Development and validation of functional markers from Dw1 and Dw2 loci to assistant 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)

China Agriculture University

Research article

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

Posted Date: January 14th, 2021

DOI: <https://doi.org/10.21203/rs.2.24669/v4>

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’(*Malus × domestica* Borkh.) trees grafted on 1123 hybrids from ‘*Malus baccata* Borkh.’ × ‘M9’, the intervals of *Dw1* and *Dw2* were narrowed down. *MdLBD3* (Md Lateral organ boundaries domain 3) and *MdARF6* (Md Auxin response factor 6) were predicted as potential candidate genes from *Dw1*, while *MdG3OX3* (Md Gibberellin 3-beta-dioxygenase 3) 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* (Md WRKY transcription factor 2) 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* (Md Absciscic acid insensitive 5), leading to higher expression of *MdG3OX3*. At -954 bp of the *MdARF6* promoter, a 14 bp insertion destroyed the binding ability by *MdGAMYB* (Md transcription factor GAMYB) 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 genomic assisted prediction of dwarfing ability in apple rootstock breeding. The data also suggested that hormone (ABA, Auxin, GA, and zeatin signals) is one of the regulation pathways in controlling apple rootstock dwarfing mechanisms.

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 inter-stem 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 used for genetic transformation of over express *MdPIN1b* as inter-stem 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 potential candidate genes involving dwarfing were identified from the intervals of these QTLs.

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

Results

Inheritance of dwarfing traits

Phenotype of dwarfing traits, tree height, trunk diameter, and canopy width segregated significantly among 'Red Fuji'(*Malus × domestica* Borkh.)scions grafted on the 1123 hybrids of '*Malus 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).

Genetic of ultra affinity and extremely dwarfing group selection

The major segregation give the present of metaphase trend in those three dwarfing phenotype, but there still a minor group cover the range from 4.5% to 5.4% holding an ultra affinity in genetic(Fig. 2 A-C), which will help the selection of extremely dwarfing group both in validation of dwarfing markers and new strain of dwarfing rootstock. An intersection used to cross three dwarfing phenotype, 41 and 44 of individuals

were selected between plant height cross trunk diameter and canopy width cross trunk diameter, lastly we get 34 of individuals gathering three dwarfing phenotype in dwarfing group(Fig. 3 A,B,E), and 36 of individuals in vigorous group(Fig. 3 C,D,F). By each group, the top of 30 were selected as extremely dwarfing/vigorous (Fig. 3 B,D,G)respectively to validate the molecular markers and genetic linkage analysis.

Markers validation and genetic linkage analysis

Three SSR markers, Hi01c04, Hi04a08, and CH03a09 near *Dw1* on chromosome 5, and the other two markers, Hi07d11 and CH02d08 close to *Dw2* on chromosome11, were genotyped in 30 hybrids where the scion exhibited the dwarfing phenotype [2]. All five of these markers were heterozygous in 'M9' rootstock, homozygous in '*Malus baccata*' rootstock, and displayed a distorted segregation ratio in dwarfing F1 hybrids indicating the association of these markers with dwarfing effects (Fig. 4).

Ratio of partial segregation will give more information between the genetic marker and the functional candidate dwarfing genes. There were explanation of dwarfing phenotype in terms of 86.7%, 76.7% and 73.3%, means the recombination of 13.3%, 23.3% and 26.7% addressed in Hi01c04, Hi04a08, and CH03a09 respectively in *Dw1* section(Fig.5 A,C), and the genetic distance were of 12.9cM, 25.2cM and 29.8cM convert into physical distance were 0.15Mb, 0.29Mb and 0.35Mb which were used to cut from the position of SSR marker for compressing. For example, Hi01c04 at the top of the QTL section with the physical position of 4.58Mb, exploration of 86.7%, recombination of 13.3%, genetic distance of 12.9cM, and physical distance of 0.15Mb. Position of 4.58Mb(location of Hi01c04) add up with 0.15Mb is the new top of the section(Fig.5 A,C); Hi04a08 is in the middle of the QTL section with the physical position of 5.15Mb, exploration of 76.7%, recombination of 23.3%, genetic distance of 25.2cM, and physical distance of 0.29Mb, afterwards 5.15Mb cut off with 0.29Mb is the new bottom of the section, then a new small sized section from 4.73Mb to 4.86Mb was cut out from *Dw1*(Fig.5 A,C). As state above, the intervals were narrowed down based in this protocol, the next section cut out from *Dw1* was from 5.44Mb to 7.27Mb, and *Dw2* section was compressed from 2.35Mb to 8.63Mb(Fig.5 B). Size of intervals of *Dw1* and *Dw2* were narrowed down which will assist the prediction of candidate dwarfing genes next step.

Re-sequencing analysis and candidate gene prediction

There were a mount of variation in sequence level in terms of SNPs, InDels and SVs, and the mutants were 4103 and 7507 in interval of *Dw1* and *Dw2* respectively(Table 2), which give a lot of information which troubled in validate sites selection related with dwarfing trait, fortunately the pipe line mentioned in method fulfill the duty to assist validate selection as follows: There were 1350 and 2135 of mutant in narrowed down section of *Dw1*and *Dw2* respectively(Table 2); then 216 and 407 of mutant after constraint by M9(0/1) × *Malus baccata*(0/0); in turn 43 and 85 of mutant constraint by B9(0/1) × *Malus robusta*(0/0); 16 and 30 of mutant after confined in promoter and exonic; lastly, four and nine InDel mutant respectively in *Dw1* and *Dw2* were selected after excluded Synonymous(Table 2), and markers explored on the InDel variation were also genotyped in the 60 hybrids(Fig.6), 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. 6). 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 potential candidate genes

The relative expression of *MdLBD3* and *MdG3OX3* was significantly higher in the fine roots of 'M9' than in '*Malus baccata*', but *MdARF6* was expressed at higher levels in the roots of '*Malus baccata*' than in 'M9' (Figure 7A). The 0~1104 bp, 0~1289 bp, and 0~1176 bp segments upstream of the transcription start site (TSS) of *MdLBD3*, *MdARF6*, and *MdG3OX3*, respectively, were cloned from the DNA of 'M9' and '*Malus baccata*' and Sanger sequenced (Fig. S1; Fig. 7C). Besides some SNPs that were heterozygous on 'M9', an 11 bp 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. 7C). 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 '*Malus baccata*' × 'M9' were sampled for a gene relative expression assay (Table 3). 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. 7B). 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 '*Malus baccata*', whilst the promoter activity of *MdARF6-pro-Ins* from 'M9' was lower than that of *MdARF6-pro-Del* from '*Malus baccata*' or 'M9' (Fig. 7D).

To further examine the functional variations on the promoter sequences of these potential 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. 7 A, B and D). Consequently, InDels on the promoter of *MdLBD3*, *MdARF6*, and *MdG3OX3* were utilized as functional markers for dwarfing effects.

Motif analysis and transcription factor genes prediction

Full length of 1104 bp sequence of *MdLBD3* promoter were analyzed through net software, revealing that the 11 bp deletion 'TCAACAACATA' located -339 bp from TSS weren't broken TATA box or other motifs (Fig. 8A), but may have caused the generation of a new cis-acting element with the critical motif 'TGGAATGGGTATAG', which could be bound by some transcription factor of list with MPD numbers, but

only MDP0000144203 was checked and validated confer to MD04G1244700 that was *MdWRKY2* transcription factor (Fig. 8B).

Full length of 1289 bp sequence of *MdARF6* promoter were analyzed through net software, revealing that the 14 bp insertion 'GAGAGAGCGAGAGA' located -954 bp from TSS was nearly to a GC box for dozens of bps (Fig. 8C), but the insertion had destroyed a motif 'GGCGGTTGTG' bound by the transcription factor of MDP0000147309 corresponding with MD07G1085000 that was *MdGAMYB* transcription factor (Fig. 8D).

Full length of 1176 bp sequence of *MdG3OX3* promoter were analyzed through net software, revealing that the 10bp deletion 'ATCATATTTT' located -278 bp from TSS was in the middle of two TATA box (Fig. 8E), but may have caused the generation of a new cis-element with the critical motif 'ACGTTTTTGT', which could be bound by transcription factor of MDP0000144105 corresponding with MD12G1024200 that was *MdABI5* transcription factor (Fig. 8F).

Functional analysis of allelic variation of the potential candidate genes

Prediction revealed that the 11 bp deletion of *MdLBD3* may create a new cis-acting element could be bound by *MdWRKY2* transcription factor, and Yeast one hybrid assay (Y1H) and co-expressing confirmed the binding of *MdWRKY2* protein to the promoter of *MdLBD3-Del* in 'M9' but not to *MdLBD3-Ins* in '*Malus baccata*' (Fig. 9B; Fig. 7D). No differences in *MdWRKY2* relative expression were identified between either 'M9' and '*Malus baccata*' nor their F1 hybrids with dwarfing and non-dwarfing effects (Fig. 9A).

To the promoter of *MdARF6* and its transcription factor *MdGAMYB* as predicted previous (Fig. 8C, D), by Y1H showed that the binding ability was completely absent between AD-*MdGAMYB* and BD-*MdARF6-Ins* (Fig. 9C), and the co-expressing revealed that the *MdGAMYB* protein could promote the activity of *MdARF6-Del-GUS*, but with no effect on *MdARF6-Ins-GUS* (Fig. 7D), indicated that the 14 bp insertion specially on 'M9' broken the co-expressing between *MdARF6-ins* and *MdGAMYB*. Expression levels of *MdGAMYB* were quite analogous among 'M9', '*Malus baccata*', and their F1 hybrids, irrespective of the dwarfing effects (Fig. 9A).

Y1H assay and co-expressing also confirmed that the 10 bp deletion on the *MdG3OX3* promoter created a new cis-acting element on *MdG3OX3* bind by *MdABI5* (Fig. 9D; Fig. 7D), and the expression levels of *MdABI5* were observed among 'M9', '*Malus baccata*', and their F1 hybrids, irrespective of the dwarfing effects (Fig. 9A). 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 '*Malus 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 3). AdAi had the largest marker genotype effect, and LdLi had the least (Table 3). 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. 10A, B and C). Five-fold cross validation produced an average correlation coefficient of $r = 0.93$ ($P < 0.01$) (Fig. 10A, B and C), 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 (Table 4). The GEGVs were consistent with the accession dwarfing ability reported by previous literature, implying that the GEGV model should be applicable for a broad spectrum of apple rootstocks (Table 4).

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 '*Malus baccata*' × 'M9' in this study. *Dw1* explains 43% of the total phenotypic variance in 'M9' × 'Robusta 5' [2]. Consistently in '*Malus 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 '*Malus 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. *GAMYB*, one of myb-related genes, which was first found to be regulated by GA in barley, and is strongly induced by GA and repressed by ABA. [29], it's always act as a transcription factor related with plant's growth and embryo generation [30], but in this study there no differences in *MdGAMYB* expression were detected between '*Malus 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 [31].

G3OX3 oxidizes gibberellin and therefore represses vegetative growth or elongation of roots or shoots [32, 33], and ABA can change a variety of plant physiological and growth processes to adapt to environment changes [34], they are both sensitive in growth mediation. *ABI5* is the transcription factors can directly bind to the ABA-responsive element (AREB) cis-element in the promoter sequence of ABA-responsive

genes, and play positive roles in ABA signaling and is identified as an ABA receptor[34], which will allow seedlings to escape from ABA inhibition of growth[35]. 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 [36, 37]. *AtABI5* in *Arabidopsis* functions as the main transcription factor in abscisic acid signal transduction [38, 39]. 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, 40].

The lateral organ boundaries domain (*LBD*) transcription factor family promotes lateral root or shoot formation and lateral growth function of a crucial role in shaping the plant architecture through coordinating cell fate[41]. It's a gene of homozygous to *AtLBD3/AtLBD9* which is induced by exogenous cytokinins and effected by CTKS [43]. In rice (*Oryza sativa* L.), *OsLBD3-1* is expressed only in roots, and *OsLBD3-2* is expressed in roots, shoots, and petioles[44], however it's competitive or resisted between root vertical growth and lateral branches extension, and there are wide angles of organs from roots and shoots exist in dwarfing combinations of rootstock and scions [45, 46]. 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 and evidence showing that angles between roots and vertical line are 69.30° from 'M9', but 37.27° from '*Malus robusta*' [47]. *WRKY* proteins are a recently identified class of DNA-binding proteins that recognize the TTGAC(C/T) W-box elements found in the promoters of a large number of plant defense-related genes[48], and most of them are in many aspects of physiological processes and adaption to environment, besides it's also a key components in some signal transduction processes mediated by plant hormones such as abscisic acid (ABA) and salicylic acid (SA) [49]. Besides Auxin is synthesis in roots and transport to upward, the more vertical of the root angle will get the stronger of polar transport capacity, and the more Auxin content obtained from the upper part of the root. On the contrary, the more horizontal of the root angle with the weaker the polar transport capacity, then less of the Auxin content is obtained [50, 51] concludes that *MdLBD3* indirectly regulated apple rootstock dwarfing level and scions growth by hormone through morphological formation in this study. But to this study, 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 hormone including cytokinin, Auxin and CTKs with different pathways, 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[52, 53]. Another example is that the GEGV of 'GM256' was relatively large, but the dwarfing potential is much greater than 'MM106' or 'M7' [54, 55]. 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 most of the apple rootstock dwarfing ability prediction especially in 'Malling-series' and some *Malus* wild resource. Though there exist other factor induce rootstock's dwarfing such as early-bloom and nutrition et al[56, 57], in this study, these functional markers also suggest that ABA, Auxin, GA, and zeatin signals are involved in the regulation of apple rootstock dwarfing mechanism addressed in QTL sections of *Dw1* and *Dw2*. Then there must be more genetic variations contribute to the dwarfing traits which needs further study, but molecular marker of InDels involved with dwarfing potential in rootstocks explored in this paper will enrich the selection tools assisted in apple rootstock breeding practice.

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 '*Malus 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'/'*Malus 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 [58]. 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 '*Malus 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].

Genetic linkage analysis and *Dws* interval narrow down

Whole segregation composed by 1123 of population were analyzed with dwarfing phenotype in terms of plant height, trunk diameter and canopy width, and intersection composed by these three dwarfing phenotype was taken into account lower than 'M9', further extremely dwarfing group nearly 30 of individuals was analyzed for linkage and recombination. Formula of $x = (1/4) \ln[(1+2r)/(1-2r)]$ account for genetic distance, and "r" means ratio of recombination, "x" means value of exchange. 1cM $\approx$ 12kb based on the genome of *Malus* and referenced on other species[59], and the genetic distance was converted into physical distance, then the intervals were narrowed down contrasted to the location of Markers.

Parental re-sequencing and candidate gene prediction

High quality genomic DNA of the two parents, '*Malus baccata*' , 'M9', and two contrast rootstocks '*M. robusta*' , 'B9', was extracted from leaves using the CTAB method, by each sample were taken tissues from 3 graft-reproduction individuals as biological repeat. 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 $\times$ the genome size. The reads were aligned to the reference apple genome: 'Golden Delicious' *Malus $\times$ domestica* v 3.0 from the Genome Database for Rosaceae (GDR) (<https://www.rosaceae.org/>)[60]. Selection pipe line as follows: Narrow down the intervals of *Dws* by genetic linkage; Mutant were heterozygous (0/1) in 'M9' and 'B9', but were homozygous (0/0) in '*Malus baccata*' and '*M. robusta*'; Locate in Exonic or Up-streams; SNPs of Non-synonymous or InDels of Frameshift.

Mutants after selected were explore into molecular markers by the method of addressed on the critical sequence in terms of ride on the InDels for 5 bp to 15bp or flank beside the SNPs(to this study, none validate SNPs were assessed). InDels were identified easily by agarose electrophoresis with a special

sized PCR production, and SNPs will be identified by screened using the High Resolution Melting technique.

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 vigour phenotypes from '*Malus baccata*' × 'M9' population and the parenal 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 tissue of fine root by each samples taken from 3 cut-reproduction individuals as biological repeat, and cDNA was synthesized using the RNAiso kit (Prime ScriptTM RT, Takara, Dalian, China), following the manufacturer's instructions. RT-qPCR was performed for 3 times as technical repeat by 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-treated 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 potential 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.

Motif analysis and transcription factor genes prediction

The sequence of promoter of -1300 bp from TSS with different types of these three genes were input the net of (<http://plantpan2.itps.ncku.edu.tw/> and <https://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/>) to analyze the motifs bind by transcription factors of upstream in a network. Two aspect were considered in terms of a motif was broken or created addressed to the sequence attributed by the Insertion or Deletion. It was outlined whether the motifs being a cis-acting element bind by a transcription factor detailed with MDP number in *Malus* Genome. It's obviously that a cis-element will broken if there exist a Insertion specially in the middle of a motif, to an other aspect a new cis-acting element will be re-build by two sections of sequence joint together if a Deletion was taken place in a promoter.

Promoter activity and co-expressed assay

Promoter fragments of 1104 bp, 1289 bp, 1235 bp upstream of *MdLBD3*, *MdARF6*, *MdG3ox3*, and full length CDS of the fragment of 2181bp, 1656bp and 1413bp corresponded to *MdWRKY2*, *MdGAMYB* and *MdABI5* were cloned from the genomic DNA of 'M9' and '*Malus baccata*', respectively, and their sequences were validated by Sanger sequencing. The promoter of each gene was digested and cloned into the pCAMBIA1391-GUS plant transformation vector, and the CDS of transcription factor were digested and cloned into the pCAMBIA1301 plant transformation vector.

Promoter activity was identified in full length and truncated level by pCAMBIA1391-GUS constructs transformed into leaf epidermal cells of 4-week-old *Nicotiana benthamiana* plants by GV3101 *Agrobacterium* infiltration. Co-expressed was identified with a mixture *Agrobacterium* infiltration of 1:1 composed by Promoter-pCAMBIA1391-GUS vector and CDS-pCAMBIA1301vector. Assess of promoter activity and co-express were both 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) [61].

Estimation of marker genotype effect and GEGV

One hundred and eight randomly chosen F1 hybrids from '*Malus baccata*' × '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[62]. The prediction ability of the GEGV was examined by using five-fold cross-validation[63].

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; *CDS*:Coding Sequence; *Gibberellin*; *GEGV*: Genotype estimated genetic values; *GUS*: Glucuronidase;*G3OX3*: Gibberellin 3-beta-dioxygenase 3; *InDel*: Insertion/Deletion; *LBD3*:Lateral organ boundaries domain 3; *OPV*: Observed phenotype values; *ranscription Start Site*; *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: MD05G1279200; 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 Presedent'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 verification 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.

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Tables

Please see the supplementary files section for Tables 1-4.

Figures

Figure 1

A photograph (A) and a diagram (B) showing segregation in dwarfing tree architecture of 4-year-old trees of ‘Red Fuji’ (*Malus × domestica* Borkh.)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' x '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

Selection and division of extremely segregation group of dwarfing/vigorous in F1 hybrids from the cross of 'Malus baccata'× 'M9'

Figure 4

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

Figure 5

Analysis of genetic linkage and dwarfing locus compress in Dw1 and Dw2 through F1 hybrids of ‘Malus baccata’ × ‘M9’. (A) Dw1; (B) Dw2; (C) Formula of genetic distance, physical distance and method of narrowing down of the interval.

Figure 6

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 7

Validation of genetic variations on MdLBD3, MdARF6 and MdG3OX3 as potential 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 'Malus 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

[illegible]

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Figure 9

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 10

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

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- [Additional2Table.S.2..xlsx](#)
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