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Overexpression of *MsGH3.5* inhibits shoot and root development through the auxin and cytokinin pathways in apple plants

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SUMMARY

Phytohormonal interactions are crucial for plant development. Auxin and cytokinin (CK) both play critical roles in regulating plant growth and development; however, the interaction between these two phytohormones is complex and not fully understood. Here, we isolated a wild apple (*Malus sieversii* Roem) *GRETCHEN HAGEN3* (*GH3*) gene, *MsGH3.5*, encoding an indole-3-acetic acid (IAA)-amido synthetase. Overexpression of *MsGH3.5* significantly reduced the free IAA content and increased the content of some IAA-amino acid conjugates, and *MsGH3.5*-overexpressing lines were dwarfed and produced fewer adventitious roots (ARs) than the control. This phenotype is consistent with the role of *GH3* in conjugating excess free active IAA to amino acids in auxin homeostasis. Surprisingly, overexpression of *MsGH3.5* significantly increased CK concentrations in the whole plant, and altered the expression of genes involved in CK biosynthesis, metabolism and signaling. Furthermore, exogenous CK application induced *MsGH3.5* expression through the activity of the CK type-B response regulator, MsRR1a, which mediates the CK primary response. MsRR1a activated *MsGH3.5* expression by directly binding to its promoter, linking auxin and CK signaling. Plants overexpressing *MsRR1a* also displayed fewer ARs, in agreement with the regulation of *MsGH3.5* expression by *MsRR1a*. Taken together, we reveal that *MsGH3.5* affects apple growth and development by modulating auxin and CK levels and signaling pathways. These findings provide insight into the interaction between the auxin and CK pathways, and might have substantial implications for efforts to improve apple architecture.

Keywords: adventitious roots, auxin, cytokinin, *Malus sieversii* Roem, MsGH3.5, MsRR1a.

INTRODUCTION

Auxin is a well-characterized phytohormone required for many aspects of plant development, including tropism, apical dominance and root patterning (Zhao, 2010). Mounting evidence suggests that auxin is a central player in the stimulation of adventitious root (AR) formation in many woody species (Fett-Neto *et al.*, 2001; Xu *et al.*, 2017; Shu *et al.*, 2019). The rooting competence of a plant is likely dependent on the presence and concentration of auxin, particularly the levels of free auxin, which is the active form in plants (Pacurar *et al.*, 2014b).

Auxin homeostasis is maintained by the conversion of excess auxin to inactive forms of the compound. Some members of the *GRETCHEN HAGEN3* (*GH3*) family of auxin-responsive genes are involved in maintaining auxin homeostasis, and thereby contribute to the regulation of the auxin pool (Staswick *et al.*, 2005; Mellor *et al.*, 2016). For instance, some *GH3*s encode indole-3-acetic acid (IAA)-amido synthetases, which conjugate excess free active IAA to amino acids (Staswick *et al.*, 2005). Recent research has shown that some members of the *GH3* family are involved in plant development; for example, in *Arabidopsis thaliana*, the overexpression of *AtGH3.6/DFL1* resulted in a shorter hypocotyl, a dwarf phenotype and decreased root growth (Nakazawa *et al.*, 2001). The *AtGH3.2*-overexpressing mutant *YDK1-D* produced a shorter primary root and hypocotyl than the control as well as fewer lateral roots (LRs), and displayed reduced apical dominance (Takase *et al.*,

2004). *AtGH3.5/WES1* and *AtGH3.9* have also been shown to play important roles in root development (Khan and Stone, 2007; Park *et al.*, 2007). In rice (*Oryza sativa*), the constitutive activation of *TLD1/OsGH3.13* in the *tld1-D* mutant reduced the number of LRs and dramatically altered the plant architecture (Zhang *et al.*, 2009). Similarly, *OsGH3-2*-overexpressing rice plants had significantly fewer but longer crown roots with a reduced density of root hairs (Du *et al.*, 2012). *BpGH3.5* overexpression in birch (*Betula platyphylla* × *Betula pendula*) trees grown *in vitro* resulted in short primary roots and LRs due to a smaller root apical meristem (Yang *et al.*, 2014).

Cytokinin (CK) functions alongside auxin to regulate plant growth and development. In the shoot, CK promotes cell proliferation, including the activity of the apical and axillary meristems (Kieber and Schaller, 2018). CK also inhibits root growth and branching; for example, exogenously applied CK inhibited LR initiation but stimulated LR elongation in Arabidopsis (Kuderova *et al.*, 2008) and rice (Rani Debi *et al.*, 2005). CK treatment strongly inhibited AR formation during the induction phase of cutting growth (De Klerk *et al.*, 1999). The current model of CK signaling predicts that CK is perceived by receptors, which transfer the signal via a phosphorelay to the nucleus, activating two primary classes of response regulators, the type-A and type-B response regulators (RRs; Hwang *et al.*, 2002). Type-B RRs are positive regulators of the CK response that induces the transcription of the CK primary response genes, including the type-A RRs, which negatively regulate CK signaling (To *et al.*, 2004; Mason *et al.*, 2005; Hill *et al.*, 2013). Overexpression of the type-B RR, *ARR1*, in Arabidopsis resulted in an increased sensitivity to CK, while the *arr1* loss-of-function mutant exhibited reduced sensitivity to CK in terms of its shoot regeneration and root elongation (Sakai *et al.*, 2001). Recent studies have shown that *ARR1*, *ARR10* and *ARR12* are the most critical regulators of the CK response, functioning redundantly in the control of root development (Ishida *et al.*, 2008; Hill *et al.*, 2013). A previous study showed that the type-B RR *PtRR13* acts downstream of CK to repress AR formation in poplar (Populus; Ramirez-Carvajal *et al.*, 2009); however, the potential role of CK in regulating AR development remains obscure.

The crosstalk between auxin and CK is complex. Strong evidence has recently emerged to indicate that these hormones interact at the level of hormone perception and signal transduction (Cheng *et al.*, 2013; Street *et al.*, 2016). In most cases, a perturbation of the level of one of these phytohormones influences the other (Jones *et al.*, 2010). The crosstalk of auxin and CK is tissue- and context-specific; these phytohormones generally act synergistically in the shoot apical meristem but antagonistically in the determination of root architecture (Chandler and Werr, 2015; Kong *et al.*, 2018). A spatiotemporal auxin gradient was found to regulate the spatial CK response during *de novo* shoot induction (Cheng *et al.*, 2013). In the root meristem of Arabidopsis, CK promotes cell differentiation by repressing both auxin signaling and transport, thereby removing the stimulatory effect of auxin on cell division (Chandler and Werr, 2015). Auxin and CK also have opposing effects on the regulation of LR organogenesis; CK has been shown to affect the establishment of the local auxin gradient during LR development, in addition to altering the expression of the *PIN-FORMED* (*PIN*) genes, which encode auxin efflux carriers (Laplaze *et al.*, 2007; Simaskova *et al.*, 2015). Despite substantial evidence indicating that CK and auxin jointly coordinate plant growth and development, the complexity of this relationship means that additional mechanisms underlying their interactions continue to be discovered.

Apple (*Malus domestica*) is a major fruit tree cultivated worldwide. In recent decades, dwarf cultivation has become a global trend in apple production, and there has been a major breakthrough in the development of dwarf planting techniques (Zhu *et al.*, 2008; Li *et al.*, 2015; Harrison *et al.*, 2016). 'M26', a semi-dwarf apple rootstock, is frequently used (Holefors *et al.*, 1998; Welander *et al.*, 1998). However, the plant architecture of 'M26' should be further optimized, including the plant height and AR formation. Therefore, studying the molecular mechanisms of shoot and root development in apple rootstock has important theoretical and practical value for apple rootstock breeding.

*GH3*s play vital roles in root development in Arabidopsis and rice; however, the functions of *GH3* in woody plants are unclear. Previously, we performed a genome-wide analysis of the *GH3* family in cultivated apple (*Malus* × *domestica*), and observed that *MsGH3.5* was significantly induced in *Malus sieversii* under various phytohormone and abiotic stress treatments (Yuan *et al.*, 2013). In this study, we further analyzed the function of *MsGH3.5* in *M. sieversii* Roem., one of the most drought-tolerant wild apple rootstocks in China, which is mainly distributed in the Tianshan Mountains of Central Asia (Chen *et al.*, 2007). We characterized the roles of *MsGH3.5* in the auxin–CK interaction, and in the resulting regulation of shoot and root development in apple plants.

RESULTS

MsGH3.5 is highly responsive to exogenously applied IAA, and *MsGH3.5* overexpression represses transgenic apple growth and development

Our previous observation suggesting that *MsGH3.5* plays an important role in the crosstalk between auxin and other phytohormones (Yuan *et al.*, 2013) prompted us to select *MsGH3.5* for further functional study. Phylogenetic analysis showed that MsGH3.5 belongs to Group II of the GH3 family, and was evolutionarily close to AtGH3.1 (Figure S1).

Considering that the expression levels of most members of Group II *AtGH3*s are upregulated by auxin (Staswick *et al.*, 2005), we analyzed *MsGH3.5* expression in wild apple following an exogenous IAA treatment. *MsGH3.5* was rapidly upregulated in both roots and shoots following a 0.5-h treatment with IAA (Figure S2a). After a 2–4-h treatment with IAA, *MsGH3.5* was upregulated more than 40-fold in the roots. We next examined the expression pattern of *MsGH3.5* in different tissues and organs under normal conditions, and observed that expression was highest in the roots (Figure S2b). These results indicate that *MsGH3.5* is highly responsive to auxin and may be involved in modulating root architecture.

To investigate the function of *MsGH3.5* in apple plants, we overexpressed this gene in an apple rootstock variety (M26). We identified two representative transgenic lines, with high levels of *MsGH3.5* expression, which were used for the functional study (Figure 1a). Compared with the control plants, the *MsGH3.5*-overexpressing lines were smaller in both above-ground and below-ground parts, with decreased plant heights and AR number (Figure 1b–e). The transgenic plants were 59% shorter than the control plants (Figure 1c) and produced 46% fewer ARs (Figure 1d). By contrast, the mean root lengths of *MsGH3.5*-overexpressing lines were not significantly different from those of the control plants (Figure 1e). These results support the hypothesis that *MsGH3.5* inhibits plant growth.

To investigate the mechanism by which *MsGH3.5* retards plant growth, we measured the contents of free IAA, some IAA-amido conjugates, CK and gibberellic acid (GA) in the shoots and roots of the transgenic and control apple plants. The free IAA levels in the transgenic plants were 25% lower in the roots and 28% lower in the shoots than in those of the control (Table 1). However, the IAA-Ala and IAA-Asp levels in the transgenic plants were significantly higher than in the control plants. The contents of the four different active CKs were all higher in the roots of the transgenic plants than in those of the control plants, while isopentenyladenine (iP), the primary active CK, was higher in the shoots of the transgenic plants. The GA contents were not significantly different in the transgenic and control plants in either the roots or the shoots. These results indicate that *MsGH3.5* overexpression reduces the free IAA contents and increases the CK contents of the transgenic plants.

Next, we explored whether the phenotypes of the transgenic and control plants are affected by an exogenous application of IAA or CK. We applied a concentration series of IAA and/or the inhibitor of CK biosynthesis (lovastatin) to the micropropagated transgenic and control apple plants (Figure 2a). The height of the transgenic plants and the number of ARs they produced were both gradually restored with increasing levels of IAA (Figure 2b,c). The mean root length of the transgenic and control plants remained similar following the IAA treatment, but root growth was significantly repressed in both groups of plants after lovastatin treatment (Figure 2d), which also reduced the number of ARs produced in the control and transgenic lines (Figure 2c). A concurrent application of lovastatin and IAA treatment increased the number of ARs compared with the lovastatin treatment alone, but did not remove the repression of root growth. These results suggest that changes in the IAA and CK contents of the transgenic plants caused their phenotypic characteristics.

MsGH3.5 overexpression affects the expression of hormone-related genes and those involved in adventitious rooting

The molecular components involved in the auxin pathway were analyzed to determine the effect of *MsGH3.5* overexpression on auxin accumulation and the auxin response. Some auxin biosynthesis and receptor genes were downregulated in the transgenic plants (Figure S3). Similar expression patterns were observed in these transgenic lines for several *PIN* genes, encoding auxin efflux carriers, and auxin influx carrier genes (Figure 3a–d), which are involved in polar auxin transport. Furthermore, we detected the transcript levels of some members of the auxin signaling genes, including the *AUXIN RESPONSE FACTOR*s (*ARF*s) and *SHORT HYPOCOTYL2/INDOLE-3-ACETIC ACID3* (*SHY2/IAA3*). The expression levels of *MdARF6*, *MdARF8* and *MdARF19*, which were positive regulators of AR formation in Arabidopsis (Okushima *et al.*, 2007; Gutierrez *et al.*, 2009), were approximately half that of the control in the roots of the transgenic plants and even lower in the shoots (Figure 3e,f). Inversely, *MdARF17* expression, which negatively regulates adventitious rooting (Gutierrez *et al.*, 2009), showed an almost twofold upregulation in the roots of the transgenic plants (Figure 3e). By contrast, the expression levels of the *MdSHY2*s, which belong to the *AUX/IAA* gene family and transcriptionally repress auxin-regulated gene expression (Tiwari *et al.*, 2001), were significantly elevated in the transgenic plants (Figure 3g,h). These results imply that *MsGH3.5* overexpression likely suppresses auxin signaling and transport systems by changing auxin homeostasis.

To determine the molecular mechanisms by which the CK levels were altered in *MsGH3.5*-overexpressing apple plants, we analyzed the expression of *ISOPENTENYL TRANSFERASE* (*IPT*), which functions in CK biosynthesis, and *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*), which functions in CK metabolism, in the transgenic and control plants. The expression levels of most *MdIPTs* were increased in the roots of the transgenic plants (Figure 4a). However, in the shoots of the transgenic plants, only *MdIPT3* and *MdIPT5* were upregulated (Figure 4b). By contrast, almost all of the *MdCKX*s were decreased in the roots and shoots of the *MsGH3.5*-overexpressing plants

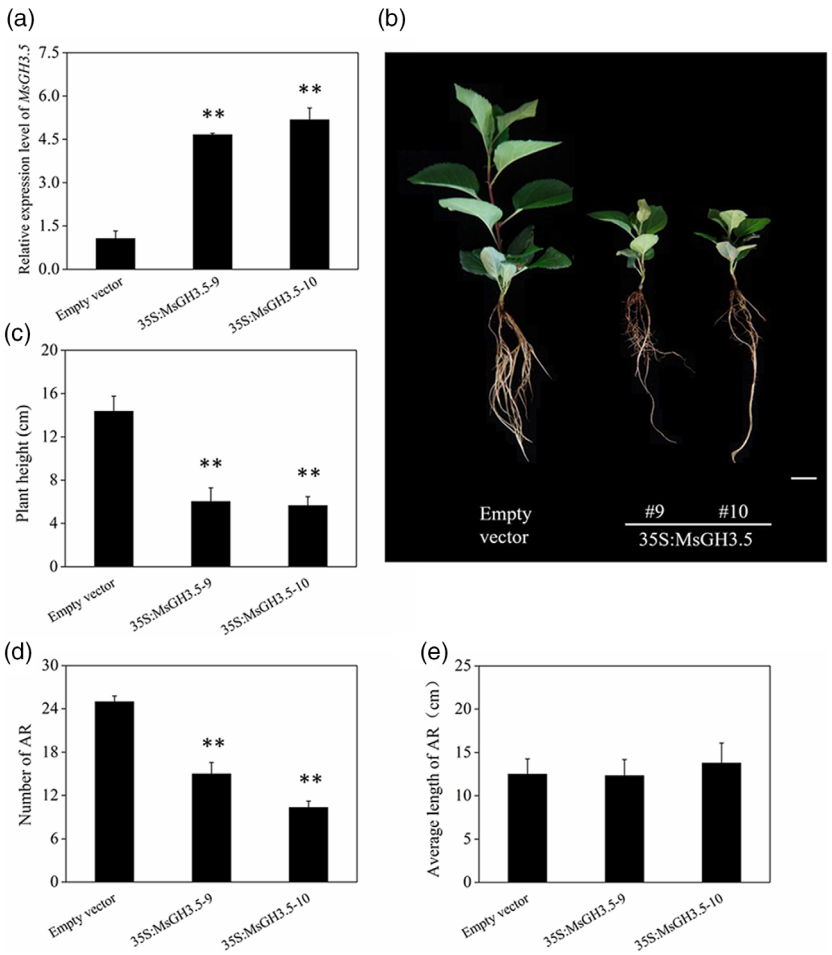

Figure 1. Phenotypic characterization of 30-day-old *MsGH3.5*-overexpressing and control apple plants.
(a) Expression levels of *MsGH3.5* measured using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).
(b) Morphological phenotypes of transgenic and control apple plants grown in hydroponic culture for 30 days. Scale bars: 2 cm.
(c) Heights of transgenic and control apple plants.
(d,e) Number (d) and average length (e) of adventitious roots (ARs) in transgenic and control apple plants. The plants were analyzed at the same time point as those imaged in (b). Data are the means (± SE) of three biological replicates. Asterisks indicate significant differences from the control apple plants (**$P < 0.01$, Student's *t*-test).

relative to their expression levels in the control (Figure 4c,d). We therefore concluded that *MsGH3.5* overexpression might increase the CK levels in the plants by up- and downregulating the genes responsible for CK biosynthesis and metabolism, respectively. The altered CK levels in the transgenic plants prompted us to investigate whether the expression levels of the CK response genes were also affected. We analyzed the expression of a series of CK signaling genes, including the *MdHK*s (encoding histidine kinases), *MdHP*s (encoding His phosphotransfer proteins) and *MdRR*s (encoding RRs), in the transgenic and control plants. Most of the *MdHK*s and *MdHP*s were upregulated in the transgenic plants compared with the control plants (Figure S4). Among the type-A *MdRR*s, *MdRR3* was downregulated in the roots, while *MdRR5* and *MdRR9b* were downregulated in the shoots. *MdRR9a* was downregulated in both the roots and shoots (Figure 4e,f). In addition, most of the type-B *MdRR*s were upregulated in the transgenic plants, particularly *MdRR1a*, which was most strikingly upregulated in both the roots and shoots (Figure 4g,h). These results indicate that *MsGH3.5* overexpression had a large effect on CK signaling.

To investigate whether the change in CK contents in *MsGH3.5*-overexpressing lines is due to a disturbance in auxin homeostasis, we exogenously applied IAA to the wild apple plants. The expression levels of the *MsIPT*s and type-B *MsRR*s were suppressed (Figure S5a,d), whereas those of the *MsCKX*s, and type-A *MsRR*s were induced (Figure S5b,c) after IAA treatment. These observations suggest that the auxin level could affect the CK pool.

Previous studies showed that *WUSCHEL-RELATED HOMEOBOX11* (*WOX11*) is required for the *de novo* regeneration of ARs from leaf explants and functions by upregulating *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) and *LBD29* (Liu *et al.*, 2014b). We examined the expression of several *WOX* and *LBD* family genes; the transcript levels of *MdWOX11*, *MdLBD16* and *MdLBD18* were dramatically reduced in the transgenic plants (Figure 5a,b). Further analysis showed that the transcript levels of most cell cycle-related genes were distinctly repressed in the transgenic plants (Figure 5c). We therefore propose that *MsGH3.5* indirectly downregulates some of the genes involved in adventitious rooting. Consistent with this hypothesis, *MsGH3.5* overexpression caused an abnormal plant

Table 1 Quantification of phytohormones in the *MsGH3.5*-overexpressing lines and in control apple plants

Hormone concentration (ng g^{-1} FW)		Root			Shoot		
		Empty vector	35S:MsGH3.5-9	35S:MsGH3.5-10	Empty vector	35S:MsGH3.5-9	35S:MsGH3.5-10
Auxin	IAA	0.835 ± 0.041	0.620 ± 0.031**	0.641 ± 0.007**	2.103 ± 0.184	1.402 ± 0.080**	1.594 ± 0.048**
	IAA-Ala	0.280 ± 0.025	0.399 ± 0.029*	0.407 ± 0.034*	0.278 ± 0.016	0.365 ± 0.020*	0.350 ± 0.009*
	IAA-Leu	None detected	None detected	None detected	0.036 ± 0.002	0.034 ± 0.006	0.038 ± 0.005
	IAA-Glu	0.216 ± 0.040	0.209 ± 0.018	0.208 ± 0.012	0.068 ± 0.009	0.074 ± 0.010	0.075 ± 0.007
	IAA-Asp	0.097 ± 0.006	0.164 ± 0.015**	0.159 ± 0.014**	0.035 ± 0.008	0.075 ± 0.004**	0.073 ± 0.002**
CK	iP	0.080 ± 0.001	0.116 ± 0.014**	0.102 ± 0.005**	0.112 ± 0.004	0.189 ± 0.002**	0.274 ± 0.006**
	tZ	0.022 ± 0.002	0.064 ± 0.002**	0.042 ± 0.006**	None detected	None detected	None detected
	cZ	0.016 ± 0.001	0.035 ± 0.001**	0.026 ± 0.006**	None detected	None detected	None detected
	DZ	0.011 ± 0.001	0.054 ± 0.003**	0.031 ± 0.003**	0.125 ± 0.004	0.135 ± 0.005	0.108 ± 0.019
GA	GA_{1+3}	5.061 ± 0.167	5.336 ± 0.280	5.815 ± 0.480	4.039 ± 0.120	3.950 ± 0.133	3.988 ± 0.079
	GA_{4+7}	3.578 ± 0.125	3.923 ± 0.142	3.720 ± 0.145	4.921 ± 0.197	4.741 ± 0.174	4.814 ± 0.108

Phytohormone contents in the shoot and root of 30-day-old transgenic and control apple plants.
CK, cytokinin; GA, gibberellic acid; IAA, indole-3-acetic acid; iP, isopentenyladenine; tZ, trans-zeatin; cZ, cis-zeatin; DZ, dihydrozeatin.
Units are ng g^{-1} of fresh weight (FW). Data are the means (± SE) of three biological replicates. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

morphology and retarded AR growth and development through regulating the expression of genes involved in these processes.

Heterologous expression of *MsGH3.5* in Arabidopsis results in an abnormal phenotype that is altered by exogenous auxin and CK treatments

To further investigate the function of *MsGH3.5*, we heterologously expressed this gene in Arabidopsis plants. The transgenic lines were shorter than the wild-type (WT) and produced more shoot branches (Figure S6a,c,d). The root architecture of the transgenic seedlings also differed from that of the WT plants (Figure S6b); while the lengths of the primary roots of both genotypes were similar, the transgenic seedlings produced fewer LRs than the WT plants (Figure S6e,f).

The decreased number of ARs in the *MsGH3.5*-overexpressing apple plants was related to the level of CK and IAA; therefore, we explored the effects of exogenous CK and IAA treatments on the transgenic Arabidopsis plants expressing *MsGH3.5*. IAA restored the number of LRs produced by the transgenic seedlings, with higher concentrations of IAA having a greater effect (Figure S7a). The lower concentration of benzylaminopurine (BA) did not restore the reduced LR phenotype of the transgenic seedlings, although the higher concentration of BA resulted in phenotypically similar transgenic and WT plants (Figure S7b). The number of LRs decreased under the high concentration of IAA when BA was concurrently added; however, adding IAA and high levels of BA did not restore LR growth (Figure S7c). These results suggest that *MsGH3.5* plays a conserved role in the inhibition of LR and AR formation by altering the IAA and CK contents.

As primary root growth is inhibited by excess amounts of exogenous auxins, we examined the effect of exogenously applied auxin on the primary root elongation of the transgenic Arabidopsis. When plants were exposed to a low concentration of IAA (0.001 and 0.01 μM), no significant difference in primary root elongation was observed between transgenic and WT plants (Figure S8). However, at a moderately high concentration of IAA (0.01–1 μM), the primary root growth of the WT was strongly inhibited and the degree of inhibition was not as severe in the transgenic plants. At a high concentration of IAA (1 and 10 μM), the primary root growth of both transgenic and WT was strongly inhibited (Figure S8). Thus, the transgenic lines had a lower sensitivity to the exogenously applied auxin than did the WT plants.

MsGH3.5 is induced by exogenous CK application and is directly regulated by *MsRR1a*

We showed that the CK content was increased in transgenic apple overexpressing *MsGH3.5*; therefore, we next explored whether *MsGH3.5* could act as a signaling factor in the CK pathways. Exogenous application of 6-BA induced *MsGH3.5* expression in *M. sieversii* plants by 30 min of treatment, with expression peaking at 4.6- and 5.5-fold that of the control in the roots and shoots, respectively, at 1 h after treatment (Figure S9). Thus, CK induces *MsGH3.5* expression.

As reported previously (Xie *et al.*, 2018), the type-B *ARR*s are positive regulators of the CK response, which induces the transcription of the CK primary response genes. We therefore investigated whether *MsGH3.5* is a CK-responsive gene directly targeted by the type-B RRs. We analyzed the expression of several type-B *MsRR*s in *M. sieversii* and

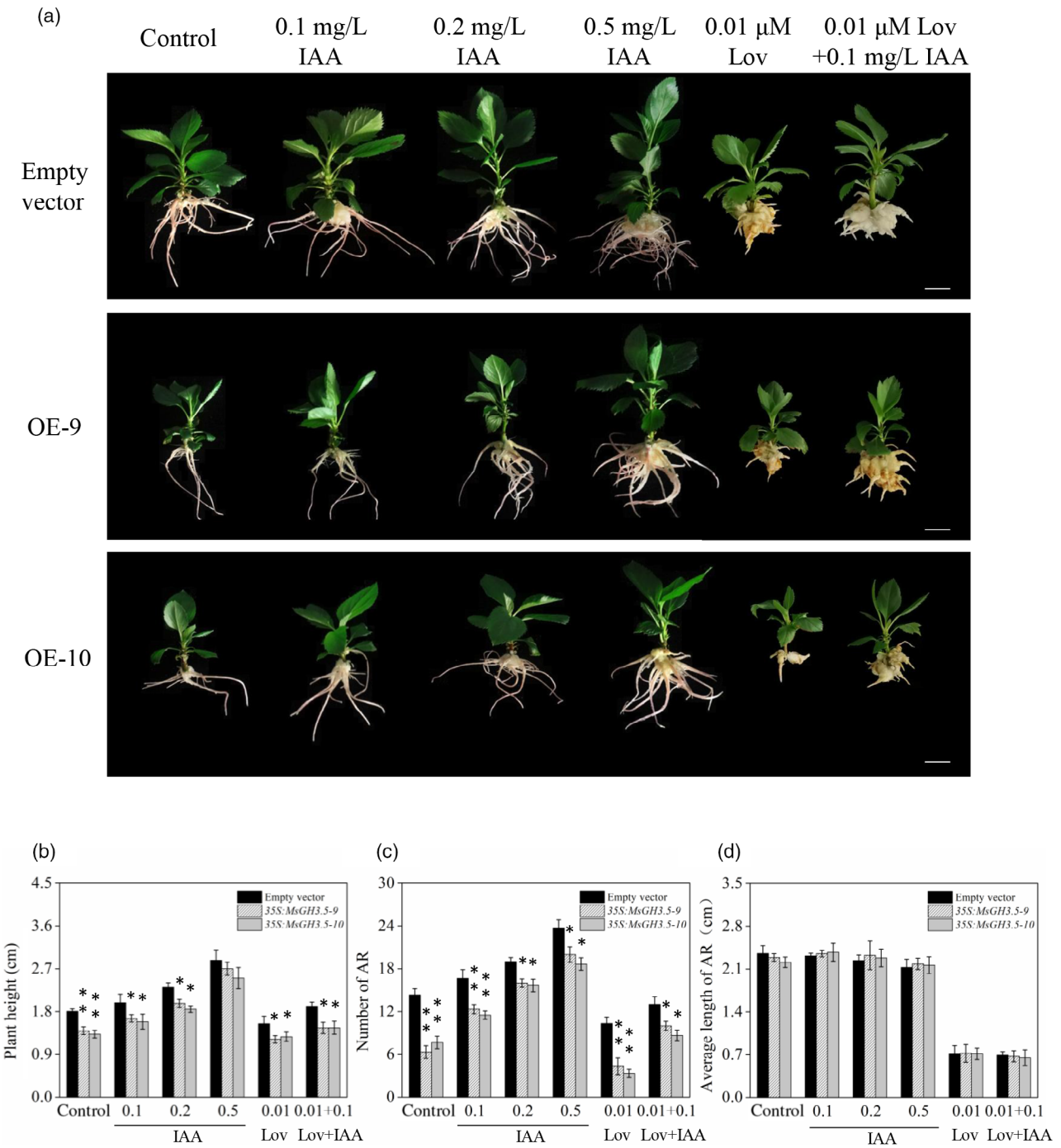

Figure 2. Phenotypes of *MsGH3.5*-overexpressing and control apple plants treated with indole-3-acetic acid (IAA) and/or the cytokinin (CK) biosynthesis inhibitor lovastatin (Lov).
(a) Morphologies of micropropagated plants grown in rooting medium without any additional hormone (control); with 0.1, 0.2 or 0.5 mg L^{-1} IAA; with 0.01 μM Lov; or with 0.01 μM Lov and 0.1 mg L^{-1} IAA for 30 days. Scale bars: 1 cm.
(b) Plant heights under the indicated treatments.
(c,d) The number (c) and average length (d) of the adventitious roots (ARs) produced. Data are the means (± SE) of the three biological replicates. Asterisks indicate significant differences from the control apple plants (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

observed that *MsRR1a*, which has the highest similarity to *ARR1* in Arabidopsis, was the most strongly induced by the CK treatment (Figure S10). *MsRR1a* expression peaked 30 min after the 6-BA treatment in both the roots and shoots, and then decreased to the initial level at 4 h after treatment in the roots and 8 h in the shoots.

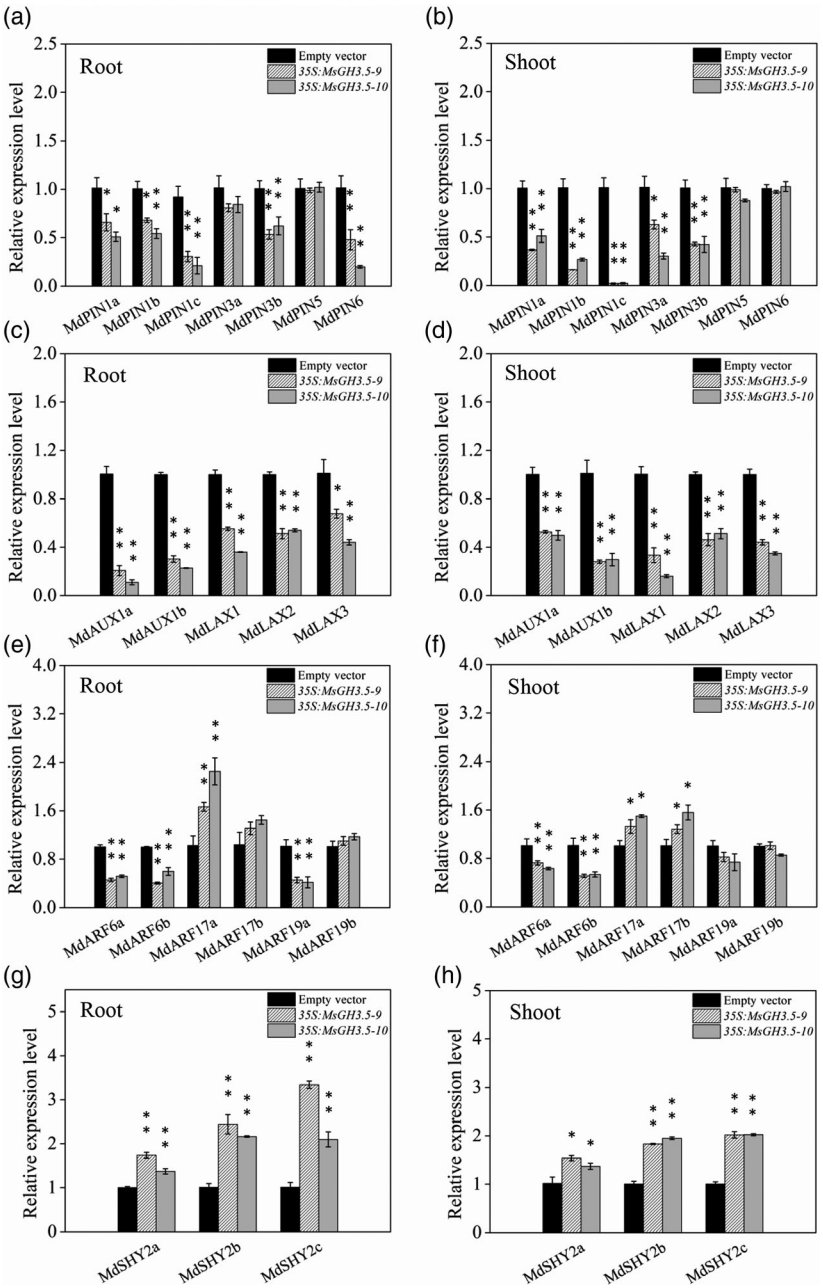

Figure 3. Expression of genes encoding the auxin transporters and proteins involved in the auxin signaling pathways in *MsGH3.5*-overexpressing and control apple plants.
The expression levels of auxin efflux carriers in the roots (a) and shoots (b); of auxin influx carrier in the roots (c) and shoots (d); and of auxin signaling genes in the roots (e, g) and shoots (f, h), as measured using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Data are the means (± SE) of three biological replicates. Asterisks indicate significant differences from the control apple plants (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

We next investigated whether *MsGH3.5* is the target of MsRR1a. First, we examined whether there were any ARR-binding motifs in the promoter of *MsGH3.5* (http://www.dna.affrc.go.jp/PLACE/signalscan.html). We identified two AGATT motifs located in the *MsGH3.5* promoter region (a 1348-bp genomic fragment upstream of the start codon). To determine whether *MsGH3.5* is directly regulated by MsRR1a, we next performed transient expression experiments in which tobacco (*Nicotiana benthamiana*) leaves were co-transformed with *ProMsGH3.5:LUC* and *Pro35S:MsRR1a* as the reporter and effector, respectively (Figure 6a). MsRR1a activated the expression of the reporter driven by the *MsGH3.5* promoter, which displayed a fourfold greater LUC/REN signal than the control (Figure 6b). Furthermore, when BA was concurrently applied with the effector, the LUC activity increased to more than five times the level in the control. When BA was applied in the absence of the effector, the LUC activity was twofold greater than that of the control.

Next, we performed an electrophoretic mobility shift assay (EMSA), which revealed that the purified MsRR1a-His fusion protein bound to the binding element in the

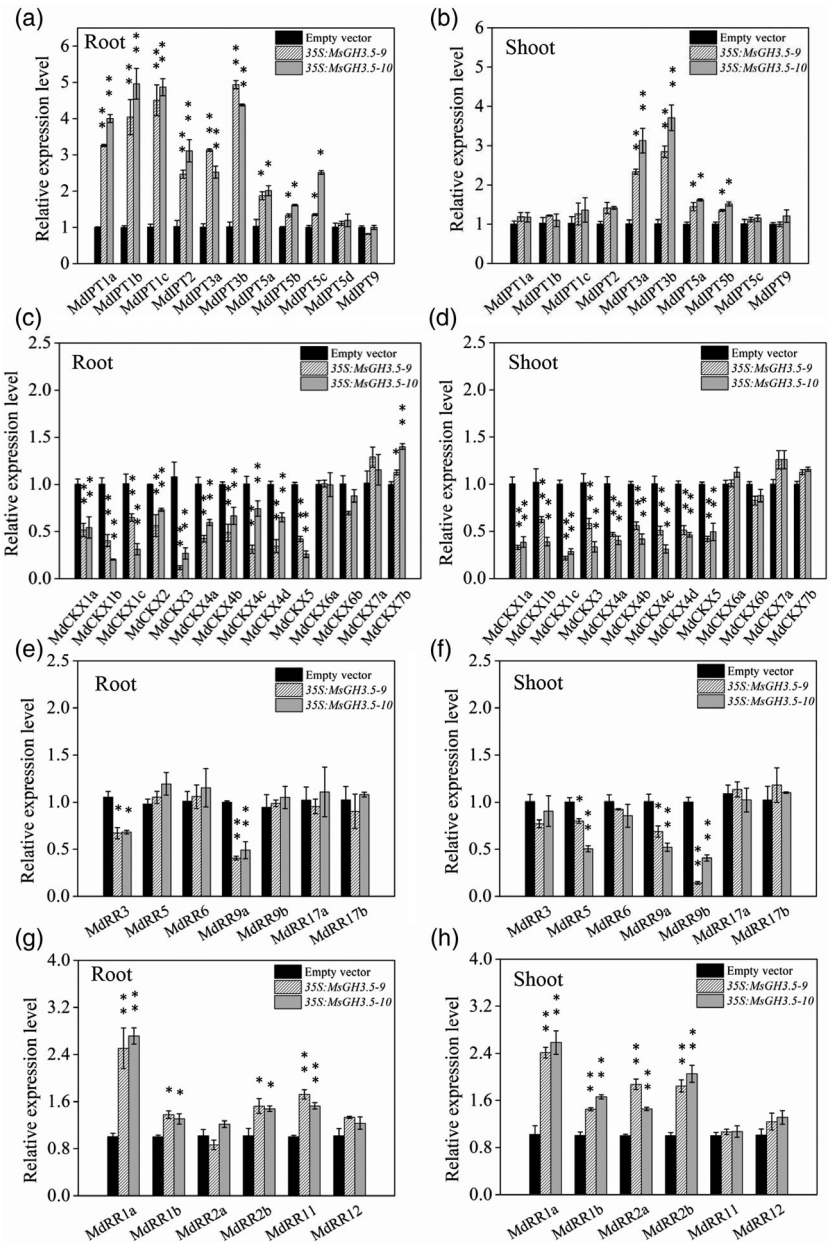

Figure 4. Expression of genes related to the cytokinin (CK) biosynthesis, metabolism and signaling pathways in *MsGH3.5*-overexpressing and control apple plants.
The expression levels of CK biosynthesis genes in the roots (a) and shoots (b); of CK degradation genes in the roots (c) and shoots (d); of the type-A response regulators (RRs) in the roots (e) and shoots (f); and of the type-B RRs in the roots (g) and shoots (h) were measured using qRT-PCR. Data are the means (± SE) of three biological replicates. Asterisks indicate significant differences from the control apple plants (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

promoter of *MsGH3.5 in vitro* (Figure 6c). The extent of binding was reduced and eventually abolished with the addition of increasing concentrations of an unlabeled core probe (competitor 1), but was not affected by the addition of a mutant probe (competitor 2). These results indicate that MsRR1a specifically binds to the MsRR1a-binding site in the promoter of *MsGH3.5 in vitro*. A chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction (qPCR) analysis revealed that MsRR1a bound to cis-elements in the promoter of *MsGH3.5 in vivo* (Figure 6d). Based on our combined analyses, we conclude that MsRR1a can directly bind to the *MsGH3.5* promoter and regulate its expression.

MsRR1a negatively regulates AR formation

To elucidate whether increasing the expression of *MsRR1a* would result in similar phenotypes to that of the *MsGH3.5*-overexpressing apple plants, we produced transgenic *MsRR1a*-overexpressing transgenic apple lines. Three lines were selected for a detailed phenotypic analysis (Figure 7a). Compared with the WT, *MdGH3.5* expression was increased in the transgenic plants (Figure 7b). Significantly fewer ARs were produced by the transgenic plants (Figure 7c,f), indicating that the level of *MsRR1a* expression was negatively associated with AR formation in apple. However, the plant height and mean root lengths of the

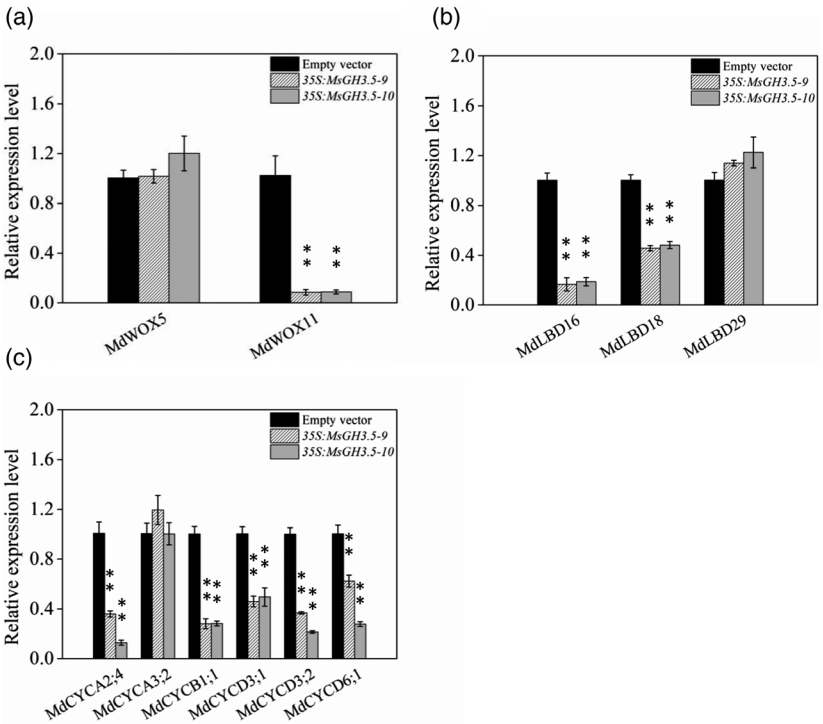

Figure 5. Expression of genes involved in adventitious rooting in *MsGH3.5*-overexpressing and control wild apple plants.
Expression levels of *MdWOXs* (a), *MdLBDs* (b) and *MdCYCs* (c) in the roots, as measured using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Data are the means (± SE) of three biological replicates (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

MsRR1a-overexpressing lines were not significantly different from those of the WT (Figure 7c–e). We therefore conclude that *MsRR1a* negatively regulates AR formation in apple, which is consistent with our observation that this gene regulates *MsGH3.5* expression.

Exogenously applied CK activates *MsGH3.5* expression in an *ARR1*-dependent manner in transgenic Arabidopsis tissues

To further determine the role of *MsGH3.5* in plant development, especially in root development, we analyzed the tissue-specific expression patterns of *MsGH3.5* in transgenic Arabidopsis lines. We transformed WT (Col-0) and *arr1* mutant Arabidopsis plants with a *ProMsGH3.5:GUS* vector and then treated these transgenic plants with 0 or 10 μM 6-BA before histochemical analysis of *GUS* expression.

First, we determined the effect of 6-BA on *GUS* expression in different tissues in *ProMsGH3.5:GUS*-expressing plants in the WT background. In the absence of 6-BA, GUS staining was obvious in the roots, particularly in the root stele, but was inconspicuous in other tissues (Figure 8a), consistent with our previous analysis (Figure 2b). GUS staining was more extensive in both the cotyledon and primary root when 6-BA was applied (Figure 8a). Second, we evaluated GUS staining during LR development and found that, in the absence of the 6-BA treatment, the GUS signal was weaker during the induction of LR primordia (LRP; Figure 8b). However, as the LRP emerged, the GUS signal grew stronger. After the emergence of the LR, GUS staining became weak again and eventually disappeared from the fully developed LR. By contrast, the 6-BA treatment greatly increased GUS staining at all LR developmental stages as well as in the emerged LRs (Figure 8b). Third, we analyzed GUS staining in *arr1* mutant Arabidopsis plants expressing *ProMsGH3.5:GUS*. No obvious difference in the pattern or intensity of GUS staining was detected between the WT and *arr1* plants expressing *ProMsGH3.5:GUS* in the absence of 6-BA; however, while the 6-BA treatment greatly increased GUS staining in the WT plants, it had no effect on GUS staining in the *arr1* mutant (Figure 8c). These results suggest that CK activates *MsGH3.5* expression in different tissues and that its activation depends on *ARR1* in transgenic Arabidopsis lines.

DISCUSSION

MsGH3.5 participates in the regulation of both shoot and root development

The conjugation of auxin to amino acids is an important step in the auxin-degradation pathway in plants. Members of the *GH3* family are involved in this process. Some *GH3* genes are upregulated in response to auxin (Abel and Theologis, 1996), providing a negative feedback loop that regulates auxin levels, indicating that these genes function in auxin homoeostasis and may be vital regulators of plant development. Previous studies have shown that the overexpression of *GH3* in Arabidopsis resulted in plants with short hypocotyls and roots (Takase *et al.*, 2004; Khan and

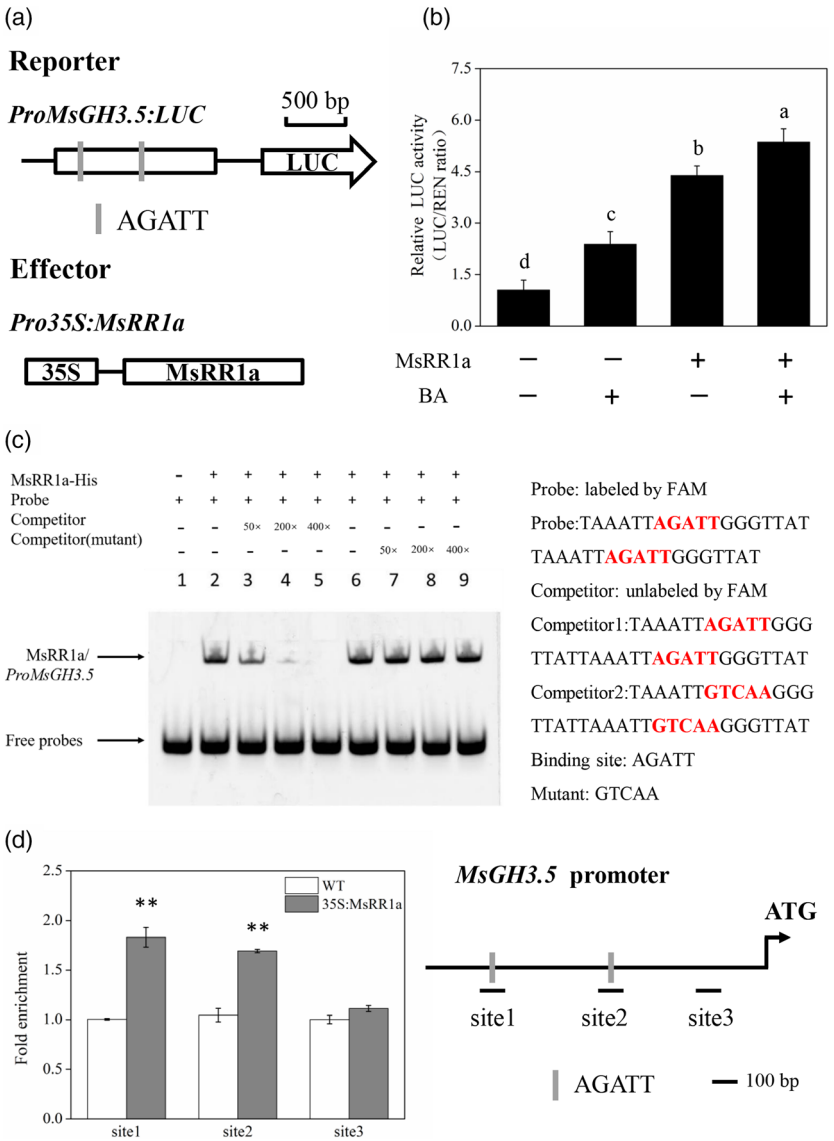

Figure 6. MsRR1a binds to the *MsGH3.5* promoter.
(a) Schematic diagrams of the *MsGH3.5* promoter and the constructs used in the dual-luciferase assay. LUC, firefly luciferase. Gray lines in the *MsGH3.5* promoter region indicate the MsRR1a binding site (AGATT) elements.
(b) MsRR1a activates the *MsGH3.5* promoter. Promoter activities are presented as the ratio of LUC/REN activity. Data are the means (± SE) of three biological replicates. Different letters indicate significant differences, according to Duncan's multiple range test ($P < 0.05$).
(c) Electrophoretic mobility shift assay (EMSA) showing the binding of MsRR1a to the *MsGH3.5* promoter. Each 5-carboxyfluorescein (FAM)-labeled DNA probe was incubated with MsRR1a-His. An excess of unlabeled normal and mutated probes was added as competitor 1 and competitor 2, respectively. The red font presents binding element and mutant binding element. The EMSA was performed using increasing concentrations (50, 200 and 400 ×) of competitors. FAM-labeled probes incubated with the His protein served as the negative control.
(d) Chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction (qPCR) showing the binding of MsRR1a to the *MsGH3.5* promoter. Sites 1 and 2 contained the binding site of the promoter region, while site 3 did not include the binding site as a negative control. Data shown are means ± SE ($n = 3$). Asterisks indicate significant differences from the wild-type (WT) apple plants (**$P < 0.01$, Student's *t*-test).

Stone, 2007). Moreover, several rice lines overexpressing various *OsGH3*s had reduced free IAA contents and similar morphological phenotypes, including dwarfism and the production of fewer lateral or crown roots (Zhang *et al.*, 2009; Fu *et al.*, 2011; Du *et al.*, 2012). Inversely, the *osgh3* mutant had more roots (Mao *et al.*, 2019a). However, few studies have explored the functions of the *GH3*s in woody plants, particularly in apple.

In this study, we cloned *MsGH3.5* from *M. sieversii* micropropagated plants. Overexpressing this gene resulted in shorter plants with fewer ARs than the control (Figure 1b–d), similar to the phenotype of transgenic Arabidopsis plants heterologously expressing *35S:MsGH3.5* (Figure S6). Primary root growth is inhibited by excess amounts of exogenous auxin. The transgenic Arabidopsis plants were resistant to exogenous IAA (Figure S8). As IAA is a substrate of *GH3*-encoded IAA-amido synthases, *MsGH3.5* upregulation might partially contribute to the weaker sensitivity to exogenous IAA in the transgenic lines. Similar results were found in *AtGH3.6/DFL1*, *AtGH3.5/WES1* and *OsGH3.8* transgenic lines (Nakazawa *et al.*, 2001; Park *et al.*, 2007; Li *et al.*, 2016b). We propose that *MsGH3.5* functions as an auxin response gene that suppresses plant shoot and root development.

MsGH3.5 suppresses plant development by regulating the expression of auxin- and CK-related genes

Plant height and root development are tightly regulated by auxin concentration (Lu *et al.*, 2015; Fendrych *et al.*, 2018). CK is also an important regulator of plant root systems, as it suppresses AR and LR formation in many species (Kuderova *et al.*, 2008; Ramirez-Carvajal *et al.*, 2009; Werner

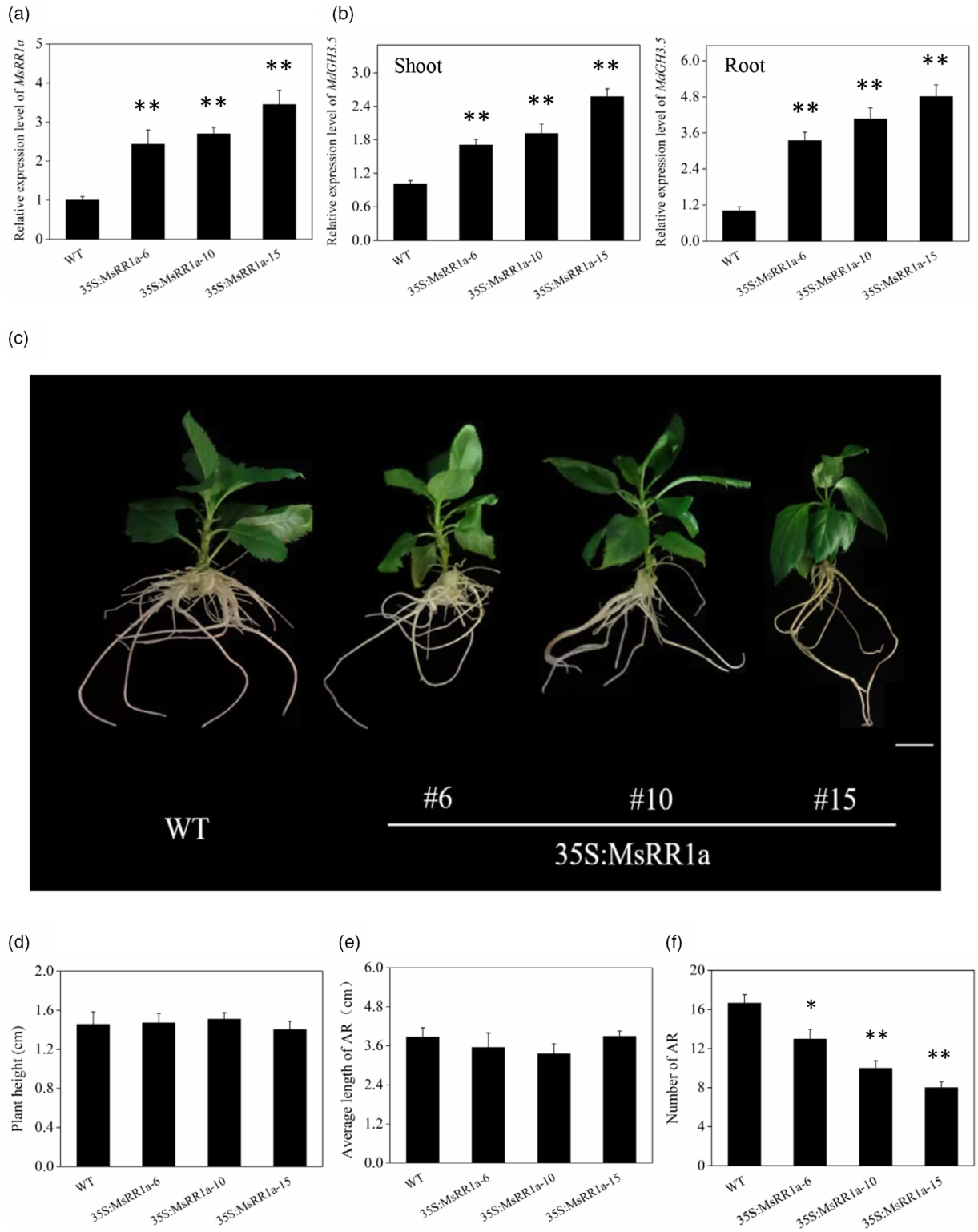

Figure 7. Phenotypic characterization of micropropagated *MsGH3.5*-overexpressing and control apple plants.
(a,b) Expression levels of *MsRR1a* (a) and *MsGH3.5* (b), measured using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).
(c) Morphological phenotypes of plants grown in rooting medium for 30 days. Scale bars: 1 cm.
(d) Plant heights.
(e,f) Average length (e) and number of adventitious roots (ARs) (f). Data are the means (± SE) of three biological replicates. Asterisks indicate significant differences from the wild-type (WT) apple plants (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

et al., 2010; Jiang *et al.*, 2016). Here, we demonstrated that *MsGH3.5* overexpression inhibited AR formation by reducing the free IAA content and increasing the CK content of the transgenic plants (Table 1). This result is consistent with previous observations that low IAA concentrations had a negative effect on AR formation in Arabidopsis (Pacurar *et al.*, 2014a), while higher CK concentrations inhibited AR formation in Populus (Ramirez-Carvajal *et al.*, 2009).

Furthermore, we observed that the height of the transgenic *MsGH3.5*-overexpressing apple lines and the number of ARs they produced were restored by the application of exogenous IAA (Figure 2). However, both shoot and root development of the transgenic lines were severely repressed by the application of a CK biosynthesis inhibitor, indicating that inhibition of CK did not restore the phenotype. It was previously reported that low CK concentrations are required to enhance rooting in apple (Klerk, 2002) and Monterey pine (*Pinus radiata*) cuttings (Ricci *et al.*, 2008). These results support previous reports that auxin and CK play antagonistic roles in root growth, and that *MsGH3.5* affects AR formation by regulating the level of these hormones.

Auxin homoeostasis has a major influence on plant organogenesis and morphogenesis (Woodward and Bartel, 2005). Several studies have shown that the conjugation of auxin with amino acids affects the distribution of the hormone and consequently alters plant architecture (Yang *et al.*, 2014; Lu *et al.*, 2015). A comparable outcome was observed in our *MsGH3.5*-overexpressing lines, which had reduced expression levels of some auxin transport genes (Figure 3a–d). Furthermore, *SHY2*, which represses the expression of the *PIN* genes, appears to modulate root meristem size by influencing the crosstalk between CK and auxin (Dello Ioio *et al.*, 2008). The gain-of-function *shy2-101* mutant has markedly inhibited LRP development and emergence (Goh *et al.*, 2012). Consistent with previous observations, the expression levels of the *MdSHY2*s were significantly elevated in the transgenic lines overexpressing *MsGH3.5* (Figure 3e–f), which may inhibit AR development. The *ARF*s are regulators of AR and LR formation. Overexpressing *ARF17* in Arabidopsis resulted in plants with fewer ARs than the WT, confirming that *ARF17* is a negative regulator of adventitious rooting (Sorin *et al.*, 2005). By contrast, *ARF6* and *ARF8* have been previously reported to promote AR formation (Gutierrez *et al.*, 2009). *ARF7* and *ARF19* function redundantly in the regulation of both LR and AR development (Huang *et al.*, 2018). In our study, the positive regulators of AR formation, including *MdARF6*, *MdARF8* and *MdARF19*, were downregulated in the transgenic plants overexpressing *MsGH3.5*, while the negative regulator *MdARF17* was upregulated in the transgenic plants (Figure 3g,h). These results provide further evidence that overexpression of *MsGH3.5* inhibited the formation of ARs in transgenic apple plants by partially suppressing auxin signaling and transport systems.

Auxin was previously shown to mediate a rapid negative effect on the CK pool by suppressing the expression of the *IPT* genes (Nordstrom *et al.*, 2004), consistent with the finding that the overproduction of IAA in *Nicotiana tabacum* plants decreased their CK contents (Eklof *et al.*, 2000). Our results similarly demonstrated that exogenous IAA treatment suppressed the expression of most *MsIPT*s and induced the expression of some *MsCKX*s in wild apple plants (Figure S5a,b). Correspondingly, the expression levels of several *MdIPT*s were markedly increased in the transgenic apple plants with low levels of endogenous IAA, while the expression of almost all *MdCKX*s was decreased (Figure 4a–d). Furthermore, auxin antagonizes CK output by directly transcriptionally activating the negative regulators of CK, *ARR7* and *ARR15* (Muller and Sheen, 2008), which is consistent with our observations here (Figure S5c,d). This might explain why the expression levels of several type-A *MdRR*s were decreased, while those of the type-B *MdRR*s were increased in the transgenic apple plants (Figure 4e–h). We propose that the overexpression of *MsGH3.5* decreased the abundance of active auxin, and consequently changed the expression of genes involved in CK biosynthesis, metabolism and signaling. However, considering that treating of plants with exogenous CK resulted in changes in gene expression, including of genes involved in CK signal transduction and metabolism (Bhargava *et al.*, 2013), it is also possible that altered CK levels affected the expression of these genes in transgenic apple plants. We cannot rule out the possibility that an unknown factor in the auxin and CK signaling pathways might also influence the expression of these genes.

Phytohormones play critical roles in AR formation; however, the key factors by which hormonal actions result in AR initiation are not well understood. Auxin promotes the fate transition of a cell near a wounded site into the AR founder cell by upregulating the expression of several *WOX* genes. The overexpression of *WOX5* and *WOX11/12* promotes AR regeneration (Liu *et al.*, 2014a,b; Li *et al.*, 2018), while *WOX11* expression is upregulated by wound-induced auxin and expressed in AR founder cells, where it activates *LBD16* expression during AR initiation (Sheng *et al.*, 2017). *LBD16* and *LBD29* exhibited functional redundancy in their regulation of LR formation (Okushima *et al.*, 2007; Feng *et al.*, 2012). Furthermore, *LBD18* controls *ARF* activity through a positive feedback loop to ensure continued LR growth (Pandey *et al.*, 2018). Our results indicate that *MdWOX11*, *MdLBD16* and *MdLBD18* expression were dramatically reduced in the transgenic plants (Figure 5a,b), which might partially inhibit AR formation.

As plant growth and development are driven by the continuous generation of new cells, there is mounting evidence to suggest that some key cell cycle regulators

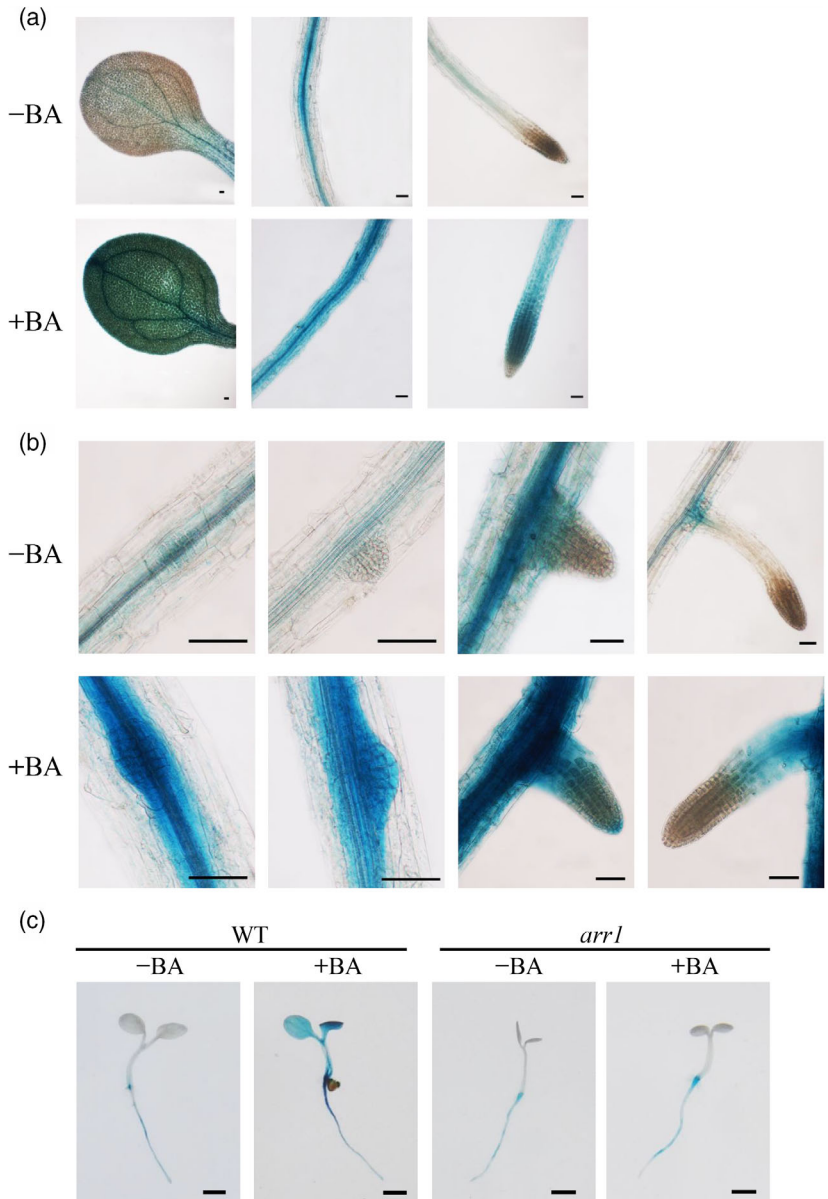

Figure 8. *GUS* expression pattern of *ProMsGH3.5:GUS*-expressing transgenic Arabidopsis is regulated by cytokinin (CK). Plants were grown for 5 days on a ½ Murashige and Skoog (MS) agar plate and then transferred to 10 μM 6-benzylaminopurine (6-BA) culture solution (or a solution lacking 6-BA as the control) for 1 h, followed by GUS staining.
(a) GUS staining of the cotyledon (left), primary root stele (center) and primary root tip (right) of *ProMsGH3.5:GUS* transgenic plants in the WT background in the presence or absence of 10 μM 6-BA. Scale bars: 50 μm.
(b) GUS staining of *ProMsGH3.5:GUS* transgenic plants in the WT background during lateral root development, in the presence or absence of 10 μM 6-BA. Scale bars: 50 μm.
(c) GUS staining of plants expressing *ProMsGH3.5:GUS* in the WT or *arr1* mutant background, in the absence or presence of 10 μM 6-BA. Scale bars: 0.1 cm.

function as intrinsic factors for the formation of new tissues and organs (De Veylder *et al.*, 2007; Li *et al.*, 2018). Cell cycle progression requires various cyclins, including *CYCA*, *CYCB* and *CYCD*, which function at different stages of the process. In Arabidopsis, *AtWOX7* regulates LR development through the direct regulation of *AtCYCD6;1* (Kong *et al.*, 2016). In poplar (*Populus tomentosa*), *PtoWOX5a* plays a role in root development through the regulation of *CYCD* expression (Li *et al.*, 2018). In birch, overexpression of *BpGH3.5* inhibits root development by decreasing the expression of *CYCB* and *CYCD* (Yang *et al.*, 2014). In our study, *MdCYCA2;4*, *MdCYCB1;1* and three *MdCYCD* genes were distinctly repressed in the *MsGH3.5*-overexpressing plants (Figure 5c), indicating that the decreased root growth in the transgenic lines may be associated with the inhibition of the root cell cycle. Our data suggest that *MsGH3.5* changes the expression of auxin- and CK-related genes, which represses apple development.

MsGH3.5 is targeted by MsRR1a in the CK signaling pathway

Previous reports have revealed the importance of the *GH3*s in plant root development (Khan and Stone, 2007; Zhang *et al.*, 2009), while others have demonstrated that CK is also a key regulator of root systems (Werner *et al.*, 2010; Jiang *et al.*, 2016). Until recently, however, little was known about the relationship between the *GH3*s and CK. Using a microarray meta-analysis and RNA-seq,

researchers identified 226 genes differentially regulated by CK, including some *GH3* genes (Bhargava *et al.*, 2013). Similarly, some *GH3*s were identified as potential CK-dependent targets for ARR10 in Arabidopsis using ChIP-seq (Zubo *et al.*, 2017). Another study established that *GH3.17* was a direct target of ARR1 in Arabidopsis, and was induced in an *ARR1*-overexpressing line and by CK treatment but not by auxin treatment (Di Mambro *et al.*, 2017). Furthermore, CK was shown to regulate meristem size by activating the expression of *GH3.5*, *GH3.6* and *GH3.17*, and in the LR cap, CK, via ARR1, controlled auxin levels by directly controlling the transcription of both *PIN5* and *GH3.17* (Di Mambro *et al.*, 2019; Pierdonati *et al.*, 2019).

Our results expand on these prior observations by directly linking the effect of a type-B CK RR, MsRR1a, to the activity of *MsGH3.5*. We further determined that MsRR1a directly binds to the promoter of *MsGH3.5*, positively regulating its expression (Figure 6). The *MsRR1a*-overexpressing lines produced fewer ARs, consistent with the phenotype of the *MsGH3.5*-overexpressing lines (Figure 7). Furthermore, our data show that CK induces *MsGH3.5* expression in an ARR1-dependent manner in Arabidopsis (Figure 8), further suggesting that *MsGH3.5* is involved in the crosstalk between auxin and CK.

The relationship between auxin and CK is complex. Researchers are beginning to elucidate the molecular mechanisms underlying the crosstalk between these phytohormones at the levels of hormone metabolism, transport and signaling (Simaskova *et al.*, 2015; Street *et al.*, 2016; Yan *et al.*, 2017). The transcript levels of several auxin-signaling genes are affected by CK treatment; for instance, there were strong increases in the abundance of *SHY2* transcripts in response to CK (Jones *et al.*, 2010), consistent with our observations for *MsGH3.5*. These results suggest that there may be some overlap between the auxin and CK signaling pathways, and that these two hormones regulate some genes in common, such as *MsGH3.5*.

As auxin and CK both have a strong capacity to regulate critical plant growth and developmental processes, some researches have indicated that root growth and development of apple rootstock are mainly influenced by auxin and CK signaling pathways (Li *et al.*, 2016a, 2019; Mao *et al.*, 2019b). Therefore, overlaps in their functional mechanisms have consistently attracted attention, especially in fruit trees. Our results complement the known network of mechanisms underpinning auxin–CK interactions, indicating that *MsGH3.5* plays an important role in regulating apple growth and development not only in the modulation of auxin homeostasis but also in the regulation of endogenous CK levels. Furthermore, we have shown that exogenous CK activates *MsGH3.5* expression by causing MsRR1a to directly bind to the *MsGH3.5* promoter. These results, and the identification of *MsGH3.5* as a key mediator of auxin–CK interaction in plant growth, may have agronomically valuable applications.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Rooted, micropropagated *M. sieversii* Roem plants were hydroponically precultured in ½ Hoagland nutrient solution for 15 days and then transferred to a full-strength Hoagland nutrient solution for hydroponic growth. *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used as the WT and background for the transformation of Arabidopsis. The *arr1* loss-of-function Arabidopsis mutant was kindly provided by Dr Xiansheng Zhang (Shandong Agriculture University, China). Sterilized seeds were cold treated for 4 days at 4°C, germinated on ½ Murashige and Skoog (MS) medium containing 0.7% (w/v) agar and 3% (w/v) sucrose for 10 days, and then transferred into containers of Hoagland nutrient solution. All plants were grown at 22°C and 60% relative humidity under a photoperiod of 16 h light/8 h darkness.

RNA extraction and real-time qPCR

The total RNAs of various Arabidopsis and apple tissues were extracted using a Quick RNA Isolation Kit (CWBIO, Beijing, China, https://www.cwbiotech.com/), and used as a template to synthesize cDNA with the M-MLV Reverse Transcriptase Kit (Takara Bio, Shiga, Japan, https://www.takarabiomed.com.cn/). Real-time qPCR was performed in the Rotor-Gene Q system (Qiagen, Hilden, Germany, https://www.qiagen.com/us/) using Ultra SYBR Mixture (CWBIO). *AtACTIN2* and *Histone H3* were used as the internal controls for the quantification of Arabidopsis and apple genes, respectively. The primers are listed in Table S1.

Plasmid construction and genetic transformation

According to the predicted cDNA sequence and genome-wide analysis of the *GH3* family in cultivated apple (Yuan *et al.*, 2013), the full-length open reading frame (ORF) of *MsGH3.5* was amplified from apple RNA using reverse transcriptase (RT)-PCR and cloned into the pMD19-T vector (Takara Bio). To generate the *MsGH3.5* and *MsRR1a* overexpression vectors, the full-length ORFs of *MsGH3.5* and *MsRR1a* without the termination codons were amplified and inserted into the pCAMBIA1302 vector, which was tagged with GFP under the control of the cauliflower mosaic virus 35S promoter. To generate the *ProMsGH3.5:GUS* (β-glucuronidase) vector, the promoter region of *MsGH3.5* (1348 bp) was amplified and inserted into the pCAMBIA1301 vector containing the *GUS* reporter gene. All these constructs were introduced into *Agrobacterium tumefaciens* strain EHA105.

For the dual-luciferase transient expression assays, the reporter construct was generated by inserting the *MsGH3.5* promoter into the pGreenII0800-LUC vector. The *MsRR1a* coding fragment was amplified using PCR and inserted into the pGreenII 62-SK vector (Hellens *et al.*, 2005). The *35S* promoter driving full-length *MsRR1a* cDNA was subsequently co-transformed alongside the helper plasmid pSoup into GV3101 and was used as an effector; the empty vector was used as a control.

For the EMSAs, the coding region of *MsRR1a* was amplified and inserted into the pET-28a vector containing a His tag. The recombinant plasmid was transformed into *Escherichia coli* BL21. All the primer sequences are shown in Table S1.

For the transformation of Arabidopsis, the resulting constructs were transformed into Col-0 plants using the floral dip method

with *A. tumefaciens* liquid cultures (Clough and Bent, 1998). The *ProMsGH3.5:GUS* vector was transformed into the backgrounds of both Col-0 and the *arr1* mutant. The seeds of positive transgenic plants were individually harvested, and the homozygous transgenic lines were used for further investigation. Leaf explants of the clonally propagated apple 'M26' were used for the apple transformation (Liao *et al.*, 2017). Transgenic apple shoots were regenerated as described previously (Holefors *et al.*, 1998). Independent transgenic shoots were micropropagated and rooted from each transgenic line, and the rooted plantlets were transferred to pots containing Hoagland nutrient solution.

Hormone treatment assays

Micropropagated *M. sieversii* plants with 20-cm roots were transferred into Hoagland nutrient solution supplemented with 10 μM IAA, 10 μM 6-BA, or a control solution for 0.5, 1, 2, 4 or 8 h. The shoots and roots were then collected, frozen in liquid nitrogen, and stored at −80°C. Micropropagated transgenic and control apple plants were grown in rooting medium (with 0.5 mg L^{-1} IBA) containing IAA (0.1, 0.2 or 0.5 mg L^{-1}), 0.01 μM lovastatin, or 0.01 μM lovastatin with 0.1 mg L^{-1} IAA for 30 days.

The seeds of WT and transgenic Arabidopsis lines expressing *MsGH3.5* were grown in ½ MS medium with different concentrations of IAA or 6-BA (0, 0.01 or 1 μM) combined with 1 μM 6-BA or 1 μM IAA, respectively, for 11 days.

The seeds of WT and transgenic Arabidopsis lines expressing *MsGH3.5* were grown in ½ MS medium with different concentrations of IAA. After 11 days, the lengths of the primary roots of seedlings were measured. Thirty seedlings of each line were analyzed with three biological replicates.

Quantification of endogenous hormones

The content of endogenous hormones (IAA, IAA-amido conjugates, CK and GA) in the shoots and roots was determined in 30-day-old transgenic and control plants grown under the control conditions described above. Three replicates of samples (600 mg) were harvested from each line, immediately frozen in liquid nitrogen, and stored at −80°C. The standards IAA, IAA-Ala, IAA-Leu, IAA-Glu, IAA-Asp were purchased from Sigma-Aldrich, https://www.sigmaaldrich.com/china-mainland.html (America). A quantitative analysis of these endogenous hormone contents was performed using high-performance liquid chromatography-mass spectrometry (HPLC-MS). The IAA and IAA-amino acid conjugate samples were extracted and quantified as previously described (Ding *et al.*, 2008). The CK and GA samples were extracted and quantified as previously described (Pan *et al.*, 2010).

Dual-luciferase transient expression assays

The reporter and effector constructs were co-transformed and infiltrated into *N. benthamiana* leaves. Firefly and Renilla luciferase activities were quantified using a Dual Luciferase Assay Kit (Promega, https://www.promega.com.cn/?renqun_youhua=213852). Three biological repeats were measured for each sample. The primers used to generate the various clones are listed in Table S1.

Electrophoretic mobility shift assays

The recombinant proteins were subsequently extracted from the cells and purified using Ni Sepharose High-Performance medium according to the manufacturer's instructions (GE Healthcare, https://www.gehealthcare.com/). The EMSA was performed using a Chemiluminescent EMSA Kit (Beyotime, https://www.beyotime.com/index.htm). 5-Carboxyfluorescein (FAM)-labeled DNA fragments were synthesized and used as probes, while unlabeled DNA with the same sequence or a mutant sequence was used as competitors. The probe and primer sequences are shown in Table S1.

ChIP assays

About 3 g of leaves of 3-week-old *35S:MsRR1a*-expressing transgenic and WT apple plants were taken and cross-linked in 1% formaldehyde for 20 min before being quenched in glycine for 5 min. The chromatin was pelleted by centrifugation (4, 4000 rpm, 20 min) and sonicated into 100–300-bp DNA fragments. An anti-GFP antibody (ab290; Abcam, https://www.abcam.cn/) was used to pull down DNA–protein complexes. The precipitated DNA fragments were recovered and quantified using qPCR with SYBR Premix Ex Taq Mix (Takara Bio). qPCR was conducted in triplicate for the input DNA sample, the control sample containing no antibodies, and the antibody-bound DNA sample. The primers used for qPCR are listed in Table S1.

GUS staining

The positively transformed GUS reporter lines driven by the *MsGH3.5* promoter were germinated on ½ MS medium and histologically stained after 5, 7 and 10 days. To detect CK-inducible GUS staining, the *MsGH3.5:GUS* seedlings were treated with 10 μM 6-BA for 1 h before the histological staining. The seedlings were incubated in 0.5 mg ml^{-1} X-Gluc staining solution at 37°C for 12 h in darkness, and then the chlorophyll was removed using 70% (v/v) ethanol. The stained seedlings were observed using a Nikon ECLIPSE Ti microscope and imaged with a camera (SONY, DSC-TX66).

ACCESSION NUMBERS

The accession numbers are shown in Table S2.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Di Zhao and Tianhong Li designed experiments; Di Zhao analyzed experimental data; Yantao Wang, Xiao Guo, Xinwei Guo, Zefeng Zhai, Jian Li and Xiaoshuai Shen performed some experiments; Chen Feng, Yan Wei and Xiang Peng carried out cultivating plant materials, Di Zhao and Tianhong Li wrote the manuscript.

SUPPORTING INFORMATION

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

Figure S1. Phylogenetic analysis of GH3 proteins in wild apple (*Malus sieversii*), *Arabidopsis thaliana* and rice (Oryza sativa).

Figure S2. The effect of exogenous IAA treatment on the expression level and pattern of MsGH3.5 in wild apple.

Figure S3. Expression of genes related to auxin biosynthesis in MsGH3.5-overexpressing and control apple plants.

Figure S4. Expression of genes related to the CK signaling pathways in MsGH3.5-overexpressing and control apple plants.

Figure S5. Expression of CK-related genes in wild apple plants following a treatment with auxin (IAA).

Figure S6. Phenotypic characterization of transgenic Arabidopsis plants heterologously expressing MsGH3.5.

Figure S7. The phenotype of transgenic and WT Arabidopsis plants treated with auxin (IAA) and/or CK (BA).

Figure S8. Primary root growth inhibition in response to exogenous IAA.

Figure S9. Effect of exogenous CK on the expression level of MsGH3.5 in wild apple plants.

Figure S10. Effect of exogenous CK on the expression level of the type-B MsRRs in wild apple.

Table S1. Primer sequences.

Table S2. Accession numbers.

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