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Exogenous melatonin improved potassium content in *Malus* under different stress conditions

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Abstract

Melatonin mediates many physiological processes in plants. We investigated its role in regulating growth, potassium uptake, and root system architecture under three types of stress: salinity or a deficiency of all nutrients in *Malus hupehensis* Rehd., as well as a K deficiency in *Malus rockii* Rehd. Each treatment caused a reduction in growth rates and disrupted the absorption of potassium. However, pretreatment with 0.1 μmol/L melatonin significantly alleviated such inhibitions. The addition of melatonin also upregulated genes for antioxidant enzymes involved in the ascorbate–glutathione cycle (*MdcAPX*, *MdDHAR1*, *MdDHAR2*, *MdMDHAR*, and *MdcGR*) and helped decrease the accumulation of H_2O_2 while improving the expression of K transporters and genes for the CBL1–CIPK23 pathway. These results indicated that melatonin can regulate the ROS signal and activate the CBL1–CIPK23 pathway to regulate the expression of a potassium channel protein gene, thereby promoting the absorption of potassium ions. Our findings demonstrate that inducing melatonin production is an important mechanism for plant defenses that can serve as a platform for possible applications in agricultural or related fields of research.

KEYWORDS

CBL–CIPK pathway, *Malus*, melatonin, potassium transporters, root system architecture, stress

1 | INTRODUCTION

Plant roots acquire essential nutrients from the soil and utilize various mechanisms to adapt to fluctuations in nutritional conditions. Salinity is a major abiotic factor that limits plant growth and productivity. Globally, more than 800 million ha are affected by excess salt in the soil.[1] Crop performance may be adversely affected by salinity-induced nutritional disorders, including alterations in nutrient contents, as well as their transport or partitioning within the plant.[2] When plants are deprived of nutrients such as potassium, roots activate two mechanisms that involve either deploying other processes for acquisition and remobilization, for example, transporters[3,4] or channels,[5] or else altering root structure. In response to environmental stresses, particularly nutrient deficiencies, the architecture of a root system can be modified to increase uptake capacity.[6] The particular assembly and properties of different root segments have a vital role in plant development because they drive the expansion, direction, and senescence of older roots as well as the production of new roots.[7,8] The shape of a root system also reflects its spatial distribution and major functions, such as resource capture, anchorage, and plant hydraulics.[9]

Potassium (K^+), the most abundant monovalent cation in cells, has essential roles in plant growth and development.[10,11] Concentrations of K^+ in the cytosol are generally

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maintained at approximately 100 mmol/L.[3] This relatively high, stable level supports many physiological processes, including enzyme activation, protein biosynthesis, and the maintenance of membrane potential.[12] Under starvation conditions, K^+ uptake is stimulated in the roots by regulating the activity of potassium transport proteins.[3] The *Arabidopsis* K^+ transporter (AKT) subfamily includes AKT1, AKT2/3, AKT5, and AKT6/SPIK while the subfamily of K^+ transporters of *Arabidopsis thaliana* (KAT)-type transporters comprise KAT1, KAT2, and KC1.[13] Ion homeostasis is also regulated by the salt overly sensitive (SOS) pathway. High-sodium stress initiates a calcium signal that activates the SOS3–SOS2 protein kinase complex. This then stimulates the Na^+/H^+ exchange activity of SOS1 and regulates, transcriptionally and post-transcriptionally, the expression of some genes. This complex may also stimulate or suppress the activities of other transporters involved in ion homeostasis, including the vacuolar Na^+/H^+ exchanger (NHX) as well as plasma membrane K^+ and Na^+ transporters (e.g., those in the HKT gene family).[14]

Calcineurin B-like (CBL) proteins are a unique family of calcium sensors in plants.[15,16] They interact with and regulate a unique family of plant protein kinases: CBL-interacting protein kinases, or CIPKs.[16] In root cells, low-potassium stress is associated with the production of reactive oxygen species (ROS), leading to Ca^{2+} fluctuations that are sensed by CBLs.[17] The AtCBL1/AtCBL9–AtCIPK23 complex can directly activate the plasma membrane-localized potassium channel AtAKT1, enhancing K^+ uptake under low-K^+ conditions.[18,19] In addition to the specific relationship between AtAKT1 and AtCIPK23, AtCIPK9, the closest homolog to AtCIPK23, interacts with AtCBL3 to regulate K^+ homeostasis under low-K^+ stress in *Arabidopsis*.[20] Similarly, AtCIPK6 and AtCIPK16 both activate AtAKT1 by interacting with certain At*CBLs*.[21,22]

Melatonin (N-acetyl-5-methoxytryptamine) is an animal hormone that modulates sleep, mood, sexual behavior, reproductive physiology, and circadian rhythms while also acting as an antioxidant.[23–25] This molecule is ubiquitous and highly conserved in the plant and animal kingdoms[26] and has been identified in insects, arthropods, planarians, mollusks,[27] dinoflagellates,[28] and brown algae.[29] Physiologically, melatonin has roles in signaling environmental changes, inhibiting cancerous cell growth, and detoxifying free radicals, other ROS, and related products.[30,31] In plants, this hormone regulates root development,[31–33] seed germination,[31,34] leaf senescence,[35,36] and circadian rhythms.[37]

Melatonin can also alleviate the effects of abiotic stresses in plants, primarily serving as the first line of defense against environmental challenges that include extreme temperatures,[38,39] heavy metals,[40] drought,[41] UV radiation,[42] and elevated salinity.[43–45] Its antioxidant activity seems to function via the following means: (i) directly scavenging free radicals, (ii) stimulating antioxidant enzymes, (iii) augmenting the activities of other antioxidants, (iv) protecting antioxidant enzymes from oxidative damage, or (v) increasing the efficiency of the mitochondrial electron transport chain, thereby easing electron leakage and reducing the generation of free radicals.[26,46] In apple (*Malus* sp.), melatonin enhances the activities of ascorbate peroxidase (APX), catalase, and peroxidase and increases tolerance to stress.[43] However, little is known about its possible role in directing the absorption of K^+ elements by plants under stress conditions. Therefore, the objective of our research was to examine the relationships among melatonin status, potassium absorption, and root system architecture in apple plants exposed to salinity, a K deficiency, or an insufficient supply of all nutrients.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growing conditions

All experiments were conducted at the Northwest A & F University, Yangling (34°20′N, 108°24′E), China. Seeds of *Malus hupehensis* and *Malus rockii* were collected from their native regions in China (Table 1). Both were reproduced by apomixis. *Malus hupehensis* was chosen for testing responses to NaCl stress or starvation of all nutrition while *M. rockii* was used for K deficiency treatments, as described previously.[47,48] Those species were selected because they are either salt sensitive or K inefficient. After cold stratification, seeds of each genotype were sown in individual plastic tubs filled with sand, which were sprinkled with distilled water to maintain a moist environment for supporting good germination and proper seedling establishment. For the hydroponics experiments, seeds were stratified for 50 days at 4°C. After germination, three seeds each were planted in plastic pots (12 × 12 cm) filled with sand and then placed in a greenhouse under natural light and temperature conditions. The design for our hydroponics system followed that described by Bai et al.[49] Briefly, plants of similar size (8–9 leaves, about

TABLE 1 Origins in China for apple rootstock genotypes

Genotype	Origin	Elevation (m)	Mean annual precipitation (mm)	Mean annual temperature (°C)
Malus hupehensis Rehd.	Pingyi, Shandong	154–1156	849	14.1
Malus rockii Rehd.	Lijiang, Yunnan	2400–3800	1000	18.1

8 cm tall) were selected after 40 days of growth and transferred to plastic tubs (52 × 37 × 15 cm) containing 20 L of a half-strength Hoagland's nutrient solution.[50] The tubs, wrapped with black plastic to block light exposure to the root systems, were placed in a growth chamber (23–25°C/15–18°C day/night). Light was provided by sodium lamps during a 14-h photoperiod (photon flux density of 160 μmol/m^2/s). The nutrient solution was continuously aerated with an air pump, and dissolved oxygen (DO) concentrations were maintained at 8.0–8.5 mg/L by a DO controller (FC-680; Corporation of Super, Shanghai, China). The pH was adjusted to 6.5±0.1 with H_3PO_4, and the solution was refreshed every 3 days.

2.2 | Precultivation

Seedlings were precultured for 10 days to allow them to adapt to the new conditions. From the sixth day of precultivation, half of the seedlings were treated with a half-strength nutrient solution containing 0.1 μmol/L melatonin. [The concentration of melatonin used in this experiment was determined in a previous trial that showed this level to be quite effective in alleviating the effects of salinity stress.]

2.3 | Salt treatment

After 10 days of precultivation, normally grown *M. hupehensis* seedlings and those that had received melatonin pretreatment were assigned to four new experimental groups: (i) control: continued use of the half-strength nutrient solution (CK); (ii) salinity treatment: half-strength nutrient solution and 200 mmol/L NaCl (ST); (iii) melatonin control: half-strength nutrient solution and pretreatment with 0.1 μmol/L melatonin (MT); and (iv) melatonin and salinity: half-strength nutrient solution with 0.1 μmol/L melatonin pretreatment followed by 200 mmol/L NaCl (MT + ST). Each treatment contained three replicates of 50 plants. The seedlings were treated for 12 days.

2.4 | K deficiency trials

Our normal half-strength nutrient solution contained 2.5 mmol/L Ca $(NO_3)_2$, 1 mmol/L $MgSO_4$, 0.5 mmol/L $(NH4)H_2PO_4$, 0.2 μmol/L $CuSO_4$, 1 μmol/L $ZnSO_4$, 0.1 mmol/L Fe-Na-EDTA, 20 μmol/L H_3BO_3, 5 pmol/L $(NH_4)_6$ Mo_7O_{24}, and 1 μmol/L $MnSO_4$. The concentration of K, in the form of potassium sulfate (K_2SO_4), in this solution was 3.0 mmol/L for the K-sufficient treatment and 0.1 mmol/L for the K-deficient treatment. After 10 days of precultivation, the normally grown *M. rockii* seedlings and melatonin-pretreated ones were randomly assigned to four new groups: (i) control: half-strength nutrient solution (CK); (ii) K-deficient treatment: 0.1 mmol K_2SO_4 (−K); (iii) melatonin control: half-strength nutrient solution with 0.1 μmol/L melatonin (MT); and (iv) melatonin precultivation and K-deficient treatment: half-strength nutrient solution with 0.1 μmol/L melatonin and 0.1 mmol/L K_2SO_4 (−K + MT). Each treatment contained three replicates of 50 plants. These trials spanned 56 days.

2.5 | All-nutrition deficiency trials

Our normal half-strength nutrient solution was as described above. After 10 days of precultivation, the normally grown *M. hupehensis* seedlings and melatonin-pretreated ones were randomly assigned to four new groups: (i) control: half-strength nutrient solution (CK); (ii) all-deficient treatment: twentieth-strength nutrient solution (−All); (iii) melatonin control: half-strength nutrient solution with 0.1 μmol/L melatonin (MT); and (iv) melatonin precultivation and all-deficient treatment: twentieth-strength nutrient solution with 0.1 μmol/L melatonin (−All + MT). Each treatment contained three replicates of 50 plants. These trials spanned 20 days.

2.6 | Growth measurements

After each treatment was finished, shoot lengths were measured. Their dry weights were determined after oven-drying at 80°C for at least 72 h.

2.7 | Assessing potassium concentrations

To evaluate their mineral components, we collected root, stem, and leaf tissues at the end of the hydroponics experimental period and washed all samples three times with distilled water. They were then fixed at 105°C for 30 min, dried at 80°C for 48 h, and ground into powder. The potassium element was digested in solutions containing HNO_3–$HClO_4$. The materials were then filtered, diluted with distilled water, and analyzed for K^+ concentrations on an atomic absorption spectrophotometer (Z-2000; Hitachi Instrument, Tokyo, Japan).

2.8 | Investigation of root architecture

The root systems were carefully cleared of substrates with tap water and further rinsed with distilled water. After they were arranged for image capture with a scanner, their architecture was studied with the WinRHIZO® image analysis system (V4.1c; Régent Instruments, Quebec, Canada).[51] From there, we could obtain total lengths, surface area, and volume; plus the number of root tips and

forks; and average root diameters in different size categories.

2.9 | QRT-PCR analysis

Total RNA was extracted from leaves per the method described by Chang et al.[52] Sequences of the primers (Table 2) for antioxidant enzyme genes, K transporter genes, the CBL1–CIPK23 pathway gene, and the *Malus* elongation factor-1 alpha gene (*EF-1a*; DQ341381) were designed by Primer Premier 6 software (Biosoft International, Palo Alto, CA, USA). The poly(A)+ RNA was purified with a poly(A)+ Ttract® mRNA Isolation Systems III kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed on an iQ5.0 instrument (Bio-Rad, Hercules, CA, USA) using SYBR Green qPCR kits (TaKaRa, Dalian, China) according to the manufacturer's instructions. To test the suitability of these primers, we monitored the specificity and identity of the PCR products after each reaction by conducting melting-curve analysis of the products.

Transcripts of *EF-1a* were used to standardize the cDNA samples for different genes. We had previously compared apple *EF-1*α, actin, and 18S rRNA as internal controls[53] and found that *EF-1a* is more stable than the others as a reference gene under saline conditions. Three independent biological replications were performed for each experiment.

TABLE 2 Sequences of primers used in quantitative real-time RT-PCR

Gene	Primer sequence (5′–3′)
MdHKT1	F: TCGTTCGCTATTTCGTGTCCTGCT R: TGGGCCTGAAAGAAGTGTTTGTGC
MdSOS1	F: TCCGGTTAATCCATCACACACCGT R: TTTGCTGCCCTGGAGGATTTGTTG
MdNHX2	F: ATGCGTGGCTCTGTTTCAAT R: AACTGTGATGGTGCTGGTGA
MdNHX4	F: ATCACCAAAACCACCAACCA R: GCCACACTTCTTAGGCAACG
MdNHX6	F: AGCACAGCGTCATTCACAG R: ATGGAAACCCCCTCTTGTAG
MdAKT1	F: GCGGAGACGAAAAGTCCTAA R: AGTGGGAGCAGCACAAGTTT
MdAKT2/3	F: TTCAAGGGAAACACTTCTGC R: TCTCTCTCCATCTCACAATCAA
MdKEA2	F: GCTGTCAATCAGGGAATAATGA R: CACCTCAAAACGAGAAGCAA
MdKAT1	F: ATGGGCAAGATCAAGTAAGTCACA R: GTCAGAGCCAATCGACCAAGTAT
MdcAPX	F: AACTACAAGGGATGAAGCC R: CAACGAGGATGATAACCAG
MdMDHAR	F: CCATACTTCTATTCCCGCTCCT R: CGACCACCTTCCCGTCTTT
MdDHAR1	F: AGTGGACGGTTCCAGCAGA R: TTCCCATCCCGCAATCAC
MdDHAR2	F: CCACCATCAAACACCACCTT R: TTGGGAACAGTAACGGAAGC
MdcGR	F: GTTCAGCGACAAGGCGTAT R: TCAACCGATTTCCATTTCC
MdCBL1	F: CTGTGATTGACGATGGGTTG R: GGCATTGGGATGAAAGACAT
MdCIPK23	F: AGTGGAGAGGCGAGAAGAGC R: GCGTTTGGATGTGAACCTTG
*EF-1*α	F:ATTCAAGTATGCCTGGGTGC R:CAGTCAGCCTGTGATGTTCC

2.10 | Statistical analysis

Data were expressed as means±standard deviation (SD). The data were analyzed via one-way ANOVA, followed by Tukey's tests. A *P*-value of <.05 indicated a significant difference.

3 | RESULTS

After 12 days of treatment, our data confirmed that, whereas salinity conditions significantly decreased the rate of seedling growth, a pre-application of 0.1 μmol/L melatonin to the roots noticeably reversed that inhibition. Heights and dry weights were 34.8% and 48.5%, respectively, lower in ST plants than in the CK vs respective reductions of only 22.7% and 36.6% for MT plants when compared with the untreated control (Fig. 1A,B). After 56 days of exposure to K-deficient conditions, plant growth was significantly inhibited but that response was markedly alleviated by the addition of melatonin. When compared with the adequate-K group, respective reductions for nonmelatonin and melatonin-pretreated plants were 29.5% vs 7.2% for height and 34.3% vs 24.3% for dry weight (Fig. 1C,D). After 20 days of exposure to all-nutrition deficiency conditions, plant growth was also significantly inhibited but that response was markedly alleviated by the addition of melatonin. When compared with the CK, respective reductions for nonmelatonin and melatonin-pretreated plants were 37.0% vs 9.1% for height and 36.8% vs 18.5% for dry weight (Figs 1E,F and 2).

Potassium contents were lower in the roots, stems, and leaves after plants were exposed for 12 days to 200 mmol/L NaCl. However, pretreatment with melatonin significantly buffered that drop (Fig. 3A–C). In the roots, stems, and leaves, the average decrease over levels in the nonmelatonin plants under salt stress was less for the melatonin-applied group (by 31.8% for roots, 3.8% for stems, and 22.1% for leaves) than for plants not receiving melatonin (41.9% for roots, 10.5% for stems, and 25.2% for leaves) (Fig. 3A–C).

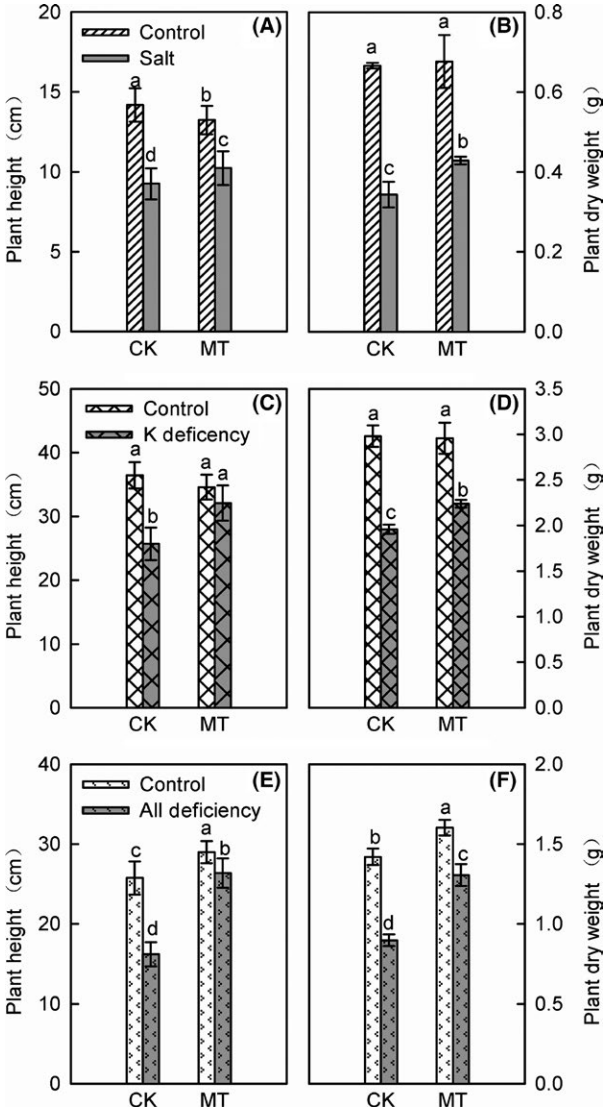

FIGURE 1 Effects of melatonin pretreatment on heights and dry weights of plants grown hydroponically under 200 mmol/L NaCl (A and B), K-deficient (C and D), and all-nutrients-deficient (E and F) conditions. Data are means of 30 measurements (3 × 10). Values not followed by same letter denote significant differences by Tukey's multiple-range tests ($P<.05$). CK, control plants without melatonin pretreatment; MT, plants pretreated with melatonin

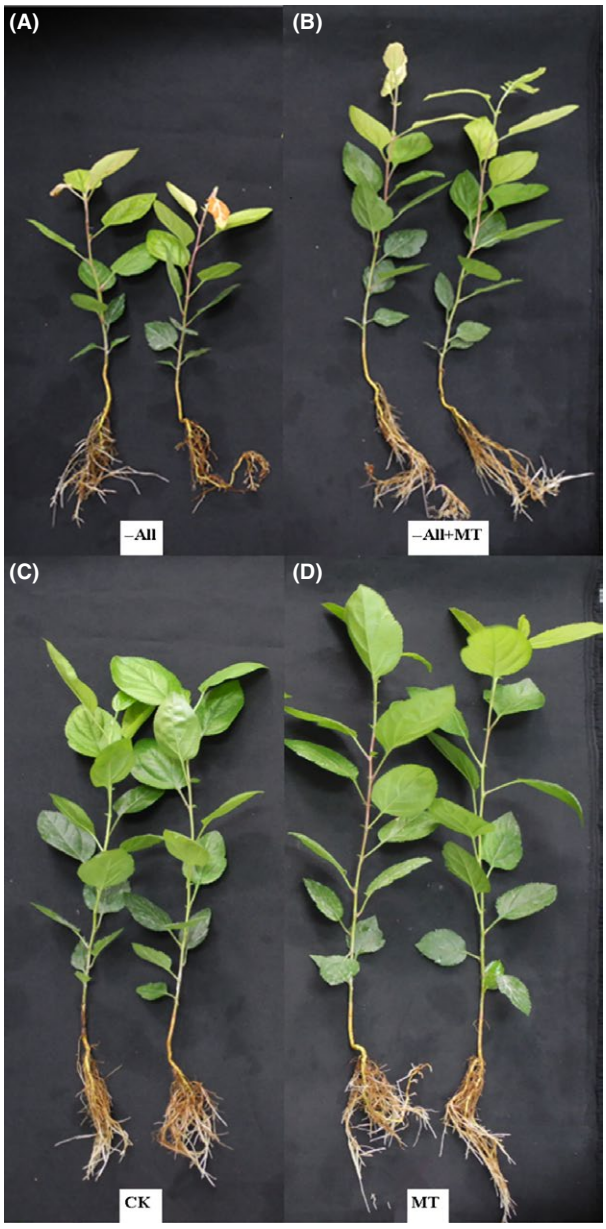

FIGURE 2 Plants after 20 days exposure to all-nutrients-deficient conditions. (A) −All, treatment with solution deficient in all nutrients; (B) −All + MT, melatonin-pretreated plants under nutrient deficiency; (C) CK, control plants not pretreated with melatonin; (D) MT, plants pretreated with melatonin

Potassium contents were also decreased in the roots, stems, and leaves after 56 days of exposure to K-deficient conditions. However, in the roots and leaves, K levels were significantly higher in MT plants than in CK tissues under such stress (Fig. 3D,F). Those contents were also decreased in the roots, stems, and leaves of treated plants after 20 days of exposure to the twentieth-strength nutrient solution (all-nutrition deficiency trials). However, the average decline was greater in plants that had not received melatonin pretreatment (Fig. 3G–I).

Compared with the controls, Na contents in the roots, stems, and leaves were dramatically elevated after 12 days of salinity treatment. In the roots and stems, the average increase over the normal control was greater in the melatonin-applied group (by 951.3% for roots and 869.8% for stems) than for plants not receiving melatonin (802.1% for roots and 726.4% for stems) (Fig. 4A,B). The opposite trend was found with the leaves (Fig. 4C). Sodium contents were also altered after 56 days of the K deficiency, albeit to different extents (Fig. 4D–F). For example, in the roots, those levels were dramatically elevated in response to stress, and the average increase over the normal control was greater in the nonmelatonin group than in plants receiving melatonin (Fig. 4D). In stems and leaves, Na contents in the untreated group were almost the same while

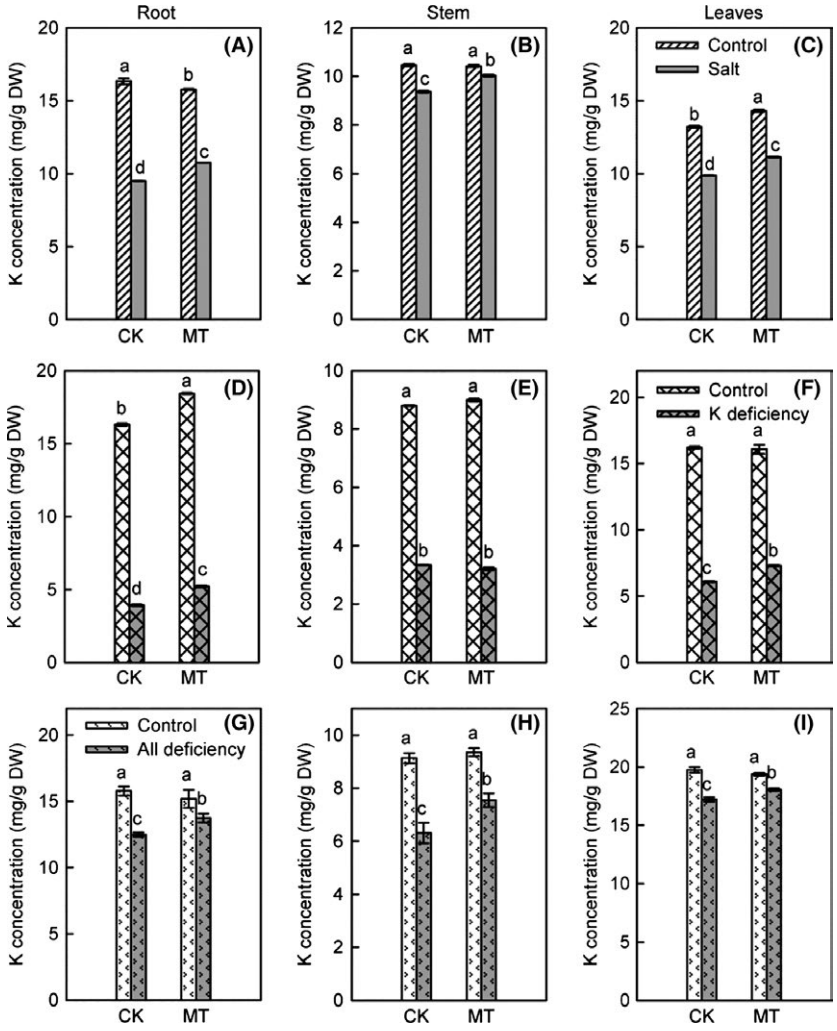

FIGURE 3 Effects of melatonin pretreatment on K^+ contents in roots, stems, and leaves under 200 mmol/L NaCl (A, B, and C), K-deficient (D, E, and F), and all-nutrients-deficient (G, H, and I) conditions. Data are means of 30 measurements (3 × 10). Values not followed by same letter denote significant differences by Tukey's multiple-range tests (P<.05). CK, control plants without melatonin pretreatment; MT, plants pretreated with melatonin

the application of melatonin significantly decreased that level when compared with the control (Fig. 4E,F). In all-nutrition deficiency conditions, Na contents were lower in roots, stems, and leaves in melatonin-applied group.

Pretreatment with melatonin enabled those plants to maintain significantly higher K levels in leaves compared with plants that received no additional melatonin. Because the level of Na was lower in melatonin-applied leaves, the K/Na ratio was relatively greater as a result of this pretreatment.

After 12 days of exposure to NaCl, root growth was significantly inhibited. However, applying 0.1 μmol/L melatonin to the roots beforehand noticeably alleviated this response. When compared with the normal control, respective reductions for nonmelatonin and melatonin-pretreated plants were 24.50% vs 13.16% (root lengths), 13.64% vs 6.82% (average diameter), 37.50% vs 21.88% (volume), 48.11% vs 40.14% (number of root tips), 35.33% vs 20.77% (number of forks), and 30.33% vs 6.99% (surface area). In addition, almost all values calculated for root system architecture, except the average diameter from pretreated plants, were higher in normal melatonin-pretreated plants than in plants with no melatonin (Table 3).

With regard to architecture, the same trend was found for K-deficient *M. rockii* plants as for salt-stressed *M. hupehensis* plants. That is, root growth was significantly inhibited in untreated plants but was restored in response to melatonin pretreatment. For example, root length and volume, the numbers of tips and forks, and total surface area for non-melatonin plants were significantly reduced by K-deficient stress while those declines in pretreated plants were minor (Table 4).

After 20 days of exposure to the twentieth-strength nutrient solution, root growth was also significantly inhibited. However, applying 0.1 μmol/L melatonin to the roots noticeably alleviated this response. When compared with the normal control, respective reductions for nonmelatonin and melatonin-pretreated plants were 28.04% vs 4.78% (root lengths), 15.39% vs 0.00% (average diameter), and 17.62% vs 2.74% (number of root tips). In addition, values for root volume, number of root forks, and surface area were lower under the all-nutrition-deficient conditions for plants without pretreatment but were higher in the pretreated plants (Table 5).

All of the genes for antioxidant enzymes analyzed here, that is, *MdcAPX*, *MdDHAR1*, *MdDHAR2*, *MdMDHAR*, and

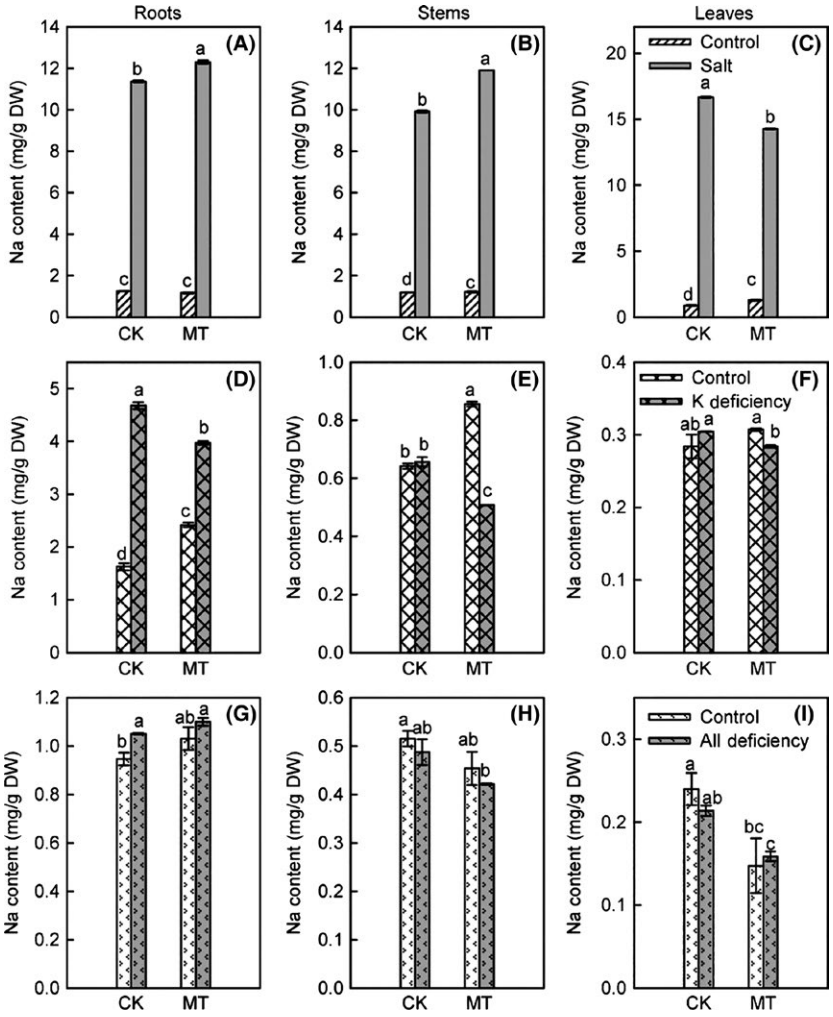

FIGURE 4 Effects of melatonin pretreatment on Na contents in roots, stems, and leaves under 200 mmol/L NaCl (A, B, and C), K-deficient (D, E, and F), and all-nutrients-deficient (G, H, and I) conditions. Data are means of 30 measurements (3 × 10). Values not followed by same letter denote significant differences by Tukey's multiple-range tests ($P<.05$). CK, control plants without melatonin pretreatment; MT, plants pretreated with melatonin

MdcGR, were markedly upregulated in control and stressed plants midway through the experiments with deficient hydroponics solutions (Fig. 5A–E). This finding supported our conclusion that ROS has an important role in low-nutrition signaling. In fact, the addition of melatonin upregulated all of those genes in stressed plants when compared with the control.

The expression of genes involved in regulating potassium transport, that is, *MdHKT1*, *MdSOS1*, *MdNHX2*, *MdNHX4*, *MdNHX6*, *MdAKT1*, *MdAKT2/3*, *MdKEA2*, and *MdKAT2*, was significantly upregulated in response to a lack of adequate nutrients in the solution (Fig. 6). However, their relative expression was higher in melatonin-pretreated plants during the first 5 days of those deficit conditions. Transcript levels peaked between days 5 and 10 of treatment before returning to normal or below-normal levels.

TABLE 3 Effects of melatonin on root system architecture of *Malus hupehensis* under salt-stress conditions

	CK	ST	MT	MT + ST	ST/CK (%)	MT + ST/CK (%)	CK/MT (%)
Length (cm)	419±17.3b	295±18.4d	490±21.1a	370±18.3c	75.50	86.84	85.52
Diam (mm)	0.44±0.03a	0.38±0.02b	0.42±0.04ab	0.41±0.03ab	86.36	93.18	104.76
Volume (cm^3)	0.64±0.04b	0.40±0.02d	0.69±0.04a	0.50±0.02c	62.50	78.12	92.75
Number of root tips	1881±183b	976±111d	2144±166a	1126±75c	51.89	59.86	87.73
Number of root forks	3034±151b	1962±125d	3781±185a	2404±139c	64.67	79.23	80.24
Surface area (cm^2)	57.7±2.9b	40.2±2.8d	64.6±4.4a	47.9±3.5c	69.67	93.01	89.32

CK, control plants not pretreated with melatonin; ST, plants exposed to 200 mmol/L NaCl; MT, plants pretreated with melatonin; MT + ST, melatonin-pretreated plants exposed to 200 mmol/L NaCl. Data represent means ± SD of 10 replicate samples. Different letters indicate significant differences according to Tukey's multiple-range tests ($P<.05$).

TABLE 4 Effects of melatonin on root system architecture of *Malus rockii* under K-deficient conditions

	CK	−K	MT	−K + MT	−K/CK (%)	−K + MT/CK (%)	CK/MT (%)
Length (cm)	1063±74.9b	909±50.7c	1224±77.8a	1121±67.7b	85.45	105.39	86.88
Diam (mm)	0.52±0.04a	0.46±0.02b	0.48±0.02ab	0.46±0.03b	88.46	88.46	108.33
Volume (cm^3)	2.31±0.24a	1.48±0.18c	2.27±0.24a	1.90±0.15b	64.07	82.25	101.76
Tips	2713±184b	2312±151c	3236±143a	2636±186b	85.20	97.12	83.86
Forks	6467±350b	5280±231c	8240±246a	6818±417b	81.70	105.49	78.43
Surface area (cm^2)	175.8±8.9a	130.0±7.0c	185.7±14.6a	163.0±7.0b	73.91	92.69	94.69

CK, control plants not pretreated with melatonin; −K, K deficiency treatment; MT, plants pretreated with melatonin; −K + MT, melatonin-pretreated plants under K deficiency conditions. Data represent means ± SD of 10 replicate samples. Different letters indicate significant differences according to Tukey's multiple-range tests ($P<.05$).

TABLE 5 Effects of melatonin on root system architecture of *Malus hupehensis* plants grown in hydroponics solution deficient in all nutrients

	CK	−All	MT	−All + MT	−All/CK (%)	−All + MT/CK (%)	CK/MT (%)
Length (cm)	709±13.5b	510±96.9c	856±5.2a	676±14.906b	71.96	95.22	82.85
Diam (mm)	0.52±0.04a	0.44±0.03b	0.54±0.04ab	0.52±0.03b	84.61	100.00	96.30
Volume (cm^3)	1.31±0.10a	1.08±0.11c	1.37±0.17a	1.35±0.03b	82.44	103.05	95.62
Number of root tips	931±30b	767±39c	1021±44a	906±27b	82.38	97.26	91.19
Number of root forks	5204±180b	4006±83c	5753±148a	5318±167b	79.73	105.84	87.33
Surface area (cm^2)	108.8±8.9a	95.0±7.0c	119.7±14.6a	109.0±7.0b	87.24	100.14	90.93

CK, control plants not pretreated with melatonin; −All, treatment with solution deficient in all nutrients; MT, plants pretreated with melatonin; −All + MT, melatonin-pretreated plants under nutrient deficiency. Data represent means ± SD of 10 replicate samples. Different letters indicate significant differences according to Tukey's multiple-range tests ($P<.05$).

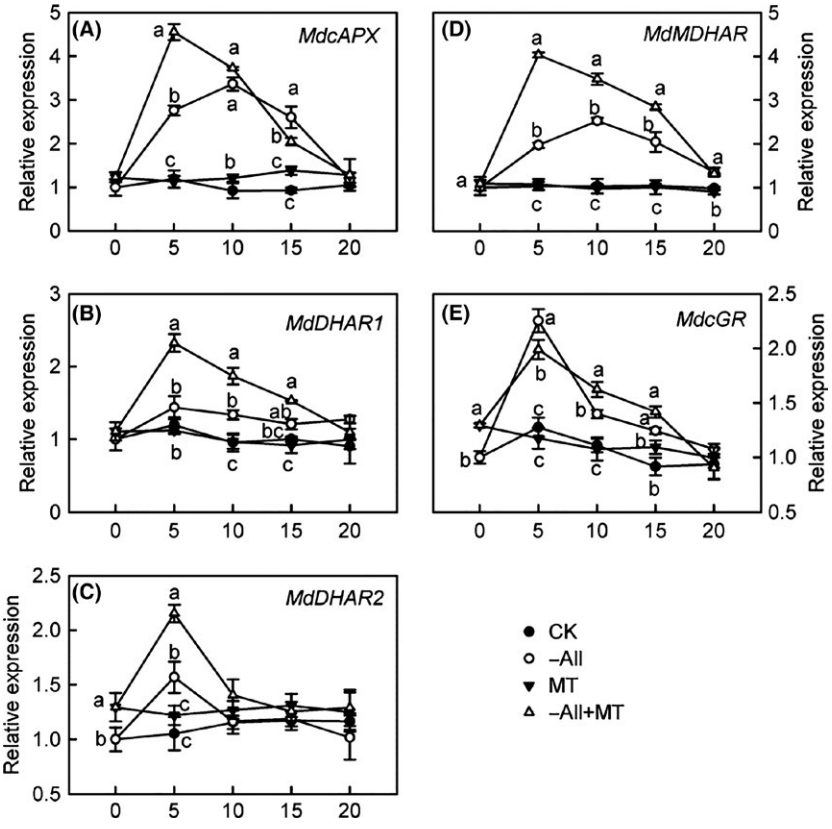

FIGURE 5 Effects of melatonin on expression of *MdcAPX* (A), *MdDHAR1* (B), *MdDHAR2* (C), *MdMDHAR* (D), and *MdcGR* (E) in leaves of *Malus hupehensis* grown in hydroponics solution deficient in all nutrients. Total RNA was isolated from samples at different time points, converted to cDNA, and subjected to qRT-PCR. Expression levels were calculated relative to expression of *Malus EF*-1a mRNA. Values are means of 5 replicates±SD. Different letters indicate significant differences according to Tukey's multiple-range tests ($P<.05$)

To determine how melatonin might regulate potassium transporter genes, we performed qRT-PCR analysis to measure the transcript levels of two CBL–CIPK pathway genes. Both *MdCBL1* and *MdCIPK23* were upregulated when nutrient levels were low, with that change in expression being more dramatic in samples from pretreated plants (Fig. 7).

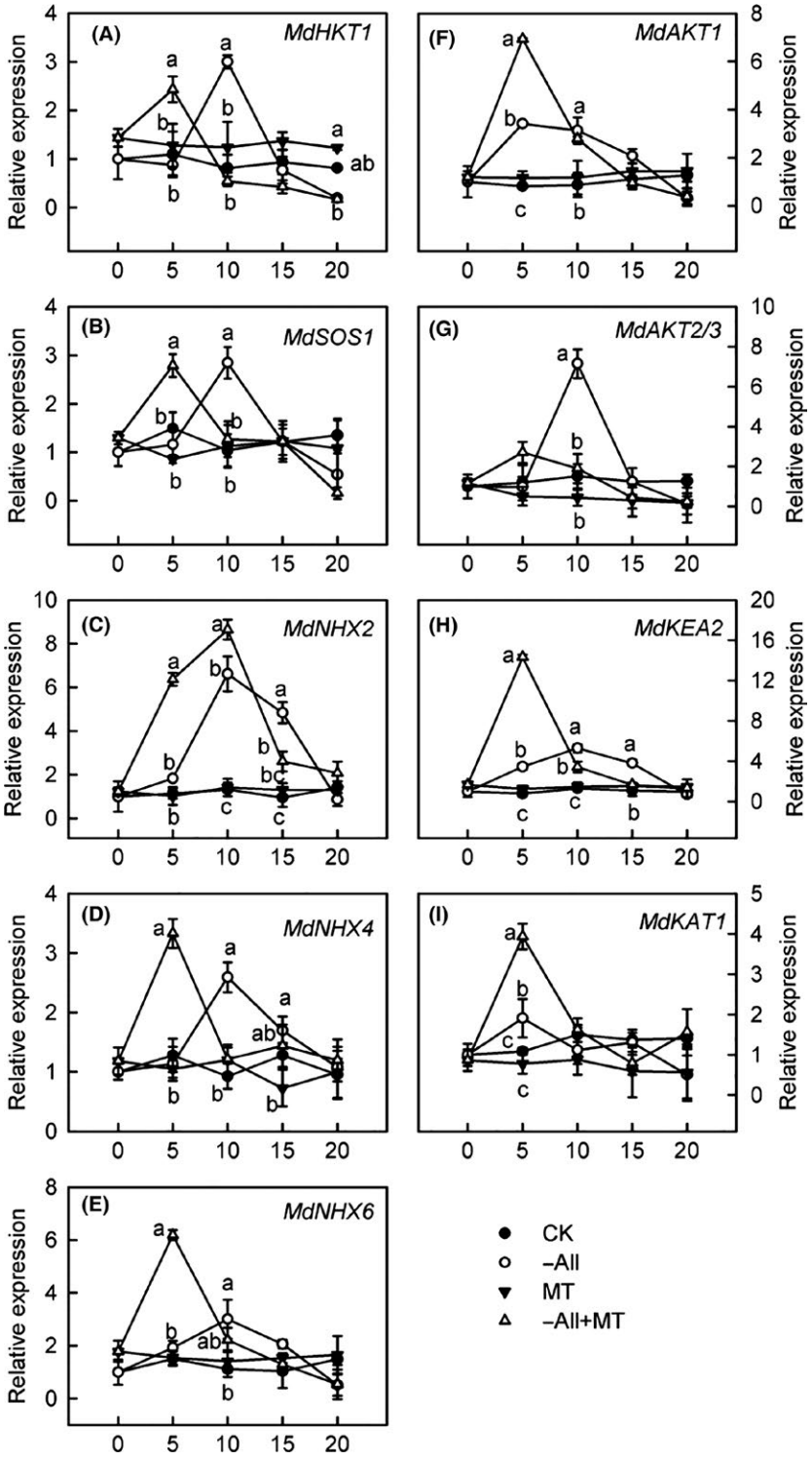

FIGURE 6 Effects of melatonin on expression of genes involved in K transport: *MdHKT1* (A), *MdSOS1* (B), *MdNHX2* (C), *MdNHX4* (D), *MdNHX6* (E), *MdAKT1* (F), *MdAKT2/3* (G), *MdKEA2* (H), and *MdKAT1* (I) in leaves of *Malus hupehensis* grown in hydroponics solution deficient in all nutrients. Total RNA was isolated from samples at different time points, converted to cDNA, and subjected to qRT-PCR. Expression levels were calculated relative to expression of *Malus EF*-1a mRNA. Values are means of 5 replicates±SD. Different letters indicate significant differences according to Tukey's multiple-range tests ($P<.05$)

4 | DISCUSSION

Melatonin primarily functions in plants as the first line of defense against internal and environmental oxidative stressors.[26] This is accomplished when melatonin directly scavenges free radicals and upregulates the expression of genes for antioxidant enzymes. Because of those properties, this molecule is applied to plants to protect them when exposed to various stress conditions, including excess salt.[31,39,41,43,54] Our experiment results showed that exogenous melatonin can improve plant growth under salinity or when the supply of either potassium or all nutrients is deficient in the solution. Although exogenous melatonin can enhance plant development,[26,32,43,55,56] no data have explained how it accomplishes this by affecting root system architecture and influencing potassium absorption. Here,

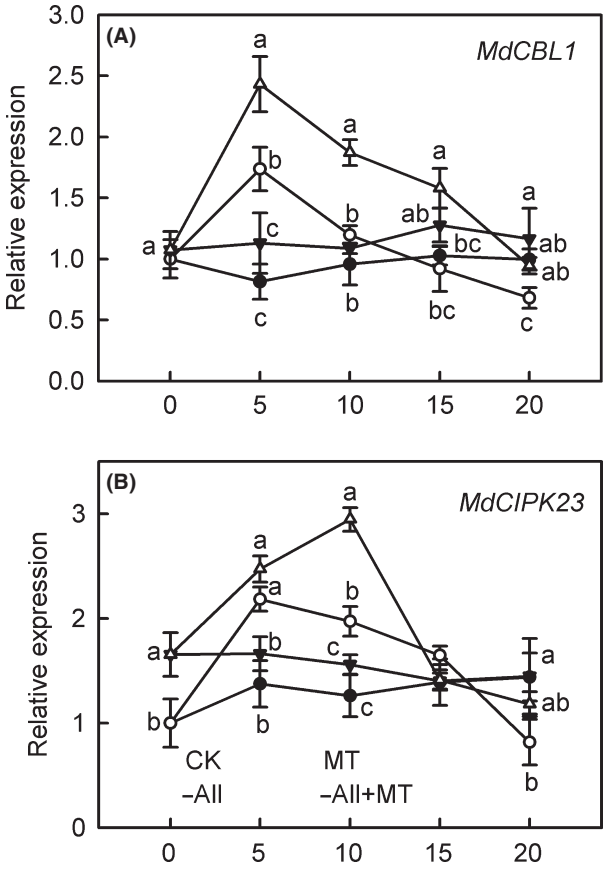

FIGURE 7 Effects of melatonin on expression of *MdCBL1* (A) and *MdCIPK23* (B) in leaves of *Malus hupehensis* growing in hydroponics solution deficient in all nutrients. Total RNA was isolated from samples at different time points, converted to cDNA, and subjected to qRT-PCR. Expression levels were calculated relative to expression of *Malus EF*-1a mRNA. Values are means of 5 replicates±SD. Different letters indicate significant differences according to Tukey's multiple-range tests ($P<.05$)

salt stress and nutrient deficiencies caused reductions in growth parameters. However, supplemental melatonin significantly eased those inhibitory effects. Therefore, our research is the first to demonstrate that melatonin can mitigate the negative influence of induced nutrient deficiency stress in *Malus*.

Mineral nutrients that are taken up by the roots are essential for plant growth and crop production. Salt stress disturbs ion homeostasis.[57] Plants respond to elevated Na^+ by maintaining low levels of cytosolic Na^+ and a high cytosolic K^+/Na^+ ratio. Our findings revealed a significant decrease in K^+ in the roots, stems, and leaves after 12 days of salinity exposure. However, pretreatment with melatonin enabled those plants to maintain significantly higher K levels in their leaves when compared with plants that received no melatonin. Likewise, under a K deficiency or all nutrients deficiency, the application of melatonin was associated with significantly higher K levels in the shoots and leaves when compared with plants not exposed to melatonin. Potassium influences cellular homeostasis by contributing to the charge balance, osmotic adjustments, and enzyme catalysis.[58,59] Increasing evidence suggests that improvement of the K nutritional status in plants can greatly lower ROS production by reducing the activity of NAD(P)H oxidases and maintaining photosynthetic electron transport. By contrast, a lack of sufficient potassium can cause a severe decline in photosynthetic CO_2 fixation and an impairment in the partitioning and utilization of photosynthates.[59]

Plant root system architecture is essential for nutrient acquisitions from the soil. We showed here that melatonin can affect the uptake of potassium because this growth regulator influences root structure. Chen et al.[55] have found that melatonin stimulates the growth of roots from etiolated seedlings of *Brassica juncea*. Furthermore, Zhang et al.[31] have reported with cucumber (*Cucumis sativus*) that melatonin stimulates the development and vigor of roots and also increases the root:shoot ratio. Our results are in accord with those from previous investigations. Alterations in plant growth and root system architecture in response to melatonin might occur through a signaling mechanism similar to that of mineral nutrients. Pelagio-Flores et al.[33] have suggested that melatonin regulates the architecture of the *Arabidopsis* root system, probably independent of auxin signaling. Therefore, the existence of a signaling pathway responsible for this developmental influence by melatonin is an interesting possibility that merits further research.

To study the mechanism by which melatonin regulates potassium uptake in plants under stress conditions, we conducted trails with hydroponics solutions in which all nutrients were deficient. Northwestern China is an important region for apple cultivation, even though poor soils are common there. Therefore, it is critical that we improve our understanding of how melatonin regulates potassium uptake under conditions where all nutrients are lacking. In the ascorbate–glutathione (AsA–GSH) cycle, the enzymatic action of APX reduces the accumulation of H_2O_2, using AsA as an electron donor. Oxidized ascorbates, for example, the monodehydroascorbate radical and dehydroascorbate, are then enzymatically reduced back to AsA by NADPH- or NADH-dependent monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), respectively. Oxidized glutathione is reduced back to GSH by an NADPH-dependent glutathione reductase (GR).[36] Thus, the activity of antioxidant enzymes and the redox state of primary antioxidants play important roles in protecting plant cells against free radical damage. Our results demonstrated that *MdcAPX*, *MdDHAR1*, *MdDHAR2*, *MdMDHAR*, and *MdcGR* were markedly upregulated in control and stressed plants during the middle stage of our experiments where supplies of all nutrients were insufficient. Moreover, the addition of melatonin upregulated all of these antioxidant enzyme genes

in stressed plants when compared with the control, thereby indicating that melatonin and ROS have opposing roles in low-nutrition signaling.

In plant roots, K^+ absorption from soils is mainly mediated by K^+ channels and transporters for which transcription may be induced and activities enhanced in response to K^+-deficient stress.[60] We measured the transcript levels of such transporters—*MdHKT1*, *MdSOS1*, *MdNHX2*, *MdNHX4*, *MdNHX6*, *MdAKT1*, *MdAKT2/3*, *MdKEA2*, and *MdKAT2*—and found that all were significantly induced under the all-nutrition deficiency conditions. Furthermore, the addition of melatonin upregulated all of those transporters, suggesting that melatonin has a critical function in controlling K transporters under such deficits.

Although these results provide evidence that melatonin has a unique role in regulating these transporters, further research is required to clarify the exact mechanism by which this is achieved. Reactive oxygen species are involved in pathways for low-potassium signaling and may also serve as an upstream regulator of calcium signaling, as sensed by calcineurin B-like proteins.[5,19] These CBLs act as membrane-anchored Ca^{2+} sensors that, when activated, recruit a specific set of Ser-Thr protein kinases, that is, CIPKs, to their sites of action.[21,61] Xu et al.[18] have found that the *AtCBL1/AtCBL9–AtCIPK23* complex can directly activate the plasma membrane-localized potassium channel *AtAKT1*, enhancing K^+ uptake under low-K^+ conditions. Here, *MdCBL1* and *MdCIPK23* were not regulated when all nutrients were deficient, and upregulation of the two related genes was more dramatic in melatonin-pretreated plants. This demonstrates that melatonin can regulate the CBL1–CIPK23 pathway and may influence the expression of the potassium channel protein gene. Further research is required to clarify the exact mechanism by which melatonin affects potassium uptake.

In conclusion, when apple plants are pretreated with melatonin, they show greater tolerance and adaptability to future salt and nutrition deficiency stresses. Our data also provide evidence that melatonin has comprehensive physiological actions in various tissues, such as improved plant development and greater potassium absorption, possibly as a consequence of changes to the root system architecture. The qRT-PCR results indicate that melatonin has an important role in regulating K uptake. When nutrient concentrations are low, ROS are produced that trigger calcium fluctuations. However, *MdCBL1* binds Ca and interacts with *MdCIPK23*, a gene that activates *MdAKT1* and possibly other K channels involved in the control of K uptake and turgor. Therefore, we propose that the mode of action by melatonin involves regulation of the ROS signal, followed by activation of the CBL1–CIPK23 pathway to regulate the expression of the potassium channel protein gene, which then promotes K^+ absorption.

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CONFLICT OF INTEREST

The authors declared that they have no competing financial interests.

AUTHOR CONTRIBUTIONS

CL and BWL performed and analyzed most of the experiments in this study, with assistance from CC, ZWW, and SSZ. FWM provided all financial support and critical intellectual input in the design of this study and preparation of the manuscript. CL designed this study and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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