Development and validation of functional markers from *Dw1* and *Dw2* loci to accurately predict apple rootstock dwarfing ability

Feng Yang

China Agriculture University, Liaoning Institute of Pomology

Fei Shen

China Agriculture University, Beijing Academy of Agriculture and Forestry

Zhi Liu

Liaoning Institute of Pomology

Mingli He

Liaoning Institute of Pomology

Yi Wang

China Agriculture University

Ting Wu

China Agriculture University

Xuefeng Xu

China Agriculture University

Wei Li

China Agriculture University

Changpeng Qiu

China Agriculture University

Xinzhong Zhang

China Agriculture University

Zhenhai Han (✉ rschan@cau.edu.cn)

Research article

Keywords: Malus, dwarfing rootstock, diagnostic marker, genomic assisted prediction

Posted Date: June 12th, 2020

DOI: <https://doi.org/10.21203/rs.2.24669/v2>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: 'M9' is a widely used apple dwarfing rootstock due to the outstanding effects on both precocity and vigorous control. Two quantitative trait loci (QTLs), Dw1 and Dw2, for the dwarfing effect were previously mapped on 'M9', but the genetic variations that underpin the dwarfing ability have not been elucidated to date.

Result: By using 'Red Fuji' trees grafted on 1123 hybrids from 'Malus baccata' × 'M9', the intervals of Dw1 and Dw2 were narrowed down. MdLBD3 and MdARF6 were predicted as potential candidate genes from Dw1, while MdG3OX3 was a possible candidate gene from Dw2. An 11 bp deletion at -339 bp upstream of the transcription start site (TSS) of MdLBD3 generated a new cis-element binding site with MdWRKY2 and caused increased expression of MdLBD3. Coincidentally, a ten bp deletion at -278 bp upstream of the TSS of MdG3OX3 created an additional binding site of MdABI5, leading to higher expression of MdG3OX3. At -954 bp of the MdARF6 promoter, a 14 bp insertion destroyed the binding ability by MdABI5 and reduced MdARF6 expression. The genotype effects of these insertion and deletions as diagnostic markers on dwarfing traits (tree height, trunk diameter, and canopy width) were estimated in 108 F1 hybrids. The genomic predicted genetic values (GEGV) were calculated by adding up the genotype effects of the three markers and the population mean phenotype. The GEGV of the dwarfing traits exhibited high correlation coefficients of 0.93, 0.94, and 0.93 in terms of tree height, trunk diameter, and canopy width for the observed phenotype values, respectively. The predictability of GEGV was validated in 64 Malus accessions.

Conclusion : The development of the three functional markers, Ld/Li, Ad/Ai, and Gd/Gi, ensures the accurate genomic assisted prediction of dwarfing ability in apple rootstock breeding. The data also suggested that ABA, auxin, GA, and zeatin signals may be involved in the regulation of apple rootstock dwarfing mechanism.

Background

'M9' is a widely used apple (*Malus domestica* Borkh.) dwarfing rootstock across the world due to the outstanding effects on both precocity and vigorous control [1, 2]. Many other popularly used apple rootstocks are descendants of the 'Malling' series. 'M9' is still one of the most important core parents in the dwarfing rootstock breeding programs [3, 4]. However, the dwarfing mechanism and genetic control of 'M9' are not yet fully understood.

Phytohormone levels, distribution, and hormonal signaling are extremely relevant to the dwarfing effect in rootstocks. It was reported that the more vertical plant architecture will the plant produce more auxin resulting more vigorous growth [5, 6]. The dwarfing ability of 'M9' used as an interstem is related to the lower expression level of *MdPIN* genes that encode auxin efflux carrier proteins, which restricts IAA basipetal transport to the roots [7, 8]. Tobacco plants that use transformants that overexpress *MdPIN1b* as interstem show precocity and dwarfing architecture [9]. On the other hand, when 'M9' is used as the

rootstock, the low root zeatin content, caused by the lower expression of *MdIPT* gene encoding isopentenyl transferase, contributes to the weakened vegetative growth of the scion that has been grafted onto 'M9' rootstock [10]. Gibberellins (GA) has been discovered as a rate-limiting hormone in the process of dwarfing control of plants such as apples (*Malus pumila* Mill), breadfruits (*Artocarpus altilis*) and bean (*Phaseolus coccineus*)[11-14].

Recently, many efforts have been exerted in mapping quantitative trait loci (QTLs) to facilitate practical marker assisted selection (MAS) in apple rootstock [15-17]. In a F1 hybrid population of 'M9' × 'Robusta 5', a QTL spanning the dwarfing locus *Dw1* from 'M9' was mapped on the top of chromosome 5 and positioned at 4.74-7.62Mb including 547 genes. Another QTL *Dw2* was mapped at 1.88- 8.98Mb in chromosome 11 including 1141 genes [2, 18]. Soon after, the third dwarfing locus *Dw3* associated with root bark ratio was discovered at 6.0-9.8cM of chromosome 13 using 257 F1 hybrids of the 'M27' × 'M116' family [19]. However, no functional markers or candidate genes involving dwarfing were identified from the intervals of these QTLs.

In this study, F1 hybrids derived from '*Malus baccata* L.' × 'M9' were used as a segregating population to predict and validate candidate dwarfing genes in M9 rootstock. '*M. baccata*' was once popularly used as seedling rootstock in apple nurseries owing to its strong winter hardiness and good grafting compatibility with most apple cultivars [20].

Results

Inheritance of dwarfing traits

Phenotype of dwarfing traits, tree height, trunk diameter, and canopy width segregated significantly among 'Red Fuji' scions grafted on the 1123 hybrids of '*M. baccata*' × 'M9' (Fig.1 A and B; Table 1). None of the frequency distributions of these dwarfing traits showed a Gaussian distribution (Fig. 2 A-C). Broad sense heritability was estimated to be 73.0%, 79.7%, and 82.3% for tree height, trunk diameter, and canopy width, respectively (Table 1).

Significant linear regressions were shown between tree height, diameter and canopy width. The Pearson's correlation coefficients were 0.9421, 0.8663, and 0.9388 ($P < 0.01$) for tree height/trunk diameter, tree height/canopy width, and trunk diameter/canopy width, respectively (Fig. 2 D-F).

Marker validation and candidate gene prediction

Three SSR markers, Hi01c04, Hi04a08, and CH03a09 near *Dw1* on chromosome 5, and the other two markers, Hi07d11 and CH02d08 close to *Dw2* on chromosome 11, were genotyped in 30 hybrids where the scion exhibited the dwarfing phenotype [2]. All five of these markers were heterozygous in 'M9' rootstock, homologous in '*M. baccata*' rootstock, and displayed a distorted segregation ratio in dwarfing F1 hybrids, indicating the association of these markers with dwarfing effects (Fig. 3).

To further narrow down the intervals of *Dw1* and *Dw2*, four and nine InDel markers flanked by the above-mentioned SSRs near *Dw1* and *Dw2*, respectively, were selected from the parental resequencing data set (Table S1). All markers were heterozygous in 'M9', and were homologous in '*M. baccata*'. These InDel markers were also genotyped in the 60 hybrids, the scion grafted on each of the 30 hybrids showed extremely dwarfing or extremely vigorous tree architecture. Whenever a marker recombined with the phenotypes, it's considered not to be a diagnostic marker at all and the corresponding gene should not be the candidate gene. Of these markers, the segregation ratio of C10 and B06 near *Dw1* and M90 close to *Dw2* was 30:0 in dwarfing hybrids and 0:30 in vigorous hybrids (Fig. 4). According to the apple genome, C10, B06, and M90 were located in the upstream regions of *MdLBD3*, *MdARF6*, and *MdG3OX3*, respectively. Therefore, these three genes were selected as potential candidate genes for further validation.

Validation of allelic variation of the candidate genes

The relative expression of *MdLBD3* and *MdG3OX3* was significantly higher in the fine roots of 'M9' than in '*M. baccata*', but *MdARF6* was more extensively expressed in the roots of '*M. baccata*' than in 'M9' (Figure 5A). The 0~1104 bp, 0~1289 bp, and 0~1176 bp segments upstream of the transcription start sites (TSS) of *MdLBD3*, *MdARF6*, and *MdG3OX3*, respectively, were cloned from the DNA of 'M9' and '*M. baccata*' and Sanger sequenced (Fig. S1; Fig. 5C). Besides some SNPs that were heterozygous on 'M9', an 11bp and a 10 bp deletion were found -339 bp and -278 bp upstream of the TSS of *MdLBD3* and *MdG3OX3*, respectively, while a 14 bp insertion was located -954 bp prior to the TSS of *MdARF6* (Fig. 5C). Non-synonymous variations were not found on the coding sequences of these three genes (Fig. S1). To test whether the gene expression differs between genotypes of these InDels in *MdLBD3*, *MdARF6*, and *MdG3OX3*, fine roots of 108 F1 randomly chosen F1 hybrids of '*M. baccata*' × 'M9' were sampled for a gene relative expression assay (Table 2). The relative gene expression of hybrids with *MdLBD3* Del:Ins, *MdG3OX3* Del:Ins, and *MdARF6* Del:Del genotypes was significantly greater than that of hybrids with *MdLBD3* Ins:Ins, *MdG3OX3* Ins:Ins, and *MdARF6* Del:Ins genotypes, respectively (Fig. 5B). These promoter variants of each gene were then constructed fusion with a GUS reporter and transiently expressed in *Nicotiana benthamiana* leaf epidermal cells. The promoter activity of *MdLBD3-pro-Del* and *MdG3OX3-pro-Del* from 'M9' was significantly stronger than that of *MdLBD3-pro-Ins* and *MdG3OX3-pro-Ins* from 'M9' or '*M. baccata*', whilst the promoter activity of *MdARF6-pro-Ins* from 'M9' was lower than that of *MdARF6-pro-Del* from '*M. baccata*' or 'M9' (Fig. 5D).

To further examine the functional variations on the promoter sequences of these candidate genes, the promoter activity was re-examined by promoter truncation. Truncating off segments of promoter sequences, including the InDels of *MdLBD3*, *MdARF6*, and *MdG3OX3* in 'M9', destroyed the promoter activity completely. The SNPs did not interfere with promoter activity, but the InDels on the each gene's promoter altered the transcription activity significantly (Fig. 5 A, B and D). Consequently, InDels on the promoter of *MdLBD3*, *MdARF6*, and *MdG3OX3* were utilized as functional markers for dwarfing effects.

Functional analysis of allelic variation of the candidate genes

Prediction revealed that the 11bp deletion 'TCAACAACATA' located -339 bp from the TSS of *MdLBD3* may have caused the generation of a new cis-element with the critical motif 'TGGAATGGGTATAG', which could be bound by *MdWRKY2* transcription factor (Fig. 6A). Yeast one hybrid assay (Y1H) confirmed the binding of *MdWRKY2* protein to the promoter of *MdLBD3-Del* in 'M9' but not to *MdLBD3-Ins* in '*M. baccata*' (Fig. 6B). No differences in *MdWRKY2* relative expression were identified between either 'M9' and '*M. baccata*' nor their F1 hybrids with dwarfing and non-dwarfing effects (Fig. 6A).

The 14 bp insertion 'GAGAGAGCGAGAGA', on the promoter of *MdARF6* in 'M9', destroyed the *MdGAMYB* binding motif 'GGCGGTTGTG' based on the prediction from PlantCare software (Fig. 6C). Y1H showed that the binding ability was completely absent between AD-*MdGAMYB* and BD-*MdARF6-Ins* (Fig. 6C). The *MdGAMYB* expression levels were quite analogous among 'M9', '*M. baccata*', and their F1 hybrids, irrespective of the dwarfing effects (Fig. 6A).

The 10 bp deletion 'ATCATATTTT', on the *MdG3OX3* promoter, produced a new *MdABI5* binding motif 'ACGTTTTTGT', which was confirmed by Y1H assay (Fig. 6D). Similar *MdABI5* expression levels were observed among 'M9', '*M. baccata*', and their F1 hybrids, irrespective of the dwarfing effects (Fig. 6A). These data suggested the use of the InDel variations *MdLBD3*, *MdARF6*, and *MdG3OX3* as potential markers for dwarfing effects.

Prediction of dwarfing ability with marker genotype estimated genetic values (GEGV)

To estimate the genotype effects of the functional markers, *MdLBD3(Del/Ins)* / *MdARF6(Del/Ins)* / *MdG3OX3 (Del/Ins)* (abbreviated as Ld/Li, Ad/Ai, and Gd/Gi), 108 F1 hybrids of '*M. baccata*' × 'M9' were randomly chosen and genotyped. The marker genotype effects and effects of marker genotypic combinations on tree height, trunk diameter, and canopy width of the grafted trees were estimated (Table 2). AdAi had the largest marker genotype effect, and LdLi had the least (Table 2). The marker GEGV of the F1 hybrids were calculated by adding up the genotype effects of the three functional markers and the overall mean phenotype value. The Pearson's correlation coefficients for tree height, trunk diameter, and canopy width between GEGV and observed phenotype value (OPV) were all significant (Fig. 7A, B and C). Five-fold cross validation produced an average correlation coefficient of $r = 0.93$ ($P < 0.01$), indicating a very high predictability of GEGV in the current hybrid population. To test the applicability of this GEBV in *Malus* germplasm, 64 *Malus* rootstock accessions were genotyped for Ld/Li, Ad/Ai, and Gd/Gi markers. The GEGVs were consistent with the accession dwarfing ability reported by previous literatures, implying that the GEGV model should be applicable for a broad spectrum of apple rootstocks (Table 3).

Discussion

Polygenic control of dwarfing traits and the stability of dwarfing QTLs

Relatively high broad sense heritability was detected in this study for dwarfing traits such as tree height, trunk diameter, and canopy size, which is consistent with many previous reports [21-23]. The loci *Dw1* and *Dw2* were formerly identified from a hybrid population of 'M9' × 'Robusta 5' [2]. The markers from these

loci segregated with perfect distortion in '*M. baccata*' × 'M9' in this study. *Dw1* explains 43% of the total phenotypic variance in 'M9' × 'Robusta 5' [2]. Consistently in '*M. baccata*' × 'M9', relatively large marker effects of 1.09 and 0.66 on tree height were estimated for *MdARF6* and *MdLBD3* as examples, respectively. Not only in the population of '*M. baccata*' × 'M9', but also in *Malus* accessions, the GEGVs of tree height, trunk diameter, and canopy size estimated by the three functional markers were very consistent with dwarfing ability. These consistency was likely because most dwarfing apple rootstocks are descendants of the East Malling series rootstocks [24, 25].

Genetic variations in phytohormone signaling genes control dwarfing traits

MdARF6, the functional marker gene with the largest effect on dwarfing effect, negatively regulates its target genes involved in auxin signaling [26-28]. In 'M9' and other dwarfing rootstocks, the *MdGAMYB* binding site of *MdARF6* promoter was eradicated by an insertion on one allele of the locus, and the expression of *MdARF6* was reduced by half. No differences in *MdGAMYB* expression were detected between '*M. baccata*' and 'M9' nor between hybrids with different dwarfing abilities. Therefore, the change in *MdARF6* expression caused by the deletion in its promoter may lead to an increase in the expressions of the target genes, such as the Gretchen Hagen3 (GH3) genes [29].

G3OX3 oxidizes gibberellin and therefore represses vegetative growth or elongation of roots or shoots [30, 31]. Though *MdABI5* expression did not vary with dwarfing ability, an extra *MdABI5* binding site was formed by a deletion on the *MdG3OX3* promoter of 'M9' and other dwarfing rootstocks, which caused a rise in *MdG3OX3* expression. Both root and leaf GA levels were much lower in apple trees grafted onto dwarfing rootstocks than that onto standard rootstocks [32, 33]. *AtABI5* in *Arabidopsis* functions as the main transcription factor in abscisic acid signal transduction [34,35]. Thus, *MdABI5* may act to integrate gibberellins and abscisic acid signals with dwarfing effect by interacting with *MdG3OX3*, INDUCER OF CBF EXPRESSION1 (ICE1), and DELLA protein [11, 36].

The latera organ boundaries domain (*LBD*) transcription factor family promotes lateral root or shoot formation and lateral growth [37]. *MdLBD3*, a gene homologous to *AtLBD3/AtLBD9*, is induced by exogenous cytokinin [38]. In rice (*Oryza sativa* L.), *OsLBD3-1* is expressed only in roots, and *OsLBD3-2* is expressed in roots, shoots, and petioles [39]. We found that a deletion on *MdLBD3* promoter produced an additional *MdWRKY2* binding motif and created a rise in *MdLBD3* expression. Though, fine root density, root length, and lifespan were significantly lower in dwarfing rootstocks than that in standard rootstocks [40]. The similar expression levels of *MdWRKY2* were detected in roots of both dwarfing and non-dwarfing materials. Therefore, the genetic variation of *MdLBD3* linked dwarfing effect with cytokinin signaling, but the molecular function of *MdLBD3* still requires further investigation.

There must be more genetic variations contribute to the dwarfing traits

In the *Malus* accessions, rootstocks with the same GEGV exhibited varied dwarfing ability, for example 'M27' is sometimes referred to as hyper-dwarfing, but 'M26' performs as semi-dwarfing in certain environments [41, 42]. Another example is that the GEGV of 'GM256' was relatively large, but the dwarfing

potential is much greater than ‘MM106’ or ‘M7’ [43, 44]. The concomitant absence of a 41 bp deletion on the *MdIPT5b* promoter and presence of a 6 bp deletion on the *MdAUX/IAA114* promoter of ‘M9’ were closely correlated to an decrease in fine root zeatin content, which contributed to dwarfing ability in *Malus* accessions, as well as ‘Baleng Crab’ × ‘M9’ F1 hybrids [10]. The expression of *MdPIN8* was much lower in the stem tissue of ‘M9’ interstem than in ‘Red Fuji’ scion and ‘Baleng Crab’ rootstock, which induced the dwarfing effect [9]. However, no QTLs other than *Dw1*, *Dw2*, and *Dw3* have been mapped yet.

Conclusion

Taken together, the development of the three functional markers, *Ld/Li*, *Ad/Ai*, and *Gd/Gi*, ensures the accurate apple rootstock dwarfing ability prediction. These functional markers also suggest that ABA, auxin, GA, and zeatin signals are involved in the regulation of apple rootstock dwarfing mechanism. Then there must be more genetic variations contribute to the dwarfing traits.

Methods

Plant material

All the plant materials were possessed by Liaoning Institute of Pomology (Xiongyue, Liaoning Province, China). Field investigation and sample collection were performed under institutional guidelines in accordance with local legislation. A hybrid cross was made between a single maternal plant of ‘*M. baccata*’ and a pollen parent ‘M9’ in 2008. The hybrid seeds were sown in a solar greenhouse in February 2009, and ‘Red Fuji’ scions were grafted onto each hybrid 20 cm above the collar in April 2010. Then all 1123 grafted trees were transplanted (2.5 m × 1.0m) into the orchard in April 2011. ‘Red Fuji’/‘M9’ and ‘Red Fuji’/‘*M. baccata*’ were planted in three replicates as dwarfing and standard tree architecture controls, respectively. Trees were not pruned to allow complete expression of the rootstock effects on scion growth. Sixty four *Malus* rootstock accessions were used to validate the genotype effects of the InDel markers. The orchard was subjected to conventional field management and pest control. Phenotype data were collected from 2014-2016 as three year-replicates when almost all trees bloomed and set fruit. Samples were gathered from root and basal sucker leaves, immediately frozen in liquid nitrogen, and stored at -80°C until needed.

Phenotyping, analysis of inheritance

The grafted trees were phenotyped in 2014-2016, 4 to 6 years after planting. Tree height, trunk diameter (20 cm above grafting union), and canopy width were measured during winter dormancy [45]. Frequency distribution of the phenotype was analyzed to show the continuity of the genetic segregation. The broad sense heritability (H^2) was estimated by the percentage of genetic variance (G) in the total phenotypic variance (P), whereas the P can be split into G and the environmental variance (E). The E was estimated by the mean phenotypic variance among replicates of ‘Red Fuji’ cultivar grafted on ‘M9’ and ‘*M. baccata*’.

Thus the equation is that $H^2 = (P - E) / P$. The data were processed using Microsoft Excel and SPSS statistical software [19].

DNA extraction and SSR markers validation

Leaf samples were gathered from the basal suckers of the grafted trees. Total genomic DNA was extracted with the CTAB method and quantified. The segregation of previously reported SSR markers on chromosome 5 (Hi01c04, Hi04a08, and CH03a09) and chromosome 11 (Hi07d11 and CH02d08) representing *Dw1* and *Dw2*, respectively, were validated in a F1 hybrid population and also validated in their parents [2].

Parental re-sequencing and candidate gene prediction

High quality genomic DNA of the two parents, '*M. baccata*' and 'M9', was extracted from leaves using the CTAB method. Re-sequencing was performed using Illumina platform (Illumina X10, Illumina, USA), with paired-ends 150 strategy, NGSQC filter, and FastQC identify. Each DNA sample was sequenced over 30× the genome size. The reads were aligned to the reference apple genome: 'Golden Delicious' *Malus × domestica* v 3.0 from the Genome Database for Rosaceae (GDR) (<https://www.rosaceae.org/>)[46]. Non-synonymous variations between the two parents were selected, which were heterozygous (0/1) in 'M9', but were homologous (0/0) in '*M. baccata*'. InDels were validated by Sanger sequencing. Cis-acting elements of gene promoters were predicted using the database (<https://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Genotyping by PCR

The primers of InDels were designed based on the 200 bp flanking sequences, the primer pairs were list in Table S1. F1 hybrids with extremely dwarfing or vigorous phenotypes from '*M. baccata*' × 'M9' population and the parental cultivars were genotyped for each InDel and SSR marker. PCR reactions were performed in a final volume of 20 µL system: an initial denaturation step of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, an annealing temperature of 53 °C - 58 °C for 35 s and 72 °C for 40 s, with a final extension step at 72 °C for 5 min, and then 4°C preserve and terminate. PCR products were assayed using agarose or polyacrylamide electrophoresis to polymorphic validation.

Gene relative expression assay

RNA was extracted from the fine root samples, and cDNA was synthesized using the RNAiso kit (Prime ScriptTM RT, Takara, Dalian, China), following the manufacturer's instructions. RT-qPCR was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR green reagents (Takara, Japan) as the following protocol: ten micro-litter Premix (2×), 0.4µL upstream primers, 0.4µL downstream primers, 2µL template (cDNA), and DEPC water up to 20µL; program: 94 °C for 2 min, 40 cycles of 94 °C for 20 s, and 60 °C for 34 s. The complete sequences of candidate genes were attained from the apple genome website (<http://genomics.research.iasma.it/>) by means of a basic local

alignment search tool (BLAST). The β -actin gene was used as a house-keeping reference and each of the sample were performed on three biological replicates, each containing three technical replicates. The primer pairs used are listed in Table S2.

Promoter truncation and activity assay

Promoter fragments of 1104 bp, 1289 bp, and 1235 bp upstream of *MdLBD3*, *MdARF6*, and *MdG3ox3*, respectively, were cloned from the genomic DNA of '*M. baccata*' and 'M9', and their sequences were validated by Sanger sequencing. The each gene's promoter was digested and cloned into the pCAMBIA1391-GUS plant transformation vector. Then the constructs were transformed into leaf epidermal cells of 4-week-old *Nicotiana benthamiana* plants by GV3101 *Agrobacterium* infiltration . After 2-3 days of transfection, the transgenic leaves were dyed using the β -Galactosidase Reporter Gene Staining Kit (Solarbio, China) for about 3 to 24 hours at 37°C under dark conditions, and then decolorized using 70% alcohol. Three biological replicates were performed for the transformation.

Yeast one hybrid assay (Y1H)

Full length CDS of the transcription factor, *MdGAMYB*, *MdWRKY2*, and *MdABI5*, was ligated into the PJG4-5 vector as ADActivation domain. The fragments of 0-1300 bp, 0-1206 bp, and 0-968 bp upstream of *MdARF6*, *MdLBD3*, and *MdG3OX3*, respectively, were cloned into the placZi vector as BD-DNA-binding domain. All primers that were used are listed in Table S1. The Y1H assay was done using the Matchmaker™ and Gold Yeast One-Hybrid Library Screening System kit (Cat. no. 630491, Clontech, Takala, Dalian, China) [47].

Estimation of marker genotype effect and GEGV

One hundred and eight randomly chosen F1 hybrids from '*M. baccata*' $\times$ 'M9' population and 64 *Malus* rootstock accessions were genotyped for each InDel marker. The marker genotype effects were calculated by the mean OPV of a subset of hybrids with the same genotype subtracted by the overall population mean phenotype value. The GEGV of an individual hybrid was calculated by adding the sum of all marker genotype effects to the overall population mean phenotype value. The prediction accuracy of the GEGV was represented as the Pearson's correlation coefficient between the GEGV and the OPV of a hybrid[49]. The prediction ability of the GEGV was examined by using five-fold cross-validation[50].

References

1. Li K, Liu Z, Xing LB, Wei YH, Mao JP, Meng Y, Bao L, Han MY, Zhao CP and Zhang D. miRNAs associated with auxin signaling, stress response, and cellular activities mediate adventitious root formation in apple rootstocks. *Plant Physiology and Biochemistry*. 2019; 139: 66-81.
2. Foster TM, Celton JM, Chagne´ D, Tustin DS and Gardiner SE .Two quantitative trait loci, *Dw1* and *Dw2*, are primarily responsible for rootstock-induced dwarfing in apple. *Horticulture Research*. 2015; 2: 1-9.

3. An N, Fan S, Yang Y, Chen X, Dong F, Wang YB, Xing LB, Zhao CP and Han MY. Identification and characterization of miRNAs in self-rooted and grafted *Malus* reveals critical networks associated with flowering. *International Journal of Molecular Sciences*. 2018; 19:1-20.
4. Chakrabarty D, Dewir YH, Hahn EJ, Datta SK and Paek KY. The dynamics of nutrient utilization and growth of apple root stock 'M9 EMLA' in temporary versus continuous immersion bioreactors. *Plant Growth Regulation*. 2007; 51: 11–
5. Oka M, Ueda J, Miyamoto K and Okada K. Actives of auxin polar transport in inflorescence axes of flower mutants of *Arabidopsis thaliana*: Relevance to flower formation and growth. *Journal of Plant Research*. 1998; 111:407-410.
6. Ueda J, Toda Y, Kato K, Kuroda Y, Arai T, Hasegawa T, Shigemori H, Hasegawa K, Kitagawa J and Miyamoto K. Identification of dehydrocostus lactone and 4-hydroxy-b-thujone as auxin polar transport inhibitors. *Acta Physiologia Plantarum*. 2013; 35: 2251-2258.
7. Li HL, Zhang H, Yu C. Ma L, Wang Y, Zhang XZ and Han ZH. Possible roles of auxin and zeatin for initiating the dwarfing effect of M9 used as apple rootstock or interstock. *Acta Physiologiae Plantarum*. 2012; 34: 235-244.
8. Zhang H, An HS, Wang Y, Zhang XZ and Han ZH. Low expression of *PIN* gene family members is involved in triggering the dwarfing effect in M9 interstem but not in M9 rootstock apple tree. *Acta Physiologiae Plantarum*. 2015; 37: 104-122.
9. Gan ZY, Wang Y, Wu T, Xu XF, Zhang XZ and Han ZH. *MdPIN1b* encodes a putative auxin efflux carrier and has different expression patterns in BC and M9 apple rootstocks. *Plant Molecular Biology*. 2018; 96: 353-365.
10. Feng Y, Zhang XZ, Wu T, Xu XF, Han ZH and Wang Y. Methylation effect on *IPT5b* gene expression determines cytokinin biosynthesis in apple rootstock. *Biochemical and Biophysical Research Communications*. 2017; 482: 604-609.
11. Foster T, Kirk C, Jones WT, Allan AC, Espley R, Karunairetnam S and Rakonjac J. Characterisation of the *DELLA* subfamily in apple (*Malus x domestica* Borkh.). *Tree Genetics & Genomes*. 2007; 3: 187–197.
12. Dijkstra C, Adams E, Bhattacharya A, Page AF, Anthony P, Kourmpetli S, Power JB, Lowe KC, Thomas SG, Hedden P, Phillips AL, Davey MR. Over-expression of a gibberellin 2-oxidase gene from *Phaseolus coccineus* L. enhances gibberellin inactivation and induces dwarfism in *Solanum* species. *Plant Cell Reports*. 2008; 27: 463–470.
13. Zhou YC, Underhill SJR. Breadfruit (*Artocarpus altilis*) *DELLA* genes: gibberellin-regulated stem elongation and response to high salinity and drought. *Plant Growth Regulation*. 2017; 83: 375–383.
14. Silva WD, Ghica ME and Brett CMA. Biotoxic trace metal ion detection by enzymatic inhibition of a glucose biosensor based on a poly (brilliant green)–deep eutectic solvent/carbon nanotube modified electrode. *Talanta*. 2020; 208: e120427.
15. Larsen B, Migicovsky Z, Jeppesen AA, Gardner KM, Andersen TBT, Myles S, Ørgaard M, Petersen MA and Pedersen C. Genome-wide association studies in apple reveal loci for aroma volatiles, sugar

- composition, and harvest date. *The Plant Genome*. 2019; 12: 1-15.
16. Chagné D, Vanderzande S, Kirk C, Profitt N, Weskett R, Gardiner SE, Peace CP, Volz RK and Bassi NV. Validation of SNP markers for fruit quality and disease resistance loci in apple (*Malus × domestica* Borkh.) using the OpenArray platform. *Horticulture Research*. 2019; 30: 1-16.
 17. McClure KA, Gardner KM, Toivonen PMA, Hampson CR, Song J, Forney CF, DeLong J, Rajcan I and Myles S. QTL analysis of soft scald in two apple populations. *Horticulture Research*. 2016; 43: 1-7.
 18. Pilcher RLR, Celton JM, Gardiner SE and Tustin DS. Genetic markers linked to the dwarfing trait of apple rootstock 'Malling 9'. *Journal of The American Society for Horticultural Science*. 2008; 133:100–106.
 19. Harrison N, Harrison RJ, Perez NB, Lopez EC, Medina MaC, Lipska M, Ruíz RC, Brain P, Gregory PJ and Fernández-Fernández F. A new three-locus model for rootstock-induced dwarfing in apple revealed by genetic mapping of root bark percentage. *Journal of Experimental Botany*. 2016; 67:1871–1881.
 20. Dunemann F, Gläss R, Bartsch S, Eldin MAS, Peil A and Bus VGM. Molecular cloning and analysis of apple *HcrVf* resistance gene paralogs in a collection of related *Malus* species. *Tree Genetics & Genomes*. 2012; 8:1095–1109.
 21. Yang W, Liu XD, Chi XJ, Wu CA, Li YZ, Song LL, Liu XM, Wang YF, Wang FW, Zhang C, Liu Y, Zong JM and Li HY. Dwarf apple *MbDREB1* enhances plant tolerance to low temperature, drought, and salt stress via both ABA-dependent and ABA-independent pathways. *Planta*. 2011; 233:219–229.
 22. Cheng J, Zhang MM, Tan B, Jiang YJ, Zheng XB, Ye X, Guo ZJ, Xiong TT, Wang W, Li JD and Feng JC. A single nucleotide mutation in *GID1c* disrupts its interaction with *DELLA1* and causes a GA-insensitive dwarf phenotype in peach. *Plant Biotechnology Journal*. 2019; 17:1723-1735.
 23. Parvathaneni RK, Spiekerman JJ, Zhou HY, Wu XM and Devos KM. Structural characterization of *ABCB1*, the gene underlying the *d2* dwarf phenotype in pearl millet, (*Cenchrus americanus* (L.) Morrone). *Genes, Genomes, Genetics*. 2019; 9: 2497-2509.
 24. Seleznyova A, Tustin DS and Thorp TG. Apple dwarfing rootstocks and interstocks affect the type of growth units produced during the annual growth cycle: precocious transition to flowering affects the composition and vigour of annual shoots. *Annals of Botany*. 2008; 101: 679–687.
 25. Zhu LH, Holfors A, Ahlman A, Xue ZT and Welander M. Transformation of the apple rootstock M.9/29 with the *rolB* gene and its influence on rooting and growth. *Plant Science*. 2001; 160: 433–439.
 26. Wójcikowska B and Gaj MD. Expression profiling of *AUXIN RESPONSE FACTOR* genes during somatic embryogenesis induction in *Arabidopsis*. *Plant Cell Reports*. 2017; 36: 843–858.
 27. Pucciariello O, Legris M, Rojas CC, Iglesias MJ, Hernando CE, Dezar C, Vazquez M, Yanovsky MJ, Finlayson SA, Prat S and Casal JJ. Rewiring of auxin signaling under persistent shade. *Proceedings of the National Academy of Sciences of the United States of America*. 2018;4: e1721110115.
 28. Mlotshwa S, Pruss GJ, MacArthur JL, Reed JW and Vance V. Developmental defects mediated by the P1/HC-Pro potyviral silencing suppressor are not due to misregulation of *AUXIN RESPONSE FACTOR 8*. *Plant Physiology*. 2016; 172: 1853–1861.

29. Gutierrez L, Mongelard G, Floková K, Păcurar DI, Novák O, Staswick P, Kowalczyk M, Păcurar M, Demailly H, Geiss G and Bellini C. Auxin controls *Arabidopsis* adventitious root initiation by regulating jasmonic acid homeostasis. *The Plant Cell*, 2012; 24: 2515–2527.
30. Silva WD , Ghica ME and Brett CMA . Biotoxic trace metal ion detection by enzymatic inhibition of a glucose biosensor based on a poly (brilliant green)–deep eutectic solvent/carbon nanotube modified electrode. *Talanta*. 2020; 208: e120427.
31. Nartop D, Demire B, Güleç M, Özkan EH, Yetim NK, Sarı N, Çeker S, Öğütcü H and Açar G. Novel polymeric microspheres: Synthesis, enzyme immobilization, antimutagenic activity, and antimicrobial evaluation against pathogenic microorganisms. *Journal of Biochemical and Molecular Toxicology*. 2019; e22432.
32. Looney NE, Pharis RP and Noma M. Promotion of flowering in apple trees with gibberellin A₄ and C-3 epi-gibberellin A₄. *Planta*. 1985; 165: 292-294.
33. Takeno K, Taylro J, Sriskandarajah S, Pharis RP and Mullins MG. Endogenous gibberellin- and cytokinin-like substances in cultured shoot tissues of apple, *Malus pumila* cv. Jonathan, in relation to adventitious root formation. *Plant Growth Regulation*. 1983; 1: 261-268.
34. Vicente IS, Albertos P and Lorenzo O. Protein shuttle between nucleus and cytoplasm: new paradigms in the *ABI5*-dependent ABA Responses. *Molecular Plant*. 2019; 12: 1425–1427.
35. Kalcsits L, Heijden G, Waliullah S and Giordani L. S-ABA-induced changes in root to shoot partitioning of root-applied ⁴⁴Ca in apple (*Malus domestica* Borkh.). *Trees*. 2019; 33: 433–442.
36. Zhou YC and Underhill SJR. Breadfruit (*Artocarpus altilis*) *DELLA* genes: gibberellin regulated stem elongation and response to high salinity and drought. *Plant Growth Regulation*. 2017; 83: 375–383.
37. Gombos M, Zombori Z, Szecsenyi M, Sandor G, Kovacs H and Gyorgyey J. Characterization of the *LBD* gene family in *Brachypodium*: a phylogenetic and transcriptional study. *Plant Cell Reports*. 2017; 36: 61-79.
38. Wang XF, Zhang SZ, Su L, Liu X and Hao YJ. A Genome-wide analysis of the *LBD* (*Lateral Organ Boundaries Domain*) gene family in *Malus domestica* with a functional characterization of *MdLBD11*. *PLoS ONE*. 2013; 8: e57044.
39. Xu CY, Cao HF, Zhang QQ, Wang HZ, Xin W, Xu EJ, Zhang SQ, Yu RX, Yu DX and Hu YX. Control of auxin-induced callus formation by *bZIP59–LBD* complex in *Arabidopsis* regeneration. *Nature Plants*. 2018; 4:108-115.
40. An HS, Luo FX, Wu T, Wang Y, Xu XF, Zhang XZ and Han ZH. Dwarfing effect of apple rootstocks is intimately associated with low number of fine roots. *HortScience*. 2017; 52: 503-512.
41. Cohen S, Naor A, Bennink J, Grava A and Tyree M. Hydraulic resistance components of mature apple trees on rootstocks of different vigours. *Journal of Experimental Botany*. 2007; 58: 4213–4224.
42. Leisso R, Rude D and Mazzo M. Targeted metabolic profiling indicates apple rootstock genotype-specific differences in primary and secondary metabolite production and validate quantitative contribution from vegetative growth. *Frontiers in Plant Science*. 2018; 9: 1-15.

43. Wang YX, Hu Y, Chen BH, Zhu YF, Dawuda MM and Svetla S. Physiological mechanisms of resistance to cold stress associated with 10 elite apple rootstocks. *Journal of Integrative Agriculture*. 2018; 17: 857-866.
44. Naija S, Elloumi N, Jbir N, Ammar S and Kevers C. Anatomical and biochemical changes during adventitious rooting of apple rootstocks MM106 cultured in vitro. *Comptes Rendns Biologies*. 2008; 331: 518-525.
45. Knäbel M, Friend AP, Palmer JW, Diack R, Wiedow C, Alspach P, Deng C, Gardiner SE, Tustin DS, Schaffer R, Foster T and Chagné D. Genetic control of pear rootstock-induced dwarfing and precocity is linked to a chromosomal region syntenic to the apple *Dw1* loci. *BMC Plant Biology*. 2015; 15: 1-16.
46. Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, Fontana P, Bhatnagar SK, Troggio M, Pruss D, Salvi S, Pindo M, Baldi P, Castelletti S, Cavaiuolo M, Coppola G, Costa F, Cova V, Ri AD, Goremykin V, Komjanc M, Longhi S, Magnago P, Malacarne G, Malnoy M, Micheletti D, Moretto M, Perazzolli M, Si-Ammour A, Vezzulli S, Zini E, Eldredge G, Fitzgerald LM, Gutin N, Lanchbury J, Macalma T, Mitchell JT, Reid J, Wardell B, Kodira C, Chen Z, Desany B, Niazi F, Palmer M, Koepke T, Jiwan D, Schaeffer S, Krishnan V, Wu CJ, Chu VT, King ST, Vick J, Tao Q, Mraz A, Stormo A, Stormo K, Bogden R, Ederle D, Stella A, Vecchietti A, Kater MM, Masiero S, Lasserre P, Lespinasse Y, Allan AC, Bus V, Chagné D, Crowhurst RN, Gleave AP, Lavezzo E, Fawcett JA, Proost S, Rouzé P, Sterck L, Toppo S, Lazzari B, Hellens RP, Durel CE, Gutin A, Bumgarner RE, Gardiner SE, Skolnick M, Egholm M, Peer YV, Salamini F, Viola R. The genome of the domesticated apple (*Malus x domestica* Borkh.). *Nature Genetics*. 2010; 42: 833-839.
47. Wang P, Nolan TM, Yin YH and Bassham DC. Identification of transcription factors that regulate *ATG8* expression and autophagy in *Arabidopsis*. *Autophagy*. 2020; 16:123-139.
48. Wang X, Cheng Y, Yang C, Yang C, Mu Y, Xia Q and Ma Q. QTL mapping for aluminum tolerance in RIL population of soybean (*Glycine max* L.) by RAD sequencing. *PLoS One*. 2019; 14: e0223674.
49. Gowda M, Das B, Makumbi D, Babu R, Semagn K, Mahuku G, Olsen MS, Bright JM, Beyene Y and Prasanna BM. Genome-wide association and genomic prediction of resistance to maize lethal necrosis disease in tropical maize germplasm. *Theoretical and Applied Genetics*. 2015; 128: 1957-1968.

Supplementary Information

Additional file 1: Table S1. Insertion and deletion (InDel) markers developed within the intervals of Dw1 and Dw2 between 'M9' and '*Malus baccata*'.

Additional file 2: Figure S1. Promoter and coding sequence comparison of *MdLBD3* (A) *MdARF6* (B) *MDG3OX3* (C) between 'M9' and '*Malus baccata*'.

Additional file 3: Table S2. Primers of qRT-PCR for identification of gene relative expression

Additional file 4: Original full-length gel screened in Fig.3 and Fig.4.

Abbreviations

ABI5: Absciscic acid insensitive 5 ; *ABA*: Absciscic acid; *AD*: Activation domain; *ARF6*: Auxin response factor 6; *BD*: DNA-Binding domain; *BLAST*: Basic local alignment search tool; *G3OX3*: Gibberellin 3-beta-dioxygenase 3; *LBD3*: Lateral organ boundaries domain 3; Gibberellin; *GEGV*: Genotype estimated genetic values; *GUS*: Glucuronidase; *InDel*: Insertion/Deletion; *OPV*: Observed phenotype values; *Y1H*: Yeast one hybrid.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All genome sequence assemblies and genomic sequence reads are freely available from <https://www.rosaceae.org/> . The accession numbers of gene ID referred in this paper as followed:

MdARF6: MD10G1257900; *MdLBD3*: MD05G1307000; *MdG3OX3*: MD11G1092600; *MdWRKY2*: MD04G1244700; *MdGAMYB*: MD07G1085000; *Mdabi5*: MD12G1024200.

Competing interests

The authors declare that they have no competing interests.

Funding

The design of the study was supported by the China Agriculture Research System(CARS-27) to Z.H.H.. Writing the manuscript and this publication were supported by the National Natural Science Foundation of China (31772262) to Z.H.H.. The collection, analysis, and interpretation of data were supported by the Construction of Beijing Science and Technology Innovation and Service Capacity in Top Subjects (CEFF-PXM2019_014207_000032) to Z.H.H.. The quantitative real time polymerase chain reaction detection were supported by the Natural Science Fund of Liaoning Province (20170540508) to F.Y.. To create and foster the plant material were supported by the BaiQianWan Talents Project of Liaoning Province (2016921048) to F.Y.. The data processing were supported by the President's Fund Project of Liaoning Academy of Agricultural Sciences(2020QN2414) to F.Y..

Authors'contributions

Z.H.H. and X.Z.Z. initiated and designed the experiments. F.Y., Z.L and M.L.H. created and fostered the plant materials, F.Y., Y.W., W.T., X.F.X., W.L., and C.P.Q. performed the experiments. F.S., performed the bioinformatic analysis. F.Y. and X.Z.Z. wrote the paper. All authors have read and approved the manuscript.

Acknowledgements

The authors thank the Institute of Horticultural Plants, College of Horticulture, China Agricultural University (Beijing 100193,People’s Republic of China), Key Laboratory of Stress Physiology and Molecular Biology for Fruit Trees in Beijing Municipality, China Agricultural University (Beijing 100193, People’s Republic of China) and Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Nutrition and Physiology) in Ministry of Agriculture, China Agricultural University (Beijing 100193, People’s Republic of China) for molecular marker screening and verificaton between genes interaction. Thank the Key Laboratory of Germplasm and Breeding of North Fruit, Liaoning Institute of Pomology (Xiongyue 115009, Liaoning Province, People’s Republic of China) for plant materials create, collect and foster.

Author details

- 1. College of Horticulture, China Agricultural University. Beijing, 100193, China.
- 2. Liaoning Institute of Pomology, Xiongyue, 115009,China.
- 3. Beijing Academy of Agriculture and Forestry, Beijing, 100089, China.

Tables

Please see the supplementary files section to view the tables.

Figures

Figure 1

A photograph (A) and a diagram (B) showing segregation in dwarfing tree architecture of 4-year-old trees of ‘Red Fuji’ scions grafted onto F1 hybrids derived from ‘Malus baccata’ × ‘M9’.

Figure 2

Frequency distributions and the linear regressions of dwarfing trait in ‘Red Fuji’ scion grafted on F1 hybrids of ‘Malus baccata’ × ‘M9’. (A) ~ (C) frequency distributions of tree height (A), trunk diameter (B) and canopy width (C). (D) ~ (F) Linear regressions between dwarfing traits: tree height/trunk diameter (D), tree height/canopy width (E) and trunk diameter/canopy width (F).

Figure 3

Segregations of SSR markers previously reported near the Dw1 and Dw2 loci in 30 F1 hybrids of ‘Malus baccata’ × ‘M9’ showing dwarfing effects on the scion grafted on them. * and ** indicate that the χ^2 value is significant.

is less than the threshold of χ^2 0.05 and χ^2 0.01, respectively.

Figure 4

Schematic diagram and genotyping gel images showing segregation of the narrowed down InDel markers near the dwarfing locus Dw1 (A) and Dw2 (B) in each 30 F1 hybrids of ‘Malus baccata’ × ‘M9’ showing dwarfing or vigorous effects respectively on the scion grafted on them. The diagrams in panels A and B were drawn by hand to show the position (in mega base pairs, Mb) of the markers on the chromosomes of apple genome (GDDH13). The genotype segregation ratio value was marked on the right side of each gel image.

Figure 5

Validation of genetic variations on *MdLBD3*, *MdARF6* and *MdG3OX3* as candidate genes for dwarfing effects of 'M9' apple rootstock and the F1 hybrids derived from '*Malus baccata*' × 'M9'. (A) Relative expressions of *MdLBD3*, *MdARF6* and *MdG3OX3* in fine roots of 'M9' and '*M. baccata*'. (B) Relative expressions of *MdLBD3*, *MdARF6* and *MdG3OX3* in fine roots of F1 hybrids with different genotypes. (C) Sketch map of variations on genes of *MdLBD3*, *MdARF6* and *MdG3OX3*. (D) Promoter activity assay for

variants of MdLBD3, MdARF6 and MdG3OX3 full length and truncated promoter:GUS fusion in tobacco leaf cells. Asterisks indicate statistical significant level by multiple comparison ($p > 0.01$).

Figure 6

Allelic variations on candidate gene promoters, relative expression of their transcription factors and the interactions of them in dwarfing and vigorous rootstock of ‘M9’, ‘Malus baccata’. and the F1 hybrid of dwarfing, intermediate and vigorous phenotypes. (A) Relative expressions of MdWRKY2, MdGAMYB and MdABI5. (B~D) Allelic variations on promoters of MdLBD3 (B), MdARF6 (C) and MdG3OX3 (D) and the interactions with transcription factors. The allelic variation were given on the left, and the images of yeast one hybrid between these promoters and transcription factors MdWRKY2, MdGAMYB and MdABI5 were shown on the right of each panel.

Figure 7

Linear regressions between functional marker genotype estimated genetic values (GEGV) and observed phenotype values (OPV) of tree height (A), trunk diameter (B) and canopy width (C) of apple trees grafted on 108 random F1 hybrids derived from 'Malus baccata' × 'M9'.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1Table.S.1..xlsx](#)
- [Additionalfile4Originalfulllengthgel.docx](#)
- [Additionalfile2Fig.S1.jpg](#)
- [Addltionalfile3Table.S.2..xlsx](#)
- [Table.1.xlsx](#)
- [Table.2.xlsx](#)
- [Table.3.xlsx](#)