

Modification of Plant Height via RNAi Suppression of *MdGA20-ox* Gene Expression in Apple

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ABSTRACT. GA20-oxidase (GA20-ox) is a key enzyme involved in the biosynthesis of gibberellic acid (GA). To investigate its role in plant growth and development, we suppressed *MdGA20-ox* gene expression in apple (*Malus domestica* cv. Hanfu) plants by RNA interference (RNAi). After 20 weeks of growth in the greenhouse, significant phenotype differences were observed between transgenic lines and the nontransgenic control. Suppression of *MdGA20-ox* gene expression resulted in lower plant height, shorter internode length, and higher number of nodes compared with the nontransgenic control. The expression of *MdGA20-ox* in transgenic plants was significantly suppressed, and the active GA content in transgenic lines was lower than that in the nontransgenic control. These results demonstrated that the *MdGA20-ox* gene plays an important role in vegetative growth, and therefore it is possible to develop dwarfed or compact scion apple cultivars by *MdGA20-ox* gene silencing.

Apple is one of the most widely cultivated fruit crops in the world. Tree size is critical for early, high yielding, and efficient apple crops. Dwarf trees require reduced spray volumes, produce higher yields per unit of land through higher planting densities (Atkinson and Else, 2001), and allow mechanical harvesting. Development of compact apples through conventional breeding may control growth (Talwara et al., 2013), but it is a costly and time-consuming approach with a high probability of adverse changes in fruit characteristics. Chemical growth retardants are used as a supplementary treatment to control excessive vegetative growth, but decrease profitability (Wiesman and Lavee, 1994). Apple trees become smaller in size when scions are grafted on dwarfing rootstocks or interstocks. Dwarfing rootstocks impart characteristics to the tree as early flowering from planting, more efficient stable yield, and reduced vegetative growth.

Most growth retardants act by inhibiting the biosynthesis of GA, a class of plant hormones. GA plays important roles in plant development, including stem growth, leaf expansion, flower development, fruit set, and seed germination (Yamaguchi, 2008). Studies have revealed that one of the most obvious functions of GA is to promote vegetative growth, including the elongation of stems and the expansion of leaves (Olszewski et al., 2002). GA-deficient mutants tend to have small and dark green leaves and reduced stem length (Wang et al., 2012a). Application

of exogenous active GA on wild plants or increased GA levels in mutants results in tall and spindly phenotypes (Fagoaga et al., 2007). Most of GA-deficient mutants have a dwarf phenotype with significantly decreased GA content and can be restored to normal phenotype by spraying with GA₃ solution (Wang et al., 2012b). The GA biosynthetic pathway has been well characterized and can be divided into three major stages according to the localization and the enzymes involved (Lee and Zeevaart, 2007). The final stages of GA biosynthesis require the action of 2-oxoglutarate-dependent dioxygenases, including the GA20-ox that catalyzes the penultimate step in the formation of bioactive GA, and has been shown to be an important regulator in GA biosynthesis pathway (Zhao et al., 2010).

The aim of this study was to develop dwarf apple lines by silencing of the *MdGA20-ox* gene.

Materials and Methods

PLANT MATERIAL AND GROWING CONDITIONS. In this study, Hanfu, an elite apple cultivar developed by Shenyang Agricultural University (Shenyang, China), was used for plant transformation and isolation of *GA20-ox* genes. Cultures were maintained at 23 °C day/18 °C night under a 16-h photoperiod. In vitro plants of *Malus hupehensis* ‘Pingyitiancha’ were maintained under field conditions and used for the isolation of *GA20-ox* genes.

NUCLEIC ACID EXTRACTION. Genomic DNA was isolated from apple shoots as described by Tai and Tanksley (1990). Total RNA was isolated from leaves using the modified cetyltrimethylammonium bromide method (Chang et al., 2007) and treated

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Table 1. Primers' sequence amplified for the fragment of *MdGA20-ox* gene, sense or antisense of RNAi vector, *nptII* gene, and *MdGA20-ox* gene in nontransgenic and the transgenic lines, and semiquantitative and quantitative PCR of 'Hanfu' apple.

Primer ^z	Sequences of sense and antisense primers (5' to 3')	Amplification
PF1	CCTCCCAACTCACATACA	<i>MdGA20-ox</i> gene fragment
PR1	TCTCCCTGCTTTCCTTCA	
PF2	GCCTCGAGCCTTCCCAACTCACATACA ^y	
PR2	CTGAATTC ^y TCTCCCTGCTTTCCTTCA	
PF3	CTAAGCTT ^y TCTCCCTGCTTTCCTTCA	
PR3	GCTCTAGACCTTCCCAACTCACATACA	Sense fragment of RNAi vector
PF4	GTTCTTTTGTCAAGACCGACC	Antisense fragment of RNAi vector
PR4	CAAGCTTCTCAGCAATATCACG	
PF5	GGACAGTAGAAAAGGAAGGT	Detecting <i>nptII</i> gene
PR5	GAAAATGTATGTGAGTTGGG	Detecting <i>MdGA20-ox</i> gene
actinF(SQ) ^x	ATCGTGGTCATTGGCCATGT	
actinR(SQ)	AGCCTGTGAGGTTCCAGTAATCAT	Semiquantitative PCR
actinF(Q) ^w	CCTTCGTCTTCGGCTTGTTT	Quantitative PCR
actinR(Q)	GGAGCATCATCACCAGCAA	

^zPF = sense primer; PR = antisense primer.

^yCharacters with underscore stand for sites of restriction endonuclease.

^xSQ = semiquantitative PCR, expression levels of the *MdGA20-ox* gene in transgenic lines and the nontransgenic lines.

^wQ = quantitative PCR, expression levels of the *MdGA20-ox* gene in transgenic lines and the nontransgenic lines.



Fig. 1. Schematic drawing of the pRNAi-GA20ox construct. An inverted repeat was generated by cloning a sense fragment (*MdGA20-ox* 466 bp) followed by the antisense orientation in the pART27 vector backbone. The conserved sequence of 466 bp was used in developing the hpRNA binary vector for RNA interference; RB = right border, Ocs = octopine synthase, NOS = nopaline synthase, LB = left border.

with 1 μ L RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) at 37 °C for more than 4 h.

ISOLATION OF MDGA20-OX GENE FRAGMENT AND CONSTRUCTION OF RNAI VECTOR. Forward and reverse primers (Table 1) were designed based on sequences of *GA20-ox* genes (GenBank accession numbers EB128368, EB132114, AB037114, EB126424, and EB127719). A fragment of the *GA20-ox* gene was amplified from the genomic DNA of 'Hanfu' and 'Pingyitiancha' by polymerase chain reaction (PCR). The 20- μ L PCR reactions included 50 ng genomic DNA, 1 \times buffer, 1.8 mM MgCl₂, 0.2 μ M of each primer, 200 μ M of dNTPs, and 1 U of *Taq* DNA polymerase (Invitrogen, Shanghai, China). The PCR program was as follows: 94 °C for 3 min; 94 at 30 s, 55 at 1 min, 72 °C 1 min, 35 cycles; 72 °C for 10 min. To make a construct encoding a single self-complementary hairpin RNA (hpRNA) of the *MdGA20-ox* gene (Fig. 1), the sense and antisense fragments of the *MdGA20-ox* gene were first inserted into the pKANNIBAL vector (Wesley et al., 2001). The recombinant pKANNIBAL vector and the plant expression vector pART27 were digested with *NotI*. The vector fragment including the *MdGA20-ox* gene sequence was ligated into the *NotI*-digested pART27.

PLANT TRANSFORMATION. The RNAi construct was transferred into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method (Wang et al., 2011). The transformation method was used with leaf segments as explants. The transgenic system for 'Hanfu' has been previously established (Yang et al., 2010). The system was as follows: a single colony from the bacterial strain was inoculated into liquid yeast extract peptone (YEP) medium supplemented with 25 mg·L⁻¹

kanamycin and 100 mg·L⁻¹ spectinomycin, and grown at 28 °C for 16 h on an orbital shaker at 165 rpm. The optical density at 600 nm (OD₆₀₀) of bacterial solution was diluted to OD₆₀₀ = 0.1 in 50 mL YEP and incubated at 25 °C with shaking for 5 to 6 h until OD₆₀₀ = 0.5. The first three apical expanding leaves were harvested from 4-week-

old proliferation shoots. Each leaf was transversely cut into 3- to 4-mm segments. Leaf segments were shaken gently in the bacterial suspension for 10 min and blotted on a sterile filter paper. They were then cocultivated in bud regeneration medium [Murashige and Skoog (MS) supplemented with 2.0 mg·L⁻¹ thidiazuran, 0.2 mg·L⁻¹ α -naphthalene acetic acid] in darkness for 3 d. Immediately after cocultivation, explants were washed three times with sterile distilled water to eliminate excess bacteria, blot-dried, and transferred into the bud regeneration medium supplemented with 250 mg·L⁻¹ cefotaxime and 25 mg·L⁻¹ kanamycin for selection culture. After selection culture, leaf explants were transferred to light conditions cultured for 4 weeks. When the regenerated buds were \approx 1 cm long, they were excised and transferred into Type I shoot development medium [MS medium supplemented with 1.0 mg·L⁻¹ 6-benzylaminopurine (6-BA), 0.2 mg·L⁻¹ indole-3-acetic acid (IAA)], 0.1 mg·L⁻¹ GA₃, 250 mg·L⁻¹ cefotaxime, and 25 mg·L⁻¹ kanamycin). They were subcultured two times and then transferred into Type II shoot development medium (MS medium supplemented with 1.0 mg·L⁻¹ 6-BA, 0.2 mg·L⁻¹ IAA, 1.0 mg·L⁻¹ GA₃, 250 mg·L⁻¹ cefotaxime, and 25 mg·L⁻¹ kanamycin). Subculturing was carried out every 3 weeks to maintain selection pressure.

PCR ANALYSIS. PCR analysis was performed on isolated genomic DNA to detect the presence of transgene in the putative transformed plants using primers (PF4 and PR4) for the *nptII* gene (Table 1), which confers resistance to kanamycin, and a pair of primers (P5F and P5R) that amplifies the 35S promoter region and the *MdGA20-ox* gene was designed

MdGA20-ox	TCTCCCCTGCTTTCCTTTGAGCTCTCTGTTTTTGGAGAG	39
MhGA20-ox	TCTCCCCTGCTTTCCTTTGAGCTCTCTGTTTTTGGAGAG	40
Consensus	tctcccctgcttttccttt agctctctgttttt ggagag	
MdGA20-ox	TGGCATTCCAAAAAAGTCATCCATGCAGCGGTGAGCGTCC	79
MhGA20-ox	TGGCATTCCAAAAAAGTCATCCATGCAGCGGTGAGCGTCC	80
Consensus	tggcattccaaaaaagtcacatgcagcggtagcgtcc	
MdGA20-ox	GCGATGAGCGTATTGTGCGACGCCGTGATTCACGATGAGGA	119
MhGA20-ox	GCGATGAGCGTATTGTGCGACGCCGTGATTCACGATGAGGA	120
Consensus	gcgatgagcgtattgtgacgcccgtgattcacgatgagga	
MdGA20-ox	AAAATCCATGCTTCTGGCATGCCTCTCCTACAGTTGCCGA	159
MhGA20-ox	AAAATCCATGCTTCTGGCATGCCTCTCCTACAGTTGCCGA	160
Consensus	aaaatccatgcttctggcatgcctctcctac agttgccga	
MdGA20-ox	GGCTTTCGCGAGCGGCTTCTTTGTGCGCCCGAGAGAAAGCCT	199
MhGA20-ox	GGCTTTCGCGAGCGGCTTCTTTGTGCGCCCGAGAGAAAGCCT	200
Consensus	ggcttttcgagcggcttctttgtgcccagagaaagcct	
MdGA20-ox	CCCAAGTCTATGAGTGGACTCGGAGCTCGGGAGTAITTT	239
MhGA20-ox	CCCAAGTCTATGAGTGGACTCGGAGCTCGGGAGTAITTT	240
Consensus	cccaagtctatgagtgg actcggagctcgggagtatttt	
MdGA20-ox	TGCAAGGCTTTTCGTCATCGGGCCATATGAACIGACICGG	279
MhGA20-ox	TGCAAGGCTTTTCGTCATCGGGCCATATGAACIGACICGG	280
Consensus	tgcaaggcttttcgtcacatcgggccatataaacigactcgg	
MdGA20-ox	AATTTTCAGTTTGGTACCTTAAAACGAGCCATCAAAAACC	319
MhGA20-ox	AATTTTCAGTTTGGTACCTTAAAACGAGCCATCAAAAACC	320
Consensus	aattttcagtttgggtaccttaaaacgagccatcaaaaacc	
MdGA20-ox	AATGGITTCIGGTCATCCTCTTGTGCTGCTGGGTTTCT	359
MhGA20-ox	AATGGITTCIGGTCATCCTCTTGTGCTGCTGGGTTTCT	360
Consensus	aatggitttcigggtacatcctcttgtgctgctgggtttct	
MdGA20-ox	GCGTTTGTGGGGAGGGAGGTTGTGCCATGGTTTIGCAIGCT	399
MhGA20-ox	GCGTTTGTGGGGAGGGAGGTTGTGCCATGGTTTIGCAIGCT	400
Consensus	gcgtttgtggggagggaggttgtgccatggtttigcaigct	
MdGA20-ox	GCTGGGTTTGTATCATGCACTCAACAGCCATTTTTCATGA	439
MhGA20-ox	GCTGGGTTTGTATCATGCACTCAACAGCCATTTTTCATGA	440
Consensus	gctgggtttgtatcatgcaactcaacagccatttttcatga	
MdGA20-ox	ACAGAAAATGTATGTGAGTTGGGGAAG	466
MhGA20-ox	ACAGAAAATGTATGTGAGTTGGGGAAG	467
Consensus	acagaaaatgtatgtgagttggggaag	

Fig. 2. Sequence alignment of the *MdGA20-ox* gene isolated from 'Hanfu' apple and the *MhGA20-ox* gene isolated from *Malus hupehensis* 'Pingyitiancha'.

(Table 1). The 20- μ L PCR reactions included 50 ng genomic DNA, 1 \times buffer, 1.8 mM MgCl₂, 0.2 μ M of each primer, 200 μ M of dNTPs, and 1 U of *Taq* DNA polymerase (Invitrogen). The PCR program was as follows: 94 °C 3 min; 94 at 30 s, 55 at 1 min, 72 °C 1 min, 35 cycles; 72 °C 10 min.

tests and the significance level was determined at 5% using SPSS (version 17.0; IBM Corp., Armonk, NY).

DETERMINATION OF GA CONTENTS. One gram fresh weight of leaves (control and transgene lines) at the same developmental stage were harvested and stored in liquid N₂ and were ground

SEMIQUANTITATIVE AND QUANTITATIVE REVERSE TRANSCRIPTION-PCR ANALYSIS. Complementary DNA (cDNA) was synthesized from 3 μ g of total RNA using Superscript II reverse transcriptase (Invitrogen), following the manufacturer's instructions with oligo-(dT)-18 primer (TaKaRa Biotechnology). The *Actin* gene was selected as a positive control (Gasic et al., 2004).

For semiquantitative PCR analysis, the 50- μ L PCR reactions included 2 μ L cDNA (all cDNAs were quantified to the same concentration), 1 \times buffer, 1.8 mM MgCl₂, 0.2 μ M of each primer, 200 μ M of dNTPs, and 1 U of *Taq* DNA polymerase. The PCR program was as follows: 94 °C for 2 min, 34 cycles of 94, 57, and 72 °C for 1 min each cycle, and a final extension step of 72 °C for 5 min. PCR products (5 μ L) were separated by 1.5% agarose electrophoresis.

Quantitative reverse transcription (RT)-PCR was conducted as described by Tan et al. (2013). Quantification of cDNA samples was performed in triplicate. Data were normalized against the value of the *Actin* gene. Relative fold changes in gene expression were calculated using the comparative Ct (2^{- $\Delta\Delta$ Ct}) method.

PHENOTYPE ANALYSIS. Transgenic and nontransgenic lines were grown in a multiplication medium (MS medium supplemented with 1.0 mg·L⁻¹ 6-BA, 0.2 mg·L⁻¹ IAA, and 0.5 mg·L⁻¹ GA₃). After 2 months, nine samples of each line were used to evaluate growth characteristics. Data on stem length, node number, and internode length were collected. All the transgenic lines and the nontransgenic control were transferred to the greenhouse at the same time. Stem height and internode length were measured after 20 weeks in the greenhouse. Stem height was measured from the bottom to the tip. Internode length was calculated as stem height divided by the number of leaves. Comparisons for multiple treatments were analyzed using Duncan's multiple range

into fine powder in a chilled mortar with chilled 80% (v/v) methanol. Extraction, purification, and immunoassay of GAs were performed as described by Wu et al. (2008) using a GA₁₊₃ enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Agricultural University, Nanjing, China). Finally, the absorbance at 490 nm was recorded using an ELISA analyzer.

Results

CONSTRUCTION OF AN RNAi VECTOR FOR THE MdGA20-ox GENE. A 466-bp fragment of the *GA20-ox* gene was amplified from both ‘Hanfu’ and ‘Pingyitiancha’ lines, and the nucleotide sequence identity of the two apple species was 99.15% (Fig. 2). The fragment from ‘Hanfu’ was used as the sense and antisense fragment to create the RNAi hairpin construct for the *MdGA20-ox* gene (Fig. 1). The RNAi vector for the *MdGA20-ox* gene was successfully constructed verified by enzyme digested and sequenced, then named pRNAi-GA20ox.

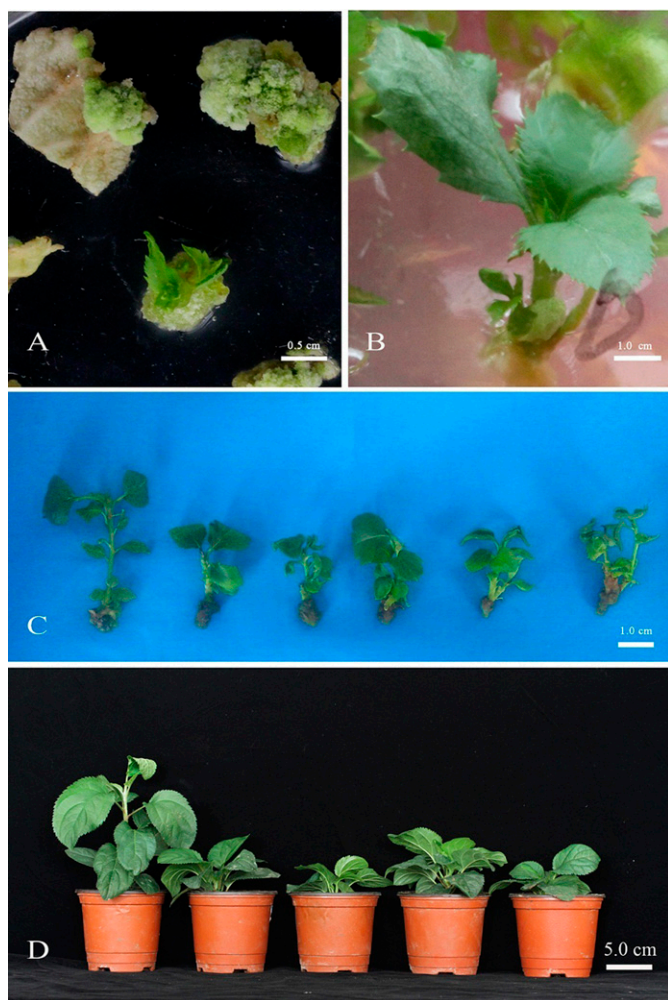


Fig. 3. Transformation of ‘Hanfu’ apple with interference RNA vector for the *MdGA20-ox* gene. (A) Kanamycin-resistant bud regenerated from leaf explants after 6 weeks of cultured; (B) kanamycin-resistant bud developed into a shoot after 2 months cultured in Type II medium; (C) phenotypes of nontransgenic control and the transgenic plants after 2 months cultured in Type II medium, from left to the right: nontransgenic control, transgenic lines (T-HF1, T-HF2, T-HF3, T-HF4, and T-HF5); (D) height of transgenic plants after 20 weeks in the greenhouse, from left to the right: nontransgenic control, transgenic lines T-HF1, T-HF3, T-HF4, and T-HF5.

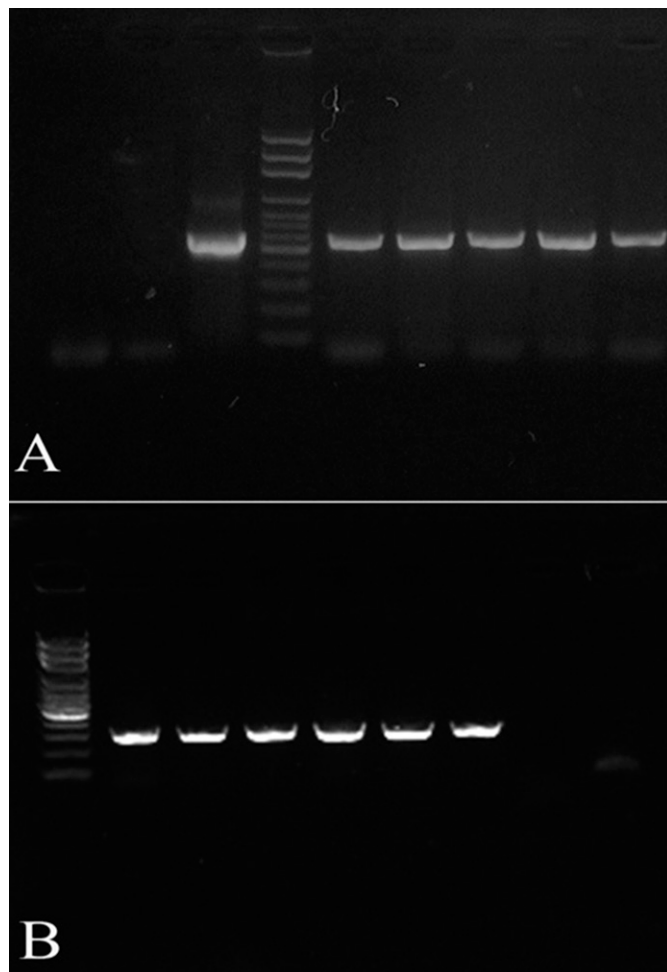


Fig. 4. PCR analysis for detection of transgenic lines. (A) PCR analysis for *nptII*, lanes from left to right: nontransgenic control, negative control (without DNA template), positive control of pRNAi-GA20ox, 100-bp molecular size marker, transgenic lines T-HF1, T-HF2, T-HF3, T-HF4, T-HF5 (M); (B) PCR analysis for the *MdGA20-ox* gene, lanes from left to right: 100-bp molecular size marker, transgenic lines T-HF1, T-HF2, T-HF3, T-HF4, T-HF5, positive control of pRNAi-GA20ox, nontransgenic control, and negative control (without DNA template).

DEVELOPMENT OF KANAMYCIN-RESISTANT PLANTS. Twenty-seven kanamycin-resistant buds (Fig. 3A) were regenerated from 9485 leaf explants, and the transformation rate was 0.28%. After 2 months of culture in Type I medium, buds were less than 1 cm long with very small leaves. Then transferred into Type II medium, they had developed into green shoots 4 months later (Fig. 3B).

MOLECULAR ANALYSIS OF TRANSGENIC PLANTS. PCR results revealed that the 562-bp fragment for the *nptII* gene (Fig. 4A) and the 400-bp fragment for the 35S promoter and *MdGA20-ox* gene (Fig. 4B) were amplified from all five kanamycin-resistant lines. No amplification products were detected in the nontransgenic control plants. So, all the five kanamycin-resistant lines, T-HF1 to T-HF5, were transgenic lines.

PHENOTYPES OF TRANSGENIC LINES. For in vitro plants, leaves of transgenic lines were small and dark green, and all transgenic plants were significantly lower in height than the nontransgenic control plants grown in the same multiplication medium (Fig. 3C). The shortest transgenic line was T-HF2, with an average height

of 2 cm, 43% lower than that of the nontransgenic control (3.5 cm). The highest transgenic line was T-HF1, with an average height of 2.9 cm, 20% lower than that of the nontransgenic control (Table 2). The node number of transgenic lines was significantly higher (1.3- to 1.5-fold) than that of the nontransgenic control (Table 2). The transgenic plants were shorter with more nodes, and therefore the length of internodes in the transgenic lines was significantly shorter than that in the nontransgenic control (Table 2).

In vitro plants were transferred into a greenhouse in early May 2014. Nontransgenic control plants and transgenic lines were survived in the greenhouse, except for T-HF2 (died of fungal infections). Phenotypes were measured after plants grew in the greenhouse for 20 weeks. No significant differences were observed in the leaf color between transgenic plants and nontransgenic control, but the height of transgenic plants [T-HF1 (5.00 cm), T-HF3 (4.20 cm), T-HF4 (6.35 cm), T-HF5 (3.43 cm)] was significantly lower than that of the nontransgenic control (16.5 cm) (Fig. 3D). No transgenic lines were over 50 cm 1 year after grew in greenhouse, while the transgenic lines were only 18% to 35% of the no transgenic lines.

EXPRESSION LEVELS OF THE MdGA20-ox GENE IN TRANSGENIC LINES. Semiquantitative RT-PCR was used to analyze the expression of *MdGA20-ox* gene in in vitro plants. As shown in Fig. 5, *MdGA20-ox* genes showed distinct patterns of transcript accumulation between transgenic plants and the nontransgenic control. The band amplified from the nontransgenic control was clear and strong, whereas the bands amplified from transgenic lines were weak. The expression of *MdGA20-ox* gene in plants that grew in the greenhouse was measured by quantitative RT-PCR. Because the plants of T-HF3 died of disease in the greenhouse, only T-HF1, T-HF4, and T-HF5 were used for quantitative RT-PCR. The results revealed that expression levels of the *MdGA20-ox* gene were 4.79% for T-HF1, 18.99% for T-HF4, and 14.76% for T-HF5, when relative quantification of the *MdGA20-ox* gene was set to 100% (Fig. 6).

EFFECT OF SUPPRESSING MdGA20-ox GENE EXPRESSION ON GA CONTENTS. GA content in leaves of in vitro plants was measured by ELISA. As shown in Fig. 7, GA content in all transgenic lines was reduced compared with that in the nontransgenic control plants, and the correlation between the GA content and the length of internodes was significantly positive ($r = 0.93$).

Discussion

GA is a phytohormone involved in many aspects of plant development. The most obvious function of GA is the promotion of vegetative growth, particularly of plant height (Hannon, 2002; Hedden and Phillips, 2000). GA biosynthetic pathway has been well characterized and can be divided into three major stages according to the localization (Lee and Zeevaart, 2007), and the enzymes involved. GA20-ox catalyzes the final steps in the biosynthesis of biologically active GA (Zhao et al., 2010). *GA20-ox* gene encoded by a gene family whose members show differential expression patterns (Hedden and Phillips, 2000), whereas in *Arabidopsis thaliana*, *GA20-ox* were encoded by five genes. *GA20-ox1*, *GA20-ox2*, and *GA20-ox3* are important to growth and fertility, which are active in flowers and seeds, whereas *GA20-ox4* and *GA20-ox5* have very minor roles. Concentrations of GA₄ in GA-deficient mutants (*ga20-ox1*, *ga20-ox2*, *ga20-ox3*) were ≈50% of

Table 2. Phenotypes change in average height, length per nodes, and numbers of nodes of control and transgenic plants of ‘Hanfu’ apple cultured in vitro.^z

Line	Avg ht	Length per node	Nodes
	[mean ± SD (cm)]		[mean ± SD (no.)]
Control	3.55 ± 0.44 a ^y	1.08 ± 0.17 a	3.38 ± 0.92 d
T-HF1	2.86 ± 0.24 b	0.67 ± 0.10 b	4.33 ± 1.22 c
T-HF2	2.36 ± 0.36 d	0.45 ± 0.10 d	4.33 ± 1.50 c
T-HF3	2.31 ± 0.37 d	0.44 ± 0.09 d	4.50 ± 1.18 b
T-HF4	2.61 ± 0.32 c	0.57 ± 0.06 c	4.57 ± 0.98 b
T-HF5	2.81 ± 0.47 b	0.58 ± 0.14 c	5.00 ± 1.51 a

^zAll samples were harvested in vitro cultured for 4 mo. The average height was measured from the bottom to the tip. The internodes length was calculated by dividing the stem height with leaf numbers. The numbers of node was statistical from the bottom to the tip.

^yDifferent letters within the same column indicate significant difference at $P < 0.05$ by Duncan’s test.

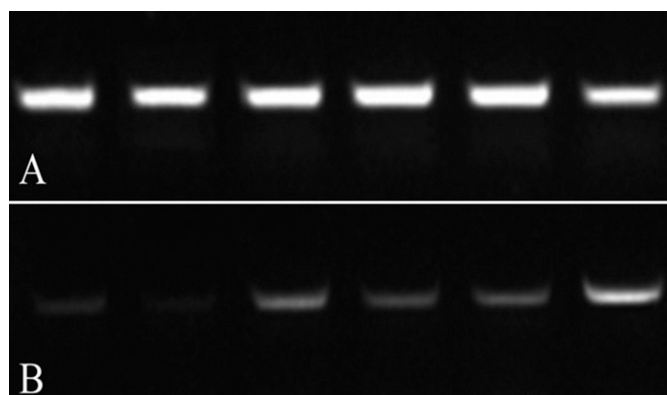


Fig. 5. Expression levels of the *MdGA20-ox* gene in ‘Hanfu’ apple transgenic lines and the nontransgenic control detected by semiquantitative reverse transcription-PCR. (A) Expression levels of *Actin* gene, lanes from left to right: transgenic lines T-HF1, T-HF2, T-HF3, T-HF4, T-HF5, nontransgenic control; (B) Expression levels of the *MdGA20-ox* gene, lanes from left to right: transgenic lines T-HF1, T-HF2, T-HF3, T-HF4, T-HF5, and nontransgenic control.

the wild types (Plackett et al., 2012). Decrease of *GA20-ox1* or *GA20-ox2* expression by RNAi resulted in shorter stems, decreased length of internodes, and small dark green leaves (Xiao et al., 2006). Leaf thickness was smaller in sense and larger in antisense *CcGA20ox1* plants (Fagoaga et al., 2007). The height of transgenic lines of *OsGA20ox2* RNAi (dwarf lines and semidwarf lines) was ≈75% to 84% and 54% to 74% of that of the control plants, and the semidwarf lines could be restored to normal plant height by applying exogenous GA₃ (Qiao and Zhao., 2011). A pair of degenerate primer was designed based on the 466-bp fragment of *MdGA20-ox* genes (common base sequences of *MdGA20-ox1*, *ox2*, and *ox3*) in this study for constructing the RNAi vector to suppress *MdGA20-ox* gene expression, and we obtained dwarf lines of ‘Hanfu’ apple with short internodes. Bulley et al. (2005) have suppressed the expression of *MpGA20ox1* gene by antisense RNA, resulting in reduced the stem height, greatly reduced contents of GA₁, and decreased number of internodes. There were some differences with our studies. First, transgenic line heights of our study were more shorter because RNAi was more efficient than antisense RNA in gene

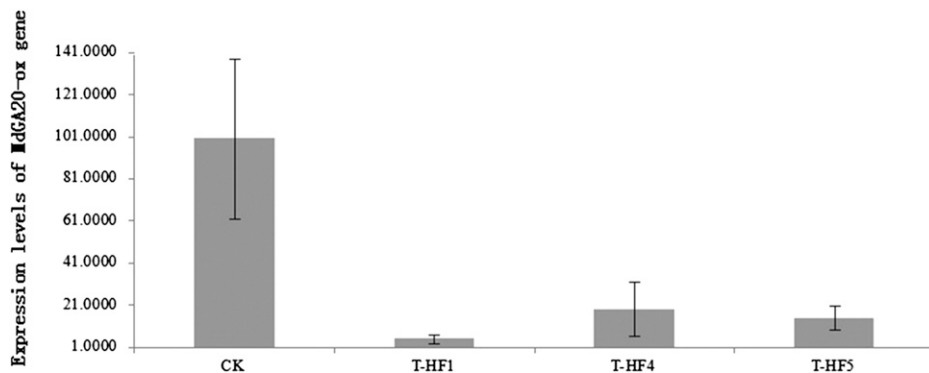


Fig. 6. Expression levels of the *MdGA20-ox* gene in 'Hanfu' apple transgenic lines and the nontransgenic control detected by quantitative reverse transcription-PCR. From left to right: nontransgenic control (CK), transgenic lines T-HF1, T-HF4, and T-HF5.

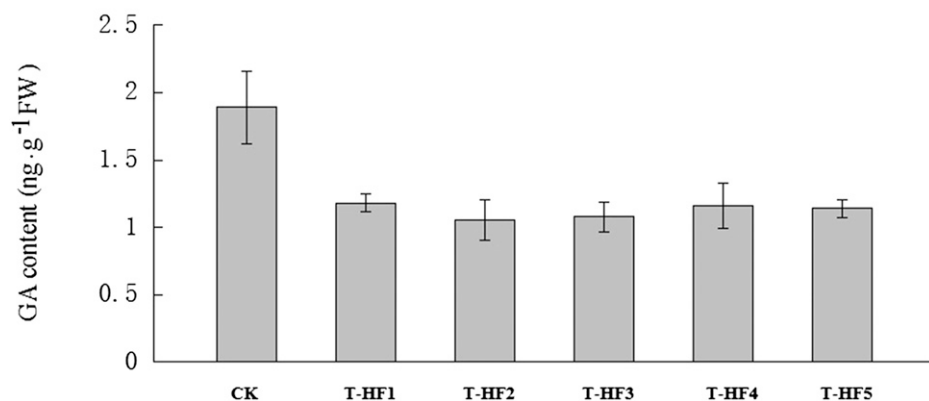


Fig. 7. Endogenous active gibberellic acid content (GA₁₊₃) in the leaves of 'Hanfu' apple nontransgenic control and the transgenic lines. From left to right: nontransgenic control (CK), transgenic lines T-HF1, T-HF2, T-HF3, T-HF4, and T-HF5.

silencing. *GA20-ox* of apple was encoding by three genes. There were several differences in base sequences of the three genes (*MdGA20-ox1*, *ox2*, and *ox3*, which are usually not active in leaves), and even more, the 466 bp for silencing was the base on the common base sequences of the three genes (a pair of primer was designed only for *MdGA20-ox1*), so we believed that silencing of *MdGA20-ox* was the cause of decrease of GA active (could unaffected other genes). Second, buds regenerated from Type I medium were less than 1 cm long cultured for 2 months, we realized that GA deficient was the main cause. Therefore, we transferred them into Type II medium cultured for 4 months. GA contents and growth parameter were measured at this time. So GA contents were higher and the number of internodes were increased in our study. And even more, the real contents of GA₃ may be more lower because we promoted GA₃ in the Type II medium, but the nontransgenic and transgenic lines were grew in the same medium, so the relative relationship between them was true. Our results demonstrated that (as also shown by Bulley et al., 2005) the *GA20-ox* gene plays an important role in vegetative growth of apple and it is possible to develop compact scions by suppressing *MdGA20-ox* gene expression. *GA20-ox* gene is tissue specific, which mainly expresses in immature seeds (Kusaba et al., 2001), leaves and flowers (Ashikari et al., 2002), and GA deficiency also impact on fertility (Plackett

et al., 2012). So, the transgenic lines obtained in this study maybe have low fertility.

RNAi is an efficient and stable transgenic method for gene silencing. The silencing effect can spread to adjacent cells or over the whole organism. It can spread from cell to cell over short distances, locally extensive or systemically via phloem (Kalantidis et al., 2008). The most promising genetic engineering technology for fruit and nut tree crops maybe produce transgenic rootstocks (Haroldsen et al., 2012a), which could be grafted with nontransgenic scions. In this case, it potentially mollifies consumer concerns with eating transgenic fruits. This approach would also address the need to affect many cultivars with one rootstock. An accumulation of small interference RNAs derived from hairpin constructs was observed in kernels of a wild-type scion grafted on a transgenic rootstock in annual crops (Haroldsen et al., 2012b). The spread of RNA gene-silencing signals through graft junctions has been evaluated in fruit trees. For example, in a preliminary experiment, virus resistance was seen in sour cherry (*Prunus cerasus*) trees grafted onto a virus-resistant rootstock that was resistant through production of sRNAs (Song et al.,

2013). A graft transmission of silencing signals in in vitro shoots was demonstrated in transgenic apple plants, over-expressing an hpRNA construct of *gusA* reporter gene. However, this could not be detected again once the scion grew in field (Flachowsky et al., 2012). In that case, lignification is generally detrimental for systemic silencing. On the other hand, it would open the possibility to down-regulate specific genes in rootstocks without influencing scions.

Our preliminary study of graft transmission in vitro (we observed that the shoots of nontransgenic apple plants grafted in vitro onto T-HF3 were shorter than those grafted onto nontransgenic 'Hanfu') has indicated that the scions are shorter suggesting that small RNAs may cross the graft junction and affect the production of GAs in the scion. Further work is being pursued on this.

Conclusions

To investigate the role of GA in the growth and development of apple, we applied RNAi technology to suppress *MdGA20-ox* gene expression. Our results showed that suppression of *MdGA20-ox* gene expression resulted in lower plant height, shorter internode length, and higher number of nodes, small and dark green leaves compared with the nontransgenic control in vitro. A significant difference in plant height was observed in

the greenhouse too. The expression of *MdGA20-ox* in transgenic plants was significantly suppressed by a small hpRNA construct, and the active GA contents in transgenic lines were lower than that in the nontransgenic control. Overall, dwarf apple plants can be developed with the application of RNAi technology.

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