Resveratrol Alleviates the KCl Salinity Stress of Malus hupenensis Rhed. Seedlings by Regulating K+/Na+ Homeostasis, Osmotic Adjustment, and Reactive Oxygen Species Scavenging

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Research Article

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Resveratrol alleviates the KCl salinity stress of *Malus hupenensis* Rhed. seedlings by regulating K^+/Na^+ homeostasis, osmotic adjustment, and Reactive Oxygen Species scavenging

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Abstract

Background: Applying large amounts of potash fertilizer in apple orchards for high apple quality and yield aggravates KCl stress. As a phytoalexin, resveratrol (Res) participates in plant resistance to biotic stress. However, its role in relation to KCl stress have never been reported.

Results: *Malus hupenensis* is a widely used apple rootstock in China and is sensitive to KCl stress. In this study, 100 μmol Res was exogenously applied to alleviate KCl stress in *M. hupenensis* seedlings. The seedlings treated with Res had higher chlorophyll content and photosynthetic index than those without Res treatment. Moreover, the molecular and physiological mechanisms of Res in ion toxicity, osmotic stress, and oxidative damage induced by KCl stress were also investigated. First, exogenous Res affects K^+/Na^+ homeostasis in cytoplasm by enhancing K^+ efflux outside the cells, inhibiting Na^+ efflux and K^+ absorption, and compartmentalizing K^+ into vacuoles. Second, this compound could respond to osmotic stress by regulating the accumulation of proline. Lastly, this polyphenol functions as an antioxidant that strengthens the activities of POD and CAT thus eliminates the reactive oxygen species production induced by KCl stress.

Conclusions: Taken together, these results reveal that resveratrol alleviates the KCl salinity stress of *M. hupenensis* seedlings by regulating K^+/Na^+ homeostasis, osmotic adjustment, and reactive oxygen species scavenging.

Keywords: resveratrol, KCl stress, *Malus hupenensis*, ion homeostasis, oxidative stress

Background

Soil salinity is one of the most harmful adverse factors restricting sustainable agricultural development [1]. Current research shows that approximately 19.5 % of irrigated land is affected by salt stress [2], and this percentage is still increasing [3]. NaCl and KCl are soluble salts that seriously damage the growth of plants. Among the different plant responses to NaCl stress, the core process is to reject sodium (Na) and absorb potassium (K) to maintain Na^+/K^+ balance in the cytoplasm [4]. As an essential nutrient element in plants, K maintains the activities of various metabolic enzymes in cells [5]. However, recent research found that high K concentration could also induce salt stress and inhibit the growth of plants [6]. Apple (*Malus domestica* Borkh.) is one of the most productive and economically valuable horticultural crops worldwide. Proper soil conditions and nutrient balance are the key factors to guarantee yield and quality [7]. However, applying large amounts of potash fertilizer for high apple yield and quality has resulted in serious KCl stress. This phenomenon damages the soil structure and limits the sustainable development of apple orchards [8]. Most studies on salt stress mainly focused on the damage of NaCl stress; however, the molecular mechanism underlying the apple's response to KCl stress remains unclear.

Salt stress includes osmotic stress and ion toxicity, the two primary reactions inducing the accumulation of reactive oxygen species (ROS) and indirectly leading to oxidative damage in plants. The presence of various stresses inhibits plant growth and energy metabolism, which results in premature aging and even death [9,10]. When plants experience KCl stress, excessive K^+ is absorbed into the cytoplasm through electrochemical potential gradient due to the large amount of K^+ in the external environment; this phenomenon breaks the original balance of cytoplasm Na^+/K^+ and generates ion toxicity [11]. For K^+ balance in the cytoplasm, ion transporters regulate the ion balance [12]. The Stellar K^+-Outward Rectifier (SKOR) is located in plasma membrane to transport K^+ from the cytoplasm to outside the cell [13]. For K^+ absorption, inward channels KAT1 and KAT2 mediate the K^+ uptake into the cell. KAT1 and HAK5 are high-affinity transporters that regulate sufficient K^+ uptake for

plant growth [14]. Except for K^+ absorption and efflux, voltage-dependent K^+ channel (TPKs) and vacuolar K^+/H^+ antiporters such as NHX1 and NHX2 are present in the tonoplast to facilitate K^+ influx and efflux in the vacuoles [15]. Furthermore, the Na^+, Ca^{2+}, and Fe^{2+} transporters are involved in the balance of K^+/Na^+, Ca^{2+}, and Fe^{2+} in the cytoplasm [16,17,18]. Excessive K^+ concentration in the soil environment reduces the water potential of the soil, impedes the water uptake of the plants, and leads to osmotic stress [19]. Osmotic stress reduces the stomatal openings through the guard cells of plant leaves, decreases plant photosynthesis, and affects plant growth and development [20,21]. Plants regulate the osmotic potential by increasing the concentrations of osmolytes such as proline, glycine betaine, soluble sugar, and soluble protein [19]. Osmotic stress and ion toxicity destroy the selective permeability of the membrane, leading to large electrolyte extravasation and excessive ROS accumulation [22]. Excessive ROS concentrations cause lipid peroxidation, protein oxidation, nucleic acid damage, and enzyme inactivation and result in programmed cell death [23,24]. To cope with this oxidative damage, plants develop enzyme and non-enzyme systems to eliminate ROS [25]. Plant antioxidant enzymes mainly include superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), and the non-enzyme system mainly involves ascorbic acid, alkaloids, carotenoids, and flavonoids [25,26]. Plants can improve the activities of their antioxidant enzymes, accumulate non-enzyme scavengers to alleviate the oxidative damage [27], and induce the expression of ROS-related genes to eliminate ROS. Rice transcription factor OsMADS25 could ameliorate salt tolerance by directly binding to the promoter of glutathione transferase gene *OsGST4* to improve its expression [28].

Applying exogenous substances effectively alleviates salt stress [29]. These substances are commonly divided into the following three categories: The first category is the plant growth regulators, such as jasmonate (JA), cytokinin (CTK), and abscisic acid (ABA) [4,30,31]. JA has a positive regulatory role in plant resistance to salt stress, and its exogenous application can improve the salt tolerance of *Arabidopsis*, tomatoes, and rice [32,33,34]. The second category is osmotic adjustment substances, such as proline, glycine betaine and sugars [35,36]. Exogenous

glycine betaine application can alleviate NaCl stress by regulating osmotic stress [36]. The third category contains substances that increase the antioxidant capacity of plants, such as NO, silicon, and melatonin [37,38,39]. Exogenous melatonin can eliminate ROS and enhance the NaCl stress resistance in horticultural crops such as grapes, apple, and cucumber [40,41]. However, most studies focused on NaCl stress; reports on substances that are effective against KCl stress are rare. As a member of the stilbene family of phenolic compounds, resveratrol (Res) has been identified in grapevines, red wine, sorghum bicolor, berries, and peanuts [42]. This antimicrobial phytoalexin contributes to plant resistance to biotic stress [43]. Grapevines could metabolize additional Res to protect themselves from *Botrytis cinerea* and *Plasmopara viticola* [44,45]. Gonzales et al. [46] reported that exogenously applying trans-resveratrol could improve postharvest resistance in fruits. Res can also help improve the resistance to *Venturia inaequalis* in apples [47]. In addition to acting as a phytoalexin, Res is involved in plant resistance to abiotic stress and plant response to ozone, wounding, or UV light [48]. In citrus seedlings, the external application of Res and α-Toc mediates salt adaptation [42]. However, the effect of Res on KCl stress and its molecular mechanism are still unclear, especially in woody plants such as apples.

In this study, the effects of different concentrations of exogenous Res on *Malus hupehensis* seedlings under KCl stress were investigated. The potential physiological and molecular mechanisms of Res on KCl stress through ion homeostasis, osmotic stress, and oxidative damage were also explored. In addition, the expression of K^+ and Na^+ transporter genes and key KCl-responsive genes under Res and KCl treatments was determined. The findings can enhance the application and examination of the physiological role of Res in apples under KCl stress.

Methods

Plant materials and growth conditions

Seeds of *M. hupehensis* after low temperature vernalization were planted in nutrient soil and sand with the ratio of 1:1. When the seedlings developed to four leaves, they were transplanted into a plastic pot, watered with Hoagland's nutrient

150 solution every 3 days, and cultivated under a greenhouse environment with the
151 temperature controlled at 25 ± 2 °C, humidity of 62 ± 2%, light intensity of 100
152 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and photoperiod of 16/8 hours light/dark. After 10 days, the seedlings
153 with similar growth status were selected for subsequent KCl and exogenous Res
154 treatment.
155
156 **KCl stress and exogenous Res treatment**
157 A total of 180 *M. hupehensis* seedlings were randomly divided into five groups.
158 Group I was treated with Hoagland's nutrient solution as control, and groups II-V
159 were treated with 50 mM KCl stress. In addition, groups III-V were sprayed with
160 exogenous Res with the concentrations of 10, 100, and 200 μM, respectively; Res
161 (Solarbio, Beijing, China) was dissolved in ethanol at a concentration of 10 mM and
162 stored at -20 °C and sprayed every 2 days. After 15 days' treatment, the wilting rate,
163 plant height, fresh weight, and dry weight of the apple seedlings were measured. Each
164 experiment was independently repeated three times.
165
166 **Determination of chlorophyll content and photosynthetic parameters**
167 Twenty apple seedlings from each group were randomly selected to determine the
168 chlorophyll content and basic photosynthetic parameters after KCl stress and
169 exogenous Res treatment for 15 days. Chlorophyll content was measured by
170 SPAD-502 Plus (Konica Minolta, Tokyo, Japan). Photosynthesis rate, transpiration
171 rate, and stomatal conductance were measured by CIRAS-3 portable photosynthetic
172 apparatus (PP Systems, Amesbury, USA). Light intensity was set at 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$,
173 humidity was 50%, and temperature was controlled at 23 °C.
174
175 **Determination of ROS level and MDA content**
176 Twenty apple seedlings were randomly selected from each group to detect the
177 ROS levels including $O_2^{\cdot -}$ and H_2O_2. $O_2^{\cdot -}$ and H_2O_2 staining was conducted as
178 described by Zheng et al. [40].
179 For MDA detection, 0.5 g of leaves from each group were ground in a pre-cooled

mortar with 5 ml of extract buffer and then centrifuged at 12000 rpm for 10 minutes. MDA content was determined using a plant MDA extraction kit (Grace, Suzhou, China). Each experiment was independently repeated three times.

Determination of antioxidant enzyme activity

In brief, 0.5 g of fresh leaves were ground in 5 ml of extract buffer to determine the activity of antioxidant enzymes including SOD, POD and CAT in apple leaves. After centrifugation at 12000 rpm for 10 minutes, the activities were determined using SOD, POD, and CAT kits (Grace, Suzhou, China). Each experiment was independently repeated three times.

Determination of osmolyte content

After KCl stress and exogenous Res treatment for 15 days, 0.5 g of leaves from apple seedlings were used for the detection of osmolytes including proline, soluble sugar, and soluble protein. Osmolyte detection was conducted as described by Su et al. [29]. Each experiment was independently repeated three times.

Quantification of mineral elements

Apple seedlings were collected after 15 days of KCl stress and Res treatment and then cleaned with deionized water. The plants were dehydrated at 105 °C for 30 minutes and then baked at 80 °C for 72 h. Afterward, 0.5 g of dried seedlings were ground into powder and added with 12 ml of HNO_3 and $HClO_4$ (ratio of 5:1). After digestion, the solution was diluted with deionized water to 25 mL for the detection of mineral elements. The contents of macronutrients (K, Ca, Na, Mg, and P) and micronutrients (Fe, Mn, Zn, and Cu) were determined by inductively coupled plasma-optical emission spectrometry (PerkinElmer, Waltham, USA). Each experiment was independently repeated three times.

RNA extraction and quantitative real-time PCR (qPCR) analysis

After 15 days of KCl and Res treatments, the total RNA of different groups was

extracted using the RNAprep pure Plant Plus Kit (Tiangen, Beijing, China). Inverse transcription and qPCR assay was conducted as described by Zheng et al. [49]. KCl-responsive genes in apple were screened from RNA-seq results (NCBI number is PRJNA588566), and apple actin (accession number: MDP0000774288) was used as the internal reference. The primers used for qPCR were designed by Primer 5 software and are shown in Table S1. Each experiment was independently repeated three times.

Results

Effects of exogenous Res on the growth of apple seedlings under KCl stress

As shown in Figure S1, the seedlings were wilted and seriously damaged by 50 mM KCl stress. When different Res concentrations were applied to the KCl-stressed apple seedlings, the growth condition improved (Fig. S1a). However, different degrees of protection were observed when spraying varying Res concentrations under KCl stress. When low (10 μM) and high concentrations (200 μM) were used, the wilting rates of the apple seedlings were significantly decreased from 68.9% to 38.9% and 41.1%, respectively (Fig. S1b), and the fresh weights were significantly increased 104% and 39.5%, respectively (Fig. S1c). However, the apple seedlings still exhibited flaccid growth condition compared with those in group I. When 100 μM exogenous Res applied, the wilting phenotype returned to normal growth condition even under KCl stress (Fig. S1a). The wilting rate was significantly decreased to as low as 15.0% compared with that of group II (Fig. 1b). In addition, the plant height, fresh weight, and dry weight were all remarkable increased in the seedling sprayed with 100 μM Res compared with that without exogenous Res under KCl stress for 15 days (Fig. 1). The plant height decreased from 6.7 cm to 3.2 cm under KCl stress but recovered to 5.2 cm after 100 μM Res application (Fig. 1c). The fresh and dry weights also increased 148% and 107%, respectively, compared with those of group II under KCl stress for 15 days (Figs. 1d, e). These results indicated that exogenous Res could protect the apple seedlings from KCl stress. The treatment of 100 μM exogenous Res exhibited the best phenotype and therefore selected for further research.

240

241 **Effects of exogenous Res on chlorophyll content and photosynthetic parameters**
242 **under KCl stress**

243 In consideration of the wilting phenotype on the leaves of apple seedlings under
244 KCl stress, the chlorophyll content and photosynthetic parameters were measured.
245 The chlorophyll content was significantly reduced in the plants after 15 days of KCl
246 stress (2.75 SPAD) and was only one-fifth that of the control group (15.85 SPAD).
247 When exogenous Res was sprayed, the chlorophyll content of apple seedlings under
248 KCl stress significantly recovered to as high as 14.4 SPAD with no significant
249 difference from that of the control group (Fig. 2a). A similar variation tendency was
250 observed for the photosynthetic parameters including photosynthesis rate,
251 transpiration rate, and stomatic conductance under KCl stress and exogenous Res
252 treatment. All values were significantly inhibited under KCl stress but increased by
253 exogenous Res application (Figs. 2b, c, and d), especially the photosynthesis rate.
254 Under KCl stress, the photosynthesis rate decreased significantly from 19
255 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to 2.85 $\mu mol \cdot m^{-2} \cdot s^{-1}$ but recovered to 14.35 $\mu mol \cdot m^{-2} \cdot s^{-1}$ when
256 exogenous Res was applied (Fig. 2b). These results indicated that exogenous Res
257 could protect the chlorophyll level and photosynthetic system against KCl stress.

258

259 **Effects of exogenous Res on the oxidative damage and antioxidant enzyme**
260 **activity of apple seedlings under KCl stress**

261 $O_2^{\cdot -}$ and H_2O_2 staining results revealed the leaves of apple seedlings were
262 seriously damaged by KCl stress for 15 days. When exogenous Res was applied, the
263 $O_2^{\cdot -}$ and H_2O_2 levels were significantly decreased (Fig. 3a). The variation tendency of
264 MDA content was similar to that of $O_2^{\cdot -}$ and H_2O_2 under KCl and Res treatment. The
265 MDA content under KCl stress (2.17 nmol/g) was more than twice than that of the
266 control group (0.83 nmol/g) but was decreased as low as 1.28 nmol/g after exogenous
267 Res was applied (Fig. 3b).

268 SOD, POD, and CAT activities were also detected. As shown in Figure 3c, SOD
269 activity was not significantly changed under KCl stress and exogenous Res treatment.

Different from that of SOD, the POD and CAT activities under KCl stress were significantly decreased from 13.2 and 2813 Unit/g to 5.09 and 905 Unit/g, respectively. However, when exogenous Res was applied, the POD activity recovered to 16.3 Unit/g, which was as high as that under normal condition (Fig. 3d), and the CAT activity significantly increased to 2177 Unit/g (Fig. 3e).

Effects of exogenous Res on the electrolyte leakage and osmolytes of apple seedlings under KCl stress

Electrolyte leakage was detected after KCl stress and exogenous Res treatment for 15 days. After KCl stress, the electrolyte leakage increased significantly from 16.07% to 59.6% but decreased to as low as 28.9% when exogenous Res was applied (Fig. 4a).

Osmolyte content under KCl stress and exogenous Res treatment was also detected. As shown in Figure 4, the contents of proline, soluble sugar, and soluble protein were all significantly increased by KCl stress. However, when exogenous Res was applied, the content of proline was decreased from 25.2 μg/g to 17.3 μg/g, and those of soluble sugar and soluble protein content had no significant changes.

Effects of exogenous Res on the mineral elements of apple seedlings under KCl stress

The mineral elements of apple seedlings were measured after KCl stress and exogenous Res treatment for 15 days. For the macronutrients (Fig. 5a), K level was significantly increased from 9.06 mg/g to 27.25 mg/g under KCl stress but decreased to 16.34 mg/g when exogenous Res was applied. The variation tendency of Ca was similar to that of K. Different from those of K and Ca, the contents of Na had no significant changes under KCl stress. However, when exogenous Res was applied, Na increased by 56.9%. For the micronutrients (Fig. 5b), Mn were reduced by KCl stress but increased from 21.27 to 28.96 mg/kg, when exogenous Res was applied. The content of Fe had no significant changes under KCl stress but increased by 100.5% when exogenous Res was applied. As an important indicator of plant tolerance to salt

stress, K:Na ratio was detected before and after KCl stress and exogenous Res treatment for 15 days. The K:Na ratio of the apple seedlings from the three groups had no significant difference before the treatment. However, after KCl stress and exogenous Res treatment for 15 days, the K:Na ratio was significantly increased 489% under KCl stress but decreased from 5.3 to 2.4 after exogenous Res treatment (Fig. 5c).

Effects of exogenous Res on the expression levels of KCl-related genes in apple seedlings under KCl stress

As shown in Figure 6, the expression levels of 18 candidate genes, which were screened out from RNA-Seq data under KCl stress, were detected under KCl and exogenous Res treatment. These genes were categorized into five groups. First, the six K^+ transporter genes including *MhSKOR*, *MhHAK1*, *MhKAT1*, *MhTPK1*, *MhNHX1*, and *MhNHX2* had significantly increased expression under KCl stress. However, those of *MhHAK1*, *MhKAT1*, *MhTPK1*, *MhNHX1*, and *MhNHX2* were down-regulated, whereas that of *MhSKOR* was further up-regulated by exogenous Res treatment (Fig. 6a). Second, three Na^+ transporter genes including *MhCAX5*, *MhCHX15*, and *MhSOS1* showed similar decreasing tendency under KCl stress and exogenous Res treatment (Fig. 6b). Third, the expression of antioxidant enzyme genes *MhGPX6*, *MhPER65*, and *MhpoxN1* was significantly induced by KCl stress, and only that of *MhGPX6* was affected by exogenous Res treatment (Fig. 6c). Finally, the expression of three selected transcription factors, namely, *MhERF017*, *MhMYB39*, and *MhWRKY28* and three kinases, namely, *MhMAPK3*, *MhANP2*, and *MhGK* was also changed under KCl and exogenous Res treatment. This finding indicated their potential important functions under plant response to KCl stress and Res signaling transduction pathway.

Discussion

K is an essential nutrient for plant growth and physiology [50] and has a regulatory function in several biochemical and physiological processes, such as

enzyme activation, carbohydrate metabolism, and photosynthesis [5]. However, K^+ at concentrations higher than 50 mM can induce salt stress and disrupt normal plant growth and metabolism [6]. *M. hupehensis* is one of the most popular rootstocks for apple production and cultivation [51], but unfortunately suffers from serious KCl stress due to the huge amount of potassium fertilizer applied to orchards. Introducing exogenous substances such as plant growth regulator, osmotic adjustment substances, and antioxidants effectively alleviates salt stress [52,53,54]. As an antimicrobial phytoalexin, Res alleviates NaCl stress in citrus seedlings [42]. However, the roles and molecular mechanism underlying Res activity on KCl stress have never been reported. In this study, the role of different Res concentrations was examined in *M. hupehensis* seedlings under KCl stress. The treatment of 100 μmol Res for KCl stress produced the lowest wilting rate and the highest fresh weight and therefore had better effect than 10 μmol (low concentration) and 200 μmol (high concentration) (Figs. 1, S1). Exogenous plant regulators usually affect plant growth and development in a dose-dependent manner. In *Malus baccata* seedlings, 600 μmol for irrigation or 200 μmol for spraying are selected as the best concentrations of melatonin to maximize its role under waterlogging stress [40]. The present result indicated that 100 μmol Res would be an appropriate concentration to alleviate KCl stress in *M. hupehensis* seedlings.

Under KCl stress, the direct injury to plants is called ion toxicity [55]. Large amounts of K^+ flow into the cytoplasm and cause an imbalance between the cations. When the apple seedlings were under KCl stress, the K content was sharply induced, and that of Ca was also substantially increased (Fig. 5). Ca is an important secondary messenger, and maintaining its concentration in cytoplasm help regulates plant signal transduction pathways under salt stress [56]. Increasing the Ca content must be the stress response of the apple seedlings to balance K^+/Ca^{2+} in the cytoplasm. When exogenous Res was applied, the K content was significantly decreased, whereas those of Na, Fe, and Mn increased (Fig. 5). These results indicated that Res could affect the ion transport under KCl stress. Fe and Mn play a key role in plant resistance to oxidative stress [17,57], and the increase in their contents after Res treatment appears

to be the response to oxidative damage caused by KCl stress. In plant responses to salt stress, the core process is the balance of K^+/Na^+ in the cytoplasm [58,59]. K^+/Na^+ ratio was significantly induced under KCl stress but decreased to the control when exogenous Res was applied (Fig. 5). The expression of K^+ transporter genes responding to KCl stress was analyzed from previous RNA-seq data (NCBI number: PRJNA588566) to explore the changes of K^+ content under KCl and Res treatment. First, SKOR family, which is located in the plasma membrane, is mainly responsible for K^+ efflux from the cytoplasm to outside of the cell [13]. The results indicated that *MhSKOR* expression was sharply induced by KCl stress and was even enhanced by exogenous Res application (Fig. 6). For K^+ absorption, the expression levels of *MhHAK1* and *MhKAT1* were induced by KCl stress due to the high K^+ concentration outside the cell. However, these values were inhibited by exogenous Res application under KCl stress (Fig. 6). These results indicated that exogenous Res could enhance K^+ efflux and inhibit K^+ influx under KCl stress. Second, for the compartmentalization of K^+ in cells, the two-pore channel TPK1 gene encodes the vacuolar K^+ conductance and plays a role in K^+ homeostasis [60]. Vacuolar K^+/H^+ antiporters NHX1 and NHX2 are present in the tonoplast to facilitate K^+ influx and efflux in the vacuoles [15]. In this study, the expression levels of *MhTPK1*, *MhNHX1*, and *MhNHX2* were induced by KCl stress and inhibited by exogenous Res treatment but remained higher than the control level (Fig. 6). *MhTPK1*, *MhNHX1*, and *MhNHX2* could function to compartmentalized K^+ into the vacuoles to balance K^+ homeostasis in the cytoplasm under KCl stress. When exogenous Res was applied, the expression of these genes was reduced to regulate K^+ influx and efflux in the vacuoles and ensure K^+ homeostasis in the cytoplasm and vacuoles. Therefore, exogenous Res could affect K^+ homeostasis in the cytoplasm by enhancing K^+ efflux outside the cells, inhibiting K^+ absorption, and compartmentalizing K^+ into vacuoles under KCl stress. For Na^+ transport under KCl stress, Na^+ balance is mainly the result of passive influx and active efflux [61]. Therefore, the expression of Na^+/H^+ and cation/H^+ antiporter genes was detected. The expression of *MhCHX15* and *MhCAX5*, which expel Na^+ from cells, was significantly inhibited under KCl and Res treatment (Fig. 6). These results

390 indicated that exogenous Res could decrease the expel of Na^+ out of the cells to ensure
391 K^+/Na^+ homeostasis in the cytoplasm under KCl stress. In summary, exogenous Res
392 could alleviate KCl stress-induced ion toxicity by regulating the transcription of K^+,
393 Na^+, and Ca^{2+} transporters and maintaining the homeostasis of K^+/Na^+, K^+/Ca^{2+},
394 K^+/Fe^{2+}, and K^+/Mn^{2+} in the cytoplasm.

395 Osmotic stress is another direct injury to plants caused by KCl stress [9]. The
396 results indicated that electrolyte leakage was significantly induced by KCl stress but
397 inhibited by exogenous Res treatment. This result was in agreement with a previous
398 study, which stated that Res could protect plants from osmotic stress [62].
399 Accumulation of osmotic substances, such as proline, soluble sugar, and soluble
400 protein, is a common defense mechanism of plants subjected to salinity [63]. In this
401 work, the contents of proline, soluble sugar, and soluble protein were significantly
402 induced by KCl stress (Fig. 4). Similar results of increased carbohydrates and proline
403 concentration were observed in citrus leaves after NaCl treatment [42]. However,
404 when exogenous Res was applied under KCl stress, the content of proline was
405 significantly lower than that under KCl treatment only. Meanwhile, the contents of
406 soluble sugar and soluble protein had no significant changes. Accordingly, these
407 damages in KCl-treated seedlings were alleviated after Res treatment (Figs. 1 and 2),
408 which might explain the low proline content (Fig. 4).

409 Oxidative damage is the subsequent injury caused by osmotic stress and ion
410 toxicity [9]. In this study, the $O_2^{\cdot-}$ and H_2O_2 contents were significantly higher under
411 KCl stress than those in the control (Fig. 3). MDA content, which represents
412 membrane lipid peroxidation damage, was also induced by KCl stress. Res can
413 scavenge ROS and alleviate oxidative damage in cell systems [42]. The data showed
414 that exogenous Res could eliminate $O_2^{\cdot-}$ and H_2O_2 and decrease the MDA content
415 under KCl stress in apple seedlings (Fig. 3). SOD, POD, and CAT are the three main
416 antioxidant enzymes in enzymatic antioxidant systems [64,65]. The POD and CAT
417 activities were inhibited by KCl stress, and the SOD activity had no change (Fig. 3).
418 This finding was consistent with that under NaCl stress [42]. Although these enzymes
419 are antioxidant, their activities could be inhibited by serious salt stress. When

exogenous Res was applied, the POD and CAT activities were significantly increased, and the SOD activity remained unchanged (Fig. 3). SOD is the major element in ROS scavenging [66]. Under NaCl stress and Res treatment, the SOD activity was only slightly induced, leading to low H_2O_2 production [59]. However, the SOD activity had no change under KCl and Res treatment. This finding explains the difference of SOD function under KCl and NaCl stresses. POD and CAT activities showed similar variation tendency under KCl and Res treatment, indicating their important role in ROS scavenging by Res under KCl stress. Furthermore, the expression levels of three ROS-related genes (*MhGPX6*, *MhPER65*, and *MhpoxN1*) screened out from RNA-seq data under KCl stress were also detected under KCl and Res treatment. The results showed that the expression levels of peroxidase gene *MdPER65* and peroxidase N1 gene *MhpoxN1* were significantly induced by KCl stress but showed no change under Res treatment. However, *MhGPX6*, the glutathione peroxidase gene, was significantly induced by KCl stress but inhibited by exogenous Res treatment (Fig. 6). Thus, exogenous Res could alleviate oxidative damage by regulating the expression of the glutathione peroxidase gene *MhGPX6* and enhancing the enzyme activities of POD and CAT under KCl stress.

In addition to the ion transporters and antioxidant enzyme genes, the expression levels of kinases (MhMAPK3, MhANP2, and MhGK) and transcription factors (MhERF017, MhMYB39, MhWRKY28), screened out from RNA-Seq data under KCl stress were also detected. MAPK3 participates in the signaling pathway of salt stress in *Arabidopsis*, soybean, cucumber, and other plants [67,68]. *ANP2* is a gene of the MAPKKK family associated with NPK1 and is an important kinase in abiotic stress in rice [69,70]. G-protein kinase plays an active role in plant response to salt stress [71]. In this study, the expression of *MhMAPK3*, *MhANP2*, and *MhGK* was induced by KCl stress but inhibited by Res treatment (Fig. 6). ERF, MYB, and WRKY transcription factors serve as connecting links between the upstream signal and the expression of functional genes under salt stress [72]. MYB46 remarkably improves the salt tolerance of *Betula platyphylla* [73], and MdWRKY28 is an important regulator in apple salt adaptation [74]. The data showed that the expression

levels of *MhMYB39* and *MhWRKY28* were induced by KCl stress and exogenous Res treatment, indicating their important role in KCl and Res signaling transduction pathway. Furthermore, the variation tendency of *MhERF017* was similar to that of the three kinase genes (*MhMAPK3*, *MhANP2*, and *MhGK*) and Na^+/K^+ transporter genes (*MhHAK5*, *MhKAT1*, *MhTPK1*, *MhNHX1*, and *MhNHX2*), and the glutathione peroxidase gene *MhGPX6*, indicating their potential relationship. These kinases, transcription factors, ion transporters, and Res-signaling genes might have complicated regulation and interaction mechanisms. Future research will focus on this relationship and the Res-signaling transduction pathway under abiotic stress.

Abbreviations

ROS: Reactive oxygen species **Res:** Resveratrol **MDA:** Malondialdehyde **SOD:** Superoxide dismutase **POD:** Peroxidase **CAT:** Catalase **ABA:** Abscisic acid **JA:** Jasmonate **CTK:** Cytokinin

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

X. Z. and C. W. planned and designed the research. T. L., Y. L., X. X., Z. S., C. M., G. S., and Y. T performed experiments, conducted fieldwork and analyzed data etc. X. Z. and T. L. wrote the manuscript.

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Figure legends

Figure 1 Phenotypes of *Malus hupehensis* seedlings treated with 50 mM KCl stress and exogenous 100 μmol Res on day 0 and day 15 (a). Effect of Res on wilting rate (b), plant height (c), fresh weight (d) and dry weight (e) of apple seedlings after KCl stress for 15 days. Bar (a) represents 4.0 cm. The data represent the mean ± SD of biological replicates. Different lowercase letters indicate significant differences, according to Fisher's LSD (P <0.05).

Figure 2 Effects of Res treatment on chlorophyll content (a), photosynthesis rate (b), transpiration rate (c) and stomatic conductance (d) of apple seedlings under KCl stress. The data represent the mean ± SD of biological replicates. Different lowercase letters indicate significant differences, according to Fisher's LSD (P<0.05).

Figure 3 Effects of Res treatment on H_2O_2, $O_2^{\cdot-}$ (a) and malondialdehyde（MDA） content (b), superoxide dismutase (SOD) activity (c), peroxidase (POD) activity (d) and catalase (CAT) activity (e) under KCl stress. Bar (a) represents 1.0 cm. The data represent the mean ± SD of biological replicates. Different lowercase letters indicate significant differences, according to Fisher's LSD (P<0.05).

Figure 4 Effects of Res treatment on electrolyte leakage (a), proline content (b), soluble protein content (c) and soluble sugar content (d) under KCl stress. The data represent the mean ± SD of biological replicates. Different lowercase letters indicate significant differences, according to Fisher's LSD (P<0.05).

Figure 5 Effects of exogenous Res treatment on macronutrients content (a), micronutrients content (b) and K:Na ratio (c) under KCl stress. The data represent the mean ± SD of biological replicates. Different lowercase letters indicate significant differences, according to Fisher's LSD (P<0.05).

Figure 6 Eighteen candidate genes are divided into K^+ transporters (*MhSKOR*, *MhHAK5*, *MhAKT1*, *MhTPK1*, *MhNHX1*, and *MhNHX2*), Na^+ transporters (*MhCAX5*, *MhCHX15*, and *MhSOS1*), antioxidant enzymes (*MhGPX6*, *MhPER65*, and *MhpoxN1*), transcription factors (*MhERF017*, *MhMYB39*, and *MhWRKY28*), and kinase (*MhMAPK3*, *MhANP2*, and *MhGK*). The expression of the 18 candidate genes under KCl stress and exogenous Res treatment for 15 days. The data represent the mean ± SD of biological replicates. Different lowercase letters indicate significant differences, according to Fisher's LSD (P<0.05).

Figures

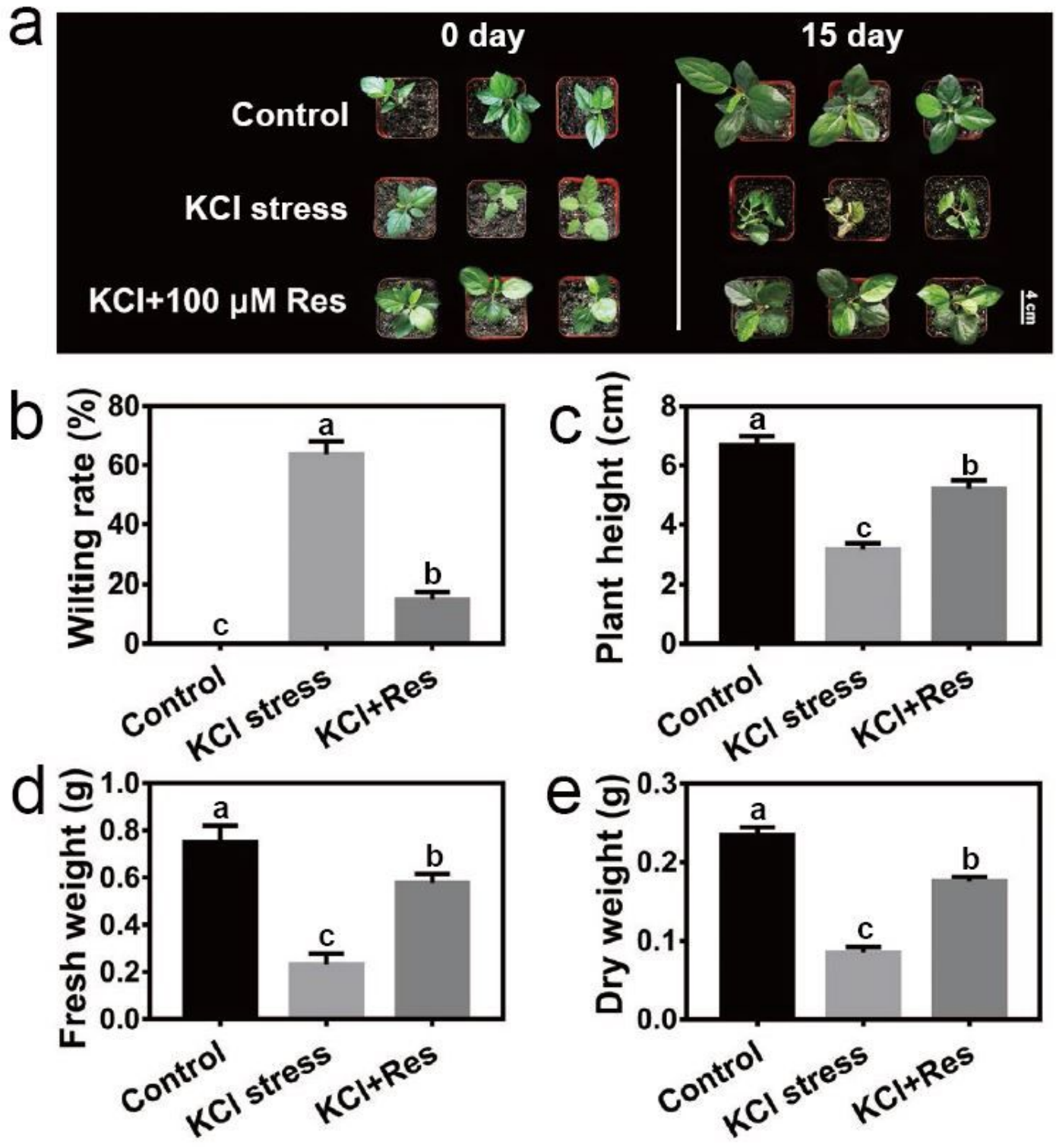

Figure 1

Full caption can be found in the manuscript PDF file

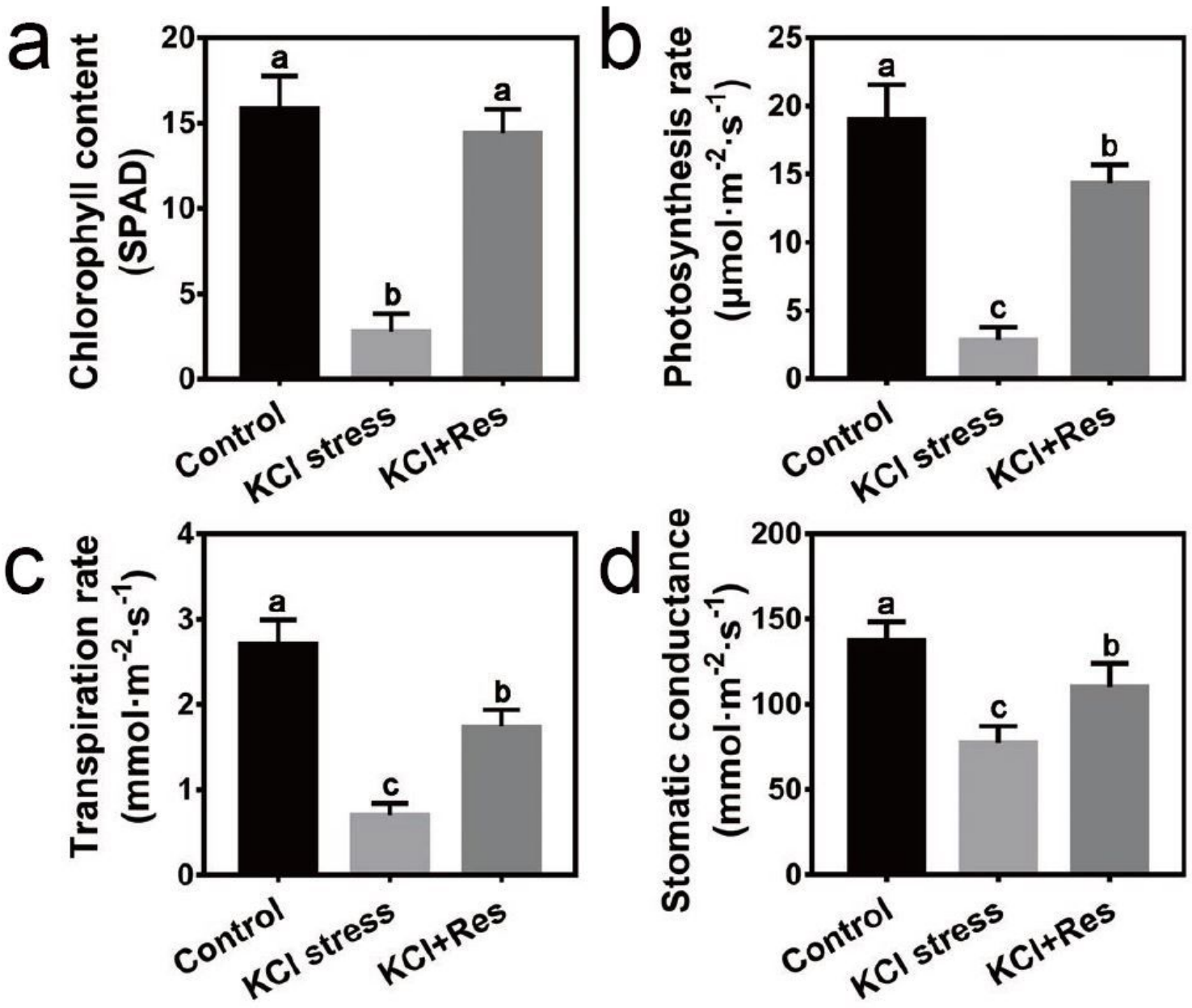

Figure 2

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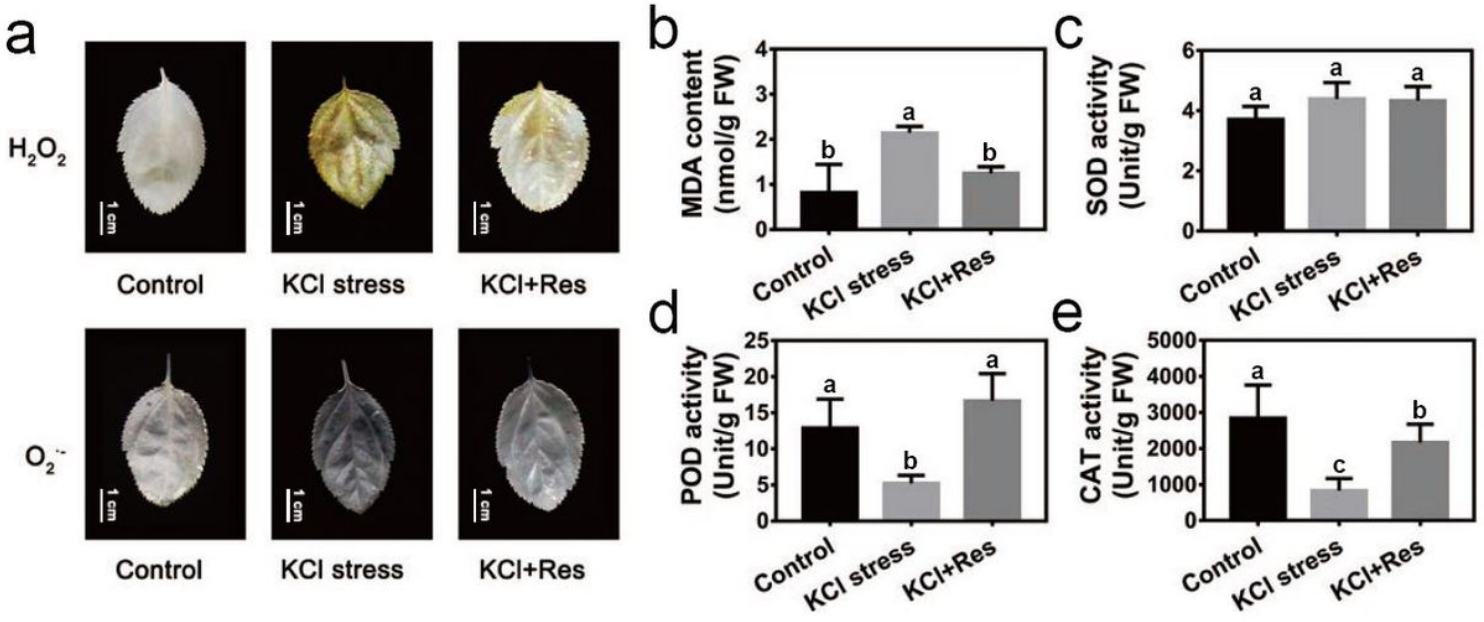

Figure 3

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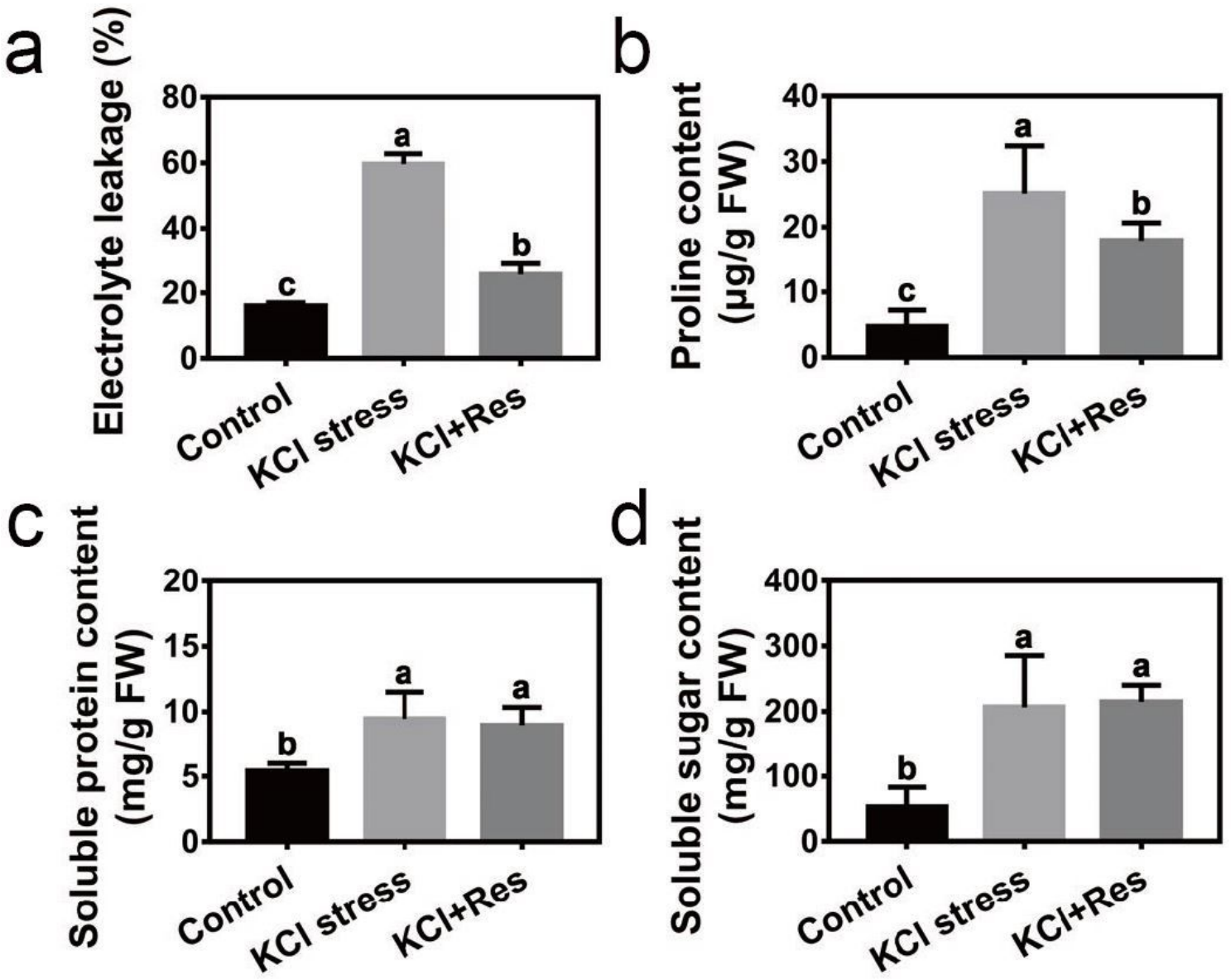

Figure 4

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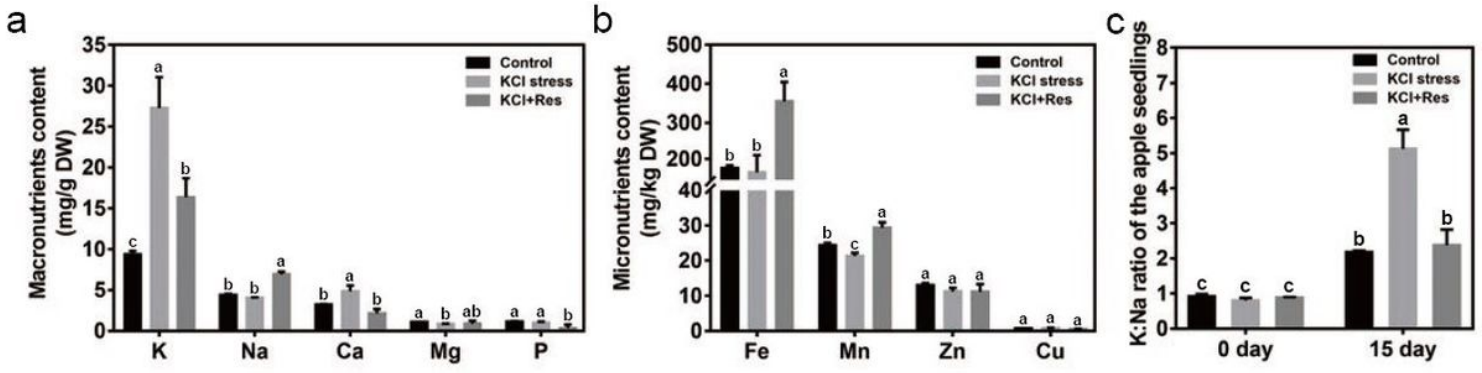

Figure 5

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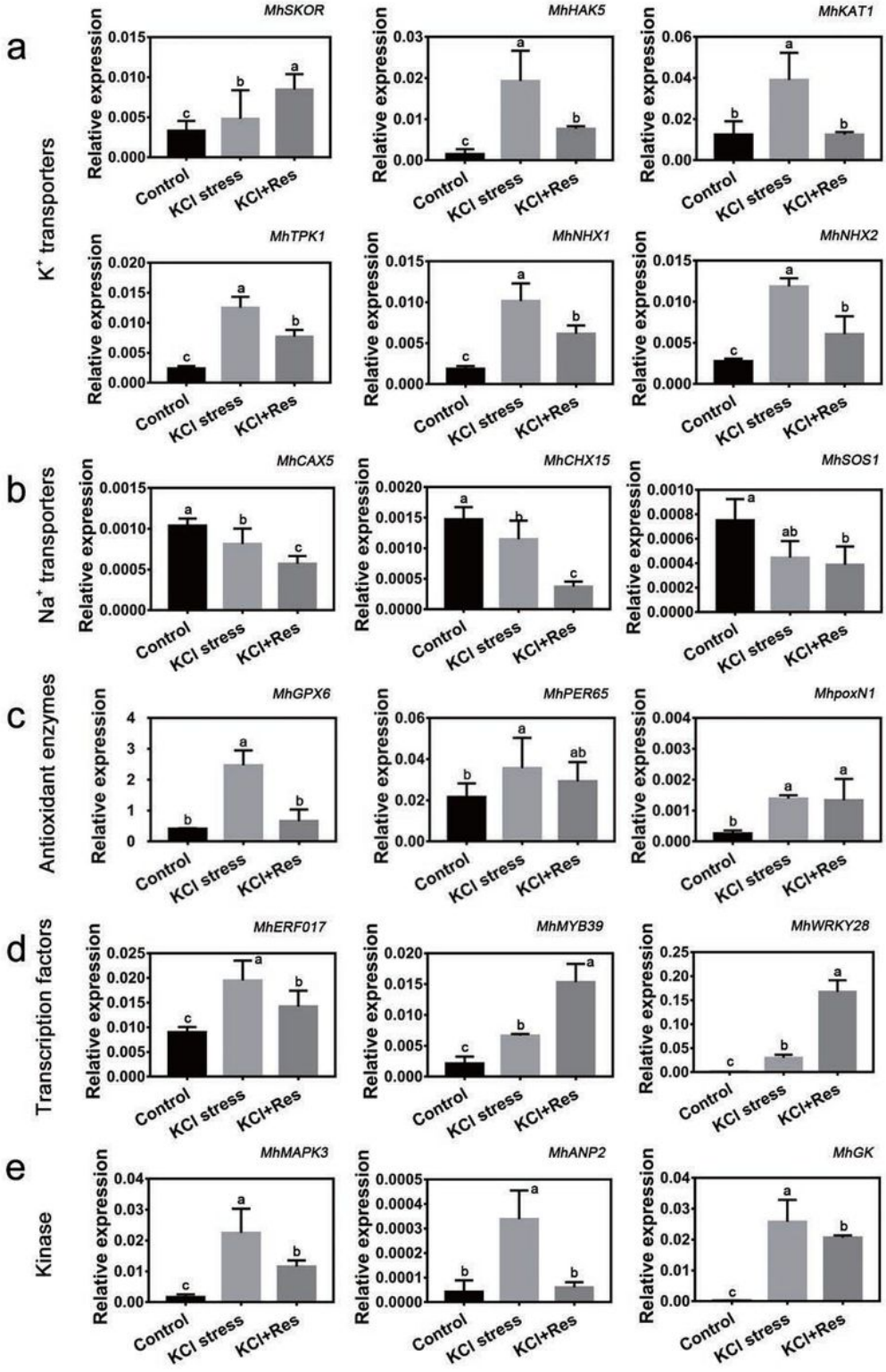

Figure 6

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