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Hydrogen peroxide acts downstream of melatonin to induce lateral root formation

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• **Background and Aims** Although several studies have confirmed the beneficial roles of exogenous melatonin in lateral root (LR) formation, the molecular mechanism is still elusive. Here, the role of hydrogen peroxide (H_2O_2) in the induction of LR formation triggered by melatonin was investigated.
• **Methods** Alfalfa (*Medicago sativa* 'Biaogan') and transgenic *Arabidopsis* seedlings were treated with or without melatonin, diphenyleneiodonium (DPI, NADPH oxidase inhibitor), *N*,*N*′-dimethylthiourea (DMTU, H_2O_2 scavenger), alone or combined. Then, H_2O_2 content was determined with 2′,7′-dichlorofluorescein diacetate (H_2DCFDA)-dependent fluorescence and spectrophotography. Transcript levels of cell cycle regulatory genes were analysed by real-time reverse transcription–PCR.
• **Key Results** Application of exogenous melatonin not only increased endogenous H_2O_2 content but also induced LR formation in alfalfa seedlings. Consistently, melatonin-induced LR primordia exhibited an accelerated response. These inducible responses were significantly blocked when DPI or DMTU was applied. Compared with the wild-type, transgenic *Arabidopsis* plants overexpressing alfalfa *MsSNAT* (a melatonin synthesis gene) increased H_2O_2 accumulation and thereafter LR formation, both of which were blocked by DPI or DMTU. Similarly, melatonin-modulated expression of marker genes responsible for LR formation, including *MsCDKB1;1*, *MsCDKB2;1*, *AtCDKB1;1* and *AtCDKB2;1*, was obviously impaired by the removal of H_2O_2 in both alfalfa and transgenic *Arabidopsis* plants.
• **Conclusions** Pharmacological and genetic evidence revealed that endogenous melatonin-triggered LR formation was H_2O_2-dependent.

Key words: Hydrogen peroxide, lateral root, melatonin, *Medicago sativa*, transgenic *Arabidopsis* plants.

INTRODUCTION

Since the first discovery of melatonin (*N*-acetyl-5-methoxytryptamine) in the bovine pineal gland in 1958, its various physiological functions have been investigated in animals (Lerner *et al.*, 1958; Stehle *et al.*, 2011; Rosales-Corral *et al.*, 2012). In higher plants, melatonin was identified in 1995 (Hattori *et al.*, 1995), and it is synthesized from tryptophan via a four-step pathway. Four enzymes – tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), serotonin *N*-acetyltransferase (SNAT) and *N*-acetylserotonin methyltransferase (ASMT) – were characterized in this biosynthetic pathway (Byeon and Back, 2014; Arnao and Hernandez-Ruiz, 2015; Lee *et al.*, 2016). Further studies demonstrated that melatonin functions in plant responses against various biotic and abiotic stresses, such as pathogen attacks (Shi *et al.*, 2015), salt (Chen *et al.*, 2017), drought (Zuo *et al.*, 2014), cold (Han *et al.*, 2017) and heavy metal exposure (Hasan *et al.*, 2015; M. Q. Li, 2016; Gu *et al.*, 2017). The promotion of root organogenesis, including lateral root and adventitious root development, by exogenous melatonin was also found in *Lupinus albus* (Arnao and Hernández-Ruiz, 2007), rice (Liang *et al.*, 2017), cucumber (Zhang *et al.*, 2013, 2014) and *Arabidopsis* (Pelagio-Flores *et al.*, 2012; Koyama *et al.*, 2013).

Lateral root formation is regarded as a critical avoidance strategy in response to unfavourable conditions and is tightly regulated by intrinsic developmental processes, environmental inputs and hormone signalling in plants (Malamy and Ryan, 2001; Casimiro *et al.*, 2003; Aloni *et al.*, 2006). Among these, auxin positively regulates lateral root development via activating asymmetrical cell division in xylem pole pericycle cells (Ivanchenko *et al.*, 2010). Previous work suggested that melatonin-promoted *Arabidopsis* lateral root formation might be independent of auxin signalling (Pelagio-Flores *et al.*, 2012). However, genome-wide expression profiling analysis in rice demonstrated that lateral root development controlled by melatonin was promoted by the modulation of auxin signalling (Liang *et al.*, 2017). Further studies found that cyclin-dependent protein kinases (CDKs), cyclins and CDK-inhibitory proteins play key roles in the above development process (Stals and Inzé, 2001; Casimiro *et al.*, 2003; Verkest *et al.*, 2005). It was observed that Kip-related protein

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(KRP1, the cyclin-dependent kinase inhibitor) interacted with the *CDKA;1/CYCD2;1* complex to regulate the G1-to-S phase transition (Verkest *et al.*, 2005). Previous results further showed that gaseous signalling molecules, including nitric oxide (NO) (Correa-Aragunde *et al.*, 2006, 2015), carbon monoxide (CO) (Cao *et al.*, 2007; Guo *et al.*, 2008) and hydrogen sulphide (H_2S) (Fang *et al.*, 2014), induced lateral root formation via the modulation of cell cycle regulatory genes (*CYCD3;1*, *KRP2* and auxin-dependent cell cycle gene).

Ample evidence showed that reactive oxygen species (ROS) act as key signalling molecules in regulating stomatal movements and biotic and abiotic stress responses, as well as many aspects of plant development, including lateral root formation (Torres *et al.*, 2002; Foreman *et al.*, 2003; Kwak *et al.*, 2003; Miller *et al.*, 2010; Mittler *et al.*, 2011; Jiang *et al.*, 2013; Ishibashi *et al.*, 2013; Orman-Ligeza *et al.*, 2016). For example, lateral root outgrowth in *Arabidopsis* was facilitated by RESPIRATORY BURST OXIDASE HOMOLOGS (RBOH)-mediated ROS production by promoting cell wall remodelling of overlying parental tissues (Orman-Ligeza *et al.*, 2016). Similarly, it was found that ROS promoted cell division through accelerating auxin-mediated cell cycle entry (G_0-to-G_1) in alfalfa (Feher *et al.*, 2008). In guard cells, hydrogen peroxide (H_2O_2) signalling mediated by *AtrbohF* was identified as a key mediator of stomatal responses to ethylene (Jiang *et al.*, 2013). Previous results showed that exogenous melatonin enhanced plant tolerance of photo-oxidative stress in an H_2O_2-dependent manner in cucumber (H. Li, 2016). Recent studies also indicated that NADPH oxidase-dependent regulation of ROS signalling was required for melatonin-induced salinity tolerance (Chen *et al.*, 2017; Gong *et al.*, 2017). Consistently, the production of ROS triggered by melatonin was previously found in animals (Radogna *et al.*, 2009). To date, although exogenous melatonin has been implicated as an inducer responsible for lateral root development, it is not clear whether and how endogenous melatonin could govern lateral root formation.

In this report, pharmacological and molecular evidence reveals that H_2O_2 was involved in exogenous melatonin-induced lateral root formation in alfalfa seedlings. By using transgenic *Arabidopsis* overexpressing *MsSNAT*, a causal link between endogenous melatonin and H_2O_2 in lateral root formation was further established. Furthermore, a molecular mechanism was preliminarily illustrated. This work may increase our understanding of the mechanisms underlying endogenous melatonin-mediated root organogenesis.

MATERIALS AND METHODS

Plant materials and growth conditions

Commercially available alfalfa (*Medicago sativa* 'Biaogan') seeds were surface-sterilized with 5 % NaClO for 5 min and rinsed comprehensively in distilled water. After soaking overnight in darkness, uniform seedlings were cultured with quarter-strength Hoagland solution in an illuminating incubator with a 14/10-h (25 ± 1/23 ± 1 °C) day/night regime with 200 μmol m^{-2} s^{-1} irradiation (Gu *et al.*, 2017).

The *SNAT* gene of *Medicago sativa* (*MsSNAT*), encoding serotonin *N*-acetyltransferase, is homologous to the Arabidopsis SNAT gene, *AtSNAT*. Visualized fluorescence indicated that the *MsSNAT-GFP* signal is localized in the chloroplast. The binary vector pCAMBIA 1302 (AF234298) was used for alfalfa *MsSNAT* overexpression. Homogenous *MsSNAT* overexpression *Arabidopsis* lines driven by the cauliflower mosaic virus were then generated and used, and showed higher levels of endogenous melatonin (Gu *et al.*, 2017). Wild-type (Col-0) and *MsSNAT*-transgenic lines (*MsSNAT-1* and *MsSNAT-2*) were surface-sterilized and rinsed three times with sterile water, then plated on solid half-strength Murashige–Skoog (MS) medium containing 1 % sucrose and 1 % agar (pH 5.8). Plates were kept at 4 °C for 2 d and then transferred into a growth chamber with a 16/8 h (23/21 °C) day/night regime with 120 μmol m^{-2} s^{-1} irradiation (Chen *et al.*, 2017; Gu *et al.*, 2017).

Chemicals and treatments

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. In the experimental conditions, 3-d-old alfalfa seedlings were treated with 0, 1.0, 10, 50, 100 or 200 μM melatonin (Mel), or treated with or without 1 μM diphenyleneiodonium (DPI) and/or 0.5 mM *N*,*N*′-dimethylthiourea (DMTU). The treatment time-points are illustrated in the corresponding figure legends. Control seedlings were grown in quarter-strength Hoagland solution alone. Uniform 4-d-old WT and *MsSNAT* transgenic seedlings (*MsSNAT-1*, *MsSNAT-2*) were chosen, and transferred to 0.1 μM DPI or 0.1 mM DMTU as described in the corresponding figure legends. Control seedlings were grown in half-strength MS medium alone.

After various treatments, photographs were taken and the number of emerged lateral roots (>1 mm) per seedling was recorded. Lateral root length, emerged lateral root density (number of lateral roots per centimetre of primary root) and primary root length were measured using Image J (supplied by NCBI and available at http://rsb.info.nih.gov/ij). Lateral root primordia were also observed in root squash preparations and the number per seedling was quantified with an optical microscope (Stemi 2000-C; Carl Zeiss, Germany). For the subsequent biochemical and molecular analyses, only lateral root-inducible segments (in the regions of root mature zone) were used. The shoots of seedlings were removed by cutting below the root–shoot junction, and the root apical meristems were cut off.

ROS detection

Reactive oxygen species in the maturation zone were detected using a laser scanning confocal microscope (LSCM; Leica Lasertechnik, Heidelberg, Germany; excitation at 488 nm, emission at 500–530 nm). After treatments, seedlings were incubated with 20 μM 2′,7′-dichlorofluorescein diacetate (H_2DCFDA; Bright *et al.*, 2006; Chen *et al.*, 2017) in 20 mM HEPES/NaOH buffer (pH 7.5) in darkness (25 °C), followed by washing three times for 15 min each. Six individual samples were randomly selected and measured per treatment. Bright-field (BF) images corresponding to the fluorescent images are shown at the bottom right or left corners of Figs 2, 3 and 5. Fluorescence of ROS accumulation in roots (an area of ~250 000 $μm^2$ in alfalfa and 50 000 $μm^2$ in *Arabidopsis*) was quantified based on 20 overlapping confocal planes using the Leica

software package. Fluorescence was expressed as relative fluorescence units (Xie *et al.*, 2011).

Quantitative analysis of H_2O_2 was performed according to a previous method (Ma *et al.*, 2014). Regions of root mature zone (0.2 g) were ground with a mortar and pestle and extracted into 2 mL of 0.2 M $HClO_4$ on ice. The combined extracts were then centrifuged (about 10 000 rpm, 4 °C) for 15 min. Briefly, an aliquot of supernatant (500 µL) was added to 500 µL of assay reagent (0.5 mM ammonium ferrous sulphate, 50 mM H_2SO_4, 0.2 mM xylenol orange and 200 mM sorbitol). The absorbance at 560 nm was determined after 1 h of incubation in darkness (25 °C). Standard curves were obtained by adding different amounts of H_2O_2.

Gene expression analysis

Total RNA was extracted from the maturation zone of roots using a TransZol Up Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. RNA concentration and quality were checked using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). cDNAs were synthesized from 1 µg of total RNA using an EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix System (TransGen Biotech, Beijing, China).

By using the gene-specific primers (Supplementary Data Table S1), real-time quantitative reverse transcription (RT) PCR was conducted using a Mastercycler® ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) with TransStart® Green qPCR SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The expression levels of corresponding genes were normalized against an internal control gene in alfalfa and *Arabidopsis* seedlings (*MSC27* and *Atactin7*, respectively). The data were based on three independent biological replicates, and each sample was prepared in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software. Means and standard errors were calculated from at least three independent experiments with at least three biological replicates for each. For statistical analysis, data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test, and P values <0.05 or <0.01 were considered statistically significant.

RESULTS

Exogenous melatonin-induced lateral root formation in alfalfa seedlings

To assess the role of exogenous melatonin in the regulation of lateral root formation in alfalfa seedlings, a dose–response study of melatonin *in vitro* was performed. As expected, the results shown in Fig. 1A–C indicate that the addition of exogenous melatonin (1.0, 10, and 50 µM) could bring about significant increases in lateral root number and length (Fig. 1B) and lateral root density (Fig. 1C), while the changes in primary root length were not so obvious. Responses to 10 and 50 µM melatonin were maximal. A similar accelerated response in lateral root primordia was also observed (Fig. 1D, E). High concentrations (100 and 200 µM) of melatonin failed to induce lateral root formation. Considering the above results and the cost of chemicals, 10 µM melatonin was used in the following experiments.

H_2O_2 accumulation in response to melatonin

To unravel the molecular mechanism underlying melatonin-mediated lateral root formation, the levels of endogenous H_2O_2, a well-known signalling molecule responsible for lateral root development, were tested. Seedlings were loaded with the ROS-specific fluorescent dye H2DCFDA, and an LSCM was used to investigate changes in ROS-related fluorescence. Time-course analysis revealed that the accumulation of ROS in the maturation zone was induced in melatonin-treated roots, with a strong and substantial peak at 36 h over a 48-h period (Fig. 2A, B). Further analysis using spectrophotography indicated that exogenous melatonin elicited an increase in H_2O_2 content as well (Fig. 2C). These results suggest that the melatonin-induced dichlorofluorescein-dependent fluorescence was, at least partially, caused by endogenous H_2O_2.

Melatonin-induced lateral root formation is impaired by removal of endogenous H_2O_2

Subsequent work investigated the causal link between melatonin and H_2O_2 in lateral root development, using the NADPH oxidase inhibitor DPI and the H_2O_2 scavenger DMTU. In the experimental conditions, both 1 µM DPI and 0.5 mM DMTU dramatically blocked the induction of endogenous H_2O_2 content triggered by melatonin, determined by fluorescence analysis and spectrophotography (Fig. 3). For example, the presence of DPI or DMTU caused significant decreases in H_2O_2 accumulation by ~59.5 % and ~60.5 % (determined by LSCM) in melatonin-treated seedlings, compared with melatonin alone (Fig. 3A, B). H_2O_2 content, checked by using spectrophotography, showed a similar tendency (Fig. 3C).

Consistently, exogenous melatonin-triggered lateral root formation was apparently impaired by the presence of DPI or DMTU in alfalfa seedlings (Fig. 4A–C). Microscopic analysis showed that the inducing effects of melatonin on lateral root primordia could be prevented by DMTU or DPI (Fig. 4D, E). Combined with endogenous H_2O_2 accumulation (Fig. 3), these pharmacological tests indicated that H_2O_2 might be required for melatonin-induced lateral root formation in alfalfa seedlings. It was noticed that DPI or DMTU alone not only decreased the corresponding fluorescence (Fig. 3), but also inhibited lateral root formation (Fig. 4).

Genetic evidence confirmed that endogenous melatonin-induced lateral root formation was H_2O_2-dependent

To further explore the function of endogenous melatonin in plants, *MsSNAT* was overexpressed in transgenic *Arabidopsis* under the control of a CaMV 35S promoter (Gu *et al.*, 2017). Two transgenic lines (*MsSNAT-1* and *MsSNAT-2*) were used to investigate the effect of *MsSNAT* on lateral root formation. The

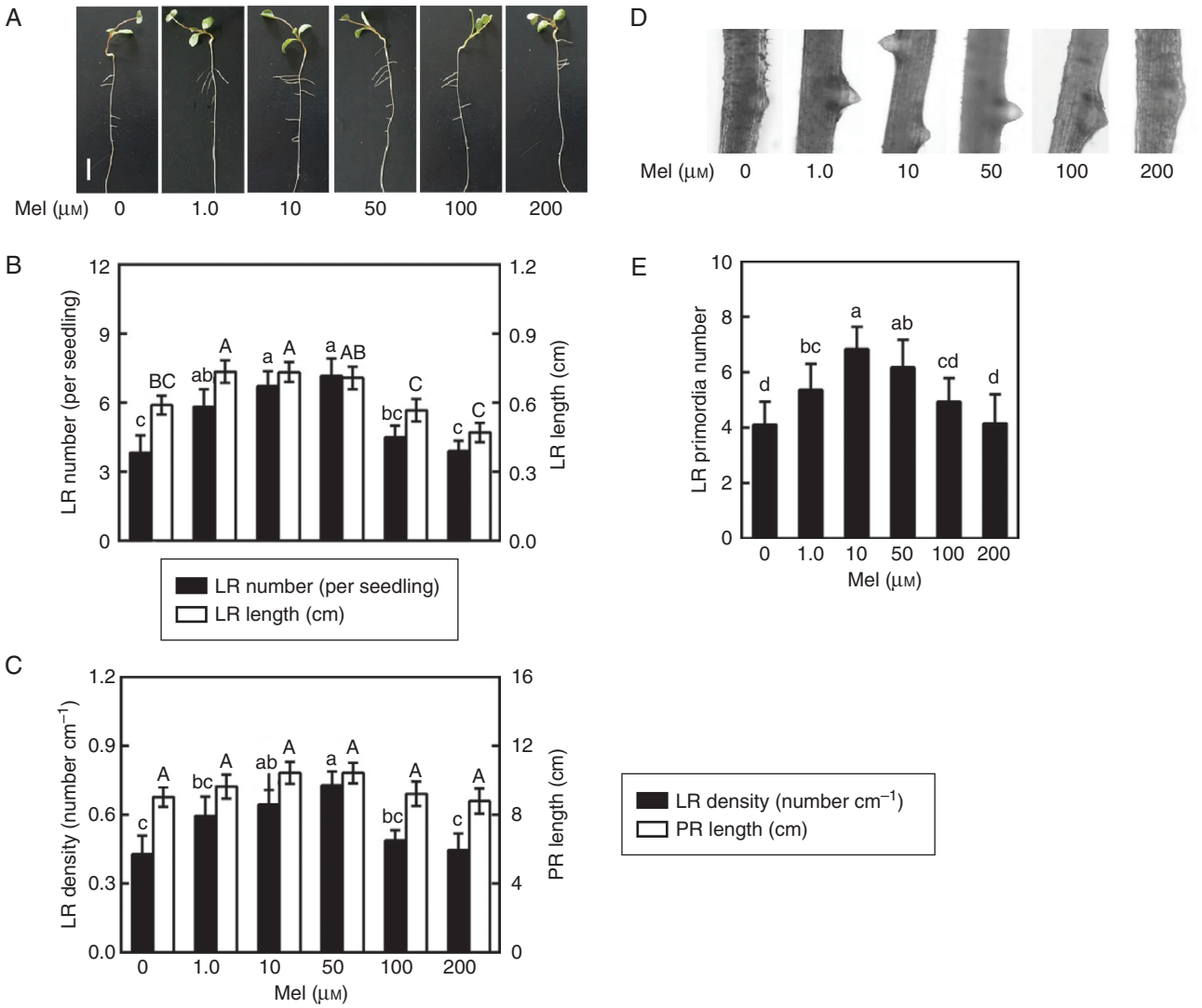

FIG. 1. Melatonin induces lateral root (LR) formation. Three-day-old alfalfa seedlings were treated with 0, 1.0, 10, 50, 100 or 200 μM melatonin (Mel). (A) Lateral root phenotypes after treatment for 5 d. Scale bar = 1 cm. (B) Number of emerged LRs (>1 mm) per seedling and LR length. (C) Density of emerged LRs and primary root (PR) length. (D, E) After treatment for 60 h, photographs of LR primordia formation were taken and the number of LR primordia was recorded. The sample without added melatonin was the control. Means and standard errors were calculated from at least three independent experiments with at least three replicates for each. Within each set of experiments (B, C, E), bars with different letters denote significant differences (one-way ANOVA followed by Tukey's multiple range test, $P < 0.05$).

dichlorofluorescein-dependent fluorescence analysis revealed the accumulation of ROS in *MsSNAT-1* and *MsSNAT-2* plants, compared with the wild-type (Fig. 5A, B). Pharmacological tests further showed that the ROS level was less impaired in two transgenic lines compared with wild-type when DPI or DMTU was added. These results further confirmed that the fluorescence was mainly caused by endogenous H_2O_2. Further results revealed that transgenic lines had more lateral roots than the wild-type seedlings, indicating that endogenous melatonin stimulated lateral root formation (Fig. 5C–E). By contrast, lateral root number and density were seriously inhibited when transgenic lines were exposed to DPI or DMTU. These results clearly suggest that endogenous melatonin-induced lateral root formation was, at least partially, H_2O_2-dependent.

Expression of cell cycle regulatory genes

To further investigate the corresponding molecular mechanism, the transcript levels of two cell cycle regulatory genes related to lateral root formation, namely *CDKB1;1* and *CDKB2;1*, were examined in alfalfa and *Arabidopsis* seedlings. Results shown in Fig. 6A, B reveal that expression of *MsCDKB1;1* and *MsCDKB2;1* was induced significantly in alfalfa seedlings treated with exogenous melatonin, and that both effects were obviously reversed when DPI or DMTU was applied. DPI and DMTU alone differentially inhibited *MsCDKB1;1* and *MsCDKB2;1* expression.

Further genetic evidence showed that *AtCDKB1;1* and *AtCDKB2;1* were upregulated in the transgenic lines compared

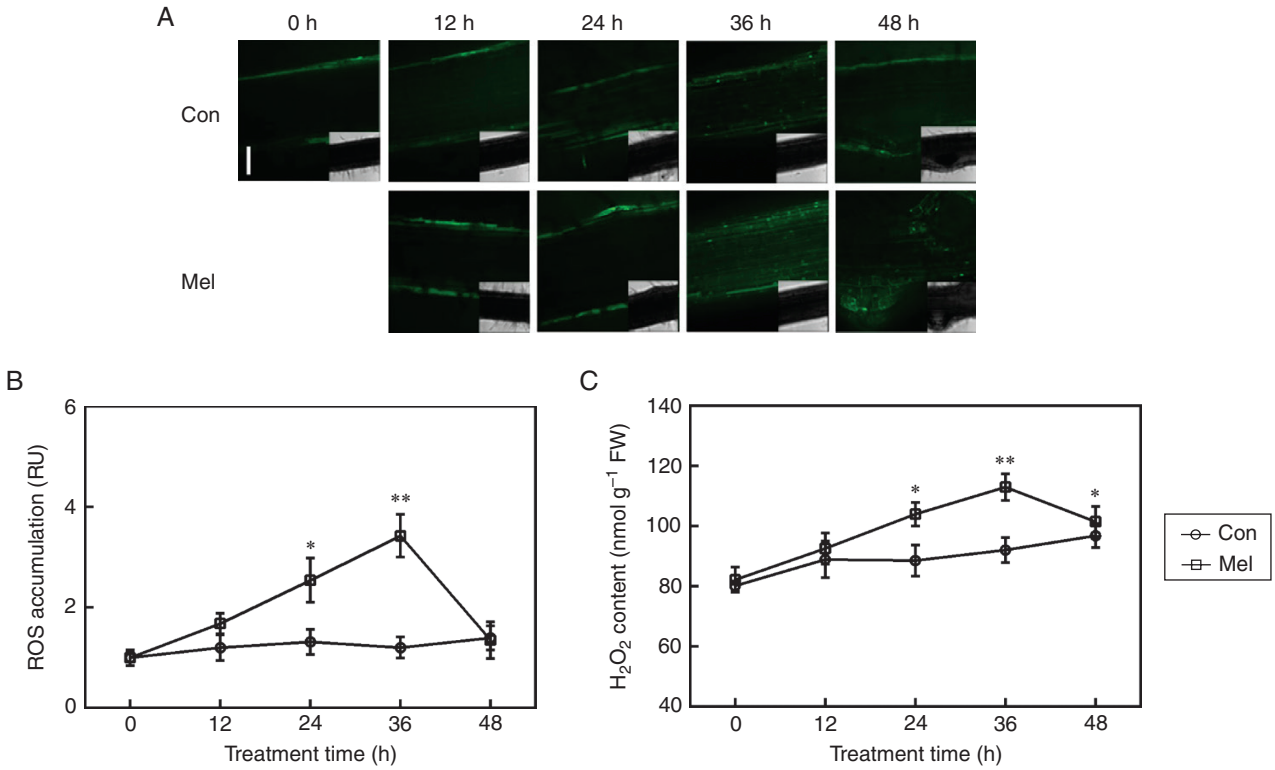

FIG. 2. ROS generation is induced by melatonin. Three-day-old alfalfa seedlings were treated with or without 10 μM melatonin (Mel) for the indicated times. (A, B) Then, root tissues were loaded with 20 μM 2′,7′-dichlorofluorescein diacetate (H_2DCFDA; a fluorescent dye for ROS) and regions of root mature zone were detected by LSCM. Scale bar (A) = 100 μm. Six individual samples were randomly selected and measured per treatment. Fluorescence is shown relative to control (Con) samples without added melatonin at 0 h. RU, relative units. (C) H_2O_2 content was determined by spectrophotography. FW, fresh weight. Means and standard errors were calculated from at least three independent experiments with at least three replicates for each. Asterisks denote significant differences in comparison with control (one-way ANOVA followed by Tukey's multiple range test, $*P < 0.05$, $**P < 0.01$).

with wild-type (Fig. 6C, D). We also noticed that the expression of these two genes decreased to a greater degree when DPI or DMTU was added, in both the transgenic lines and in the wild-type. Combined with the changes in ROS levels (Figs 3 and 5) and corresponding phenotypes (Figs 4 and 5), it can be deduced that H_2O_2 might be the downstream messenger of melatonin signalling responsible for lateral root formation by modulating the expression of cell cycle regulatory genes.

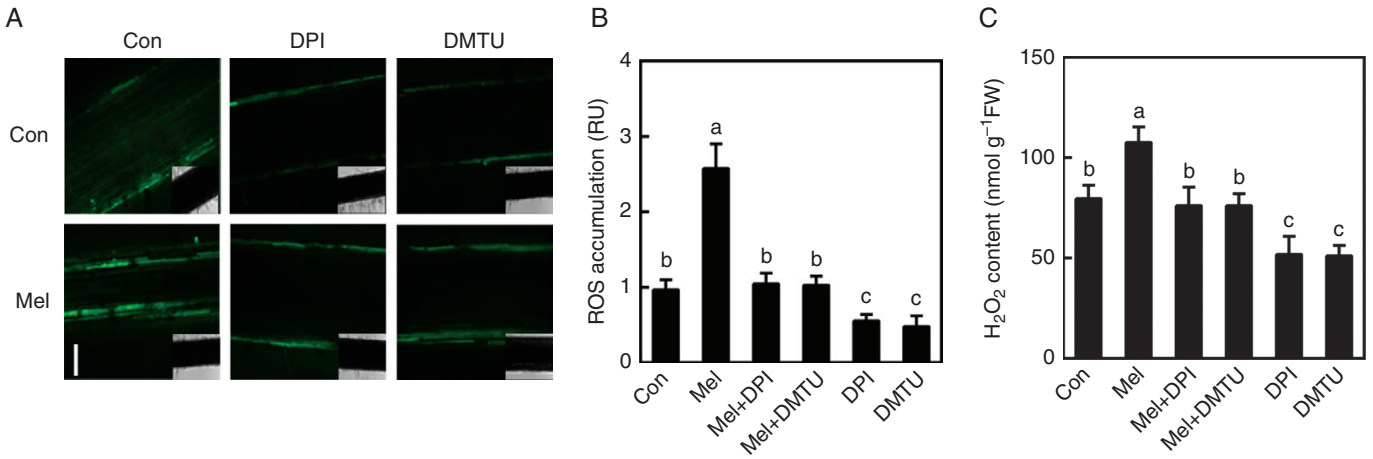

FIG. 3. Melatonin-induced H_2O_2 generation is impaired by DPI or DMTU. Three-day-old alfalfa seedlings were treated with or without 10 μM melatonin (Mel), 1 μM DPI or 0.5 mM DMTU, or a combination of Mel with DPI or DMTU. (A, B) After treatment for 36 h, root tissues were loaded with 20 μM H_2DCFDA and regions of root mature zone were detected by LSCM. Scale bar (A) = 100 μm. Fluorescence is shown relative to control samples (Con). RU, relative units. Six individual samples were randomly selected and measured per treatment. (C) H_2O_2 content was determined by spectrophotography. Means and standard errors were calculated from at least three independent experiments with at least three replicates for each. In (B) and (C) bars with different letters denote significant differences (one-way ANOVA followed by Tukey's multiple range test, $P < 0.05$).

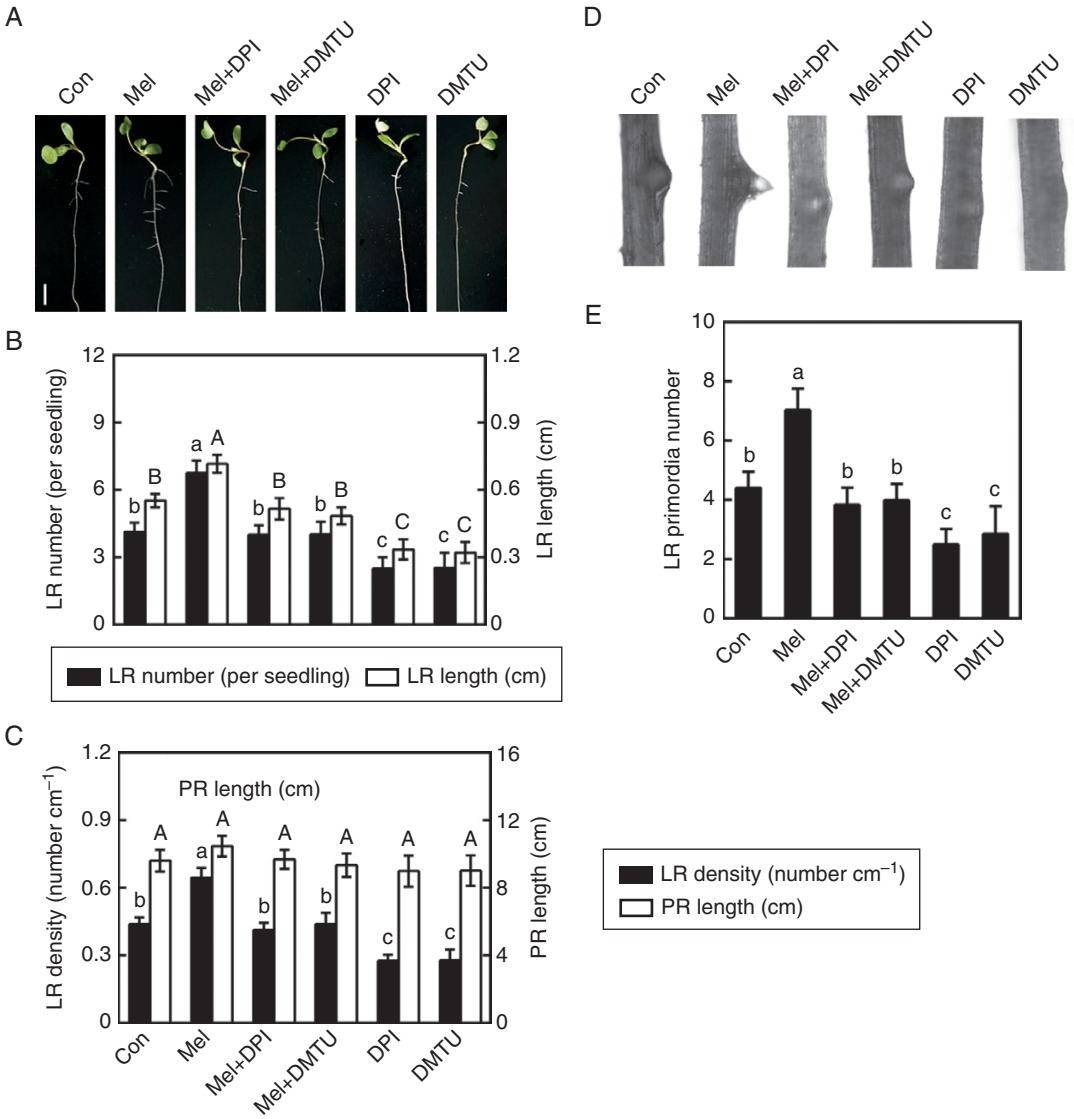

FIG. 4. Melatonin-induced lateral root (LR) formation is inhibited by DPI and DMTU. Three-day-old alfalfa seedlings were treated with or without 10 μM melatonin (Mel), 1 μM DPI or 0.5 mM DMTU, or a combination of Mel with DPI or DMTU. (A) Lateral root phenotypes after treatment for 5 d. Scale bar = 1 cm. (B) Number of emerged LRs (>1 mm) per seedling and LR length. (C) Density of emerged LRs and primary root (PR) length. (D, E) After treatment for 60 h, photographs of LR primordia formation were taken and the number of LR primordia was recorded. The sample without added chemicals was the control (Con). Means and standard errors were calculated from at least three independent experiments with at least three replicates for each. Within each set of experiments (B, C, E), bars with different letters denote significant differences (one-way ANOVA followed by Tukey's multiple range test, $P < 0.05$).

DISCUSSION

It is well established that melatonin fulfils many important roles in plants, and it is proposed to be an important regulator in controlling root development, including the induction of lateral root formation in *Lupinus albus* (Arnao and Hernández-Ruiz, 2007), rice (Liang *et al.*, 2017), cucumber (Zhang *et al.*, 2013, 2014) and *Arabidopsis* (Pelagio-Flores *et al.*, 2012). In this study, by using pharmacological, genetic and molecular approaches we extended the previous results and further discovered that (1) endogenous melatonin might modulate lateral root formation, and (2) H_2O_2 might be involved in melatonin-induced lateral root formation via modulating the expression of cell cycle regulatory genes.

Lateral root formation might be induced by endogenous melatonin

This report provides evidence that the application of exogenous melatonin was able to induce alfalfa lateral root formation, confirmed by changes in lateral root number, lateral root length and lateral root density (Fig. 1). Importantly, 10 and 50 μM melatonin exhibited maximal responses. Microscopic analyses of lateral root primordia shown in Fig. 1D, E supported the above results. This finding was consistent with previous findings in cucumber, rice and *Arabidopsis* (Pelagio-Flores *et al.*, 2012; Zhang *et al.*, 2013, 2014; Liang *et al.*, 2017). Comparatively, the most effective concentration(s) of melatonin in plants were different, which might be explained by different plant species or treatment time-points. In addition, although melatonin can act as a potential modulator of

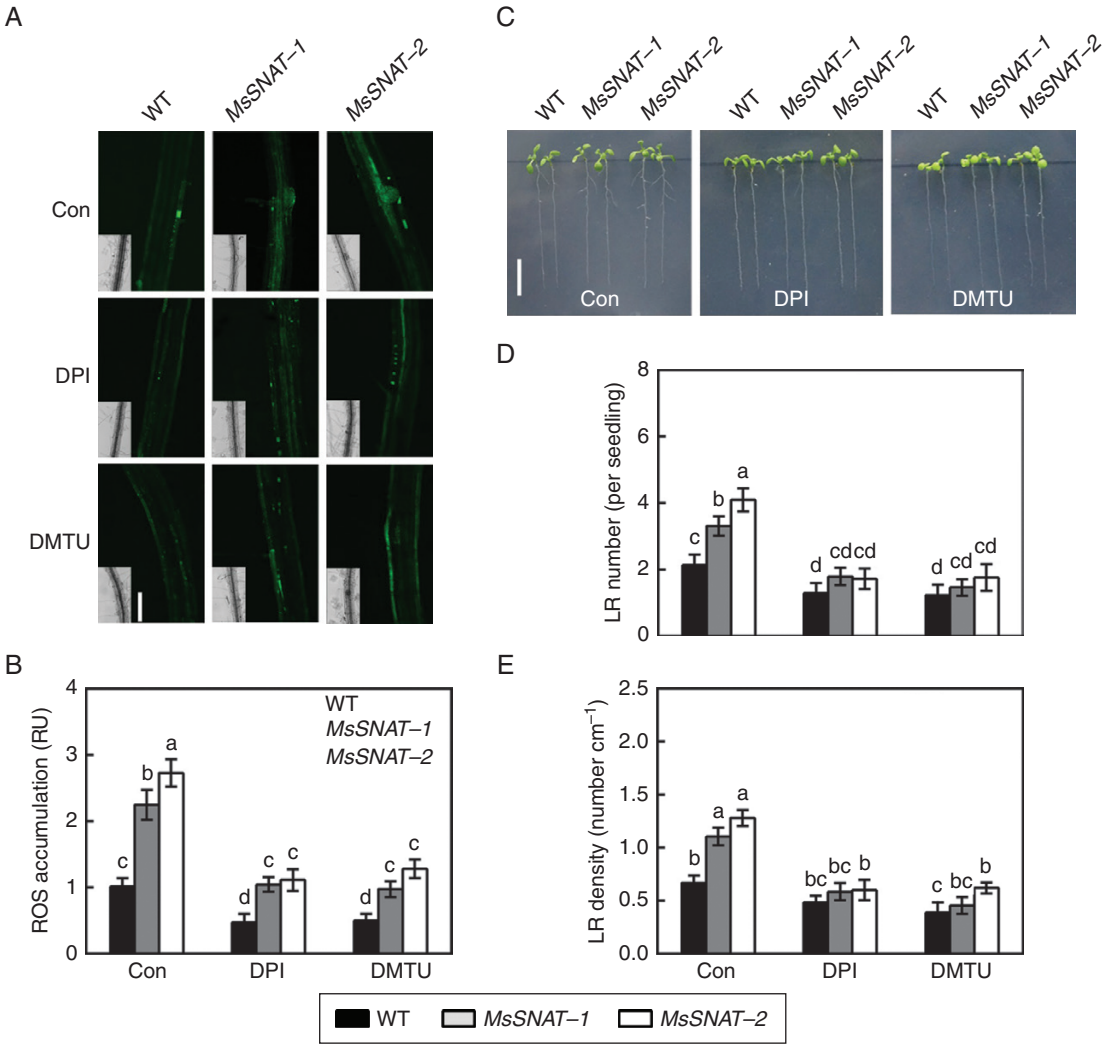

FIG. 5. Genetic evidence showed that endogenous melatonin-induced ROS generation and thereafter lateral root formation are sensitive to DPI and DMTU. The *SNAT* gene of *Medicago sativa* (*MsSNAT*), encoding serotonin *N*-acetyltransferase, is homologous to the *AtSNAT* gene. Transgenic *Arabidopsis* lines overexpressing *MsSNAT* driven by the cauliflower mosaic virus were generated, and showed higher levels of endogenous melatonin (Gu *et al.*, 2017). Four-day-old wild-type (WT) and transgenic seedlings (*MsSNAT-1* and *MsSNAT-2*) were treated with or without 0.1 μM DPI or 0.1 mM DMTU. (A, B) After treatment for 36 h, root tissues were loaded with 20 μM H_2DCFDA and regions of root mature zone were detected by LSCM. Fluorescence is shown as relative units (RU) of pixel intensity with reference to wild-type (Con). Scale bar (A) = 100 μm. Six individual plants were randomly selected and measured for each genotype per treatment. (C) Lateral root (LR) phenotypes after treatment for 5 d. Scale bar (C) = 1 cm. (D) Number of emerged LRs (>1 mm) per seedling. (E) Density of emerged LRs. Means and standard errors were calculated from at least three independent experiments with at least three replicates for each. Bars with different letters (B, D, E) denote significant differences (one-way ANOVA followed by Tukey's multiple range test, $P < 0.05$).

plant growth and development, high concentrations of melatonin (100 and 200 μM) had no obvious effect (Fig. 1) and even suppressed cell proliferation and endoreduplication in *Arabidopsis* (Wang *et al.*, 2017). These results suggest that the beneficial role of exogenous melatonin in plants might act within a narrow dose range.

Since the application of exogenous melatonin may not completely mimic the function of endogenous melatonin, two transgenic *Arabidopsis* lines, *MsSNAT-1* and *MsSNAT-2*, showing high levels of melatonin (Gu *et al.*, 2017), were used. Consistently, *MsSNAT-1* and *MsSNAT-2* transgenic lines had more lateral roots than wild-type (Fig. 5C–E). The above pharmacological and genetic evidence indicates that lateral root formation might be regulated by endogenous melatonin.

Involvement of H_2O_2 in melatonin-induced lateral root formation

Previous results showed that ROS signalling was specifically required during lateral root emergence in *Arabidopsis*, tomato and rice (Chen *et al.*, 2013; Cao *et al.*, 2014; Ma *et al.*, 2014; Manzano *et al.*, 2014). It is well established that melatonin and H_2O_2 have similar physiological roles in root organogenesis. Therefore, it is most likely that there is interaction in the process of lateral root formation.

In this subsequent study, time-course analyses by using LSCM and spectrophotography revealed that exogenous melatonin could simultaneously induce H_2O_2 generation in alfalfa seedlings (Fig. 2; reaching a maximum at 36 h of treatment). Additionally, *MsSNAT-1* and *MsSNAT-2* transgenic lines had higher endogenous H_2O_2 contents compared with the wild-type

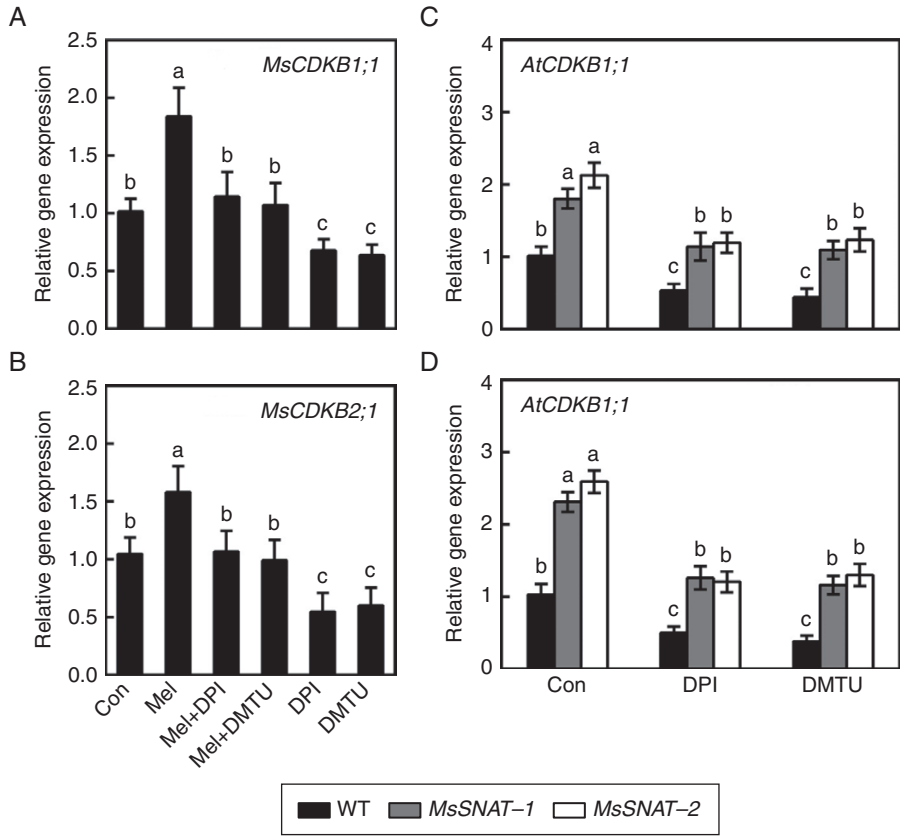

FIG. 6. Melatonin affects expression profiles of cell cycle regulatory genes in alfalfa and transgenic *Arabidopsis* seedlings. (A, B) Three-day-old alfalfa seedlings were treated with or without 10 μM melatonin (Mel), 1 μM DPI or 0.5 mM DMTU, or combinations of Mel with DPI or DMTU. After treatment for 36 h, transcript levels of *MsCDKB1;1* (X97315; A) and *MsCDKB2;1* (X97317; B) in the mature zone of the root were analysed by real-time RT–PCR. (C, D) Four-day-old wild-type (WT) and transgenic seedlings were treated with or without 0.1 μM DPI or 0.1 mM DMTU. After treatment for 36 h, *AtCDKB1;1* (At3g54180; C) and *AtCDKB2;1* (At1g76540; D) transcript levels in the mature root zone were analysed by real-time RT–PCR. Expression levels are shown relative to control samples after normalization to alfalfa *MSC27* (X63872) and *Arabidopsis Atactin7* (At5g09810). Means and standard errors were calculated from three independent experiments with three replicates for each. Bars with different letters denote significant differences (one-way ANOVA followed by Tukey's multiple range test, $P < 0.05$).

(Fig. 5A, B). Combined with the corresponding phenotypes shown in Figs 1 and 5C–E, we speculate that there is a potential interrelationship between melatonin and H_2O_2 during lateral root formation.

Further pharmacological and microscopical evidence revealed the requirement for endogenous H_2O_2 in the induction of lateral root formation triggered by melatonin. This conclusion is based on several pieces of evidence. (1) Exogenously applied DPI (NADPH oxidase inhibitor) or DMTU (H_2O_2 scavenger) inhibited exogenous melatonin-induced ROS accumulation determined by fluorescence analysis and spectrophotography (Fig. 3). (2) Melatonin-triggered lateral root formation was obviously impaired by DPI or DMTU in alfalfa (Fig. 4). (3) Similar to the beneficial responses to exogenous melatonin, the removal of endogenous ROS by using DPI or DMTU obviously blocked melatonin-triggered lateral root formation in wild-type and, in particular, transgenic plants (Fig. 5), confirming the possible role of H_2O_2 in root organogenesis elicited by melatonin. This deduction was consistent with the recent discovery that lateral root emergence was modulated by ROS accumulation in *Arabidopsis* (Orman-Ligeza *et al.*, 2016). Related signalling receptor molecules should be elucidated in future work.

Cell cycle reactivation might be mediated by H_2O_2 through regulation of the expression of multiple cell cycle genes in early lateral root initiation (Himanen *et al.*, 2004). Alfalfa *MsCDKB1;1* and *MsCDKB2;1* belong to the plant-specific CDK class, and *MsCDKB2;1* was also activated as a consequence of wounding and treatment with ethephon in a non-cell cycle-dependent fashion (Zhiponova *et al.*, 2006). The subsequent experiment found that the expression of above two cell cycle genes was upregulated by exogenous melatonin (Fig. 6A, B). These inducing effects were impaired by treatment with DPI, or DMTU, which was consistent with the reduced H_2O_2 levels (Fig. 3) and subsequently decreased lateral root formation in alfalfa seedlings (Fig. 4). Genetic evidence further revealed that, compared with the wild-type, the expression of *AtCDKB1;1* and *AtCDKB2;1* transcripts was upregulated in transgenic seedlings (Fig. 6C, D), while these increased transcript levels were significantly reversed by DPI or DMTU treatment. These results are consistent with the changes in lateral root formation (Fig. 5C–E). Therefore, this study clearly demonstrates that cell cycle regulatory genes might be the target genes of the action of H_2O_2 triggered by melatonin, thus leading to lateral root development. In agreement with the above results, an RNA-seq approach revealed that root development was modulated by melatonin via the ROS system in cucumber (Zhang *et al.*, 2014).

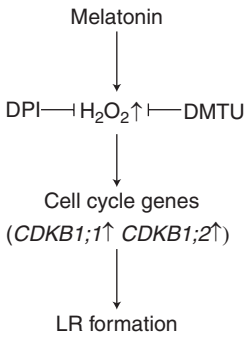

FIG. 7. Schemolving endogenous H_2O_2 during lateral root (LR) formation triggered by melatonin. Cell cycle genes (*CDKB1;1*, *CDKB1;2*) are involved in this response. T bars indicate inhibition.

A recent study has indicated that *Arabidopsis* root development processes elicited by melatonin are likely independent of auxin responses (Pelagio-Flores *et al.*, 2012). By contrast, Liang *et al* (2017) suggested that melatonin shaped root architecture by activating the auxin signalling pathway in rice. Previous findings also showed a close interaction between HY1 and H_2O_2 in auxin-induced lateral root formation in *Arabidopsis* (Ma *et al.*, 2014). Thus, whether the auxin signalling is involved in the above process should be elucidated in the future.

In summary, the ability of endogenous melatonin to induce lateral root formation is a new finding. Further pharmacological and genetic evidence demonstrated that H_2O_2 signalling might be required for melatonin-induced lateral root development, and that the regulation of cell cycle regulatory gene expression might be an indispensable and crucial strategy in this process (Fig. 7). Thus, the above results will open a new window to the understanding of molecular mechanisms related to lateral root formation induced by melatonin.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: sequences of primers for real-time RT–PCR.

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