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Melatonin regulates carbohydrate metabolism and defenses against *Pseudomonas syringae* pv. *tomato DC3000* infection in *Arabidopsis thaliana*

Abstract: Melatonin has been reported to promote plant growth and development. Our experiments with *Arabidopsis thaliana* showed that exogenous applications of this molecule mediated invertase inhibitor (C/VIF)-regulated invertase activity and enhanced sucrose metabolism. Hexoses were accumulated in response to elevated activities by cell wall invertase (CWI) and vacuolar invertase (VI). Analyses of sugar metabolism-related genes revealed differential expression during plant development that was modulated by melatonin. In particular, C/VIF1 and C/VIF2 were strongly down-regulated by exogenous feeding. We also found the elevated CWI activity in melatonin-treated *Arabidopsis* improved the factors (cellulose, xylose, and galactose) for cell wall reinforcement and callose deposition during *Pseudomonas syringae* pv. *tomato DC3000* infection, therefore, partially induced the pathogen resistance. However, CWI did not involve in salicylic acid (SA)-regulated defense pathway. Taken together, this study reveals that melatonin plays an important role in invertase-related carbohydrate metabolism, plant growth, and pathogen defense.

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Key words: *Arabidopsis*, invertase, melatonin, *Pseudomonas syringae*, sucrose metabolism

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Introduction

In most higher plants, sucrose (α-D-Glc-(1→2)-β-D-Fru) is the major transport form of reduced carbon and energy from source to sink tissues. In the sink tissues, it is either metabolized by sucrose synthase (EC 2.4.1.13), yielding UDPG and fructose in a reversible reaction, or else hydrolyzed to glucose and fructose by invertases (EC 3.2.1.26) in an irreversible reaction [1]. In all higher plants, hydrolysis by invertases may occur in the cytosol, the vacuole, and the apoplast. Based on the pH optimum, invertases are classified into neutral/alkaline or acid groups. Two enzymes in the latter group, cell wall invertase (CWI), and vacuolar invertase (VI) are evolutionarily related and belong to family 32 of the glucoside hydrolases (GH 32) [2, 3].

In addition to being active in primary carbon metabolism, invertases make a wide range of regulatory functions in plant growth and development [4, 5]. The main functions for CWIs include sucrose partitioning, responses to wounding and pathogen infections, and seed and pollen development. Roles for VIs include sucrose partitioning in fruits and storage organs; osmo-regulation and cell enlargement; and responses to drought stress, hypoxia, gravitropism, and wounding. Two CWIs and two VIs have been identified in *Arabidopsis thaliana*, with each having distinct roles during various developmental stages [6, 7].

Both CWI and VI can be regulated by environmental stimuli, for example, drought, temperature, salinity, hormones, sugars, wounding, and pathogens [5, 8, 9]. They are also subject to post-translational control by specific invertase inhibitor proteins (C/VIF) [10] that can silence invertase activity at certain developmental stages [11]. To date, cDNAs encoding two inhibitor proteins in *Arabidopsis* have been isolated and functionally identified [12].

Plant pathogens cause numerous crop diseases and are associated with yield losses and the production of mycotoxins. Plants utilize highly complex defense–response systems. The gram-negative bacterium *Pseudomonas syringae* [13] comprises more than 50 pathovars that individually infect a comparatively narrow range of plant hosts. Disease symptoms can include blights, leaf spots, and galls. In nonhost or resistant plants, a hypersensitive response occurs at the site of infection but does not lead to disease onset. *Pseudomonas syringae* pv. *tomato* strain *DC3000* infects host plants such as *A. thaliana* and tomato. The pairing between that strain and *Arabidopsis* presents an important model system for experimental characterization of the molecular dynamics of plant–pathogen interactions [14, 15].

Melatonin (*N*-acetyl-5-methoxytryptamine) is synthesized from the essential amino acid L-tryptophan [16] and is universal in mammals, bacteria, fungi, and many plants [17–20]. In plants, its functions include controlling circadian rhythms and photoperiodic reactions [21, 22], scavenging reactive oxygen species as an antioxidant [23–27]; protecting against UV and ozone damage [28, 29]; regulating photosynthetic systems and protecting chlorophyll during senescence [30–32]; promoting tolerance to cold and drought [33–36]; alleviating tissue damage upon

exposure to heavy metals [37, 38]; improving salt tolerance [39, 40]; and reducing susceptibility to diseases [41, 42]. Generally, at low concentrations, this phenomenon can promote seed formation, seedling growth, and root system development [38, 43–45]. However, its physiological and molecular roles remain unknown. In this study, we investigated how the application of exogenous melatonin (i) regulates sucrose metabolism, (ii) influences seedling development, and (iii) affects plant defenses against bacterial pathogen. Our objective was to determine whether melatonin can enhance carbohydrate metabolism and provide immunity against bacterial infections in plants.

Material and methods

Plant material and growth conditions

Seeds of *A. thaliana* (Columbia, ecotype Col-0) were sown in soil under short-day conditions (8 hr of light at 24°C, 16 hr of darkness at 18°C, and ~50% humidity). To promote their germination in Petri dishes, seeds were surface-sterilized by briefly incubating them three times in a bleaching solution containing 4% NaOCl in water supplemented with 0.02% of Triton X-100. After being washed three times with sterile ddH_2O, seeds were re-suspended in 0.1% plant agar. The 1/2 strength Murashige and Skoog (MS) medium contained 2% sucrose and 5–8 g of agar (pH 6) and was autoclaved for 20 min at 121°C. For testing the effects of exogenous pretreatment, this medium was supplemented with 0 or 50 μM melatonin (Sigma-Aldrich, St. Louis, MO, USA) that was filter-sterilized and added to the cooled MS medium (~55°C) under low light. A pipette with a cut tip was used to place sterilized seeds in Petri dishes containing one of those media types. The dishes were then sealed with Micropore surgical tape (3 M) and incubated at 4°C for 3 days before being transferred to a growth chamber set at 22°C and under a 16-hr photoperiod.

Assays of enzyme activity

Leaf and root tissues were sampled from 5 days after germination (DAG) of *A. thaliana* seedlings, ground separately in liquid nitrogen, and homogenized in 2 mL/g extraction buffer (30 mM MOPS, 250 mM sorbitol, 10 mM $MgCl_2$, 10 mM KCl, and 1 mM PMSF, pH 6.0). After centrifugation (10 min, 8500 g, 4°C), the pellets were washed once (10 min) with extraction buffer with 1% Triton X-100 and twice with only extraction buffer. The cell wall pellets were re-suspended in 1 mL/g assay buffer (20 mM triethanolamine, 7 mM citric acid, and 1 mM PMSF, pH 4.6), and used for the determination of CWI activity. For measuring VI activity, endogenous sucrose was removed by acetone precipitation of the soluble fraction with four volumes of ice-cold acetone (20 min, −20°C). After centrifugation (15,000 g, 10 min, 4°C), the pellets were re-suspended in one volume of assay buffer. Activity was monitored by mixing 20–100 μL of invertase preparation, 100 μL of sucrose (100 mM in assay buffer), and the assay buffer up to a volume of 300 μL. After incubation at 37°C for 1 hr, invertase activity was measured by enzymatic determination of the released glucose in a coupled enzymatic-optical assay with hexokinase and glucose-6-phosphate dehydrogenase, according to the method of Jansen [46].

Carbohydrate extraction and analysis

Total soluble carbohydrates were extracted by heating 200 mg of frozen, homogenized tissue in 1 mL extraction buffer (50 mM Na-acetate, pH 5, 10 mM $NaHSO_3$, 0.02% (w/v) Na-azide and 0.1% (w/v) Polyclar AT) for 15 min at 95°C, followed by centrifugation at 1000 g for 5 min and centrifugation of the supernatant at 10,000 g for 5 min. Glucose, fructose, sucrose, raffinose, stachyose, 1-kestotriose, 1,1-kestotetraose, and 1,1,1-kestopentaose were quantified via high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described by Kusch et al. [47]. For peak identifications, glucose, fructose, raffinose, stachyose, xylose, galactose (all Sigma-Aldrich), sucrose (Applichem, Darmstadt, Gemany), 1-kestotriose, 1,1-kestotetraose, and 1,1,1-kestopentaose (all Wako Chemicals, Osaka, Japan) were used as external standards.

RNA extraction and quantitative real-time PCR

Total RNA was extracted with GeneMATRIX Universal RNA purification Kit from EURx according to the manufacturer's instructions. Nucleic acid concentrations were determined spectrophotometrically (Nanodrop; Thermo Scientific, Waltham, MA, USA). Purity was controlled by calculating the ratio of OD260 nm/OD280 nm, which ideally should be 1.8–2.0. In addition, RNA quality was tested by electrophoresis on a denaturing agarose gel. The cDNA was prepared from DNase-treated RNA with AMV Reverse Transcriptase (Roboklon, Berlin, Germany) according to the manufacturer's instructions. The SYBR green (Invitrogen, Carlsbad, CA, USA) method was used to perform qRT-PCR on Rotor Gene 6000 Real-time PCR machine (Qiagen, Hilden, Germany). Three biological replications were carried out for each treatment, and three technical repeats were performed for each biological replication. Transcripts of the *Arabidopsis* Actin2/8 gene were used to standardize the cDNA samples for different genes. All primers are listed in Table S1.

Assay of pathogen infection

Colonies of *P. syringae* pv. *tomato DC3000* (*Pst DC3000*) were cultured in a liquid Luria-Bertani medium containing 50 mg/L rifampicin at 28°C until the OD600 reached 0.8–1.0. The bacterial cells were collected and re-suspended in distilled, deionized water to achieve an OD600 of 0.2 (equivalent to 10^8 CFU/mL). After further dilution to 10^5 CFU/mL, the surfactant L-77 Silwet was added to reach a level of 0.004% (40 μL/L). For testing melatonin influences bacterial growth in whole plants, 50 or 100 μM melatonin was added to the bacterial suspension (10^5 CFU/mL) and vacuum-infiltrated simultaneously with the bacteria into 5-week-old *Arabidopsis* seedlings (five leaves per plant) [48]. For testing melatonin and acarbose influences bacterial growth in whole plants, 50 μM melatonin plus 20 mM acarbose were added to the

bacterial suspension (10^5 CFU/mL) and vacuum-infiltrated simultaneously with the bacteria into 5-week-old *Arabidopsis* seedlings (five leaves per plant) [48]. To calculate bacterial populations, three fully grown leaves on 5-week-old *Arabidopsis* seedlings per plant were infiltrated with a bacterial suspension of 10^5 CFU/mL. Afterward, those bacteria were sampled by removing three leaf disks with a cork borer (Φ = 1 cm). Pathogen enumeration was conducted as described previously [48] using three replicates per sampling.

Cell wall composition assays

Cell wall composition was analyzed according to Xiong et al. [49]. In short, 500 mg fresh weight leaves from inoculated regions were ground into fine powder, washed with 70% ethanol and chloroform and methanol (1:1 v/v) mixture, then overnight in 0.1 M NaOAc buffer (pH 5.0) plus pullulanase M1 (Megazyme, Bray, Ireland) and α-amylase (Sigma, St. Louis, MO, USA). The released sugar and derivatives were assayed by HPAEC-PAD as described above.

Callose deposition analysis

For the detection of callose deposition, leaf disks were cut from inoculated regions and incubated with ethanol:acetic acid solution (1:3, v/v) overnight to completely remove leaf pigments. After rehydration, the callose deposition was detected as described previously [50].

Salicylic acid (SA) measurements

Levels of free SA were measured as described previously [51]. Briefly, 100 mg of leaf tissues was frozen in liquid nitrogen and thoroughly ground. Afterward, 250 μL of acetate buffer (0.1 M, pH 5.6) was added and the samples were then mixed and centrifuged for 15 min at 16,000 g. The supernatant was stored on ice. An Acinetobacter sp. ADPWH_lux strain was prepared to an OD600 of 0.35 and incubated on a microtiter plate for 2 hr with 20 μL of crude plant extract. Luminescence was detected on a plate reader (Fluostar Omega; BMG LabTech, Offenburg, Germany). Comparisons were made with standards containing known concentrations of SA as well as extracts taken from untreated wild-type plants.

Statistical analysis

The results presented were obtained from three independent experiments. Data were expressed as mean ± S.D. All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). Data were analyzed using independent *t*-tests at a significance level of $P < 0.05$ (*), or using one-way ANOVA Duncan's multiple range tests at a significance level of $P < 0.05$.

Results

An HPAEC-PAD chromatogram was used for carbohydrate analysis of *Arabidopsis* seedlings exposed to either mock (untreated control) condition or melatonin pretreatment. For this presentation, we focused primarily on data from experiments with 50 μM melatonin because previous analyses by our research group and others had identified it as the optimal concentration for improving the growth and development of *Arabidopsis* seedlings [45]. Our results here showed that exposure to melatonin significantly promoted sucrose metabolism in both leaves and roots. Compared with the mock, application of 50 μM melatonin significantly induced the hydrolyzation of sucrose into glucose and fructose (by 7- to 9-fold higher) in both tissue types from seedlings in 5 DAG (Table 1). Furthermore, the amount of synthesized and accumulated sucrose was 16 times greater in the roots from melatonin seedlings. The HPAEC-PAD chromatogram also showed that raffinose and stachyose were accumulated in the roots of treated plants (Fig. 1).

Measurements of enzymes indicated that activities of both CWI and VI were elevated in the leaves and roots of plants grown from seeds that had received 50 μM melatonin (Fig. 2). Real-time PCR analyses were conducted for genes that encode metabolic enzymes related to sucrose metabolism. For this, we examined seedlings from 1 to 7 DAG which represents the most important early seedling growth stage. Compared with the plants without melatonin treatment, expression of VI1 and VI2 was mainly induced in the roots. In the leaves, VI1 expression was up-regulated as observed but not for VI2 only a slightly change was found (Fig. 3A,B). Under melatonin treatment, CWI1, CWI5, and, specifically, CWI4 were up-regulated in the leaves (Fig. 3C–E). In the roots, CWI1 was not significantly affected while CWI5 was significantly down-regulated in response to melatonin (Fig. 3C–E). Expression of CWI2 was under detected level in either tissue type.

Additional transcripts analysis revealed that both C/VIF1 and C/VIF2 were down-regulated in treated leaves and roots (Fig. 3F–G). This declined level was by approximately threefold for C/VIF1 in the roots. For 6&1-fructan exohydrolase (6&1-FEH), melatonin increase its expression

Table 1. Effects of melatonin on hexose and sucrose contents in *Arabidopsis* leaves and roots at 5 DAG (days after germination). Data represent mean ± S.D. of three replicate samples. Different letters indicate significant differences according to a Duncan's multiple range test ($P < 0.05$)

Sugar contents (μmol/g)		Glucose	Fructose	Sucrose
Leaf	Control	4.12 ± 1.01b	3.38 ± 0.36ab	0.70 ± 0.07a
	50 μM	26.54 ± 3.56f	20.10 ± 3.14e	1.76 ± 0.13ab
Root	Control	2.47 ± 0.31ab	2.00 ± 0.27ab	1.23 ± 0.09ab
	50 μM	18.26 ± 2.01de	13.75 ± 1.97c	16.89 ± 2.34d

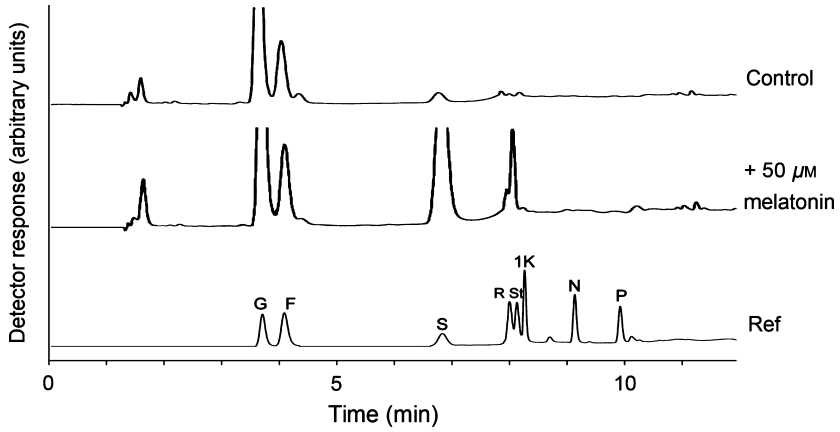

Fig. 1. Melatonin-induced synthesis of raffinose and stachyose in vivo in roots from 5 DAG *Arabidopsis thaliana* seedlings. HPAEC-PAD chromatogram was used for carbohydrate analysis. Seeds were cultured with or without 50 μM melatonin. Ref, reference; G, glucose; F, fructose; S, sucrose; R, raffinose; St, stachyose; 1K, 1-kestotriose; 6K, 6-kestotriose; N, 1,1-nystose; P, 1,1,1-kestopentaose. DAG, days after germination.

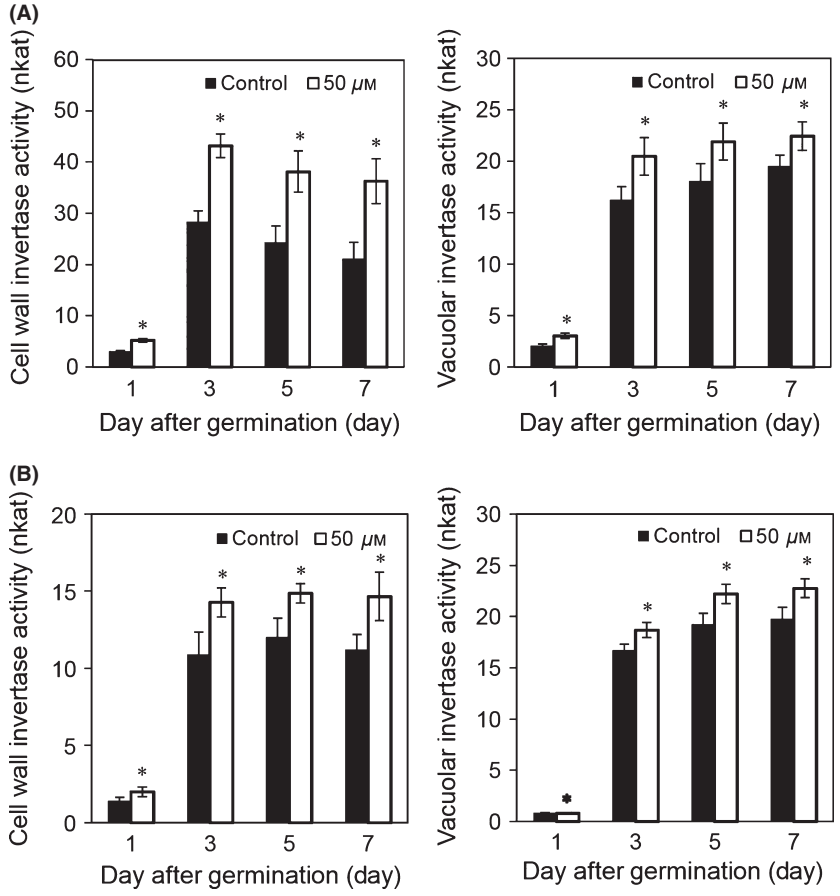

Fig. 2. Effect of activities by cell wall and vacuolar invertases in leaves (A) and roots (B) from 5 DAG *Arabidopsis* plants grown from seeds sown on half-strength MS medium with or without 50 μM melatonin. The results shown are the mean ± S.D.s (n = 3), and asterisk (*) indicates significant differences at $P < 0.05$ in comparison with the control.

only in the leaves, by threefold (Fig. 3H). The similar increase was observed for 6-FEH in the leaves, but contrasted with a significant reduction in the roots (Fig. 3I).

Previous work demonstrated that exogenous melatonin was involved in defense against the virulent bacterial pathogen *P. syringae DC3000* (*Pst DC3000*) in *Arabidopsis* [42]. Plants treated with 50 or 100 μM melatonin displayed less chlorosis and fewer necrotic lesions at 72 hours postinoculation (hpi) as compared with the mock (Fig. 4A). New leaves that emerged from the shoot-tip meristems of treated plants were not as severely affected as their mock counterparts (Fig. 4B). Furthermore, at 48 hpi, the linear stage for *Pst DC3000* reproduction in *Arabidopsis* leaves, the rate of bacterial growth was significantly slow in melatonin-treated plants (Fig. 4C).

Melatonin also induced acid invertase activity during pathogen infections. At 24 hpi, CWI activity from melatonin-treated plants was showed 3.4 and 2.8 times higher than those from the mock and without melatonin-treated controls, respectively (Fig. 5C). At 48 hpi, the activity fold changed into 3 and 2.4 times, respectively (Fig. 5C). For vacuolar invertase, its activity was induced slightly

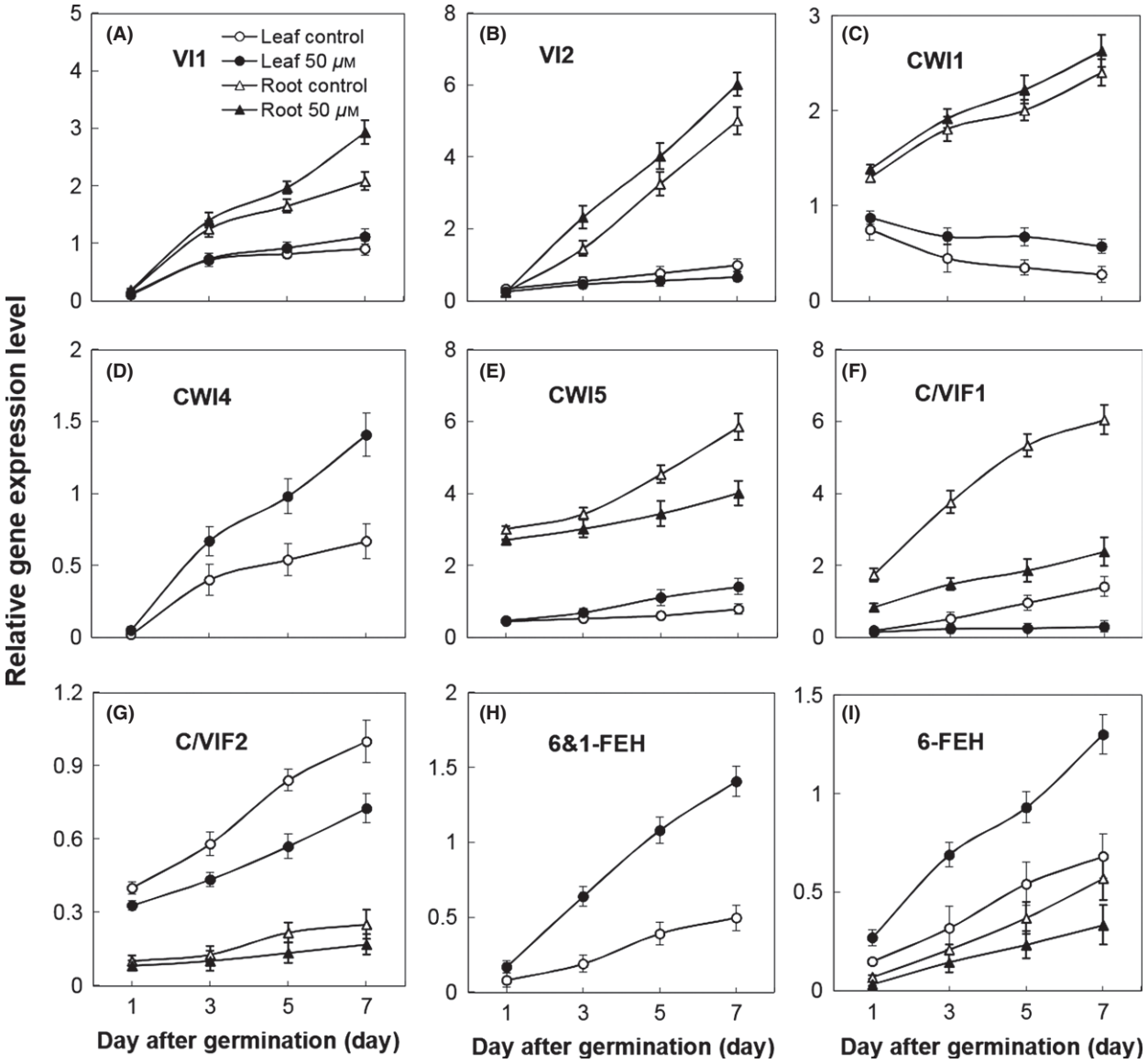

Fig. 3. Analysis of genes related to sugar metabolism in leaves and roots from 1 to 7 DAG *Arabidopsis* seedlings with or without melatonin (50 μM) treatment conditions. Expression of VI1 (A), VI2 (B), CWI1 (C), CWI4 (D), CWI5 (E), C/VIF1 (F), C/VIF2 (G), 6&1-FEH (H), and 6-FEH (I) were normalized relative to Actin2/8. Results are means of 3 biological replicates (±S.E.), each with 4 technical replicates. VI, vacuolar invertase; CWI, cell wall invertase; C/VIF, cell wall and vacuolar invertase inhibitor; FEH, fructan exohydrolase.

by melatonin (data not shown). To address the functional importance of this elevated CWI by melatonin on pathogen response, pseudo-tetrasaccharide acarbose, which has been shown to act as a competitive inhibitor of various invertase [52–54], was used for keeping CWI activity inhibited post-translationally in the presence of the pathogen. Results indicated 20 mM acarbose drastically decreased CWI activity to nearly ground level when it was co-inoculated with *Pst DC3000* and melatonin in *Arabidopsis* leaves (Fig. 5C). Symptom monitor and bacteria population test further supported that acarbose may performed as a suppressor for the increased CWI activity, and subsequently, defense response was hampered (Fig. 5A,B).

Plant cell wall mainly consists of carbohydrates, it as a major structurally barriers help plant cells limit pathogen attachment, invasion, and infection. Measurement of three important cell wall constituents (cellulose, xylose, and galactose) showed that melatonin prompted an increase in their levels on bacteria infected *Arabidopsis* leaves (Fig. 6). However, the addition of acarbose did not increase cellulose, xylose, and galactose levels and slightly decreases can be found in comparison with non-melatonin-treated and the mock controls (Fig. 6). Callose deposition deploys an effective defense barrier against pathogen attack [55, 56]. As depicted in Fig. 7, melatonin led to the increase in callose deposition, whereas acarbose abolished this promotion during *Pst DC3000* infection. Together, these findings demonstrated that melatonin trigged defense response is partially through the regulation of CWI to reinforce the cell wall-based and callose-based physical defense.

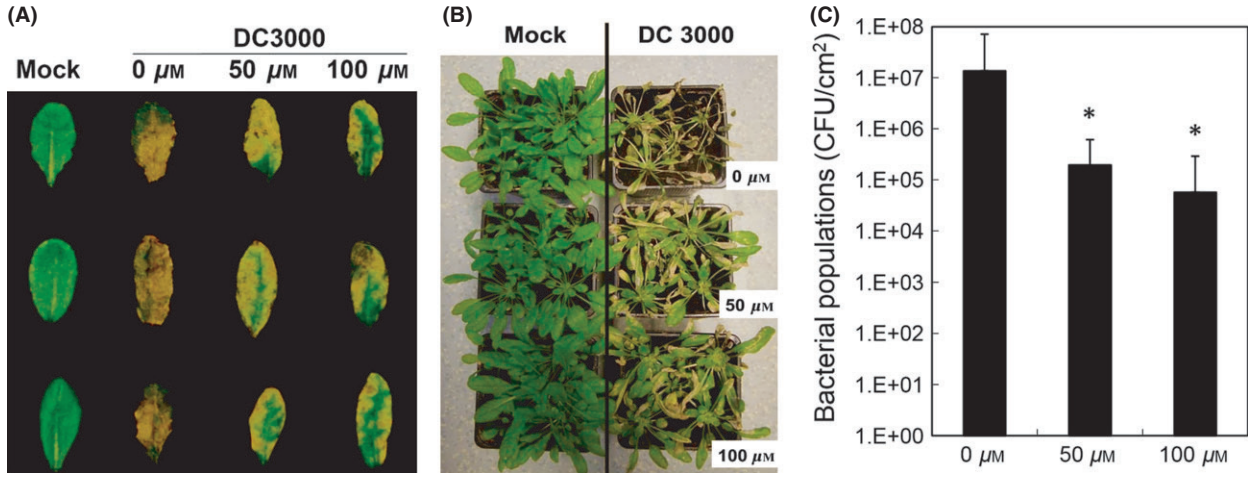

Fig. 4. Effect of melatonin on susceptibility of 5-week-old *Arabidopsis* seedlings to *Pst DC3000* after vacuum-infiltration (10^5 CFU/mL). (A) Leaf phenotypes and (B) growth performance were recorded at 72 hpi, (C) bacterial populations were calculated at 48 hpi. Results are displayed as means for 3 leaves from 3 separate plants. Bars represent mean ± S.D. Statistical differences were compared by independent *t*-tests at $P < 0.05$ (*). Asterisk (*) indicates significant differences at $P < 0.05$ in comparison with the only *Pst DC3000* infiltrated samples. hpi, hours postinoculation.

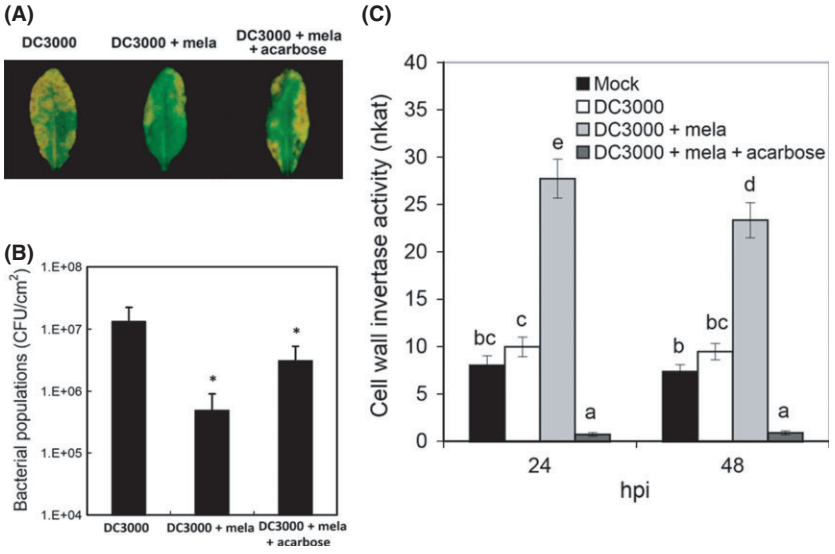

Fig. 5. Effect of invertase inhibition by acarbose on melatonin-regulated defense response during *Pseudomonas syringae DC3000* infection. Bacterial suspensions (10^5 CFU/mL) without melatonin, with 50 μM melatonin, or with 50 μM melatonin plus 20 mM acarbose, were vacuum-infiltrated in 5-week-old *Arabidopsis* leaves. (A) Leaf symptoms and (B) bacterial populations were performed at 48 hpi. (C) Cell wall invertase activities at 24 and 48 hpi. Results are displayed as means for 3 leaves from 3 separate plants. Bars represent mean ± S.D. Statistical differences were compared by independent *t*-tests at $P < 0.05$ (*) or Duncan's multiple range tests at $P < 0.05$. hpi, hours postinoculation.

The SA pathway is a major contributor to the successful defense against pathogen. *Pst DC3000* infected plants with 50 μM melatonin treatment showed higher SA accumulation than the non-melatonin-treated plants in 24 and 48 hpi (Fig. 8). Interestingly, extra acarbose input in *Pst DC3000* infection did not decrease the SA amount, adversely with slightly increase (Fig. 8), reflecting acarbose-inhibited CWI activity functionally independent of SA-related defense resistance. Expression of the SA marker gene PR1 and PDF1.2 were visualized with up-regulation by melatonin (Fig. 8). However, almost no transcript changes on PR1 and PDF1.2 were found with acarbose addition (Fig. 8). These results prompted the hypothesis that melatonin mediate SA-regulated defense pathway as a consequence to strength pathogen resistance in *Arabidopsis*. However, we have not founded any correlation between CWI and SA-regulated defense pathway during pathogen infection.

Discussion

Nonreducing disaccharide sucrose is essential for plant development and plays a pivotal role in higher plant metabolism regulation. As a major form of carbohydrate, sucrose is produced via photosynthesis in source tissues and transported through the phloem to sink tissues to maintain heterotrophic metabolism and plant growth [8]. Long-distance transport of assimilates is driven by a concentration gradient between the source and sink tissues. Sucrose also acts as a signaling molecule [57]. At low

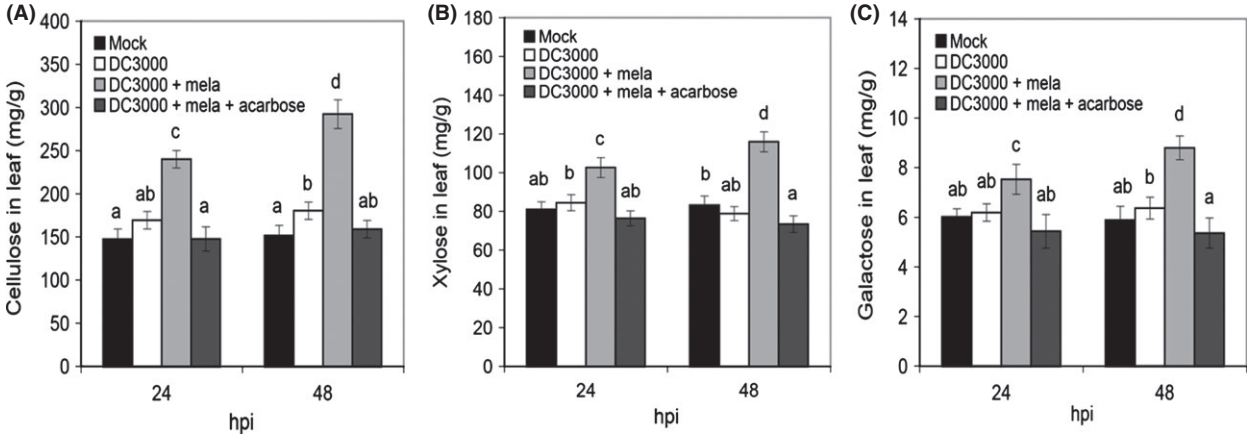

Fig. 6. Effect of invertase inhibition by acarbose on melatonin-regulated cell wall composition in *Arabidopsis* leaves during *Pseudomonas syringae DC3000* infection. 10 mM $MgCl_2$ (Mock); or bacterial suspensions (10^5 CFU/mL) without melatonin, with 50 μM melatonin or with 50 μM melatonin plus 20 mM acarbose were vacuum-infiltrated in 5-week-old *Arabidopsis* leaves, respectively. (A) Cellulose, (B) xylose, and (C) galactose levels were measured at 24 hpi and 48 hpi. Data represent mean ± S.D. of three replicate samples. Different letters indicate significant differences according to Duncan's multiple range tests ($P < 0.05$). hpi, hours postinoculation.

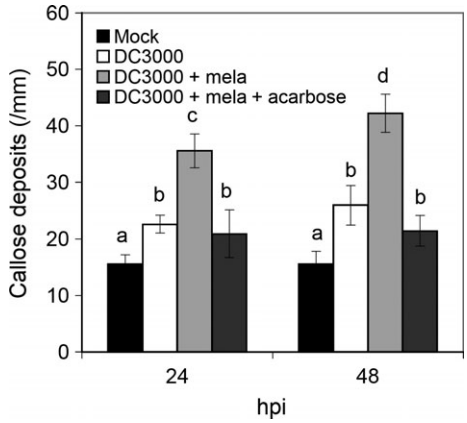

Fig 7. Effect of invertase inhibition by acarbose on melatonin-regulated callose deposition in *Arabidopsis* leaf cell walls during *Pseudomonas syringae DC3000* infection. 10 mM $MgCl_2$ (Mock); or bacterial suspensions (10^5 CFU/mL) without melatonin, with 50 μM melatonin or with 50 μM melatonin plus 20 mM acarbose were vacuum-infiltrated in 5-week-old *Arabidopsis* leaves, respectively. Callose levels were measured at 24 hpi and 48 hpi. Data represent mean ± S.D. of three replicate samples. Different letters indicate significant differences according to Duncan's multiple range tests ($P < 0.05$). hpi, hours postinoculation.

concentrations, melatonin can promote plant growth [44, 58]. This has been demonstrated in *Arabidopsis* where that molecule enhances primary-root formation and seedling fresh weights [45]. Our assays of sucrose metabolism showed that seeds treated with 50 μM melatonin resulted in higher sucrose synthesis and improved seedling development (Table 1). Two members of the raffinose family of oligosaccharides (RFOs), raffinose and stachyose, are derived from sucrose [59]; both were accumulated in the roots of melatonin-fed plants (Fig. 1). We concluded that these RFOs may protect plant cells from oxidative damage [60] and also increase tolerances to drought and salinity [61] as well as chilling stress [62].

The value of sucrose as a carbon and energy source depends upon its irreversible hydrolysis into glucose and fructose by CWI and VI. Like sucrose, glucose, and fructose are important signaling molecules participating in metabolism, cell expansion, osmotic adjustments, and vacuolar sugar storage [63, 64]. Our analysis showed that exogenous treatment with 50 μM melatonin enhanced leaf and root fresh weights in parallel with increased invertase activities (Fig. 2). Melatonin is structurally similar to Indole-3-acetic acid (IAA), and their biosynthetic pathways share the same precursor, tryptophan [65]. Because cross talk of sugar signaling and hormonal pathways (including IAA) is involved in the tight regulation of plant growth and metabolism [66], one hypothesis has suggested that melatonin serves as a type of hormone or stimulus of invertase-related sucrose metabolism. Recently, some genes in melatonin biosynthesis pathway have been cloned and well identified [67–69], but it is still unclear whether those auxin-like affects are due to the action of melatonin itself or if melatonin is instead converted into IAA.

Various hormones and stimuli can influence invertase genes expression with spatial and temporal patterns. Regulation of invertase activity is primarily found at the transcriptional level [10], and invertase transcript is highly responsive to hormones and other stimuli [70–72]. We noted that the expression of CWI1, CWI4, VI1, and VI2 were induced upon melatonin treatment (Fig. 3C,D,A,B). This was especially dominant for CWI4, with specific expression in the leaf, where transcript levels increased by approximately threefold (Fig. 3D). In addition to CWIs and VIs, another regulatory mechanism of invertase activity is subject to post-translational control by specific invertase inhibitor proteins. Those have been identified in *Arabidopsis*, where they are encoded by genes for C/VIF1 and C/VIF2, with C/VIF1 being specific to VI activity while C/VIF2 inhibits both CWI and VI [13]. Melatonin significantly suppressed the C/VIF1 and C/VIF2 transcripts was observed in *Arabidopsis* (Fig. 3F,G), indicated that the reduced expression of invertase inhibitors may

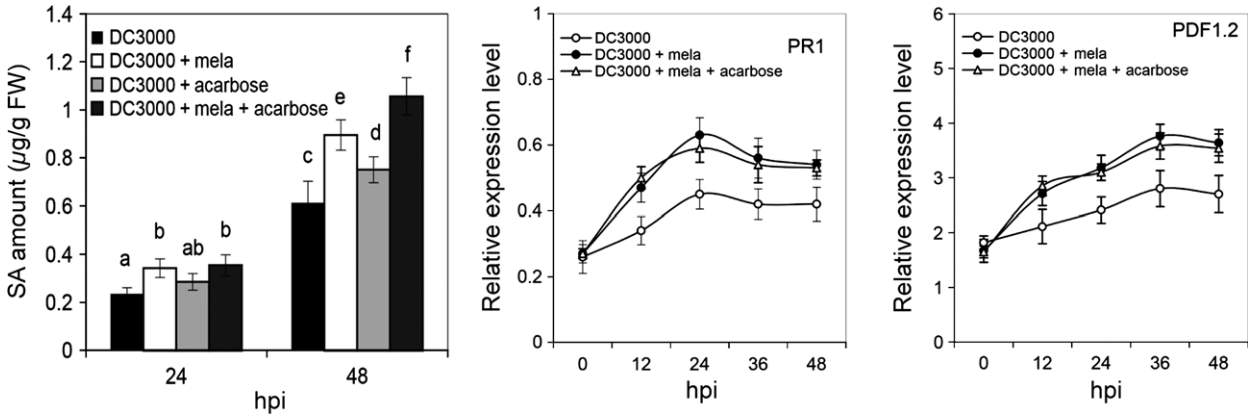

Fig. 8. Effect of invertase inhibition by acarbose on melatonin-regulated salicylic acid (SA)-related defense response during *Pseudomonas syringae DC3000* infection. Bacterial suspensions (10^5 CFU/mL) without melatonin, with 50 μM melatonin, with 20 mM acarbose, or with 50 μM melatonin plus 20 mM acarbose were vacuum-infiltrated in 5-week-old *Arabidopsis* leaves, respectively. SA levels (left), transcript of PR1 (middle), and PDF1.2 (right) were measured at 24 and 48 hpi. Data represent mean ± S.D. of three replicate samples. Different letters indicate significant differences according to Duncan's multiple range tests ($P < 0.05$). hpi, hours postinoculation.

give rise to the increase of acid invertase activity and subsequent with stress response activation.

We also postulated that plants might have an alternative mechanism for post-translational control of invertase activities. Two CWI-related fructan exohydrolases, 6-FEH and 6&1-FEH, have already been characterized from *A. thaliana*, a nonfructan plant [73]. Our findings indicated that exogenous melatonin up-regulated transcripts of both (6-FEH and 6&1-FEH) in the leaves (Fig. 3H,I). Previous research with the defective tobacco invertase Nin88 [74] has led to a proposed model in which this defective invertase prevents nonproductive binding of active CWI to the cell wall and allows for free access of its substrate sucrose and/or invertase inhibitor binding [74]. Because of the high structural similarity between CWI and FEH, we hypothesize that *Arabidopsis* 6-FEH and 6&1-FEH bind that invertase inhibitors and contribute to increasing invertase activity in *Arabidopsis*.

Exogenous melatonin was involved in defense against the virulent bacterial pathogen *P. syringae DC3000* (*Pst DC3000*) in *Arabidopsis* [42]. Plant pathogens modify the metabolism of their hosts, which causes an increase in energy levels and greater production of carbon sources [75], for example, sucrose and its cleavage products, glucose, and fructose. Studies of the link between plant responses to bacterial pathogens and CWI activity have revealed that CWI transcripts are up-regulated after infection [76]. Previous work with *Arabidopsis* has shown that post-translational inhibition of CWI activity by acarbose resulted in more pronounced growth by *Pst DC3000* [54]. Because only C/VIF2 is expressed in mature *Arabidopsis* leaves, repression of its expression can release invertase activity and strengthen the plant defense response [54]. Similar to those findings, we showed that CWI activity was elevated at postinoculation in mature *Arabidopsis* leaves (Fig. 5C). In particular, plants fed with melatonin displayed a greater CWI activity (Fig. 5C). We hypothesized, this melatonin improved resistance to *Pst DC3000* in *Arabidopsis* might due to it down-regulated C/VIF2 and/or up-regulated CWI such that CWI activity was increased, therefore, enhanced the plant defense response.

In plants, structural barriers are essential for restricting the spread of pathogens. Cell wall reinforcements, such as elevating cell wall components, are produced after an attack is recognized and create an initial obstacle to invading pathogens [77]. It increases the plant resistance to diverse pathogens, decreases susceptibility to cell wall-degrading enzymes, prevents nutrient runoff, and restricts toxins into plant cell [55, 56, 78]. Interestingly, melatonin caused CWI activity increase enhanced the cell wall components (cellulose, xylose, and galactose) levels during *Pst DC3000* infection (Fig. 6). This may explain the decreased susceptibility to *Pst D3000* (Fig. 5). Callose is a β-(1, 3)-glucan cell wall polymer, it as another physical barrier, can limit the pathogen invasion by regulating the plasmodesmata and the sieve plates permeability [79, 80]. It is well known that callose deposition enhance plant defense needs a large amount of sugars [55, 56]. CWI also triggered the callose deposition in host plants [80, 81], forming

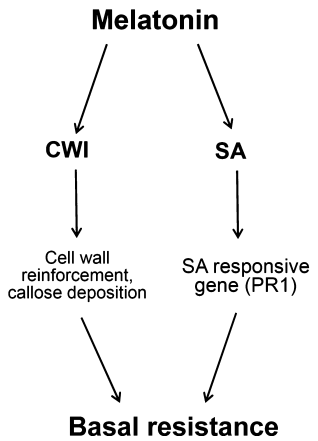

Fig. 9. Model for the melatonin-induced defense signaling pathway. CWI, cell wall invertase; SA, salicylic acid; PR1, pathogenesis-related protein 1.

an additional physical barrier against pathogen invasion through cell wall reinforcement.

Salicylic acid has crucial functions during systemic acquired resistance (SAR) induction. Its biosynthesis is promoted by multiple stimuli, for example, abiotic stresses, pathogens, and developmental triggers, except CWI (Fig. 8). The main branch of the SA signaling pathway is controlled by the NPR1 molecule, which binds to the TGA transcription factor and activates the transcription of defense-responsive genes such as PR1 [82]. It was shown that exogenous melatonin stimulated the SA synthesis and up-regulated the expression of marker genes by the pathways for SA (PR1) and JA (PDF1.2) during *Pst DC3000* infection (Fig. 8), which fit well with the previous report by Lee et al. [42]. Recently, knockout serotonin *N*-acetyltransferase in *Arabidopsis* exhibited decreased melatonin and SA levels resulting in susceptibility to an avirulent pathogen [67]. However, SA-related plant defense had no correlation with increased CWI activity by melatonin (Fig. 8), indicated that melatonin activated two different defense pathways for decreased susceptibility to *Pst DC3000* in *Arabidopsis* (Fig. 9).

Although the signaling pathways for SA form the backbone for plant defenses against pathogens, this process also includes other plant hormones and metabolites that can modulate plant immunity [83]. Whether melatonin induces the generation of phytoalexin [84] or other metabolites related to pathogen defenses need further research. In addition, melatonin may act as an antimicrobial secondary metabolite in plants. Previous reports have indicated that antimicrobial compounds camalexin [85] and glucosinolates [86] are produced in plants, and they played important roles in preventing the growth of pathogens. Interestingly, we note that the same precursor, tryptophan, is used in the biosynthesis of camalexin, glucosinolates, and melatonin.

In summary, this research provides the evidence that optimized dose of melatonin promotes the *Arabidopsis* seedlings growth and development because of its positive influence on sucrose metabolism, invertase activities, and transcription of invertase-related genes. Furthermore, melatonin decrease susceptibility to *P. syringae* pv. *tomato DC3000* in *Arabidopsis* is caused by the activation of CWI-dependent and SA-dependent pathogen defense pathways. We believe that this study may expand our understanding of the melatonin-regulated plant growth and pathogen resistance and may be useful in agricultural purposes.

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Author contributions

H.Z. and L.X. designed and performed this experiment, with assistance from T.S., Y.J., and L.H. H.Z. analyzed the data and wrote the manuscript. F.W. provided financial support, the research guidance, critical revision of the manuscript, and approval of the article.

Conflict of interest

All the authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers for qPCR.