

Melatonin promotes adventitious root formation in apple by promoting the function of *MdWOX11*

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Abstract

Background: Melatonin (MT) is important for plant growth and development; however, it is not known whether MT is involved in apple adventitious root (AR) development. In this study, we treated *Malus prunifolia* (MP) at four different stages of AR development, and analyzed the level of the endogenous hormones MT, auxin (IAA), zeatin-riboside (ZR), abscisic acid (ABA), and gibberellins (GA₁₊₃) in all four treatment groups and the untreated control group. The expression of MT, IAA biosynthesis, transport and signal transduction, the cell cycle, and root development related genes were quantified by RT-qPCR. The function of *MdWOX11* was analyzed in transgenic apple plants.

Results: The promotion of AR development by MT was dependent on the stage of AR induction between 0–2 d in apple rootstocks. MT-treatment increased the level of IAA and crosstalk existed between MT and IAA during AR formation. The expression of *MdWOX11* was induced by MT treatment and positively regulated AR formation in apple. Furthermore, transgenic lines that overexpressed *MdWOX11* lines produced more ARs than 'GL3'. Phenotypic analysis indicated that *MdWOX11* overexpression lines were more sensitive to exogenous MT treatment than 'GL3', suggesting that *MdWOX11* regulates AR formation in response to MT in apple rootstock.

Conclusions: MT promotes AR formation mainly during the AR induction stage by inducing IAA levels and upregulating *MdWOX11*.

Background

Apple (*Malus domestica*) is a major commercial fruit tree that is cultivated globally and apple fruits have a high nutritional and economic value. *Malus prunifolia* (MP) is widely known as the easiest-rooting apple rootstock. It offers advantages such as good graft compatibility, cold resistance, salt and alkali tolerance, and disease and insect resistance. Adventitious roots (ARs) induction from stem basal tissues is a major step in the vegetative propagation of apple rootstocks. ARs are post-embryonic roots that emerge from non-root organs, and AR primordia arise from interfascicular cambium cells adjoining phloem cells [1, 2]. The processes required for AR formation have been studied in different plants, including rice [3], *Arabidopsis* [4, 5], and poplar [6]; however, methods for improving AR formation in apple have not been studied.

Melatonin (MT; N-acetyl-5-methoxytryptamine) is a well-known hormone in animal and was initially discovered in plants by two groups of workers in 1995 [7, 8]. Previous studies have shown that MT functions as a regulatory signal in plants [9], and is important for the growth of roots, shoots, explants, and stress responses [10-15]. The relationship between MT and AR formation has mainly been studied in herbaceous plants; for example, the exogenous application of MT promoted adventitious rooting in tomato and rice [16, 17], but the mechanism how MT regulates AR formation remains to be elucidated in woody plants such as apple. AR formation can be categorised as a four-stage process [18-21], and the stage at which MT is important for AR development remains unknown. In this study, we observed that MT

promoted AR formation at early stages of AR induction and initiation. Previous studies have demonstrated the relationship between MT and other plant hormones such as auxin (IAA), cytokinin (CK), gibberellins (GA), abscisic acid (ABA) [22]: Treatment with MT caused the CK levels to increase during non-biological stress in the plant [23], MT also contributed to increasing the content of active GAs such as GA₃ and GA₄ [24], and exogenous MT application led to a decrease in the content of ABA [23]. However, the relationship between MT and these hormones during adventitious rooting remains to be determined. Potentially, MT acts as a growth-promoting compound by increasing the level of IAA, IAA synthesis and polar IAA transport [25-27]. Most studies have analyzed the ability of MT to stimulate root and shoot growth, in a similar way to IAA [28]. However, the effect of MT on root growth and differentiation is thought to be independent of IAA [29]. In this study, we established that MT-IAA crosstalk plays an important role in AR induction. Still, the role of plant hormone interaction and associated signaling networks during AR formation is incompletely understood in apple rootstock.

The genes involved in MT biosynthesis, such as *TDC*, *SNAT*, *HIOMT* and *ASMT*, are induced by MT [30-34], *MzSNAT5* regulates MT synthesis in the mitochondria of apple [33], and overexpression of *ASMT* increases MT production in *Arabidopsis thaliana* [34]. In this study, the expression of MT and auxin-related genes, such as *AUXIN RESPONSE FACTORS* (*ARFs*) and *PINFORMED* (*PIN*) genes, were analyzed in apples. In addition, *WUSCHEL-RELATED HOMEODOMAIN GENE 11* (*WOX11*) functions in crown root emergence and development [35] and AR development in *Arabidopsis* [36], but in woody plants, the regulation of *WOX11* during AR development is poorly understood. Furthermore, it is unknown whether AR formation in transgenic apple plants that overexpress *MdWOX11* is regulated by exogenous MT.

Currently, the mechanisms via which AR formation is regulated by MT is not well characterized in apple rootstock. In this study, we showed that exogenous MT induced AR formation at the early stages of AR induction and initiation by increasing IAA synthesis, transport, and the expression of signaling-related genes. Apple plantlets treated with MT in tissue culture showed an increase in the expression of root development-related genes, and therefore, an increase in the number of ARs. Furthermore, we demonstrated that overexpression of *MdWOX11* promoted the emergence and development of ARs, and treatment with exogenous MT induced AR development in the transgenic apple that overexpressed *MdWOX11*. The results of this study provide insights into the mechanism of how MT regulates AR formation in apple rootstock.

Results

The aim of this study was to identify the precise time at which MT promotes AR formation in tissue-culture plantlets of MP apple rootstocks. In this study, 0–2 d represents the stage of AR induction, 2–5 d represents AR initiation, and 5–20 d covers the stages of AR primordium formation and AR emergence in MP. The study consisted of five different treatment groups: MT, MT0–2, MT2–5, MT5–20, and one control group (Fig. 1A). No morphological changes were evident in any groups up to 5 d; however, ARs emerged from basal stem parts at 10 d (Fig. 1B). At 20 d, the greatest number of ARs was observed in the MT0–2 group, MT2–5 and MT5–20 groups produced more ARs than the control and MT group at 20 d,

no significant difference was observed in the number of ARs among these groups (Fig. 1B). To observe the anatomy of stems at different stages of AR formation, sections were made from paraffin-embedded samples and were viewed using light microscopy. On 0 day, cross-sections of the samples revealed the existence of competent cells. Still, mitotic cambial cell division was observed at 5 d, cell divisions were visible in the compactly arranged cells. AR appeared in sections from stem bases cultured in the medium for 10 days (Fig. 1C). No AR formation was observed in MP treated for 20 d with the auxin inhibitors N-1-naphthylphthalamic acid (NPA) or triiodobenzoic acid (TIBA), but ARs were observed following MT treatment. All phenotypes were summarised in Figure S1.

We also measured the rooting rate, number of AR, crossings, root length, root volume, and root surface area in all five treatment groups, and the data were consistent with the phenotypes of AR formation. All measured parameters were higher in the MT0–2 group than in other groups, and the minimum number of ARs and values for other root parameters were observed in the control group (Fig. 2). The results showed that MT mainly promoted AR formation at 0–2 d, during the AR induction stage.

Based on their diameter, ARs were classified into three groups: 0–2.0 mm, 2.0–5.0 mm and >5.0 mm. According to AR number, length and surface area, the 0–2.0 mm category contained the greatest percentage of the total; however, for root volume, the 2.0–5.0 mm category was the largest for all groups (Fig. 3). The MT0–2 group contained the greatest number of ARs in the 0–2.0 mm class, which was twice as many as in the control group (Fig. 3). We conclude that most ARs were fine roots (0–2.0 mm).

The levels of the hormones MT, IAA, ZR, GA_{1+3} and ABA were analyzed in MP tissue culture plantlets after treatment with MT. The MT content was higher in the MT0–2 group than in other treatment groups during the early AR developmental stage and reached a peak at 5 d in the MT0–2 group. In the MT and MT0–2 treatments, the levels of IAA, ZR and GA_{1+3} were higher than those in the control group during AR induction at 1 d and 2 d, but the levels were lower in the MT0–2 group than other groups at 10 d. The level of ABA responded opposite to that of IAA, ZR and GA_{1+3} in the treatments (Fig. 4).

Furthermore, the expression levels of MT synthesis related genes were analyzed during AR formation. Except for 0 and 20 d, the expression levels of *MdTDC1*, *MdHIOMT2*, *MdASMT1* and *MdASMT2* genes, which are involved in MT synthesis, were higher in the MT0–2 groups than those of other groups, and the expression levels of *MdSNAT* and *MdHIOMT1* were higher in MT0–2 than those of other groups at 1 d, 5 d and 20 d. These results suggested that MT treatment induced the expression of MT synthesis related genes (Fig. 5).

To determine whether an interactive effect between MT and IAA existed, we measured the expression of genes related to IAA biosynthesis and signal transduction. The expression of *MdYUCCA1*, *MdYUCCA10*, *MdARF7*, and *MdARF19* were higher in the MT0–2 treatment group than that in other groups at 1 d, 2 d and 3 d. The expression level of the IAA transport-related genes *MdAUX1*, *MdPIN1*, and *MdPIN3* were also higher in the MT0-2 than in other groups at 2 d; however, expression of the IAA signal transduction gene

MdIAA5 was lower following MT0–2 treatment than in other treatments during the AR induction stage (Fig. 6).

To investigate whether MT affects cell division, the expression of the cell-cycle related genes *MdCYCD1;1* and *MdCYCD3;1* were analyzed, these genes were more highly expressed in MT0–2 at 3 d and 10 d. Therefore, we conclude that the application of MT promoted AR formation in apple, and RT-qPCR analysis showed that the expression of root development-related genes was higher at most sampling time points in response to MT treatment. We observed that among all root development-related genes, *MdWOX11* expression following MT treatment was 5.6 times higher than that in control plants at 2 d (Fig. 7). This suggests that *MdWOX11* probably plays an important role in AR induction in response to MT treatment.

The expression of *MdWOX11* was induced by IBA treatment (Fig. 7). We generated the overexpression (OE) transgenic lines *MdWOX11-OE15#*, *16#* and *20#* in ‘GL3’, and confirmed the level of overexpression *MdWOX11* transgenic lines (Figure S2). To confirm whether *MdWOX11* transgenic lines exhibited an enhanced response to MT signaling, wild-type and transgenic apple plantlets growing in tissue culture were either treated with 3.45 μ M IBA as a control or with MT for 0–2 d (MT0-2). More ARs were observed in the MT0-2 group than in the control group, both the overexpressing *MdWOX11* transgenic lines and ‘GL3’, and the *MdWOX11* overexpressing lines produced more ARs than ‘GL3’ (Fig. 8A). Overexpression of *MdWOX11* also caused an increase in the rate of ARs (Fig. 8B). Furthermore, *MdWOX11* overexpressing plants were more sensitive to exogenous MT treatment than wild type (Fig. 8A-C), which indicates that *MdWOX11* induced AR formation in response to MT treatment

Discussion

AR formation is the key to vegetative propagation and previous studies have divided AR formation from tissue culture plantlets into four stages: induction, initiation, primordium formation, and emergence [18–21]. In this study, 0–2 d represents the stage of AR induction, 2–5 d represents AR initiation, and 5–20 d covers the stages of AR primordium formation and AR emergence in MP. MT affects many biological processes, including plant growth, flowering and stress responses, and although MT promotes AR development in tomato [16], no studies have been conducted in woody plants. Therefore, to determine when and how MT can promote AR formation, we treated MP plants with MT at different times. The phenotypes of the five treatment groups showed that there was no difference between plants in the MT2–5 and MT5–20 groups, but the MT0–2 group produced more ARs than other groups (Figs. 1, 2 and 3), which demonstrates that MT promotes AR formation mainly during the AR induction stage at 0–2 d.

The effect of interaction between MT and IAA during root development is unclear. Some studies have suggested that a low concentration of exogenous MT can induce an increase in the endogenous IAA levels in plants, and it is believed that the promoting effect of MT on growth might be caused by this increase in IAA content [25]. However, other research has shown that the regulation of root growth and differentiation by MT was independent of IAA [29]. To analyse whether MT treatment can elevate the IAA content and enhance IAA signaling, we measured the IAA content in plants in the five MT treatments. The

IAA content mainly increased during AR induction after MT application, but decreased during the AR initiation and AR emergence stages (Fig. 4). This might reflect that IAA plays an important role during the early stage of root development [37-39]. During AR formation, MP plantlets were treated with IAA inhibitors and MT (Figure S1). NPA and TIBA treatment inhibited AR formation, NPA and TIBA have the function of inhibiting IAA polar transport. We conclude that exogenous MT treatment promoted AR formation to influence the IAA distribution in the AR zone. Furthermore, it suggests that IAA may be downstream of MT to induce AR formation. However, RT-qPCR data suggest that MT is involved in IAA signaling pathways, and MT treatment can induce the expression of IAA biosynthesis, transport and signal transduction-related genes (Fig. 6). Therefore, MT potentially promotes AR induction by increasing IAA levels and IAA signaling. Previous research has shown that MT-treated plants have increased CK levels during non-biological stress [23], and MT also increased in the content of active GAs such as GA₃ and GA₄ [24]. The observed increase in the levels of GA and ZR following MT treatment also suggests that there is a link between MT and ZR or GA₁₊₃.

WOX11 regulates AR formation in response to IAA in Arabidopsis [36], and some studies have demonstrated that *WOX11* is induced by IAA and positively induces the expression of *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) and *LBD29* at an early stage of AR development [40]. However, little is known concerning the function of *MdWOX11* in woody plants such as apple, including the morphological changes that occur in transgenic apple plants in response to MT during AR formation. In this study, we generated transgenic apple plants that expressed *35S::WOX11-OE*. Their phenotype demonstrated that *MdWOX11* is a positive regulator of AR activation and that MT treatment of these *MdWOX11* overexpressing plants increased AR development (Fig. 8). Therefore, the induction of *MdWOX11* and its related genes might represent a possible mechanism by which MT promotes AR formation. This is the first study to use *MdWOX11* transgenic lines to investigate the role of *MdWOX11* in response to MT during AR induction. Collectively, the data indicate that *MdWOX11* promotes AR formation in response to MT, and provide insights into the molecular mechanisms that underlie the induction of ARs by MT.

Conclusions

Melatonin promotes adventitious root formation mainly at the stage of AR induction by increasing IAA levels and activating the function of *MdWOX11*. The results represent the potential to improve AR formation to accelerate the asexual reproduction of apple rootstock that is difficult to root.

Methods

Explant growth conditions and MT treatments

Tissue culture plantlets of MP apple rootstock were grown in tissue culture in the Yangling (108°04'E, 34°16'N), China, and were used as plantlets for AR formation. The plantlets of MP were imported from Aomori in Japan and were propagated by asexual reproduction. The tissue culture plantlets of MP were split into five groups, and plants in all groups were treated simultaneously for 20 d. Control plants were

cultured on a root-inducing medium containing half-strength MS supplemented with 3.45 μM indole-3-butyric acid (IBA) to promote root formation. The second group plants were cultivated on the medium with half-strength MS supplemented with 1.29 μM MT and 3.45 μM IBA and was designated as the MT treatment group. The third group was transferred to root-inducing medium after cultivation on MT medium for 2 d and was called MT 0–2. The fourth group (MT 2–5) was transferred to MT medium after culture on root-inducing medium for 2 d, then it was transferred to root-inducing medium after culture on MT medium for 3 d. The fifth group was transferred to MT medium after cultivation on root-inducing medium for 5 d, and was called MT5–20. The composition of the different medium used for this study was listed in Supplemental Table S1. Samples were harvested from all five groups at 0 d, 1 d, 2 d, 3 d, 5 d, 10 d and 20 d (even though some samples were collected before MT-treatment). In total, 3150 cuttings, consisting of 630 cuttings from each of the five groups were harvested, which in turn, consisted of 90 cuttings sampled at each sampling point. Samples were collected from the basal part of the stems, including the AR formation zone (approximately 0.5 cm). The plants in the NPA treatment group were continuously cultivated in 10 μM NPA and 1.29 μM MT for 20 d, and the TIBA-treated plants were continuously cultivated in 10 μM TIBA and 1.29 μM MT for 20 d, the control was the same as above. Overexpression of *MdWOX11* transgenic apple (*35S::MdWOX11-OE*) and ‘GL3’ were separated into two groups: one group was continuously cultured on root-inducing medium, which was served as the controls, and the other group was transferred to root-inducing medium after culturing in MT medium for 2 d.

Anatomical observations and morphological measurements

Anatomical observations were carried out according to previously described protocols [1, 41, 42]. The morphological parameters measured included: AR rate, AR length and mean AR number for each cutting [43]. Also, an Epson Expression 10000XL scanner (LA I600 scanner, Canada) was used to analyse other root related indicators. In total, 90 cuttings were analyzed, with 90 cuttings from each group collected at each sampling point. The harvested samples were immediately frozen in liquid nitrogen and stored at -80°C for hormone and expression analysis.

Measurement of hormone levels

Samples for hormone extraction were harvested at different time points from all five treatment groups. Hormones were purified and extracted according to previously described procedures [44]. Three biological replicates for each group at each sampling point were analyzed. The enzyme-linked immunosorbent assay (ELISA) technique was used to detect and analyse the level of hormones [44].

Extraction of RNA and synthesis of cDNA

CTAB-based extraction method was used to isolate total RNA [45], and the total RNA integrity was tested by electrophoresis of the samples on 2% agarose gels. Prime Script RT Reagent Kit with gDNA Eraser (TaKaRa Bio, Shiga, Japan) was used to synthesize cDNA.

RT-qPCR analyses

MT, IAA synthesis, transport and signal transduction, the cell cycle and root development related genes expression were quantified by RT-qPCR. The gene full names and abbreviations, the MDP annotations in apple, as well as homologous proteins and species on which the identification of the proteins in apple was based, were listed in Supplemental Table S2. Primer design was based on previous research [43], and all gene-specific primers for the analyzed genes were listed in Supplemental Table S3.

RT-qPCR was performed according to published methods [46]. The apple *EF-α* gene was used to normalize expression. Each sample contained three biological replicates and three technical replicates. The analyzed genes relative expression were calculated by the $2^{-\Delta\Delta C_t}$ method [47].

Statistical analysis

SPSS11.5 software (SPSS, Chicago, IL, USA) was used to analysis significant differences, significance differences among each sampling time point and treatment were determined using (ANOVA). SigmaPlot12.0 (Systat Software, Inc.) was used to generate figures.

Abbreviations

MT, Melatonin; AR, adventitious root; MP, *Malus prunifolia*; IAA, auxin; CK, cytokinin; GA, gibberellins; ABA, abscisic acid; ELISA, enzyme-linked immunosorbent assay; WOX11, WUSCHEL-RELATED HOMEODOMAIN GENE 11; IBA, indole-3-butyric acid; LBD16, LATERAL ORGAN BOUNDARIES DOMAIN16.

Declarations

Author contributions

J.M., D.Z., and M.H. designed the research study. J.M., C.N., K.L., and S.C. performed the research. J.M., C.N., and M.M. analyzed the data. J.M. and D.Z. wrote the paper. All authors approved the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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Figures

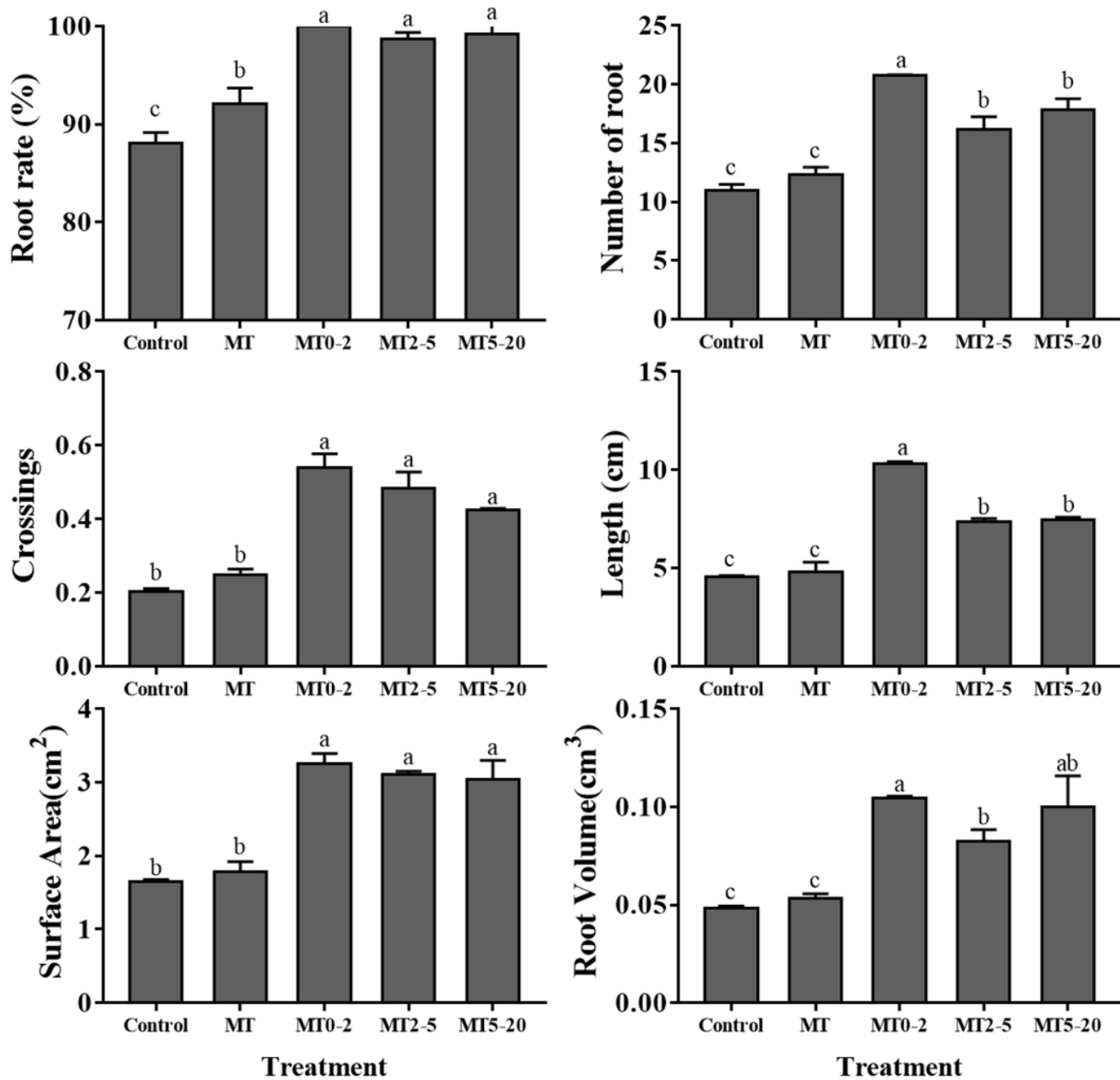


Figure 1

Anatomical and morphological observations of AR development in paraffin-embedded sections from the experimental samples generated in the study at three sampling times: 0 d, 3 d, and 10 d. Five treatment groups were analysed: (A) The control group in which apple tissue culture plantlets were continuously cultured on root-inducing medium containing 3.45 μM IBA; the MT group was continuously cultivated on 3.45 μM IBA and 1.29 μM MT, and based on different times of MT-treatment, the MT treatment group was also divided into three groups of MT0-2, MT2-5 and MT5-20; (B) Observations of morphological AR formation in the five treatment groups at 0 d, 5 d, 10 d and 20 d; (C) Anatomical observations of AR formation in the five treatment groups at 0 d, 5 d and 10 d.

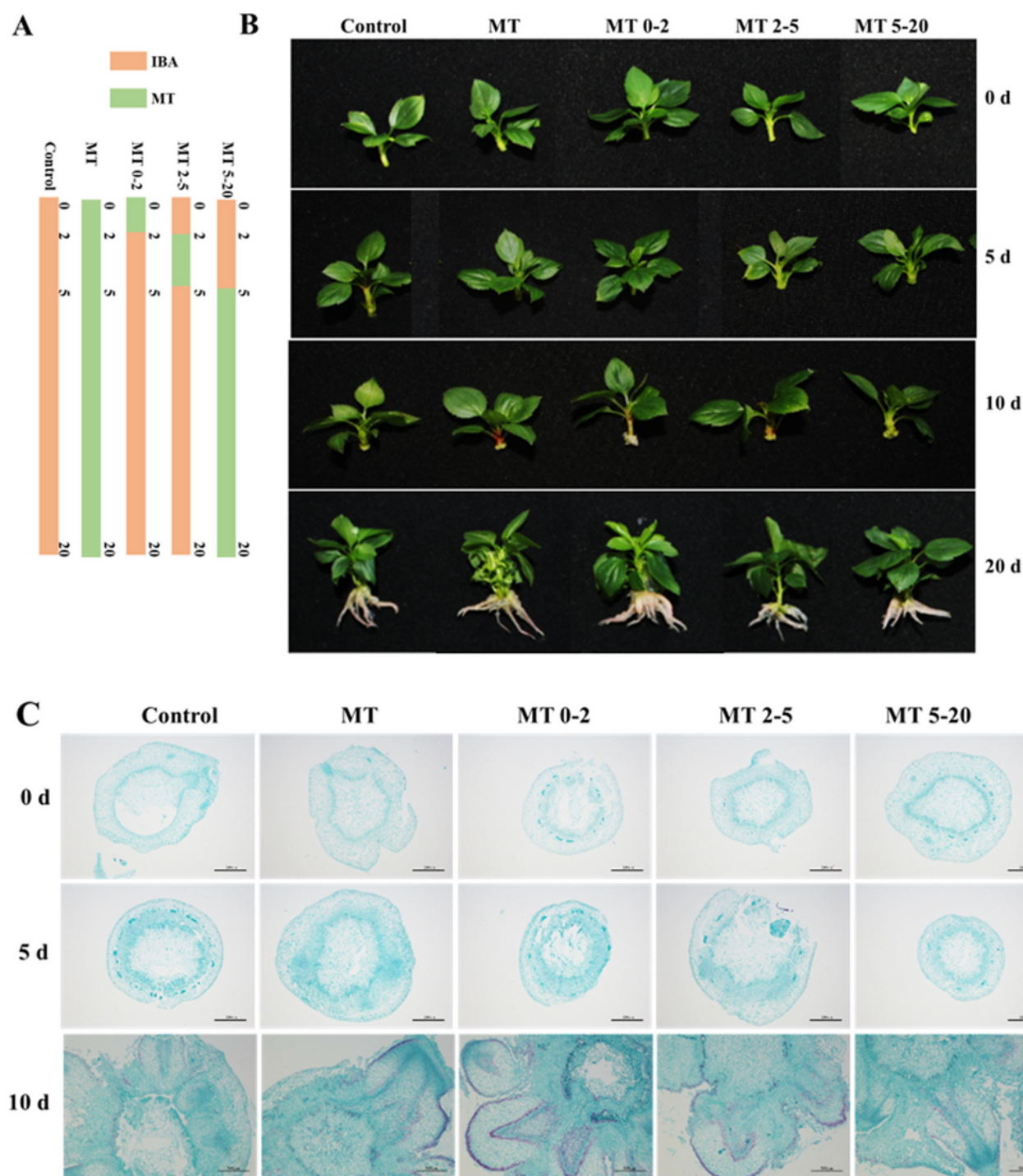


Figure 2

Morphological parameters of AR formation in tissue culture plantlets of *Malus prunifolia*. Tissue culture plantlets were divided into five groups: Control, MT, MT0–2, MT2–5, MT5–20. The number of ARs, and their length, surface area and volume were measured in the five treatment groups. Values represent the mean $\pm$ SE for three biological replicates; letters indicate significant differences between means ($P < 0.05$).

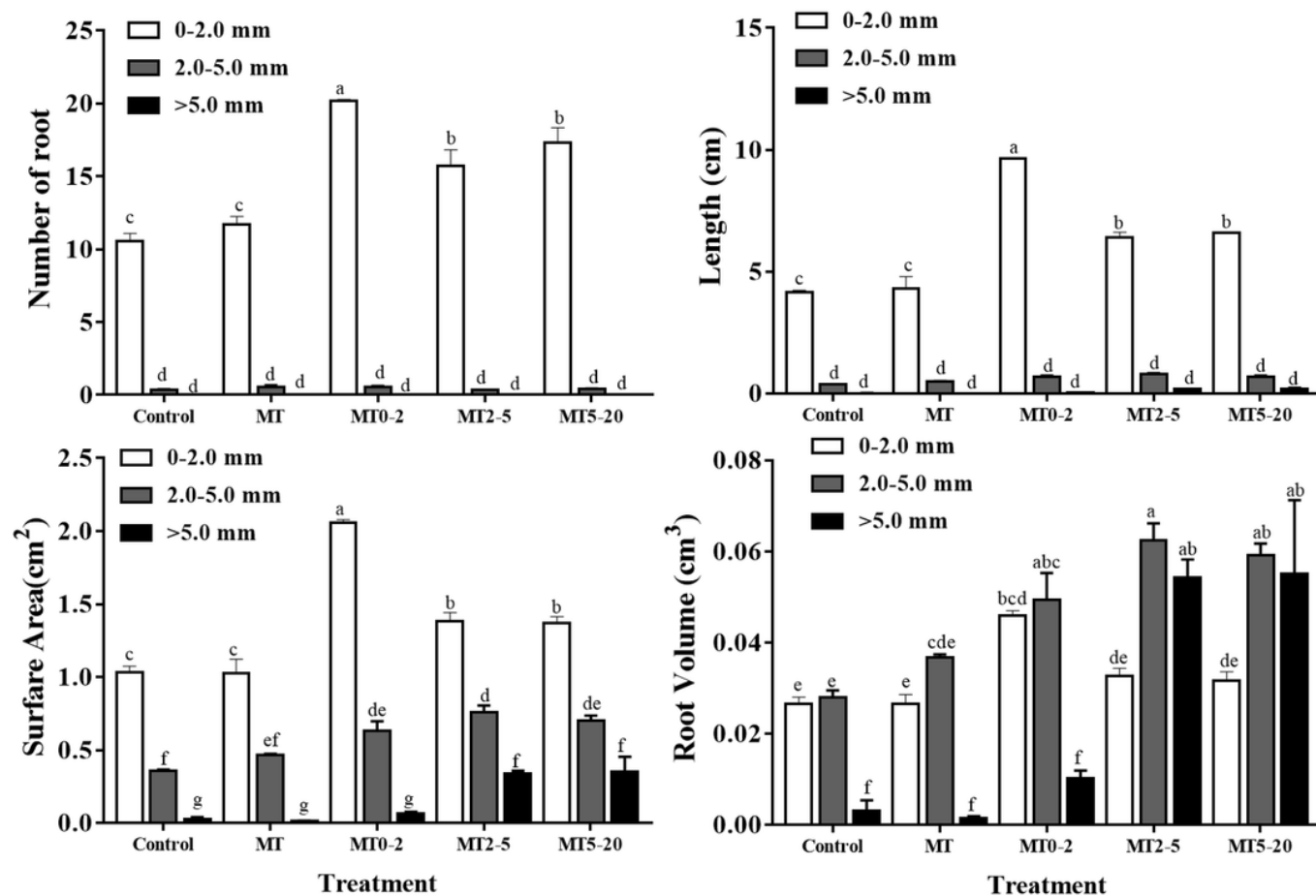


Figure 3

Morphological parameters of AR formation in tissue culture plantlets of *Malus prunifolia* apple rootstock for different categories of root diameter, according to the number, length, surface area, and volume of root. The ARs were classified into three groups based on root diameter: 0–2.0 mm, 2.0–5.0 mm and >5.0 mm.

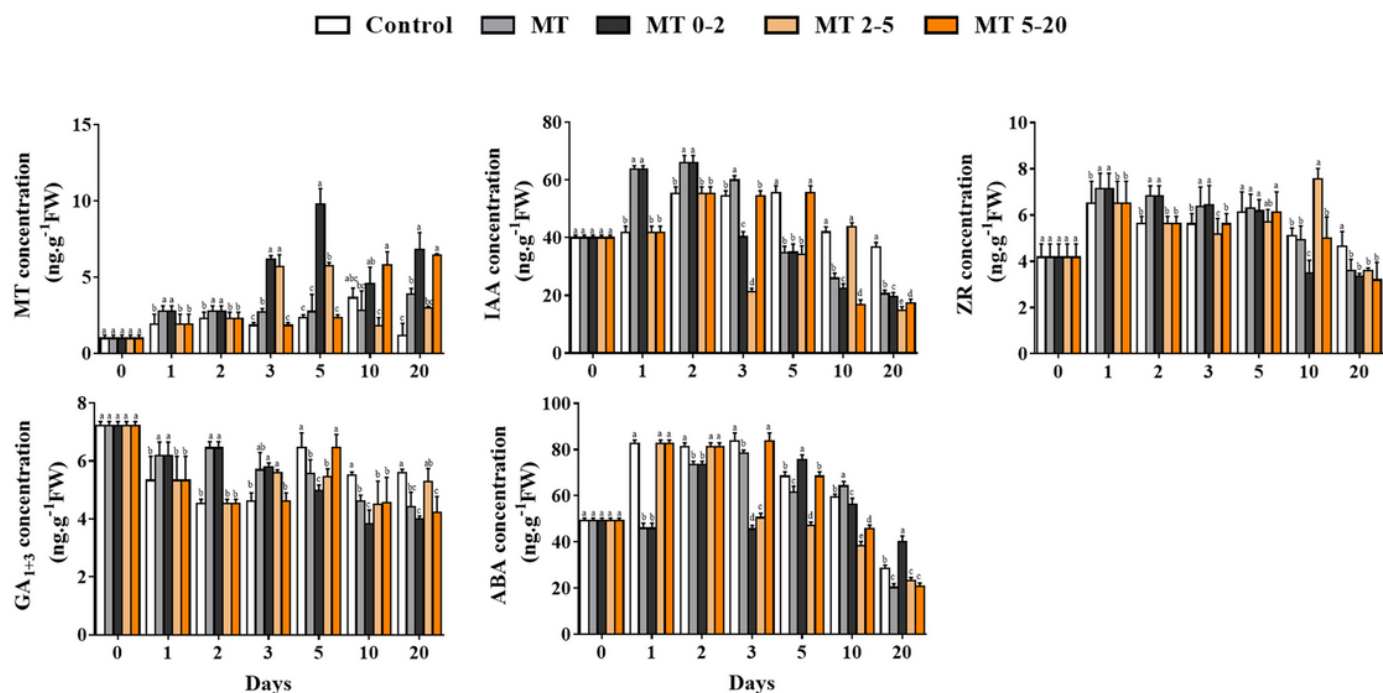


Figure 4

The effect of exogenous application of MT for different lengths of time on the contents of MT, ZR, IAA, GA₁₊₃, and ABA at different stages of AR development in five treatment groups of *Malus prunifolia* apple rootstocks.

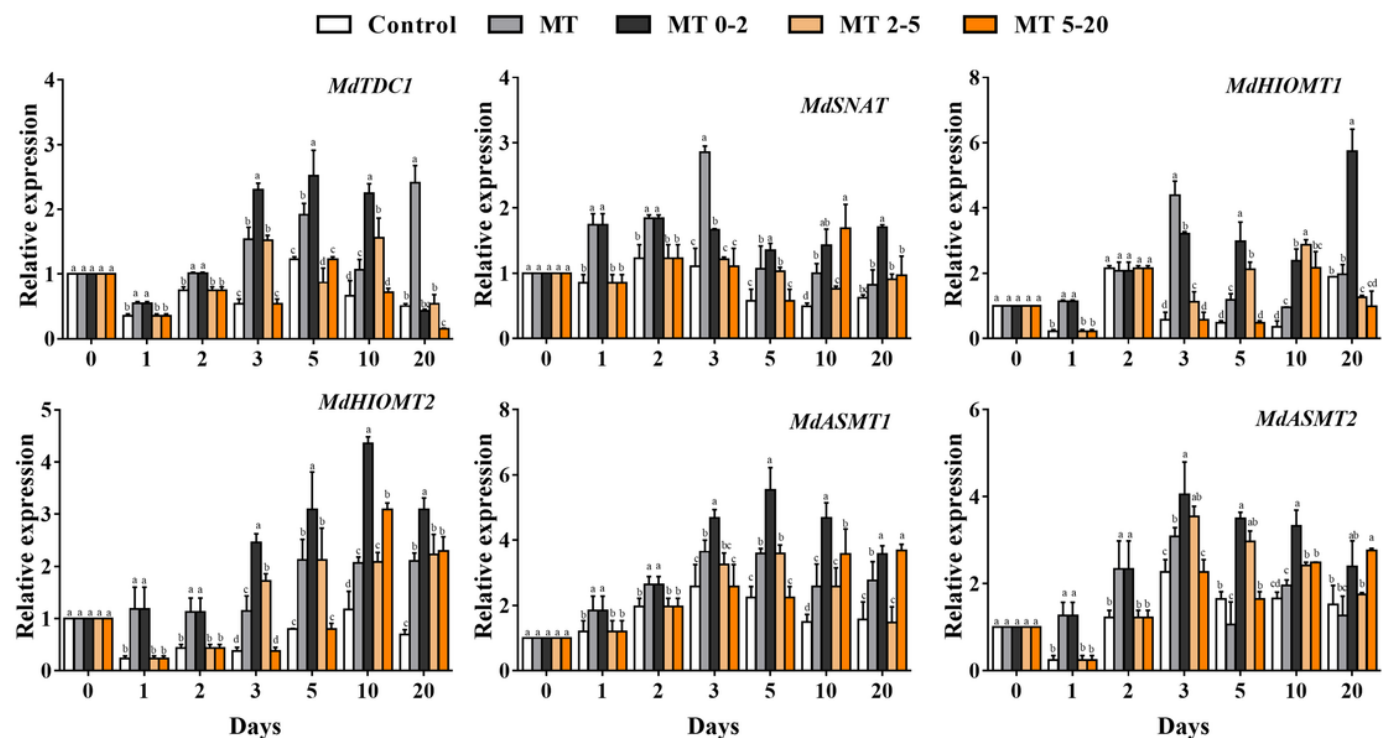


Figure 5

The effect of exogenous MT treatment on the relative expression of genes involved in MT signal transduction at different stages of AR development in five treatment groups of *Malus prunifolia*.

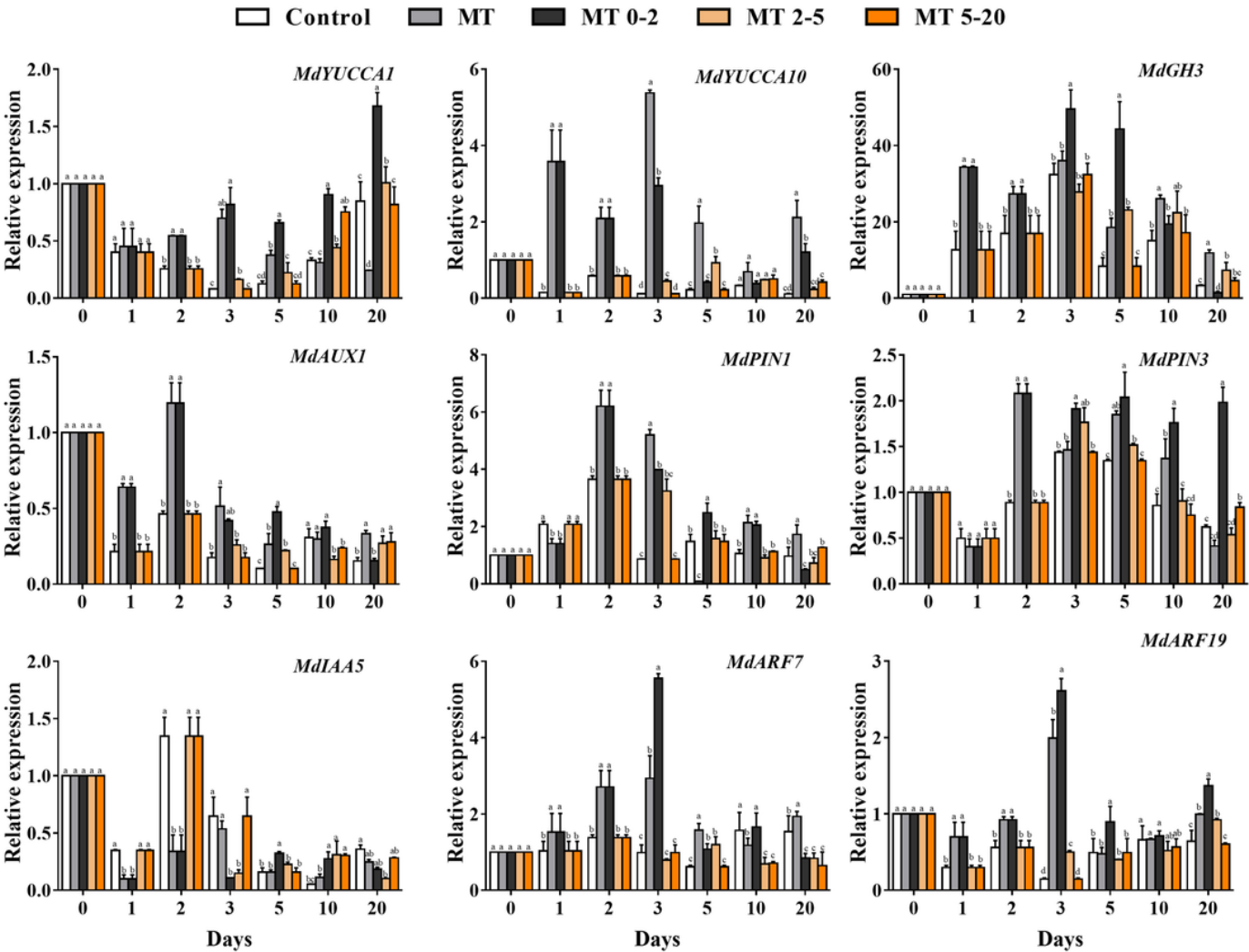


Figure 6

The effect of exogenous MT treatment on the relative expression of genes involved in auxin biosynthesis, transport, and signal transduction at different stages of AR development in five treatment groups of *Malus prunifolia*.

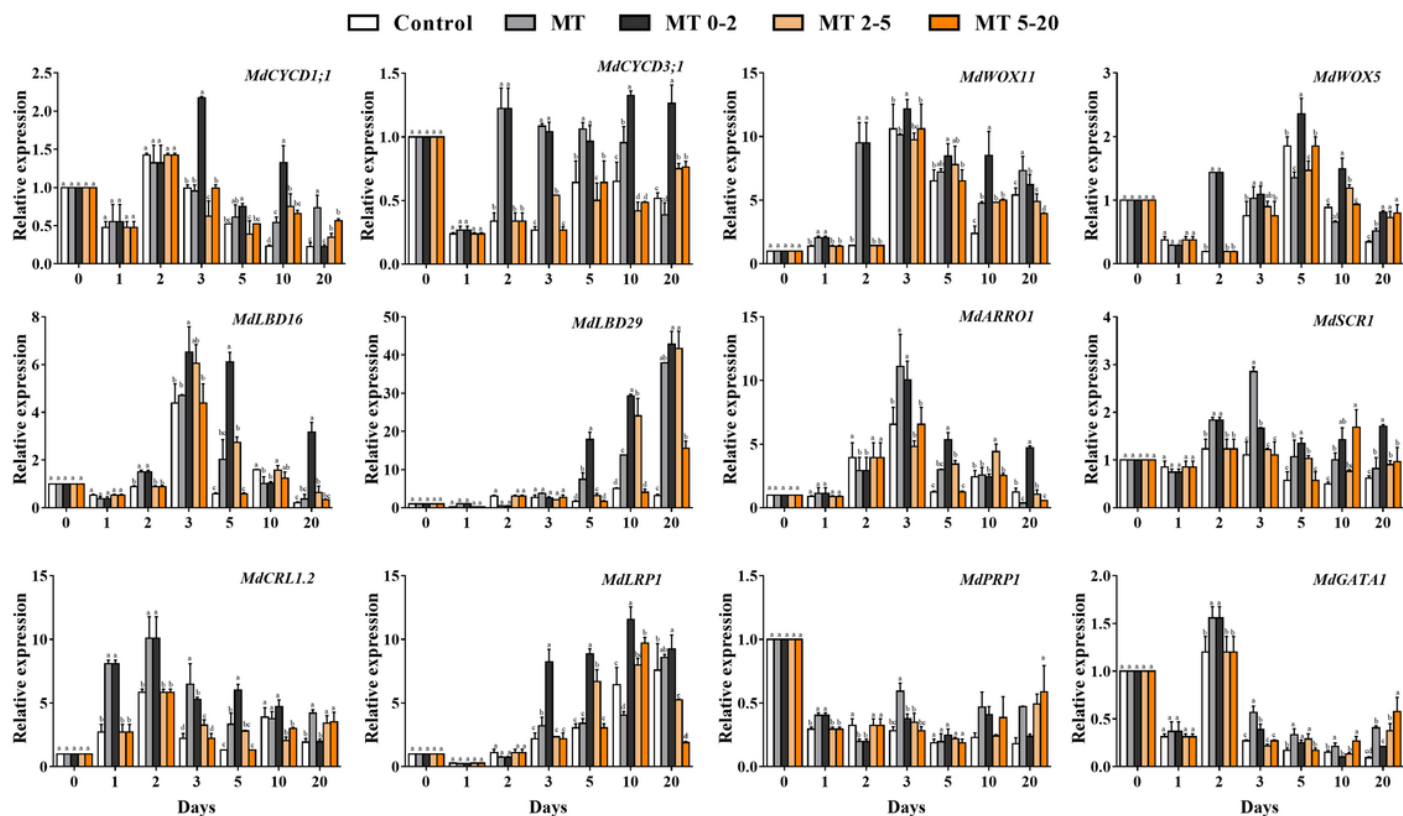


Figure 7

The effect of exogenous MT treatment on the relative expression of genes involved in the cell recycle and AR development at different stages of AR formation in five treatment groups of *Malus prunifolia*.

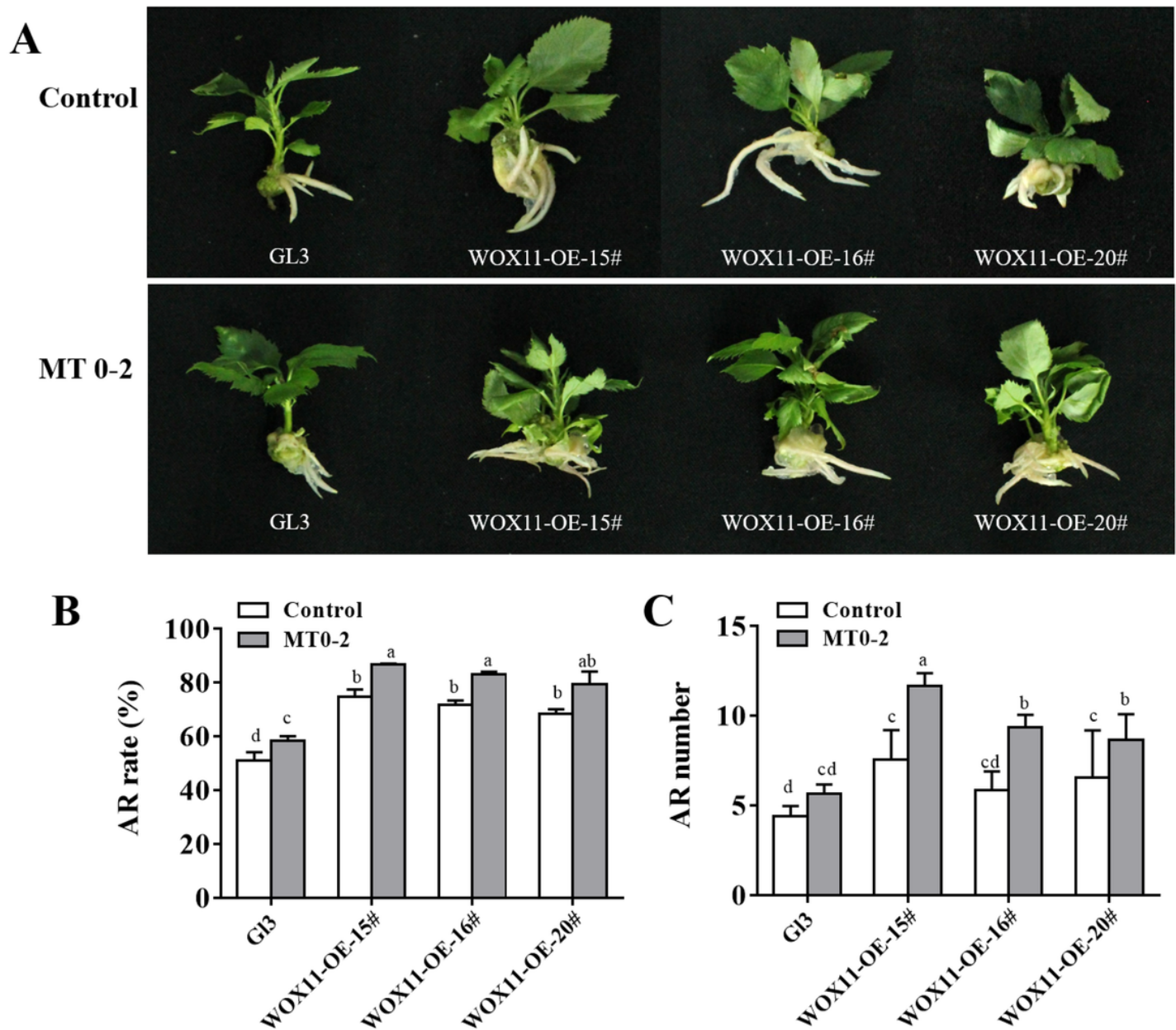


Figure 8

(A) Morphological observations, (B) AR rate and (C) AR number during AR formation in transgenic tissue-culture plantlets overexpressing MdWOX11 (35S::WOX11-OE) and wild type 'GL3' after treatment with 3.45 μ M IBA as the control. Another group was treated with MT for 0–2 d (MT0–2 group), the results represent measurements after culturing the controls and MT0–2 group for 20 d.

Supplementary Files

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