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

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Abstract

Background

Melatonin (MT) plays a key role in the plant growth and development, however, whether MT involved in apple adventitious root (AR) development is not entirely understood. In present study, we set up four MT-treated groups at different times according to the stages of AR development and one control group in *Malus domestica* (MP), endogenous hormones levels of MT, auxin (IAA), zeatin-riboside (ZR), gibberellins (GA_{1+3}), abscisic acid (ABA) were analyzed in five groups, then the expression of MT signal, IAA synthesis, transport, and signal transduction, cell cycle, and root development related genes were measured by RT-qPCR, the function of MdWOX11 was analyzes by technology of apple transgenic.

Results

Advance of AR development by MT is depended on its promotion of stage of AR induction at 0–2 d in the apple rootstocks. MT-treated increased IAA levels, there were crosstalk between MT and IAA during inducing AR formation. Expression analysis exposed that the expression of *MdWOX11* induced by MT-treatment, and positively regulated AR formation in apple. Furthermore, *MdWOX11* over-expressed lines produced more ARs than 'GL3', phenotypic analysis indicated that *MdWOX11* over-expressed lines were more sensitive to exogenous MT-treatment than 'GL3', it was also indicated that *MdWOX11* regulated AR formation in response to MT in apple rootstock.

Conclusions

MT promotes AR formation mainly during AR induction stage by inducing IAA levels as well as by up-regulation of *MdWOX11*.

Background

Apple (*Malus domestica*) is the major commercial fruit tree cultivated across the world. Apple fruits have high nutritional and economic values. *Malus prunifolia* (MP) is widely known as the easiest rooting apple rootstocks, and it has the following advantages such as good graft compatibility, cold resistance, salt and alkali resistance, disease and insect resistance and so on. The induction of AR from stem basal parts is a major step in the vegetative propagation of apple rootstocks. ARs are post embryonic roots that are emerged from non-root organs, AR primordium arise from interfascicular cambium cells adjoining to phloem cells [1, 2], the processes that are dependable for AR formation has been identified in different plants, for instance rice [3], Arabidopsis [4, 5], and poplar [6], however, the methods of improving AR formation in apple remains to be studied.

As a well-known animal hormone, MT (N-acetyl-5-methoxytryptamine) was initially discovered in plants by two groups of workers in 1995 [7, 8]. Previous studies have showed that MT exists in plants [9] and plays a major function in the growth of roots, shoots, explants, stress responses and so on [10–15], the relationship between MT and AR formation mainly studied in herbaceous plant, for example, exogenous application of MT promoted adventitious rooting in tomato and rice [16, 17], but in woody plant like apple, the mechanism of MT regulating AR formation remains to be seen. AR formation can be categorized as four-stage process [18–21]. Moreover, the stage at which MT plays a key role for AR development is still unknown. In this study, we found that MT promoted AR formation at the early stages of AR induction and initiation. Previous researches have demonstrated that the relationship between MT and other plant hormones like auxin (IAA), cytokinin (CK), gibberellins (GA), abscisic acid (ABA) [22], MT-treated plants increased CK levels during non-biological stress [23], MT contributed to high contents of active GAs such as GA_3 and GA_4 [24], exogenous MT treatment decreased the contents of ABA [23], the relationship of MT and these hormones during Adventitious rooting process still needs to be determined. MT could act as a growth promoting compound, increasing IAA levels, IAA synthesis and polar IAA transport [25–27], the great mass of studies have studied the IAA-like activity of MT which, in a similar way to IAA, was able to stimulate roots and shoots generation [28], however, there was opposite view about it, the regulation of MT on growth and differentiation of root was thought to be independent of IAA [29], in this study, we found MT–IAA crosstalk played an important role in AR induction. However in apple rootstock, the role of plant hormone homeostasis and associated signaling networks during AR formation is not completely understood.

MT biosynthetic genes, such as *TDC*, *SNAT*, *HIOMT*, and *ASMT* were induced by MT [30–34], *MzSNAT5* allows plant mitochondria to increase MT synthesis in apple [33], overexpression of *ASMT* improved MT production in *Arabidopsis thaliana* [34], in our study, the expression of MT biosynthetic genes were analyzed in apple. In addition, *WUSCHEL-RELATED HOMEOBOX GENE 11* (*WOX11*) take part in crown root emergence and development [35] and AR development in Arabidopsis [36], but in woody plant, regulatory mechanism of *WOX11* during AR development is poorly understood, in addition, whether AR formation in over expressed *MdWOX11* transgenic apple is regulated by exogenous MT remains to be seen.

At present, MT regulating mechanisms for AR formation is not well characterized in apple rootstock. In the present study, MT was involved to induce AR formation at the early stages of AR induction and initiation. Exogenous MT induced the AR formation through increasing the content of IAA, its high levels induced IAA synthesis, transformation, and signalling related gene, MT-treated tissue culture plantlets increased the expression of root development related gene, resultantly increased the number of AR. In further, we demonstrated that overexpression of *MdWOX11* promoted the emergence and development of ARs, exogenous MT induced AR development in overexpressed *MdWOX11* transgenic apple. This study was benefit to study that the mechanism of MT regulated the AR formation. This study results provided deep insights into the mechanism how MT regulates AR formation in apple rootstock.

Results

This study were conducted to identify the exact time on which MT promotes AR formation arising from tissue culture plantlets of *Malus prunifolia* apple rootstocks, in the study, we set five treatment groups: MT, MT0-2, MT2-5, MT5-20 along with one control group (Fig. 1A). From Fig. 1B, No morphological changes were evident in all groups within 5d, however on 10d ARs emerged from stem basal parts. At 20d, the number of AR was highest in the group of MT0-2 than other groups, in group of MT2-5 and MT5-20 were more conducive to AR formation than control and MT group at 20 d, but there is no difference between them. In order to observe anatomical at the different stage of AR formation, sectioning paraffin-embedded samples was made and stem sections was viewed by the light microscope. On 0 day cross sections of the samples exposed the existence of competent cells, but the capability of cambial cells to undergo mitosis was observed at 5d, now cell divisions were appeared in the agminated cells, AR appeared on transection of stem basal at cultured in medium 10 days (Fig. 1C).

Furthermore, we also measured the root rate, number of AR, crossings, root length, root surface area and root volume between five groups, it was consistent with the phenotype observations of AR formation, The results showed that all measured parameters were higher in MT0-2 group as compared to other groups, the control group was the minimum number of ARs, and other root measured parameters as compared with other groups (Fig. 2). The result showed that MT mainly promoted the AR formation at 0-2d during AR induction stage.

ARs were classified into 3 groups based on their diameter: 0–2.0 mm, 2.0–5.0 mm and > 5.0 mm. According to the data of AR number, length and surface area, the categories of 0–2.0 mm constituted most percentage of total root, differ from these indexes was root volume, the categories of 2.0–5.0 mm was the largest than other classes in all groups (Fig. 3). The data were showed that the MT0-2 group was the greatest than other groups, there were twice as many in the MT0-2 group as in the control group in the 0–2.0 mm class (Fig. 3). We can draw conclusion that the most of AR was fine root (0–2.0 mm).

The contents of hormones: MT, IAA, ZR, GA_{1+3} and ABA were analyzed in MP tissue culture plantlets after being treated with MT, as can be seen in Fig. 4, MT content was visibly greater in MT0-2 group than other treated groups during early developmental stage of ARs, and reached a peak at 5 d in MT0-2 group. In group of MT and MT0-2, IAA, ZR and GA_{1+3} content were apparently higher than control group during AR induction at 1d and 2d, but their content were apparently lower in MT0-2 than other group at 10d, ABA content was approximately contrary to content of IAA, ZR and GA_{1+3} (Fig. 4).

Expression of MT and IAA signal transduction associated gene in response to MT were analyzed, expression of *MdTDC1*, *MdHIOMT2*, *MdASMT1* and *MdASMT2* appeared the similar regulation of expression, their expression were obviously greater in MT0-2 than other groups expect at 0 and 20d, *MdSNAT* and *MdHIOMT1* expression were higher in MT0-2 than other groups at 1d, 5d and 20d (Fig. 5).

In further, to determine whether there is a link between MT and IAA, we measured the IAA related gene expression, related gene of IAA synthesis and signal transduction *MdYUCCA2*, *MdYUCCA10*, *MdARF7* and

MdARF19 were visibly greater in MT0-2 than other groups at 1d, 2d and 3d, IAA transport related gene *MdAUX1*, *MdPIN1*, and *MdPIN3* were higher in MT0-2 than other groups at 2d, however, IAA signal transduction related gene *MdIAA5* was lower in MT0-2 than other groups during AR induction stage (Fig. 6).

To investigate whether MT could affect cell division,expression of genes involved in cell cycle *MdCYCD1;1* and *MdCYCD3;1* were analysed, which were highly expressed in MT0-2 at 3 d and 10d. Now it can be concluded that application of MT promoted AR formation in apple rootstock, qRT-PCR analysis showed that all of root development related genes were higher at most time point after MT treatment. We found among all root related gene *MdWOX11* expression in MT treated group was 5.6 times higher than control group at 2d (Fig. 7). This suggests that in the stage of AR induction, *MdWOX11* are likely to have crucial roles in AR induction after treated with MT.

The expression of *MdWOX11* was induced by IBA treatment (Fig. 7). We received the over-expressed (OE) transgenic lines of *MdWOX11-OE15#, 16#, 20#* in 'GL3', identification of DNA level of overexpression *MdWOX11* transgenic lines were list in Figure S1. In order to confirm whether *MdWOX11* transgenic lines exhibited better response to MT signalling, wild-type and transgenic apple tissue culture plantlets were treated with 0.7 $mg.L^{-1}$ IBA served as a control, another group was treated with MT at 0-2d which named as MT0-2. The number of AR were higher in the group of MT0-2 than control group, both of the over-expressed *MdWOX11* transgenic lines and 'GL3', over-expressed *MdWOX11* transgenic lines showed more ARs than those of 'GL3' (Fig. 8A), the rate and number of AR were conformed to the phenotype of these apple plants (Fig. 8B and C). *MdWOX11* over-expressed plants were more sensitive to exogenous MT treatment than wild type (Fig. 8), these result indicated that *MdWOX11* induced AR formation in response to MT treatment.

Discussion

AR formation is the key to vegetative propagation, in previous studies AR formation from tissue culture plantlets is generally split into four stages such as AR induction, initiation, AR primordium formation, and emergence of AR [18–21]. In our study, according to the process of AR formation in MP, from the results of Fig. 1, 0–2d regards as the stage of AR induction, 2-5d was located at AR initiation, 5-20d regards as the stage of AR primordium formation and AR emergence. In the contrary, MT is widely known to take part in many biological process including plant growth, flowering, stress responses and so on, study suggested that MT was beneficial to AR development in tomato [16], no studies have been conducted in woody plant, however, to determine the timing and the mechanism of MT can promote AR formation, we treated plants with MT at different times, from the phenotype of five treatment groups, there is no difference between MT2-5 and MT5-20, but the MT0-2 produce more AR than other groups (Figs. 1,2 and 3), these results showed that MT facilitates AR formation mainly during AR induction stage at 0–2 d.

The relationship of MT and IAA is controversial during root development, some study suggested that low concentration of exogenous MT can induce the increase of endogenous IAA content in plants, and it is

believed that the promoting effect of MT on growth may be caused by the increase of IAA content [25], however, other research have showed that regulation of root growth and differentiation by MT was independent in the IAA [29], and to figure out whether MT-treated can improve the content of IAA and IAA signalling or not, we measure the IAA contents in five groups, IAA contents mainly increased during AR induction after MT application (Fig. 4), these results may be due to the fact that IAA play an important role in the early stage of root development [37, 38], we can draw an conclusion that exogenous MT treated group promoted AR formation rely on IAA. On the other hand, from the data of RT-qPCR, MT is involved in IAA-mediated signalling pathways, MT treated group can induce the expression of synthetic, transport and signal transduction related genes (Fig. 6), this study suggested that MT promote AR induction by increasing IAA levels and IAA - mediated signal.

WOX11 is an optimistic regulator for AR formation in response to IAA in *Arabidopsis* [36]. In Arabidopsis, some studies have demonstrated that *WOX11* is induced through IAA and that *WOX11* raised the expression of *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) and *LBD29* at the early stage of AR development [39]. However, knows little on the function of *MdWOX11* in woody plants like apple, such as morphological changes of transgenic plants in response to MT-inducing AR formation in apple. In present study, we conduct the transgenic *35S::WOX11-OE* of apple, from the phenotype of *MdWOX11* over-expressed plants, we can draw the conclusion that *MdWOX11* is a positive regulator for AR activation, as well as, MT-treated group increased the AR development in *MdWOX11* over-expressed plants (Fig. 8), this might be a possible mechanism from which MT promotes AR formation by inducing the function of *MdWOX11* and other its related genes, this is the first study to use *MdWOX11* transgenic lines to investigate the role of *MdWOX11* in response to MT during AR induction. Taken together, our research indicated the *MdWOX11* promoted AR formation in response to MT, it provided deep insights for understanding the molecular mechanisms of MT regulated induction of AR.

Conclusions

Melatonin promotes adventitious root formation mainly at the stage of AR induction by inducing auxin levels, and melatonin activated the function of *MdWOX11* promoting adventitious root formation. Our work have a massive potential to improve the ability of AR formation to accelerate the asexual reproduction for difficult-to-rooting apple rootstock.

Methods

Explant growth conditions and MT-treatments

Tissue culture plantlets of *Malus prunifolia* apple rootstock were grown under the condition of tissue culture of Northwest Agriculture and Forestry University, Yangling (108°04´ E, 34°16´ N), China and further used as plantlets for the AR formation, the plantlets of *Malus prunifolia* were imported from the Aomori in Japan and bred by asexual reproduction. The tissue culture plantlets of *Malus prunifolia* were split into five groups, plants of all groups treated with the same time for 20d. Control was cultured on inducing-root

medium having 1/2 MS supplemented with 0.7 mg.L^{-1} indole-3-butyric acid (IBA) the entire time, IBA usually used to promote formation of root, this group was designated as control. The second group was cultivated on MT medium containing ½ MS medium supplemented with 0.3 mg.L^{-1} MT and 0.7 mg.L^{-1} IBA all the time and named as MT. The third group was transferred to inducing-root medium after cultivating on MT medium for 2d, it was mentioned as MT 0-2. The forth group was transferred to MT medium after culturing in inducing-root medium for 2 d, and then transferred to inducing-root medium after culturing in MT medium for 3d, named as MT 2-5. The fifth group was transferred to MT medium after cultivation on inducing-root medium for 5d, it was served as MT5-20. The complete compositions of the different mediums used for this study is listed in Supplemental Table S1. Samples were harvested at 0 d, 1 d, 2 d, 3 d, 5 d, 10 d and 20 d from the five groups (even though some samples represented samples collected prior to the MT-treatment). A total of 2700 cuttings, 540 from each of the five groups, 90 cuttings were sampled at each sampling point. Samples gathered form basal portion of the stems including the AR formation zone (approximately 0.5cm). Overexpression of *MdWOX11* transgenic apple (*35S::MdWOX11-OE*) and 'GL3' were divided into two groups, one was cultured on 0.7 mg.L^{-1} IBA and served as controls, another was transferred to inducing-root until 20 days after culturing in MT medium for 2 d.

Observation of anatomical and measurement of morphological

Anatomical observation were processed by previously described protocols [1, 40, 41]. Morphological parameters; including AR rate, AR length and ARs average number every cutting were calculated [42], in addition, crossings, root length, root surface area, and root volume, were analysed by EPSON EXPRESSION 10000 xl type scanner (LA l600 scanner, Canada). Totally 90 cuttings were analysed, 30 for every group, and harvested at each sampling point. The collected samples were instantly immersed using liquid nitrogen and stored at −80 °C for further analysis.

Extraction and measurement of hormone levels

The samples for hormones extraction were harvested at different time points from five groups. Hormones were purified and extracted from gathered samples by previously described procedure [43]. Three biological replicates for each group at each sampling point were used. We use enzyme-linked immunosorbent assay (ELISA) technique to detect and analyse hormones [43].

Extraction of RNA and synthesis of cDNA

Total RNA was isolated through CTAB-based method [44],and the total RNA integrity was detected by running samples using 2% agarose gels, besides, cDNA was synthesized by Prime Script RT Reagent Kit with gDNA Eraser (TaKaRa Bio, Shiga, Japan).

RT-qPCR analyses

The expression of MT signal, IAA synthesis, transport, and signal transduction, cell cycle, and root development related genes were measured by RT-qPCR. In further, gene names including abbreviation and full name, MDP numbers in apple, as well as homologues proteins and species on which the identification of protein in apple is based, were showed in Supplemental Table S2. Primers design procedures were based on the previous research [42] , total of the analysed gene specific primers were listed in Supplemental Table S3.

RT-qPCR assays were referred to the methods of procedure [45]. An apple *EF-α* gene was used for normalization. Each sample was set to three biological replicates and three technical replicates. Relative expression of the analysed genes were calculated by $2^{-\Delta\Delta Ct}$ method [46].

Abbreviations

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

Declarations

Availability of data and materials

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

Author contributions

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

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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

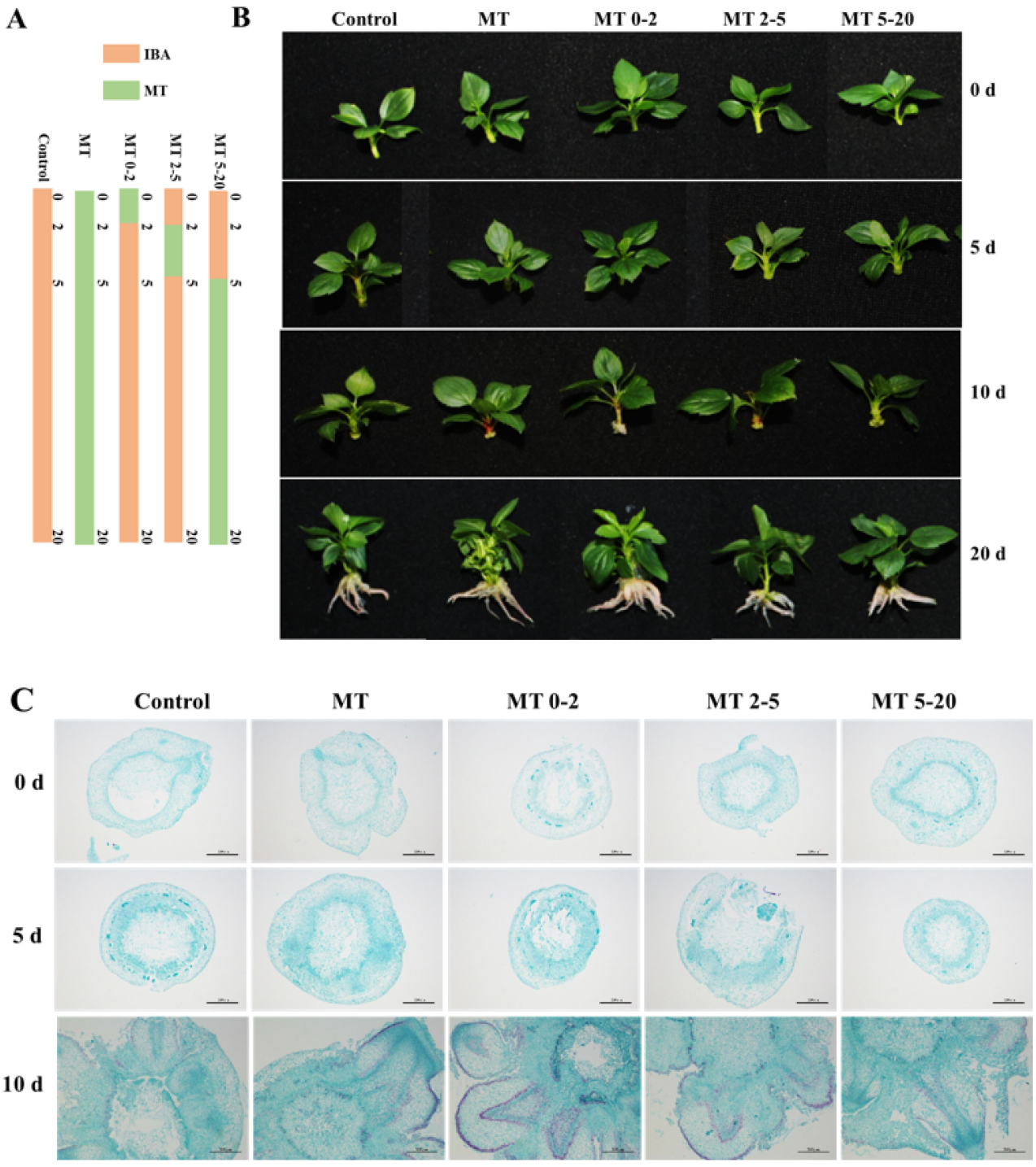

Figure 1

Anatomical and morphological observations of AR development of paraffin-embedded sections of the experimental samples generated in the present study at three sampling times, 0 d, 3 d, and 10 d. five treatment groups were used: (A) Control group in which apple tissue culture plantlets were continuously cultured on a inducing-root medium containing with 0.7 mg· L-1 IBA, MT group were continuously cultivated in 0.7 mg· L-1 IBA and 0.3 mg· L-1 MT, based on different time of MT-treated, the treatment

divide to 3 groups of MT0-2, MT2-5 and MT5-20; (B) Observations of morphological ARs formation between five treatment groups at 0d, 5d, 10d and 20d; (C) Anatomical observations of ARs formation of five treatment groups at 0d, 5d and 10d.

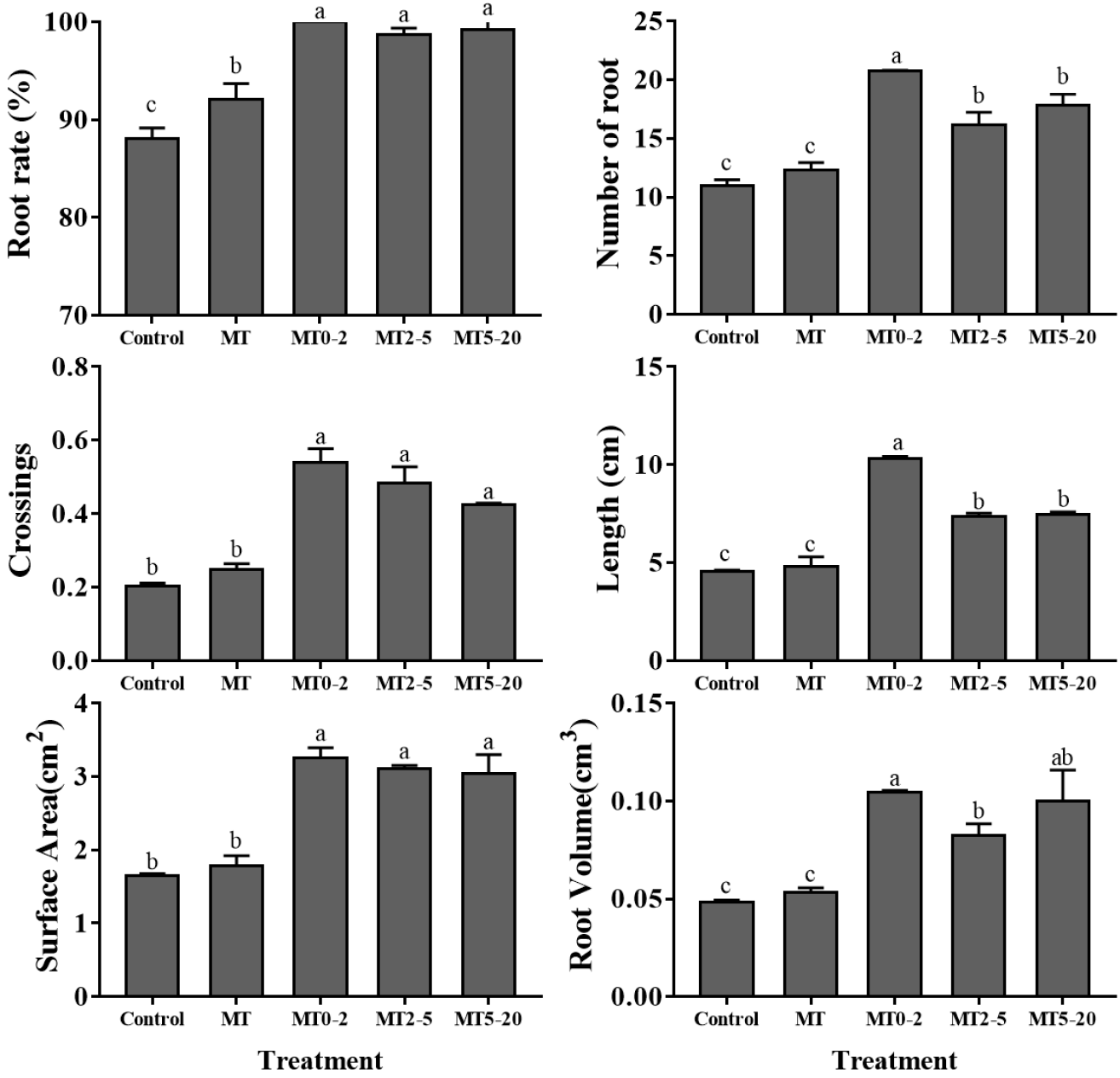

Figure 2

Morphological statistics of AR formation in tissue culture plantlets of Malus prunifolia. The tissue culture plantlets were divided into five groups: Control, MT, MT0-2, MT2-5, MT5-20, AR number, length, surface area, volume were measured in five treatment groups in Malus prunifolia tissue culture plantlets. Values represent the mean ± SE of three biological replicates, a, b, c and other letters indicate a significant difference of ($P < 0.05$), respectively, the same below.

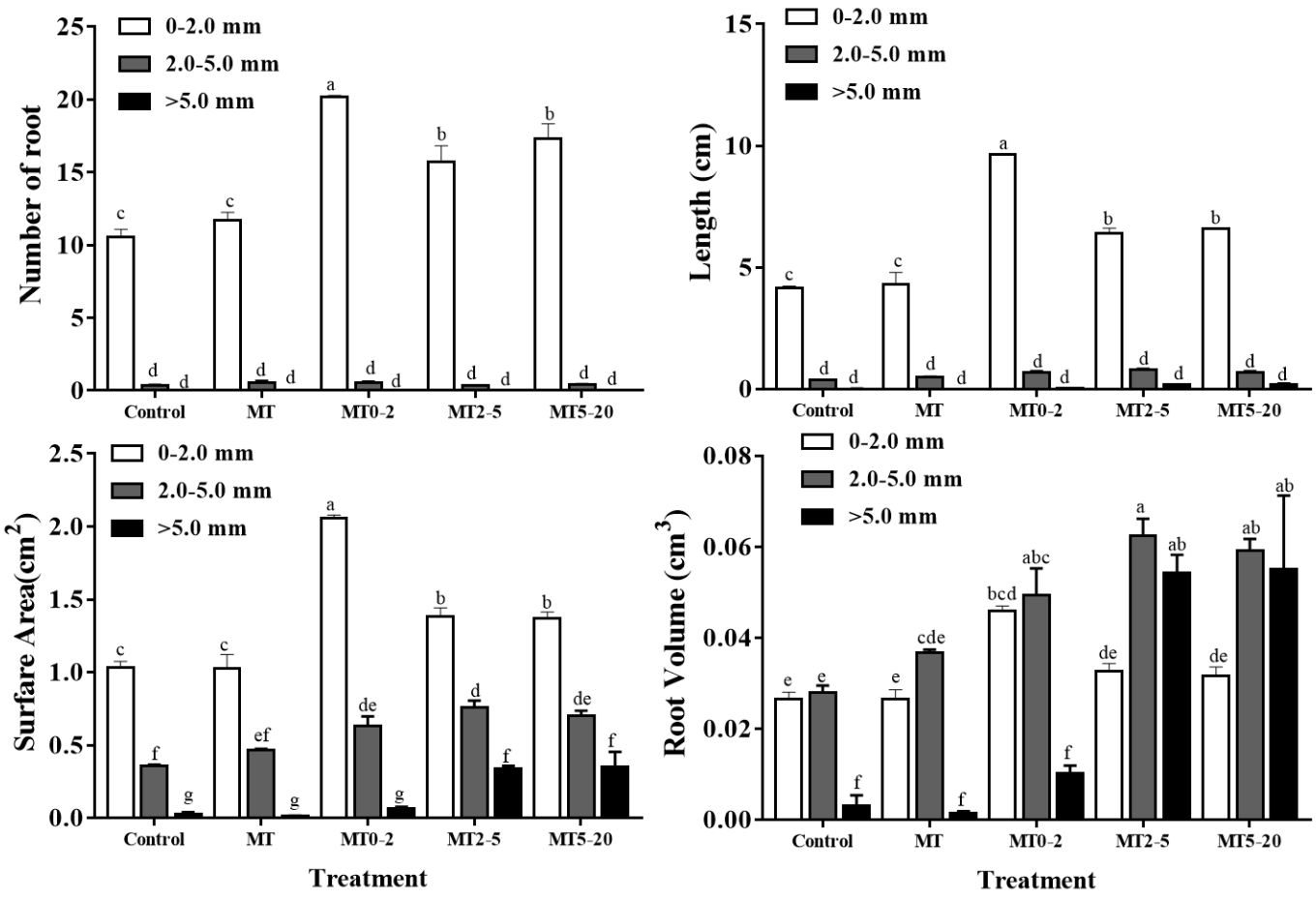

Figure 3

Morphological statistics of AR formation in tissue culture plantlets of Malus prunifolia apple rootstock in different root diameter categories on the number, length, surface area, and volume of root. AR was classified into three groups based on the diameter of root, they are 0-2.0 mm, 2.0-5.0 mm and >5.0 mm.

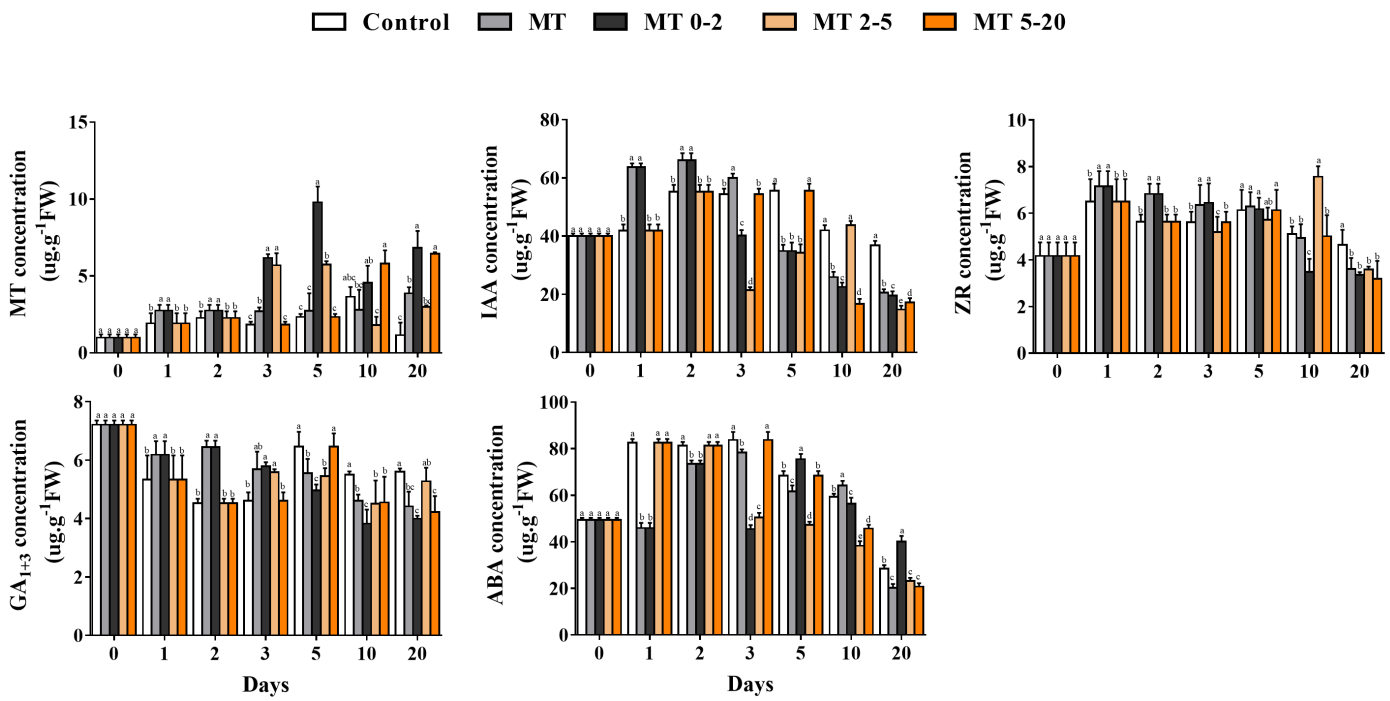

Figure 4

Effect of different time exogenous applications of MT 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.

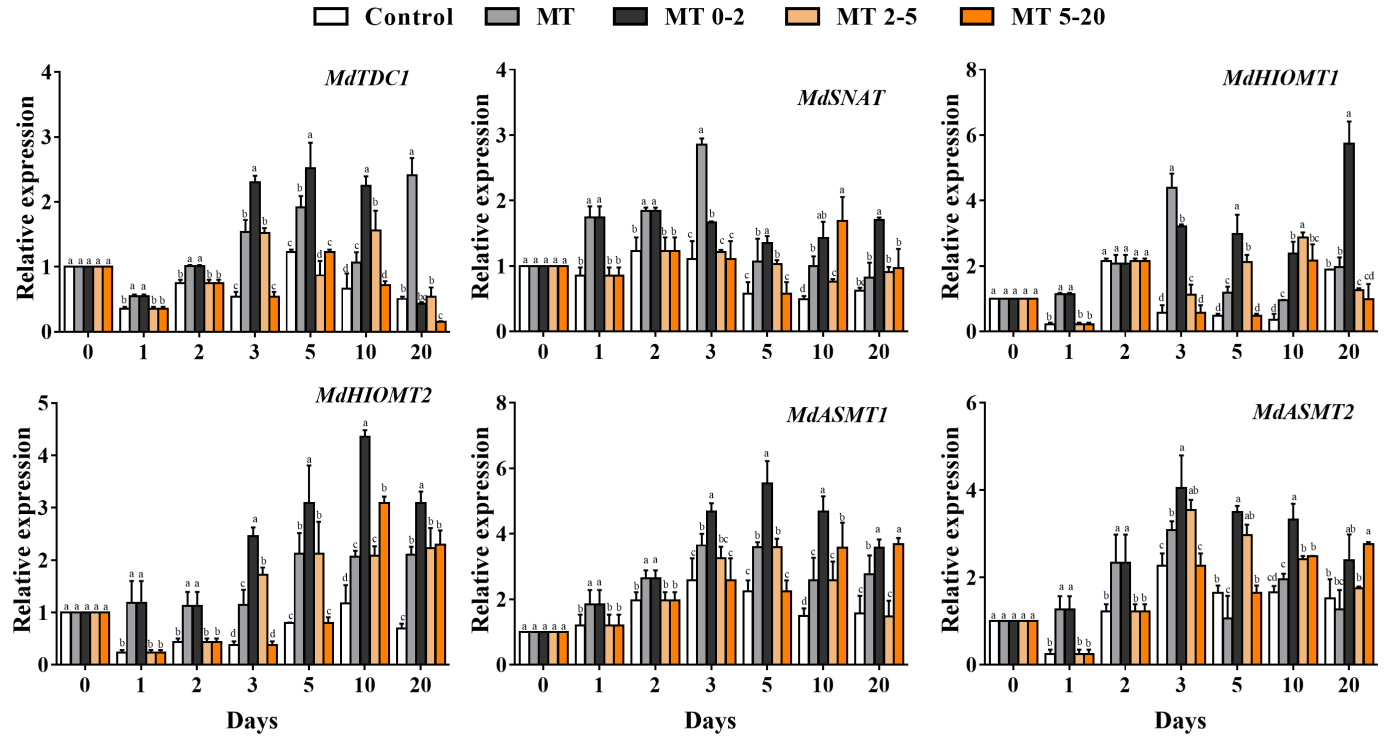

Figure 5

Effect of exogenous MT-treated on the relative expression of MT signal transduction related genes at different stages of AR development in five treatment groups of Malus prunifolia.

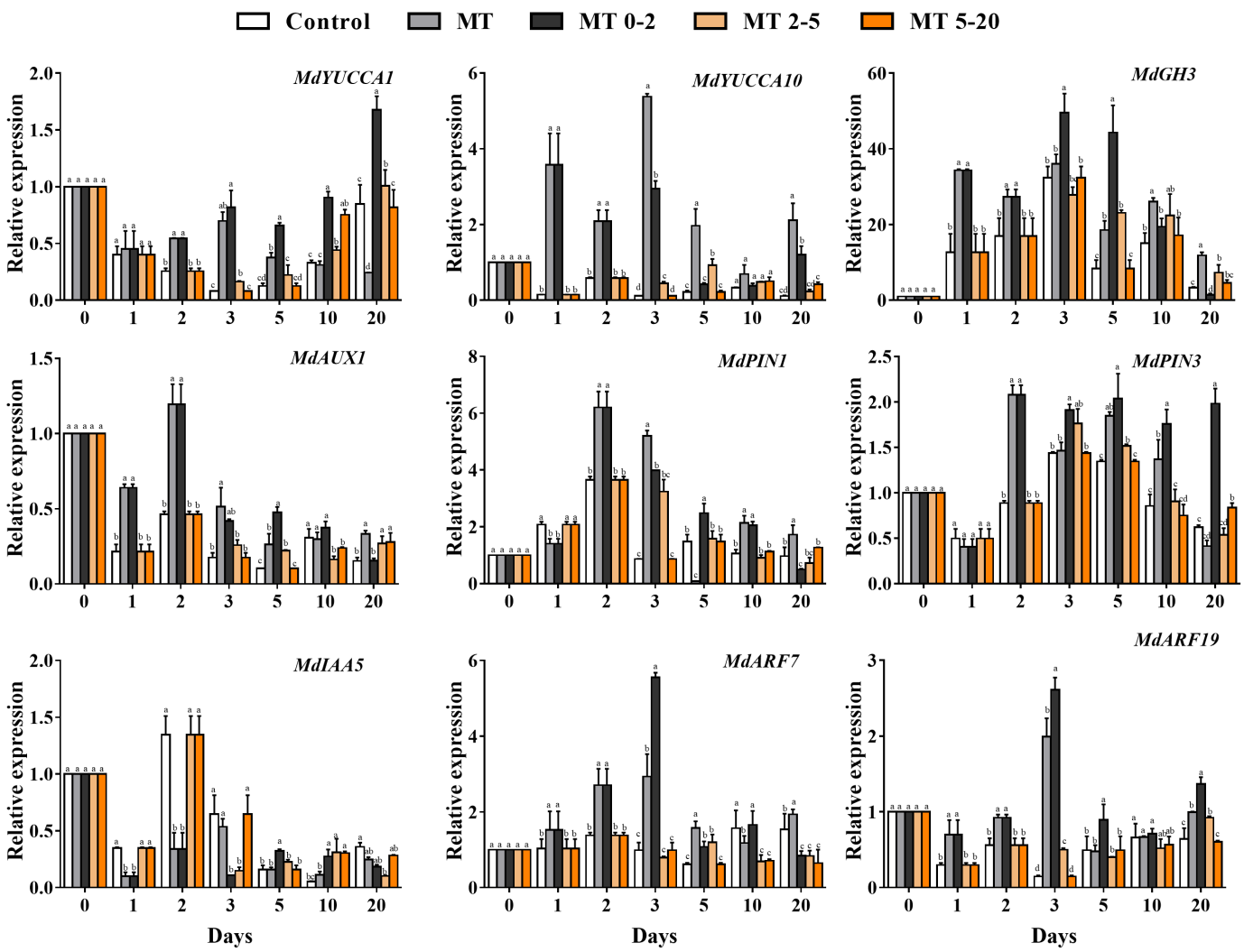

Figure 6

Effect of exogenous MT-treated on the relative expression of auxin synthesis, transport, and signal transduction related genes at different stages of AR development in five treatment groups of Malus prunifolia.

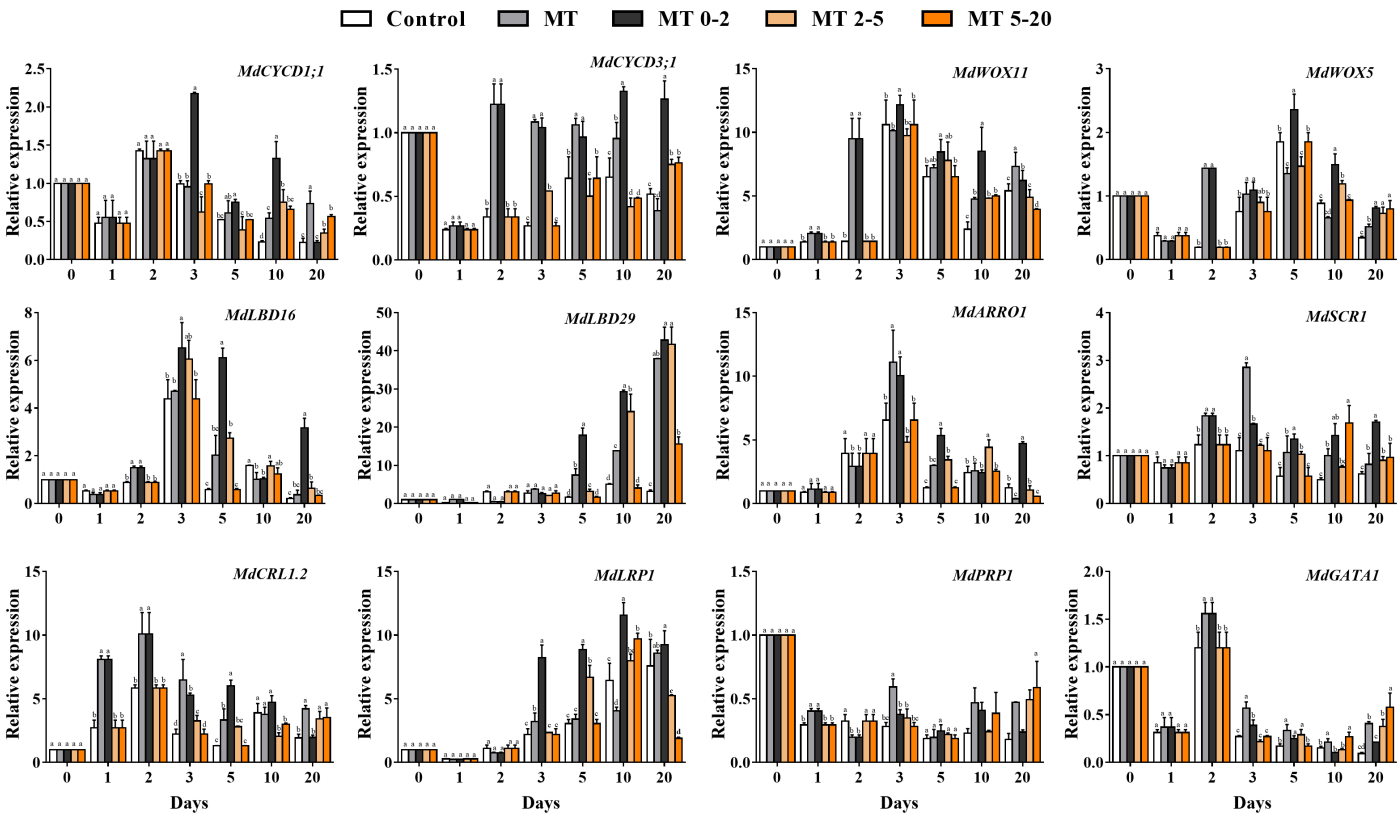

Figure 7

Effect of exogenous MT-treated on the relative expression of cell recycle and AR development related genes at different stages of AR formation in five treatment groups of Malus prunifolia.

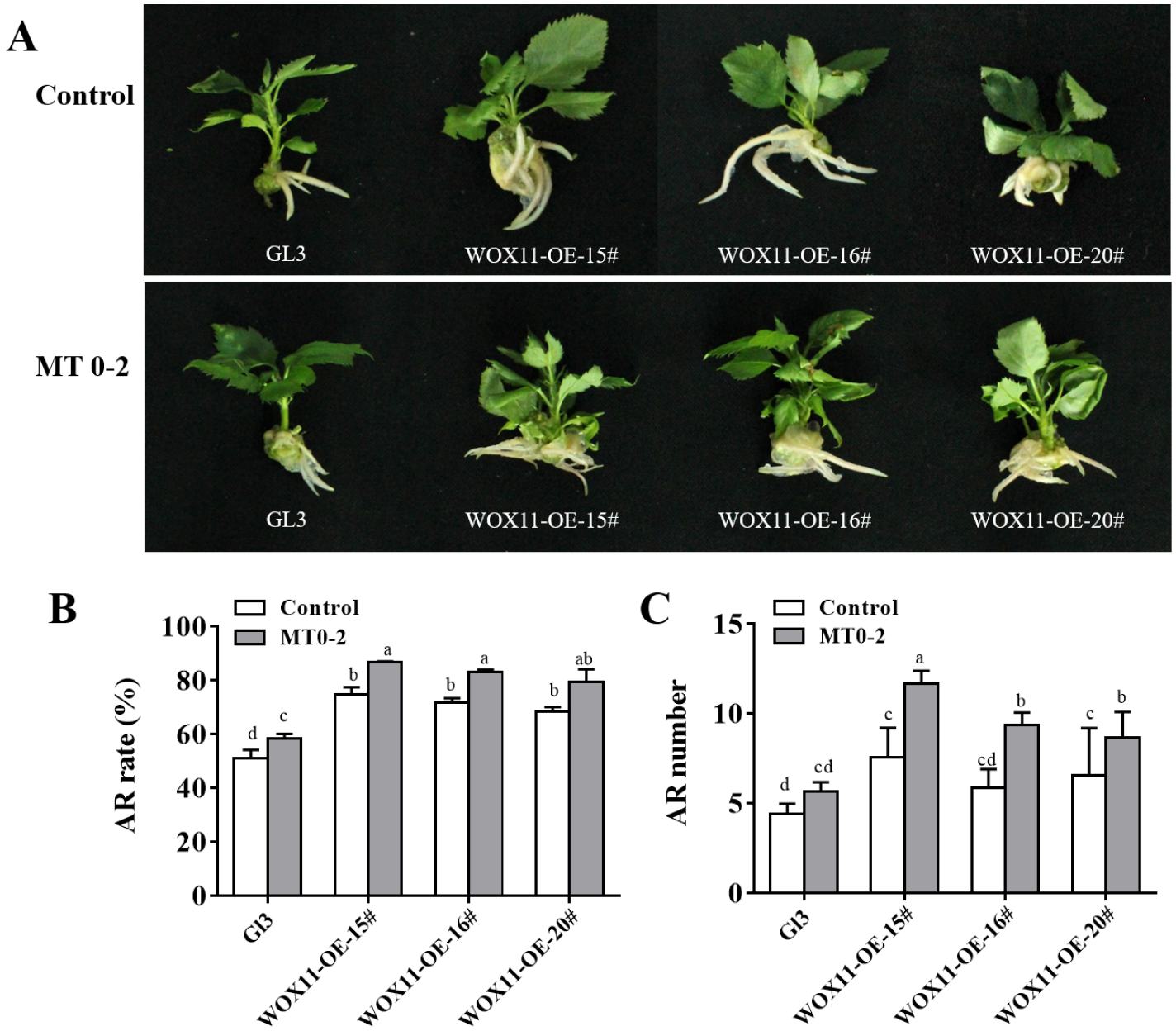

Figure 8

(A)Morphological observations, (B) AR rate and (C) AR number during AR formation in tissue culture plantlets of overexpression of MdWOX11 transgenic strain (35S::WOX11-OE) and 'GL3' after treated with 0.7 mg.L-1 IBA regarding served as the a control, another group was treated with MT during 0-2d which named as MT0-2, these results were measured after cultured the group of control and MT0-2 at 20d.

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