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# Balancing Hormones and Gene Expressions for Rooting Success: Lovastatin Unveils Cytokinin Inhibition in *Malus prunifolia* var. *ringo* Apple Stem Cuttings

**Sinuo Sun †, Muhammad Mobeen Tahir †, Zushu Xie, Pengyan Wei, Jianing Yu, Hangkong Liu, Yinnan He, Xiaoying Ren, Yuanyuan Ma and Jiangping Mao ***

College of Horticulture, Yangling Sub-Center of National Center for Apple Improvement, Northwest A&F University, Xianyang 712100, China; ssn7762023@163.com (S.S.); mubeentahir924@gmail.com (M.M.T.); xiezushu2023@126.com (Z.X.); wpy9796@163.com (P.W.); jianingyu777@outlook.com (J.Y.); lhk5223@nwsuaf.edu.cn (H.L.); neptune0526@nwafu.edu.cn (Y.H.); renxiaoying@nwafu.edu.cn (X.R.); mayuanyuan@nwafu.edu.cn (Y.M.)

* Correspondence: mjp588@nwafu.edu.cn

† These two authors contributed equally to this work.

**Abstract:** Adventitious root (AR) formation is the key to asexual reproduction; however, cytokinin (CK) hampers AR formation. But the mechanism by which CK inhibits it is still unknown. In this study, we used *Malus prunifolia* var. *ringo* apple stem cuttings that were treated with exogenous 6-benzyl adenine (6-BA) at 1 mg/L and lovastatin (CK biosynthesis inhibitor) at 1 mg/L to compare with control (untreated) cuttings. The results indicated that the control and 6-BA-treated cuttings failed to produce ARs; however, lovastatin-treated cuttings successfully produced a few ARs after 20 days (d) of treatments by increasing indole-3-acetic acid (IAA) and reducing zeatin riboside (ZR) content at several time points. The 6-BA treatment induced the expression of CK-related genes, such as *MdARR3*, *MdARR5*, *MdARR5-2*, *MdAKH4*, and *MdCKX5*, at most time points. However, lovastatin-treated cuttings reduced their expression, which favors AR formation. Furthermore, the expression of auxin-related genes, including *MdIAA23*, *MdARF7*, and *MdARF19*, was induced by lovastatin treatment. Like auxin-related genes, several root-development-related genes (*MdWOX5*, *MdWOX11*, *MdLB29*, and *MdARRO1*) were also promoted in response to lovastatin treatment that were repressed by 6-BA and control cuttings. In conclusion, lovastatin treatment supports AR formation by inhibiting CK biosynthesis inside the cuttings, as compared to the control and 6-BA-treated cuttings. This study laid the foundation for future studies on the relationship of CK biosynthesis inhibitors with adventitious rooting in apples and other crops.

**Keywords:** apple rootstock; asexual reproduction; adventitious root (AR); cytokinin (CK); lovastatin



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## 1. Introduction

Apple (*Malus domestica*) is a beneficial and highly consumable fruit worldwide, and China is the leading country in apple production. However, China's per-hectare production lags behind developed nations due to the absence of efficient breeding technologies for high-quality dwarf rootstocks. In response, the Chinese apple industry seeks innovative planting technologies, focusing on dwarf apple rootstocks to control tree structure [1,2]. Asexual reproduction by stem cuttings is used to produce parent-identical offspring. However, adventitious root (AR) formation is a bottleneck in this practice due to the gaps in understanding underlying physiological and molecular mechanisms [3]. Therefore, improving rooting ability is a major scientific question that should be solved urgently to support the apple industry. It is known that ARs are formed from non-rooted organs, such as stems and leaves. In *Arabidopsis thaliana*, AR primordia is initiated from the cells in the pericycle [4]. In contrast to Arabidopsis, AR primordia mainly develops from the ray

cells adjacent to the vascular cambium in stem cuttings from woody plants, such as Malus species [5].

Adventitious rooting in apple stem cuttings is generally divided into several stages depending on physiological and metabolic markers. Several studies indicated that AR formation is a four-stage process, as follows: 0–1 day (d), activation; 1–3 d, induction; 3–7 d, initiation; and after 7 d, emergence stage [6]. Several internal and external factors, such as phytohormone homeostasis, age, temperature, light, nutrients, sugar, etc., affect the formation of AR. Among these factors, endogenous hormones play a key role in AR regulation, affect cell physiology in response to environmental shifts, and determine the signaling network inside the plant that synchronizes cell fate and cell specialization [7]. Auxin plays a role in multiple physiological processes, such as cell proliferation, vascular differentiation, and lateral root (LR) and AR formation. Studies indicated that more indole-3-acetic acid (IAA) is required to trigger AR initiation, but it is not required for the AR emerging stage [7–9]. In addition, cytokinin (CK) is a necessary hormone that controls the cell cycle and a number of developmental processes. Several studies indicated that CK inhibits adventitious rooting [9–12], and that a low endogenous CK content promotes adventitious rooting in apples [13]. CK-auxin crosstalk also plays a key role in supporting root meristem size, with auxin, with CK showing an antagonistic action in the control of rooting [14,15]. In a previous study, apple stem cuttings were treated with 6-benzyl adenine (6-BA) for 3 d, which led to CK accumulation and the promotion of the expression of CK signaling pathway genes such as *MdRR1*, *MdRR2*, *MdRR3*, and *MdAHK4*. Furthermore, it inhibits the auxin signaling pathway, cell cycle, and root-development-related genes, which all have an adverse effect on AR formation [5]. A study indicated that *MsGH3.5*-overexpressed and control micropropagated apple plants were exogenously treated with IAA and lovastatin. AR formation was improved by increasing IAA levels but significantly repressed after lovastatin treatment in both transgenic and control plants. Furthermore, the concurrent application of IAA and lovastatin improved AR numbers compared to the lovastatin treatment alone but did not eradicate the repression of root growth [16]. But studies on the inhibition of CK biosynthesis during rooting are still lacking. Other hormones, like jasmonic acid (JA), brassinosteroids (BR), and gibberellins (GA), also have a role in regulating ARs in apples as well as other crops, as reviewed by Tahir, Mao, Li, Li, Liu, Shao, Zhang and Zhang [13]. The process of adventitious root formation is well studied in several crops, including poplar, Arabidopsis, and rice [17,18]. However, several studies have been reported on the underlying physiological and molecular mechanisms related to AR formation in apples, but they are still inadequate.

Apple is an essential horticultural crop. Asexual reproduction is commonly used for the mass propagation of apple rootstocks to generate genetically identical plants. *Malus prunifolia* var. *ringo* is the most used rootstock for cultivating apples. It has a strong tree architecture, is environmentally stress-resistant, has a high and uniform yield, and is excellent in quality. Moreover, it is easy to root in media containing IBA but hard to root in the absence of IBA. It is widely believed that CK is the main key hormone that hinders the process of adventitious rooting, and a lower CK content is usually associated with an increase in AR formation. But what is the specific effect of CK and CK biosynthesis inhibitors on AR formation? This is a bottleneck in the asexual reproduction of apples and a major scientific question that should be answered urgently to support the expansion of the apple industry. Therefore, this study was designed to identify the role of CK and CK biosynthesis inhibitors in adventitious rooting. We measured the endogenous hormone content and performed an expression analysis of CK, auxin, and root-development-related genes. Our results elucidate how lovastatin induces AR formation by modulating hormonal levels and gene expression patterns, laying the foundation for future studies to enhance AR formation abilities.

## 2. Materials and Methods

### *2.1. Plant Material*

The plantlets of *Malus prunifolia* var. *ringo* were imported from Aomori, Japan, and propagated by asexual reproduction at Northwest A&F University. 1026 morphologically homogenous stem cuttings were cultured on 1/2 MS medium (sugar, 30 g/L; agar, 7 g/L; and pH 5.8) and equally divided into three groups. The first group served as the control (untreated), the second group was treated with 1 mg/L of 6-BA and named the 6-BA-treated group, and the third group was treated with 1 mg/L of lovastatin and named the lovastatin-treated group. According to the previous studies, stem basal parts (0.5 cm) are considered the rooting zone. 162 stem cuttings were harvested at 0 d, 1 d, 3 d, 5 d, 7 d, 11 d, and 20 d from each treatment, covering the four stages of adventitious rooting, as follows: 0–1 day (d), activation; 1–3 d, induction; 3–7 d, initiation; and after 7 d, the emergence stage of AR formation. After 20 days of treatment, samples were harvested, and the number of ARs per cutting was calculated manually. Harvested samples were immediately immersed in liquid nitrogen and kept at −80 °C for endogenous hormone extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR) expression analysis.

### *2.2. Determination of Endogenous Hormone Content*

An enzyme-linked immunosorbent assay (ELISA) was used to measure the endogenous hormone content in the control, 6-BA, and lovastatin-treated groups. The hormones include IAA, BR, JA, abscisic acid (ABA), zeatin riboside (ZR), and gibberellic acid $_{1+3}$ ($GA_{1+3}$). The extraction and measurement of these endogenous hormones were conducted at 0 d, 1 d, 3 d, 5 d, 7 d, 11 d, and 20 d, covering the different stages of adventitious rooting in apples. Three biological and three technical replications were tested. The experiment was conducted at the Center of Plant Growth Regulator, China Agricultural University. The detailed description can be found in our previous study [19].

### *2.3. RNA Extraction, cDNA Synthesis, and RT-qPCR Expression Analysis*

For the gene expression analysis, stem basal parts from the control, 6-BA-, and lovastatin-treated groups were harvested at different timepoints covering the four stages of AR formation: 0 d, 1 d, 3 d, 5 d, 7 d, 11 d, and 20 d. Total RNA was extracted using the Plant RNA Purification Reagent according to the instructions (Invitrogen, Waltham, MA, USA). Total RNA integrity was determined by running samples on 2% agarose gels. Later, cDNA was prepared using a Prime Script RT Reagent Kit with a gDNA Eraser (TaKaRa Bio, Shiga, Japan). The relative expression of genes related to CK activity (*MdARR1*, *MdARR3*, *MdARR5*, *MdARR5-2*, *MdARR12*, *MdAPH1*, *MdAHK4*, *MdCKX5*, and *MdTCP17*), auxin synthesis, transport, signal transduction (*MdAUX1.2*, *MdIAA23*, *MdPIN3*, *MdARF7*, *MdARF19*, and *MdYUCCA2*), and root development (*MdWOX5*, *MdWOX11*, *MdLBD16*, *MdLBD29*, *MdHB30*, and *MdARRO1*) was measured by RT-qPCR to identify their roles in adventitious rooting. Primer 6.0 was used to design gene primers using the apple sequences that are published in GenBank, and the pair of primers is shown in the Supplementary Materials (Table S1). The RT-qPCR assay was conducted using an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hong Kong, China) with the following protocol: 95 °C for 3 min; 40 cycles at 94 °C for 15 s, 62 °C for 20 s, and 72 °C for 20 s. The actin gene was used as a reference gene. Three biological replications and three technical replications were tested for each gene, and the relative expression of genes was measured by the $2^{-\Delta\Delta Ct}$ method [20].

### *2.4. Statistical Analysis*

The data were analyzed by analysis of variance (ANOVA), and significant differences among means were measured using a least significant difference (LSD) test at the 0.05 level. The graphs were generated using GraphPad Prism version 10.0 (San Diego, CA, USA).

## 3. Results

### *3.1. Effect of 6-BA and Lovastatin Treatments on Adventitious Rooting and Endogenous Hormone Content*

The differential response of the apple stem cuttings after 20 d of treatment with 6-BA and lovastatin, as well as the control, during adventitious rooting is shown in Figure 1. The control and 6-BA-treated stem cuttings did not produce ARs; however, the lovastatin-treated cuttings produced a few ARs. We also measured the endogenous hormonal contents during the different time points of AR formation: 0 d, 1 d, 3 d, 5 d, 7 d, 11 d, and 20 d. The content of IAA was reduced in the 6-BA- and lovastatin-treated stem cuttings at 1 d and further reduced in the lovastatin-treated cuttings at 3 d. At 5 d, the 6-BA-treated group showed a sharp decline; however, the lovastatin group showed an increase. At 11 d, the control group had the highest IAA content (Figure 2). The ZR content started to decrease from 1 d to 5 d in the 6-BA- and lovastatin-treated cuttings (except lovastatin-treated cuttings at 5 d) as compared to control cuttings. The highest peak of ZR was seen in control cuttings at 11 d (Figure 2). Lovastatin-treated stem cuttings hold the highest ABA content values at most timepoints compared to the control and 6-BA-treated cuttings (Figure 2). Furthermore, the JA content was improved from 0 d to 1 d in all treated cuttings. However, at 3 d, the lovastatin-treated cuttings, and at 5 d, the 6-BA-treated cuttings, showed a downward trend (Figure 2). The $GA_{3+1}$ concentration was induced in the control cutting at 1 d and reduced in the 6-BA and lovastatin cuttings at 1 d compared to 0 d. However, $GA_{3+1}$ contents were sharply reduced in the 6-BA-treated cuttings at 5 d and improved at 11 and 20 d. However, the control cuttings held higher values of $GA_{3+1}$ content at all time points (Figure 2). The BR content showed a similar trend to $GA_{3+1}$ except at 1 d, where the content value was improved in all treated cuttings in comparison with 0 d treated cuttings (Figure 2).

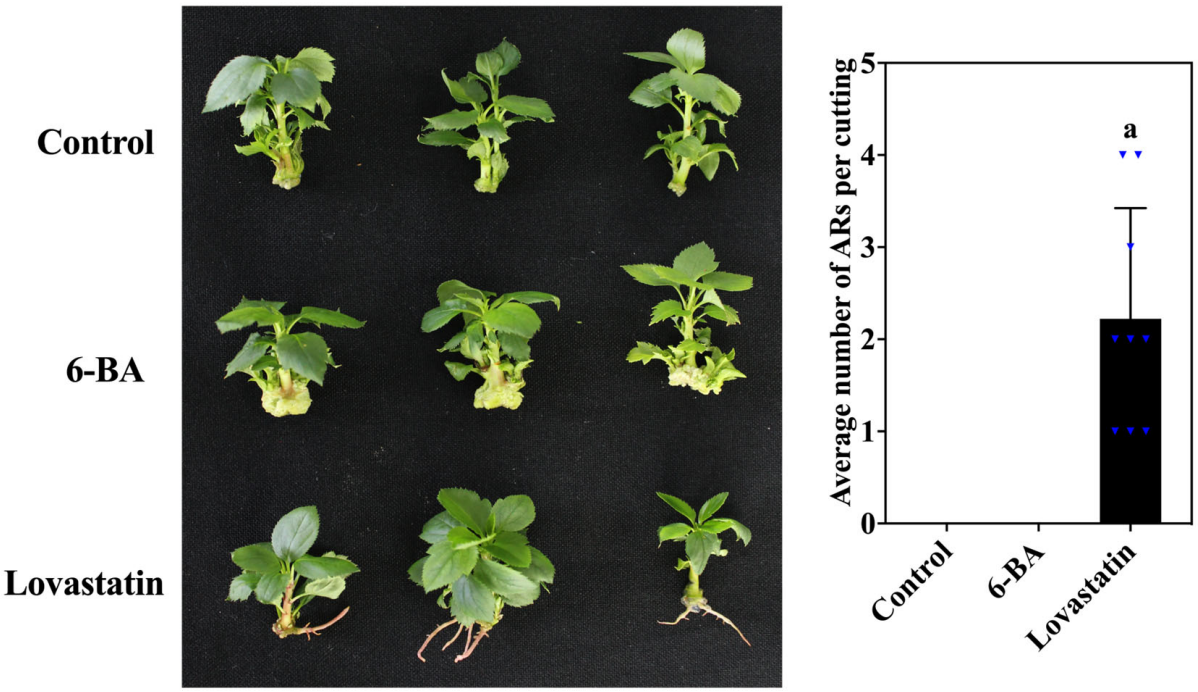


**Figure 1.** Effect of exogenous application of 6-benzyl adenine (6-BA) at 1 mg/L and lovastatin at 1 mg/L treatments compared with control (untreated) cuttings after 20 days (d) during adventitious root (AR) formation in *Malus prunifolia* var. *ringo* apple stem cuttings. The average number of AR per cutting was measured at 20 d by manually counting. Error bars refer to the average value ± SE from three biological replicates. The letter "a" above the bar indicates a significant difference ($p < 0.05$).

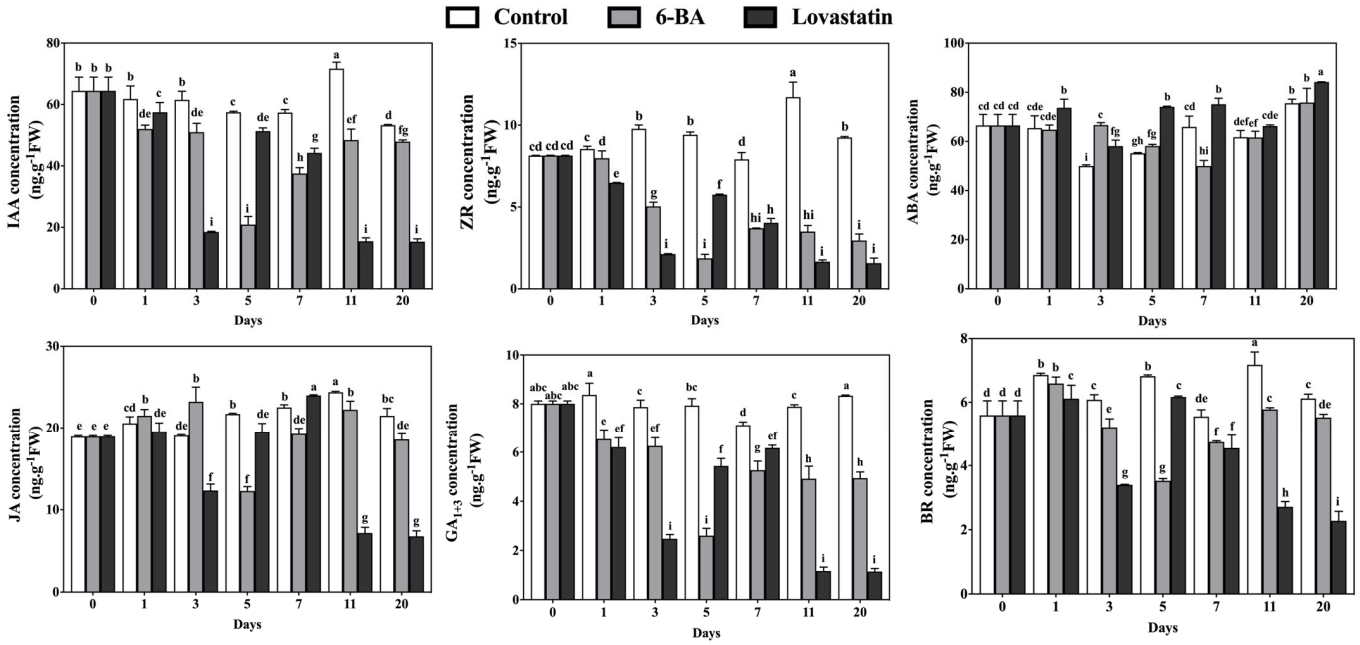


**Figure 2.** Effect of exogenous application of 6-benzyl adenine (6-BA) at 1 mg/L and lovastatin at 1 mg/L treatments compared with control (untreated) cuttings on the endogenous hormone content, including indole-3-acetic acid (IAA), zeatin riboside (ZR), abscisic acid (ABA), jasmonic acid (JA), gibberellic acid 1+3 ($GA_{1+3}$), and brassinosteroid (BR), at different time points, including 0 day (d), 1 d, 3 d, 5 d, 7 d, 11 d, and 20 d during adventitious root (AR) formation in *Malus prunifolia* var. *ringo* apple stem cuttings. Error bars refer to the average value ± SE from three biological replicates. Different letters above the bars indicate a significant difference ($p < 0.05$).

### *3.2. RT-qPCR Expression Analysis of CK Signal Transduction-Related Genes*

The expression level of *MdARR1* was higher at all time points in the control group, except at 3 and 20 d, compared to other cuttings (Figure 3). The expression levels of *MdARR3*, *MdARR5*, and *MdARR5-2* were significantly higher in the 6-BA-treated cuttings at 3 d, 5 d, 7 d, 11 d, and 20 d (Figure 3). Furthermore, *MdARR12* was expressed higher at 1 d in control cuttings, and lovastatin-treated cuttings indicated a higher expression at 20 d compared to other cuttings (Figure 3). The expression of *MdAPH1* declined from 0 d to 1 d, but at 3 d, the expression was only induced in response to lovastatin, which was further reduced at other time points and later induced at 20 d (Figure 3). Moreover, the expression level of *MdAHK4* was higher in the control and 6-BA-treated cuttings at 1 d; later, at 3 d, 5 d, and 20 d, its expression was further improved in 6-BA-treated cuttings, and control cuttings indicated a higher expression only at 11 d relative to the lovastatin-treated cuttings (Figure 3). In addition, *MdCKX5* was only expressed in response to 6-BA at about all timepoints; however, other cuttings did not exhibit its expressions (Figure 3). *MdTCP17* was highly expressed at 5 d in the control cuttings, and later, at 7 d, it was highly expressed in response to lovastatin (Figure 3).

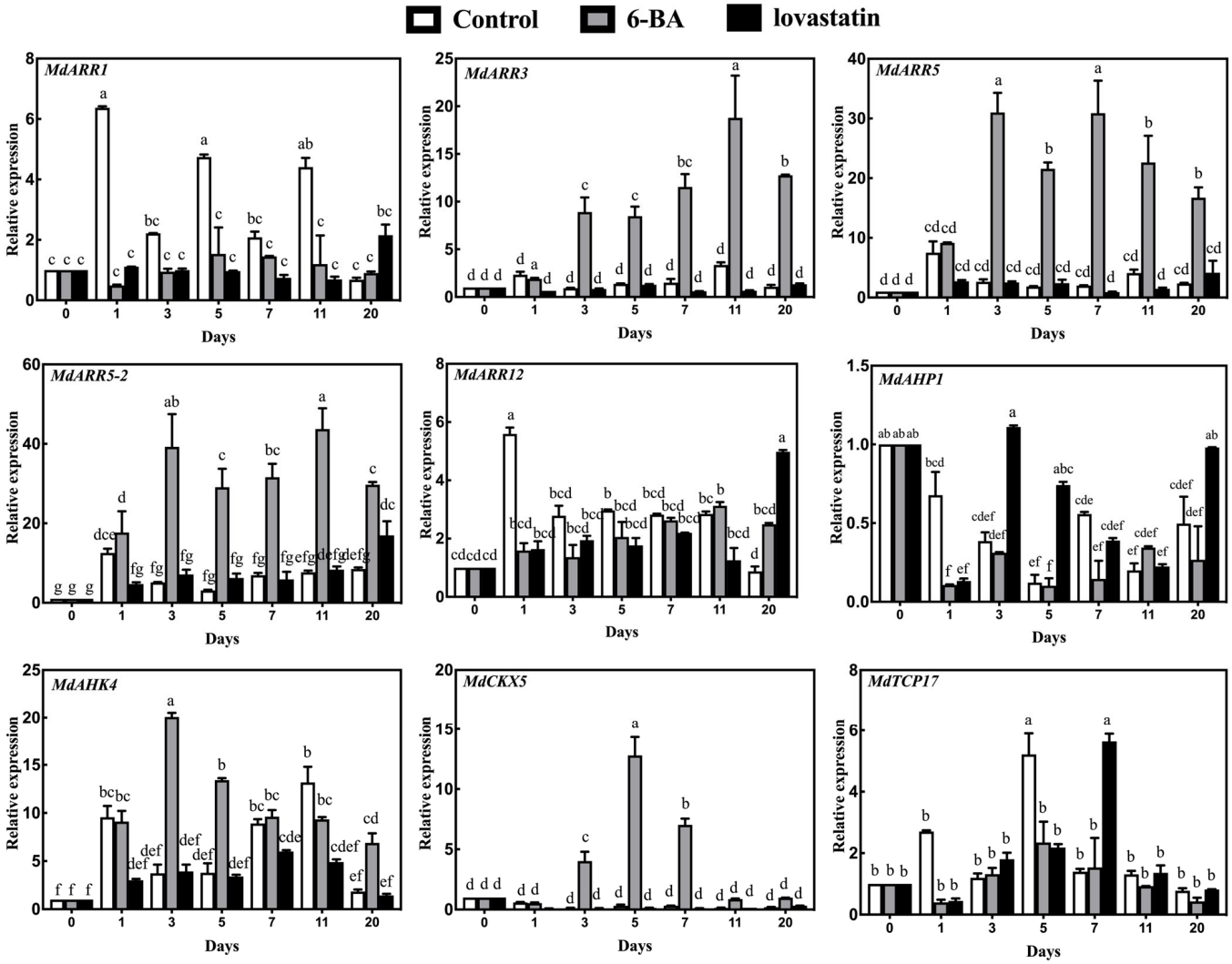


**Figure 3.** Effect of exogenous application of 6-benzyl adenine (6-BA) at 1 mg/L and lovastatin at 1 mg/L treatments compared with control (untreated) cuttings on the relative expression of cytokinin (CK) signal transduction-related genes at different time points, including 0 day (d), 1 d, 3 d, 5 d, 7 d, 11 d, and 20 d, during adventitious root (AR) formation in *Malus prunifolia* var. *ringo* apple stem cuttings. Error bars refer to the average value ± SE from three biological replicates. Different letters above the bars indicate a significant difference ($p < 0.05$).

### *3.3. RT-qPCR Expression Analysis of IAA-Related Genes*

The expression level of *MdAUX1.2* was significantly higher at 11 d; however, at 20 d, it was reduced sharply in control cuttings, where it was expressed higher in response to 6-BA (Figure 4). The expression of *MdIAA23* remained lower in 6-BA-treated cuttings at all time points, except at 7 d (Figure 4). The expression of *MdPIN3* showed an opposite trend with *MdIAA23*, where its expression was higher in response to 6-BA at most timepoints (Figure 3). Control cuttings showed an increased expression of *MdARF7* at 1 d and 7 d; at the same time, lovastatin also showed a higher expression at 7 d and 20 d (Figure 4). The expression of *MdARF19* was induced in response to the control and lovastatin at 5 d; however, *MdYUCCA2* was also highly induced at 5 d but in response to 6-BA-treated cuttings (Figure 4).

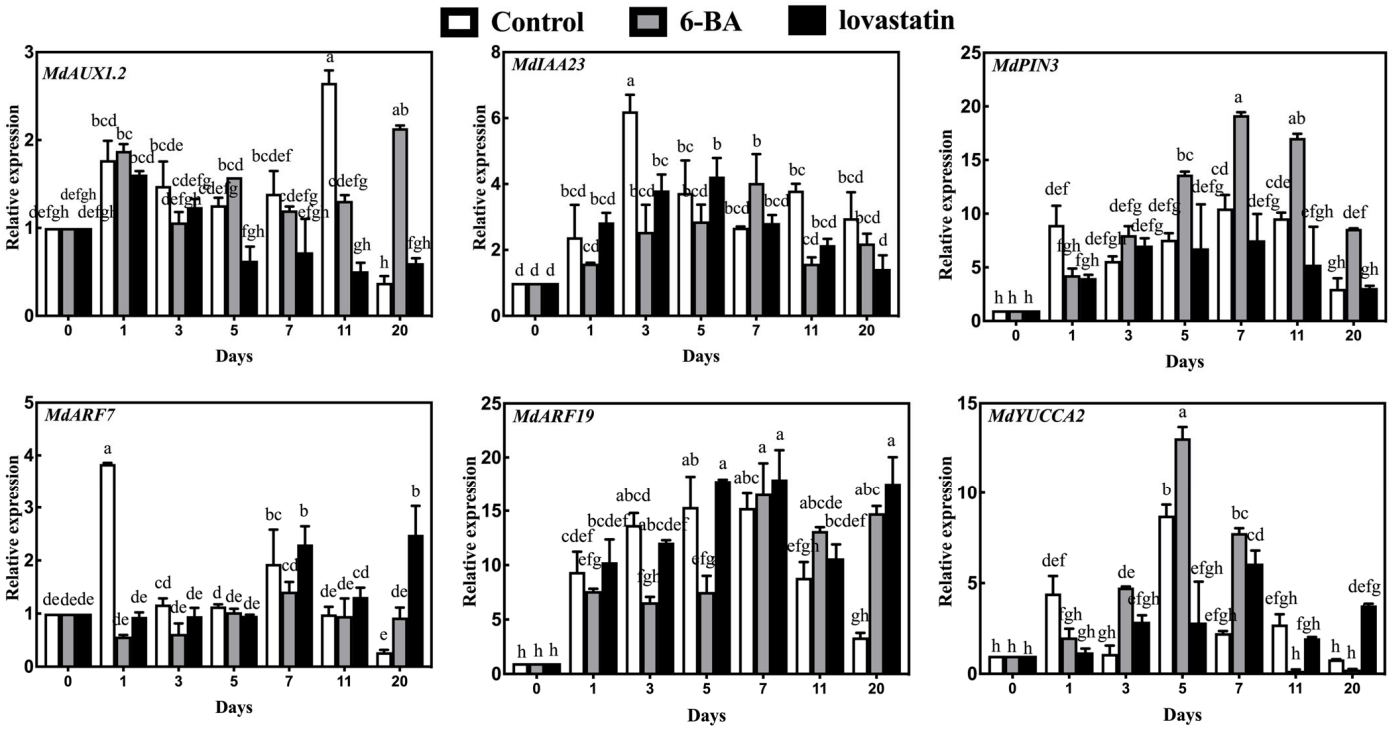


**Figure 4.** Effect of exogenous application of 6-benzyl adenine (6-BA) at 1 mg/L and lovastatin at 1 mg/L treatments compared with control (untreated) cuttings on the relative expression of auxin-related genes at different time points, including 0 day (d), 1 d, 3 d, 5 d, 7 d, 11 d, and 20 d during adventitious root (AR) formation in *Malus prunifolia* var. *ringo* apple stem cuttings. Error bars refer to the average value ± SE from three biological replicates. Different letters above the bars indicate a significant difference ($p < 0.05$).

### *3.4. RT-qPCR Analysis of Root Development-Related Genes*

The relative expression of *MdWOX5* was significantly higher in lovastatin-treated cuttings at 7 d and 11 d. Like *MdWOX5*, *MdWOX11* was also significantly higher at most time points in response to lovastatin (Figure 5). *MdLBD16* was induced at 1 d in control cuttings; at 7 d and 11 d, its expression was induced in the 6-BA-treated cuttings (Figure 4). The expression level of *MdLBD29* was higher at 1 d in the control cuttings, but later, at most time points, its expression was higher in the lovastatin-treated cuttings (Figure 5). The expression of *MdHB30* showed a reduction from 0 d to other timepoints (Figure 5). At 3 d and 5 d, *MdARRO1* expression was higher in response to lovastatin, but later, at 11 d, it was higher in the 6-BA-treated cuttings (Figure 5).

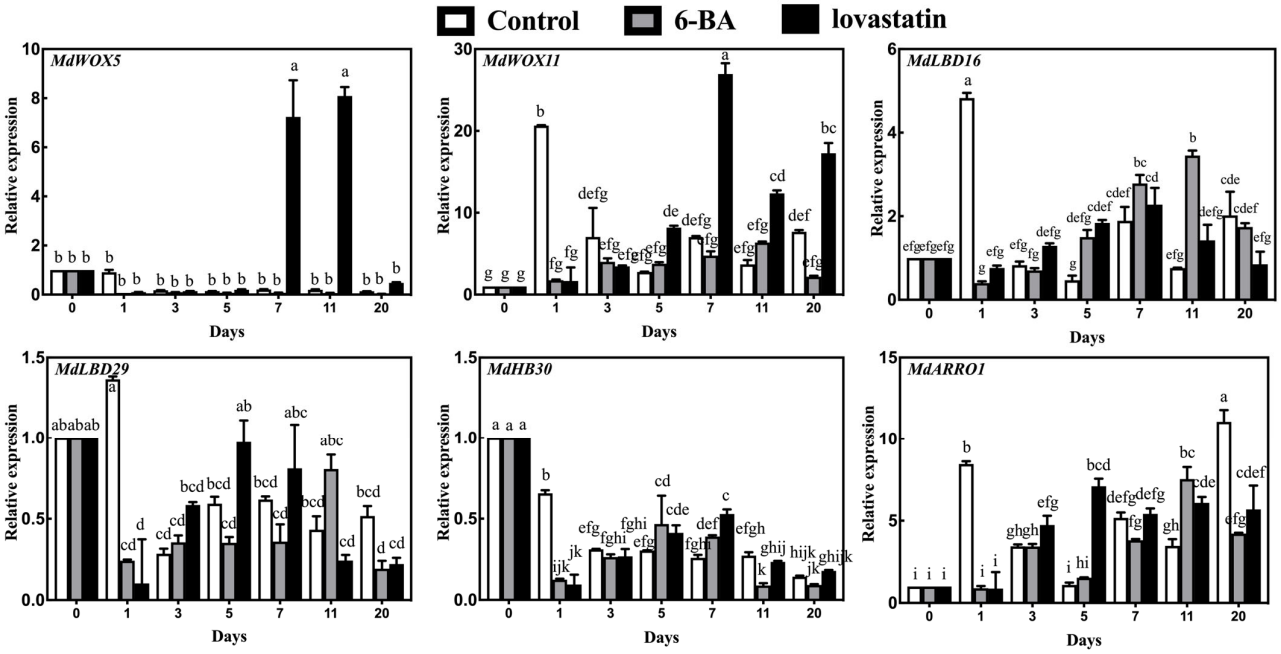


**Figure 5.** Effect of exogenous application of 6-benzyl adenine (6-BA) at 1 mg/L and lovastatin at 1 mg/L treatments compared with control (untreated) cuttings on the relative expression of root-development-related genes at different time points, including 0 day (d), 1 d, 3 d, 5 d, 7 d, 11 d, and 20 d during adventitious root (AR) formation in *Malus prunifolia* var. *ringo* apple rootstock stem cuttings. Error bars refer to the average value ± SE from three biological replicates. Different letters above the bars indicate a significant difference ($p < 0.05$).

## 4. Discussion

Adventitious rooting is crucial for herbaceous and woody horticultural plants. In apples, AR development is a complex biological process involving four distinct stages: 0–1 d, activation; 1–3 d, induction; 3–7 d, initiation; and 7–16 d, emergence [6]. A study indicated that induction of AR is an essential phase in molecular reprogramming; additionally, the initiation stage is similarly important since it creates AR primordia, and if the primordia numbers are halted, the AR number will decrease [21,22]. It is well established that AR initiation is facilitated by low endogenous CK concentrations and high auxin levels [23]. A study on poplar has revealed that CKs act as inhibitors of adventitious rooting [10]. Numerous investigations have revealed that CKs prevent adventitious rooting. At the same time, CK modulates the shift from cell proliferation to cell differentiation by restricting auxin signaling and by modulating the shift from the mitotic cycle to the endocycle by accelerating the depletion of mitotic regulators [24]. The early phases of cell differentiation necessitate changes in cell size, and these changes have been linked to the CK stimulation of expansins [25], which speeds up differentiation by controlling the mechanical control of cell walls. Cell elongation is induced by cell wall permeability, and cell differentiation occurs in the root meristem as a result. Our study elucidates the inhibitory effect of CK on AR formation in *Malus prunifolia* var. *ringo* apple stem cuttings, confirming that the application of lovastatin (a CK biosynthesis inhibitor) promotes AR formation, but not in the control and 6-BA-treated groups (Figure 1). Moreover, CK signal transduction pathways are well known to be involved in rooting. Multiple studies have pointed to a relationship between CK-related gene expression and adventitious rooting [5]. The structure of *AHK4* type-A ARR and type-B ARR CK receptors is similar, but their roles are distinct. According to a study on *AHK4*, procambial cells in freshly developing vascular tissue require CK during embryogenesis [26]. It has been demonstrated that *PtRR13* [10,17] and Type-B ARR both impede root development [27]. Root formation and growth are facilitated by the type-A ARR and CKX genes (Werner et al., 2010). In the signaling pathways of these genes, *ARR1*

and *ARR12* function downstream of *AHK3* [27], whereas type-B ARR activates type-A ARR's transcription (Taniguchi et al. 2007). The obtained outcomes are well aligned with previous studies indicating CK's negative effect on adventitious rooting, as evidenced by the limited AR formation and the induction of CK-related gene expression, including that of *MdARR3*, *MdARR5*, *MdARR5-2*, *MdAKH4*, and *MdCKX5* (Figure 3). This provides support for the thorough understanding that a higher CK content impedes AR formation processes [9–11]. Contrarily, lovastatin application exhibited a positive effect on adventitious rooting. Our study indicated that the lovastatin-treated group successfully developed a few ARs, highlighting its potential to disable the inhibitory effect of endogenous CK on AR formation by impeding the process of CK biosynthesis inside the plant body.

The intricate hormonal balance is essential for adventitious rooting. AR initiation is induced by a high auxin and low CK content [23].The investigation noted dynamic shifts in hormones at various phases of AR formation and development. Treatment with lovastatin resulted in a notable rise in the levels of IAA at key time points, which are known to stimulate root formation. On the other hand, 6-BA administration led to lower IAA levels, confirming CK's inhibitory function in the formation of AR. Control cuttings had higher IAA contents, but they were unable to produce ARs due to the higher concentration of ZR (Figure 2). Various auxin biosynthesis and signaling pathway genes, including transcription factors, were formerly classified as positive controllers for AR formation in Arabidopsis and other species [13,28,29]. Polar auxin transport (PAT) through auxin influx and efflux carriers is a prime feature in adventitious rooting. The mutation of *AUX1* limits IAA accumulation in young seedling roots; it behaves as an auxin influx carrier and promotes lateral rooting through IAA distribution between shoots and roots [30]. In the present study, IAA levels were elevated in lovastatin-treated cuttings at key time points (Figure 2); these were, however, related to the upregulation of *MdIAA23* (Figure 4). *ARF7* and *ARF19* have been found to act as positive AR formation controllers in Arabidopsis hypocotyls through the initiation of the downstream transcription factors *LBD16* and *LBD29* [31]. Furthermore, *arf7* and *arf19* single mutants lowered the number of LRs and ARs, and *arf7* and *arf19* double mutants resulted in considerably fewer roots [32,33]. The upregulated expression of auxin-related genes, including *MdIAA23*, *MdARF7*, *MdARF19*, and *MdYUCCA2* (Figure 4), in response to lovastatin application shows a pivotal role of auxin in endorsing adventitious rooting. The crosstalk between auxin and CK pathways is evident, as CK-induced inhibition is accompanied by a reduction in IAA levels.

During adventitious rooting in Arabidopsis, auxin controls *WOX11* expression at the initial stage of the cell fate transition, which endorses the *LBD16* and *LBD29* expressions [34]. Recently, we discovered that *MdWOX11* directly binds to the *MdLBD29* promoter and positively regulates its expression in apples [35]. Therefore, we can consider that increased IAA content stimulated *MdWOX11* expression, which thereby activated the expressions of *MdLBD16* and *MdLBD29*, thus promoting AR formation in response to lovastatin (Figure 5). Our study offers valuable insights into the variation of AR formation development in apples under the administration of 6-BA and lovastatin. The 6-BA inhibitory effect aligns well with existing literature, while the unexpected promotion of AR due to lovastatin in the medium unlocks opportunities for further research into the sophisticated interplay between AR formation and plant hormone signaling. Future studies should place an emphasis on elucidating the molecular mechanisms behind these observed effects and investigating the potential applications of lovastatin in horticulture.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9121341/s1. Table S1. Pair of primers used in this study.

**Author Contributions:** Conceptualization, S.S., M.M.T. and J.M.; methodology, Z.X. and P.W.; software, J.Y. and H.L.; validation, Y.H., X.R. and Y.M.; writing—original draft preparation, S.S., M.M.T. and J.M.; supervision, J.M.; funding acquisition, J.M. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** All data generated or analyzed during this study are included in this published article and its Supplementary Materials.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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