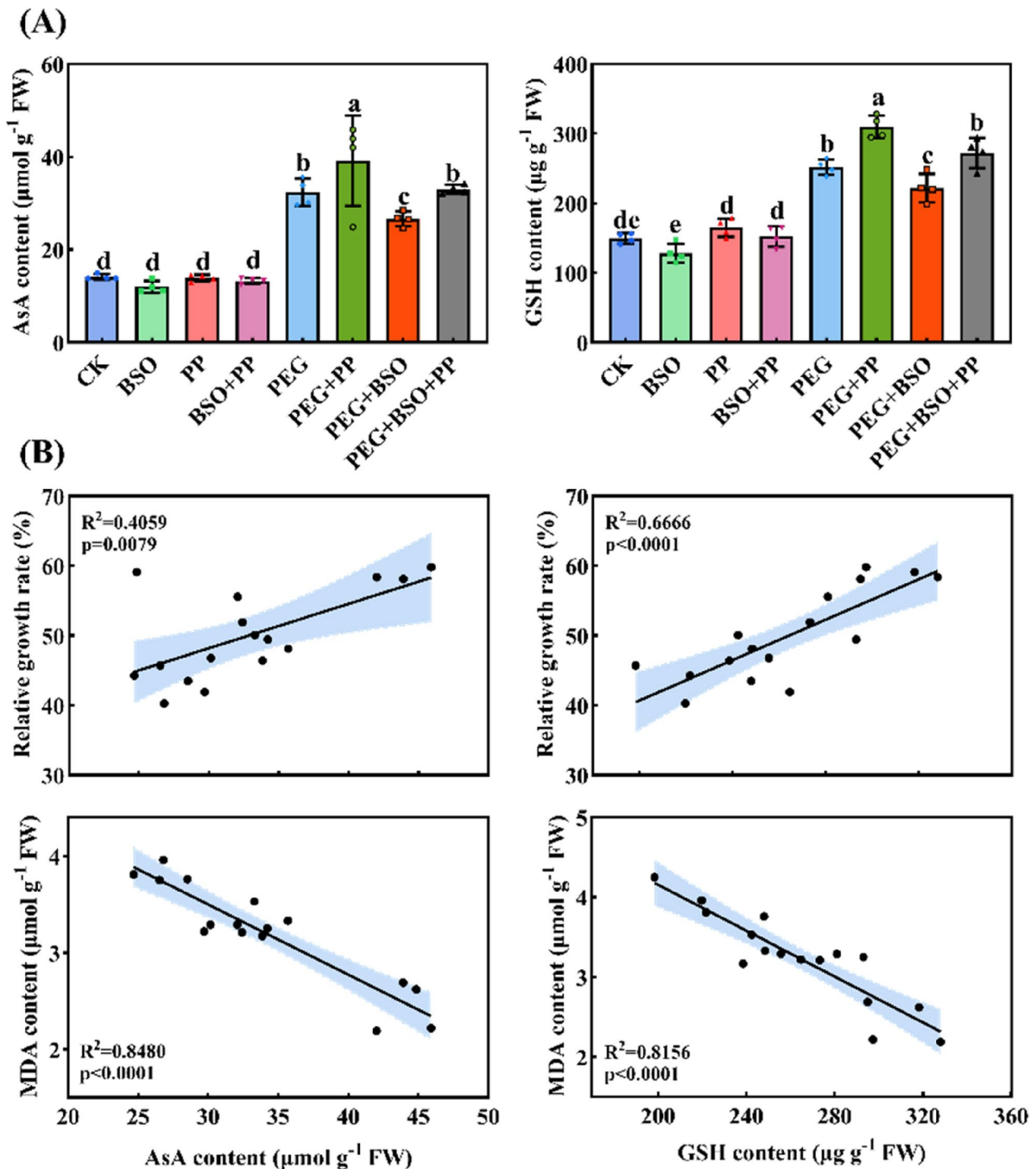


Fig. 6 AsA and GSH contents (A) and their relationship with relative growth rate and MDA (B) in tomato plants after 2 g L^{-1} PP and BSO treatment under PEG-induced osmotic stress for 3 days. Data are means $\pm$ SD. Different letters indicate significant differences ($P < 0.05$)

correlation analyses found that the AsA and GSH contents were positively correlated with relative growth rate ($R^2 = 0.41$ and 0.67) and negatively correlated with MDA content ($R^2 = 0.85$ and 0.82), respectively (Fig. 6B).

Proline content and related enzyme activities

As shown in Fig. 7A, compared with control treatment, PEG-induced osmotic stress obviously increased proline content by 98.1%, and PP treatment further significantly increased proline content by 40.69%, compared

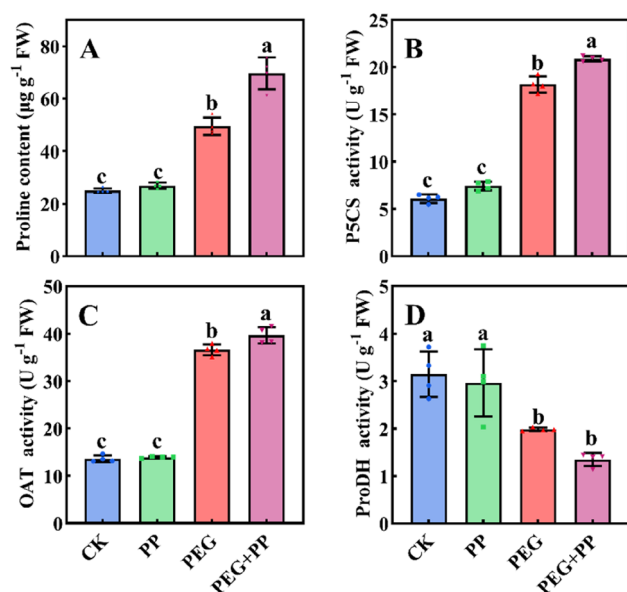


Fig. 7 Proline content (A), P5CS activity (B), OAT activity (C), and ProDH activity (D) in tomato plants after 2 g L^{-1} PP treatment under PEG-induced osmotic stress for 3 days. Data are means $\pm$ SD. Different letters indicate significant differences ($P < 0.05$)

with PEG treatment alone. The activities of the key proline biosynthetic enzymes P5CS and OAT also significantly increased by 14.91% and 8.16%, respectively, while proline degradation enzyme ProDH was significantly reduced by 20.71%, after PP treatment under osmotic stress (Fig. 7B–D). These findings suggest that PP enhances proline accumulation by promoting proline biosynthesis while suppressing its degradation under osmotic stress conditions.

Discussion

Osmotic stress triggers the excessive accumulation of ROS, such as $\text{O}_2^{\cdot -}$ and H_2O_2 , which induce severe oxidative damage to cellular structures [2, 37]. This oxidative burst promotes membrane lipid peroxidation and disrupts essential physiological and metabolic processes, ultimately resulting in significant growth inhibition and biomass reduction in various crop species, including *kiwifruit*, *Amaranthus tricolor*, maize, and tomato [15, 38–40]. Consistently, our study also indicated that PEG-induced osmotic stress initially impaired photosynthetic performance in tomato plants, which in turn increased the accumulation of ROS ($\text{O}_2^{\cdot -}$ and H_2O_2) and induced lipid peroxidation, ultimately resulting in a substantial reduction in shoot fresh weight (Fig. 1). Emerging evidence suggests that PHs can effectively alleviate oxidative stress and enhance osmotic stress tolerance in plants. For example, foliar application of a protein hydrolysate-based biostimulant has been shown to reduce H_2O_2 accumulation in pepper leaves while simultaneously enhancing plant growth and final fruit yield under severe water stress [41]. In line with these findings, our results

demonstrated that PP significantly reduced ROS accumulation and MDA content, increased photosynthetic parameters, and simultaneously enhanced biomass production under osmotic stress conditions (Fig. 1). These results clearly indicated that PP exerted protective effects against PEG-induced oxidative damage by suppressing ROS generation, attenuating membrane lipid peroxidation, and upregulating photosynthetic efficiency, thereby improving the osmotic stress tolerance of tomato plants.

Plants have evolved sophisticated non-enzymatic antioxidant systems to counteract ROS-induced oxidative stress, with AsA and GSH serving as central antioxidants in abiotic stress defense [4, 42]. Substantial evidence has demonstrated that high cellular concentrations of AsA and GSH, along with their redox ratios (AsA/DHA and GSH/GSSG), are critical indicators of plant antioxidant capacity under stress conditions [43]. In our study, osmotic stress induced a significant, time-dependent accumulation of both AsA and GSH in tomato plants (Fig. 2A and B). In addition, PEG + BSO treatment resulted in significantly higher ROS and MDA accumulation compared to PEG treatment alone (Fig. 5), suggesting that the GSH depletion severely compromises the antioxidant defense system and increases oxidative stress. Correlation analyses further revealed strong positive associations between AsA or GSH levels and relative growth rate, as well as negative correlations with lipid peroxidation (Fig. 6B), indicating the central role of AsA and GSH homeostasis in linking biomass production with ROS detoxification. Importantly, compared with PEG treatment alone, PP + PEG treatment significantly elevated both AsA and GSH levels and their AsA/DHA and GSH/GSSG ratios (Fig. 2C–H). These results indicated that PP enhances osmotic stress tolerance in tomato plants primarily through promoting AsA and GSH accumulation and maintaining cellular redox homeostasis, thereby providing effective protection against PEG-induced oxidative damage.

Under abiotic stress, maintaining high levels of AsA and GSH is closely related to the functioning of the AsA-GSH cycle in plants [7]. This cycle relies on the coordinated actions of core enzymes such as APX, MDHAR, DHAR, and GR within subcellular organelles to maintain cellular redox homeostasis. As APX detoxifies H_2O_2 , AsA is oxidized to MDHA; MDHA can be reduced back to AsA by MDHAR, while DHA is recycled to AsA via DHAR. Meanwhile, GR regenerates GSH from GSSG. In our study, the activities of APX, DHAR, MDHAR, and GR were obviously up-regulated under osmotic stress conditions (Fig. 3), consistent with the enhanced AsA and GSH levels in tomato plants (Figs. 2 and 6). This indicates an intrinsic adjustment of the AsA-GSH cycle to counteract PEG-induced oxidative damage. Similarly, Khaleghi et al. [5] reported that drought stress significantly up-regulated the APX, DHAR, and GR activities in *Maclura pomifera*, thereby reinforcing AsA-GSH cycle-mediated redox homeostasis. Importantly,

PP application under osmotic stress further enhanced the activities of APX, MDHAR, DHAR, and GR, which was accompanied by a marked reduction in ROS and MDA levels (Figs. 1 and 3). This suggests more efficient H_2O_2 detoxification via activation of the AsA-GSH cycle and alleviation of membrane lipid peroxidation. These findings are consistent with those of Hasanuzzaman et al. [44], who reported that supplementation with *Ascophyllum nodosum* extract significantly mitigated arsenic-induced oxidative stress in rice through enhancing AsA-GSH cycle enzyme activities. At the transcriptional level, exogenous PP significantly upregulated the expression of *APX*, *MDHAR*, *DHAR*, and *GR* genes (Fig. 3), demonstrating that PP enhances the antioxidant system through transcriptional regulation of key enzymes in the AsA-GSH cycle. The stimulatory effect may be attributed to the bioactive components of PP, including peptides and amino acids (Tables S1), which have been reported to modulate antioxidant systems in plants [15]. Therefore, the improved osmotic stress tolerance observed with PP application in tomato plants can be largely attributed to the upregulation of the AsA-GSH cycle at both enzymatic and transcriptional levels, leading to enhanced antioxidant capacity and maintained redox homeostasis.

GSH metabolism is essential for redox homeostasis and ROS detoxification, and plays a crucial role in enhancing plant tolerance to various stresses [45]. The rate-limiting step of GSH biosynthesis is catalyzed by γ -ECS, which condenses L-glutamate and L-cysteine to form γ -glutamylcysteine. Meanwhile, GPX and GST are key GSH-dependent enzymes involved in H_2O_2 detoxification. Kumar et al. [46] reported that overexpression of γ -ECS in transgenic tobacco plants increased the GSH content and conferred improved drought tolerance. Similarly, transgenic tobacco plants overexpressing *GST* and *GPX* exhibited enhanced growth performance, primarily by reducing lipid peroxidation under low-temperature and salt stress conditions [47]. In our study, osmotic stress increased the enzymatic activities of γ -ECS, GPX, and GST (Fig. 4), as well as ROS accumulation and oxidative damage in tomato plants (Fig. 1). Application of PP further significantly increased activities and transcript levels of γ -ECS, GPX, and GST, which correlated with reduced ROS and oxidative damage, indicating that PP reinforces GSH biosynthesis and the GSH-dependent detoxification pathways. However, treatment with BSO, an inhibitor of γ -ECS, significantly reduced both AsA and GSH contents and exacerbated PEG-induced oxidative stress, while subsequent addition of PP (PEG + BSO+PP) obviously elevated these antioxidant levels and mitigated the oxidative stress (Figs. 5 and 6). In addition, GSH and proline metabolism share the common precursor glutamate, and their metabolic pathways are interrelated [48]. GSH application has been reported to significantly enhance both the AsA-GSH cycle and proline biosynthesis through upregulation of *OsP5CS1* expression, effectively

mitigating aluminum-induced ROS accumulation and oxidative stress [49]. In our study, PP treatment also promoted proline accumulation in osmotic stress-treated tomato leaves by upregulating key biosynthetic enzymes of P5CS and OAT while downregulating ProDH activity (Fig. 7), thereby contributing to osmotic adjustment under PEG-induced stress. The high levels of amino acids in PP (Table S2) likely account for the concurrent increase in GSH and proline levels, thereby establishing a synergistic mechanism that couples redox with osmotic adjustment to enhance osmotic stress tolerance.

Compared to conventional plant growth regulators or synthetic protectants, the PP used in the present study offers a distinct advantage due to its origin as an abundant by-product of the pig industry. This ensures a large-scale, low-cost, and renewable source of raw material, aligning with circular economy principles by valorizing waste. Although numerous studies have reported stress tolerance induced by various exogenous agents, such as seaweed extracts, proline, melatonin [38, 44, 48], PP provides a unique amino acid profile and signaling properties that may complement or enhance existing biostimulant strategies. Importantly, the amino acids and peptides-based nature of PP suggest a potentially lower environmental persistence and higher biocompatibility than some synthetic alternatives. Its feasibility for field application is further supported by the development of stable, easy-to-handle powder formulations. Therefore, PP represents not only a scientifically validated biostimulant for osmotic stress mitigation but also a scalable and economically viable strategy, underscoring its potential to enhance crop resilience in practical agricultural systems. However, PP is a complex mixture of small peptides and free amino acids, and the specific components responsible for regulating the AsA-GSH cycle and conferring osmotic stress tolerance remain unclear. Further studies are therefore needed to identify its key active fractions and elucidate the underlying mechanisms.

Conclusion

Osmotic stress severely limits agricultural productivity worldwide, adversely affecting both food security and environmental sustainability. In this study, we indicated that exogenous PP application significantly enhances osmotic stress tolerance in tomato plants by mitigating oxidative damage (Fig. 8). The protective mechanism is primarily attributed to PP-induced upregulation of key enzymatic genes in the AsA-GSH cycle, leading to elevated enzyme activities and increased AsA and GSH accumulation. Furthermore, PP treatment substantially enhances the expression and activity of γ -ECS, GPX, and GST, facilitating GSH biosynthesis. This enhancement in the AsA-GSH cycle enhanced the plant's ability to scavenge ROS and maintain redox homeostasis under PEG-induced osmotic stress. BSO application further confirmed that GSH biosynthesis plays a

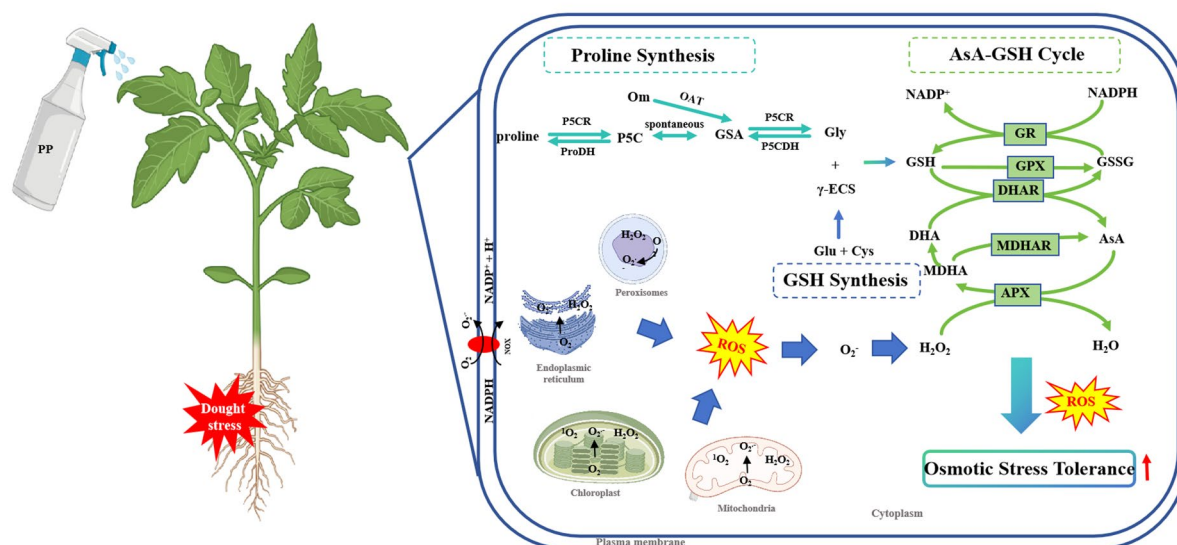


Fig. 8 A proposed model to explain the mechanism of PP treatments in alleviating PEG-induced osmotic stress in tomato by regulating AsA-GSH cycle

critical role in PP-mediated osmotic stress tolerance. These findings suggest that PP may serve as a promising biostimulant-based strategy for improving crop tolerance to osmotic stress and enhancing agricultural sustainability under increasing abiotic stress conditions.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-026-09291-2>.

Supplementary Material 1: Table. S1 The levels of amino acid polypeptide of protein hydrolysate derived pig blood. Table. S2 Amino acid profile and content of protein hydrolysate derived pig blood. Table. S3 Sequences of primers used for the quantitative real-time PCR analysis.

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Not applicable.

Authors' contributions

YHJ, YFF, and WWZ conceived the idea, designed the research and performed the experiments; YHJ wrote the main manuscript text; YHJ, YFF, XYX, WXW, and YJ determined experimental index and analyzed data. LYM, HFL, BL, and WWZ provided technical support and proofread the manuscript. WWZ obtained the funding. All authors have read and approved the final manuscript.

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Data availability

All data is presented in this manuscript. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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