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ORIGINAL PAPER

Identification and genetic characterization of a quantitative trait locus for adventitious rooting from apple hardwood cuttings

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Abstract Vegetative propagation enables the multiplication of plants that are genetically identical to the original. Hardwood cuttings have a low cost advantage and are used widely for the propagation of JM series apple rootstocks, which root well from hardwood cuttings. To breed new apple rootstocks with similarly high rooting capabilities, the development of a marker-assisted selection method is desirable. Therefore, it is important to understand the genetic mechanisms behind the rooting capabilities of hardwood cuttings. We identified quantitative trait loci (QTLs) for the rooting capability of 'JM7' using a QTL analysis with phenotypic data obtained under two environmental conditions. The QTLs for rooting rate and root quantity co-located. The QTL for rooting rate explained 66 % of the genetic variance and 57 % of the phenotypic variance. Graphical genotyping and database searching revealed candidate genes, including three related to auxin response, one related to ethylene response, and a gene encoding a WRKY transcription factor.

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Introduction

The widely used technique of vegetative propagation enables the multiplication of plants that are genetically identical to the original. In apple, which is a major fruit tree crops grown in temperate regions, both scions (*Malus* × *domestica*) and rootstocks (*Malus* spp.) are vegetatively propagated. Scion cultivars are propagated by grafting or budding on rootstocks, but rootstock cultivars must be rooted. Since the cuttings of many apple rootstocks rarely form roots, divisionary techniques, such as layering and stooling, are common (Wertheim and Webster 2003) but they require regular labor.

Malus prunifolia Borkh. is known as a vigorous rootstock and is a major apple rootstock in Japan (Arakawa and Komori 2006; Soejima et al. 2010). It roots much better from hardwood cuttings than the Malling cultivars 'M.9' and 'M.26'. JM rootstocks ('JM1', 'JM2', 'JM5', 'JM7', and 'JM8'), which are derived from *M. prunifolia* 'Morioka Seishi' (syn. 'Marubakaido' and 'Seishi') × 'M.9', also root well from hardwood cuttings (Soejima et al. 2010, 2013) and are therefore inexpensive to propagate. To date, the use of JM rootstocks has increased rapidly because of their ease of propagation, their dwarfing ability, and their successful adaptation to the climate, soil, diseases, and pests of Japan.

Methods for increasing the rooting rate (RR) of cuttings have been investigated. Severe hedge pruning of mother plants improves rooting success (Webster 1995). Hardwood cuttings taken shortly after leaf fall or before bud break root better than those taken in midwinter (Webster 1995). Additionaly, treating cuttings with synthetic auxin indole-3-butyric acid (IBA) solution improves the RR (Howard 1968),

as does the application of manure in the propagation bed and soil tamping (Soejima et al. 2002, 2004).

The physiological aspects of rooting from hardwood cuttings are considered the adventitious root formations from explant shoots. The formation of adventitious roots is regulated by the interaction of environmental and endogenous factors, among which auxins play an essential role (Pacurar et al. 2014). However, these interactions are difficult to study, as they depend on species, rooting conditions, and tissue (Pacurar et al. 2014). Therefore, molecular studies to identify genes responsible for adventitious rooting have been conducted mainly on model plant species, such as *Arabidopsis thaliana*. In woody plants, only a few genes that regulate adventitious rooting have been identified (Legue et al. 2014) and mainly encoded transcription factors (TFs). In apple, the Adventitious Rooting Related Oxygenase-1 (*ARRO-1*) gene was isolated as an up-regulated gene from auxin-treated stem discs of the 'Jork 9' rootstock (Butler and Gallagher 1999), but it may be involved in auxin homeostasis rather than rooting (Smolka et al. 2009; Li et al. 2012).

Despite the many physiological and molecular studies on root formation, the genetic background and inheritance patterns are poorly understood in woody species such as apple because the evaluation of hardwood cuttings using a segregating population requires a great investment in time, labor, materials, and space. However, we knew that the rooting capability could be inherited, since the parent *M. prunifolia* 'Morioka Seishi' and its JM rootstock progeny showed excellent rooting capabilities.

JM rootstocks are susceptible to crown gall disease caused by *Agrobacterium tumefaciens* (Moriya et al. 2008), sometimes severely (Nekoduka et al. 2001). Thus, a new breeding program was begun to introduce resistance, while maintaining the high rooting capability.

Genetic linkage maps for apple rootstocks have been constructed (Celton et al. 2009; Antanaviciute et al. 2012; Moriya et al. 2012a; Fazio et al. 2014), and DNA markers linked to several target traits have been developed (Bus et al. 2008; Pilcher et al. 2008; Moriya et al. 2010; Fazio et al. 2014; Foster et al. 2015). To use markers in rootstock breeding, developing a method for marker-assisted selection (MAS) of high rooting capability would be valuable. Therefore, it is important to reveal the genetic mechanisms responsible for rooting capability to develop new rootstocks that can be easily propagated by hardwood cuttings.

The objectives of this study were to identify quantitative trait loci (QTLs) for rooting capability in 'JM7', to estimate their contribution to genetic variance and to predict candidate genes. This information would lead to a better understanding of the genetic control of adventitious rooting in apple.

Materials and methods

Plant materials

We analyzed the apple rootstock 'JM7', the wild apple *Malus sieboldii* 'Sanashi 63', and the F_1 progeny (120 offspring) derived from their cross in 1994. Cuttings were taken from the original F_1 trees until 2009 and from trees grafted onto 'JM7' in 2008. All of the trees were grown in the orchard of the Apple Research Station of the NARO Institute of Fruit Tree Science and were cut back to 1-m height every winter. Genomic DNA was isolated according to the method described by Moriya et al. (2010). In brief, 100 mg of young leaves was ground in liquid nitrogen in a bead crusher (SH-48; Kurabo, Osaka, Japan), and the powder was incubated for 30 min at 37 °C in 1 mL of isolation buffer (10 % polyethylene glycol 6000, 100 mM Tris–HCl, pH 8.0, 350 mM sorbitol, and 50 mM EDTA, pH 8.0). Then genomic DNA was extracted using an automated extractor (PI-50α; Kurabo) according to the manufacturer's instructions.

Evaluation of rooting from hardwood cuttings

One-year-old shoots were taken from each tree in early to mid-December (winter). They were wrapped in plastic to protect them against desiccation and stored in a refrigerator at 1 °C. From mid- to late March, cuttings (20–25 cm long) were prepared. All but the topmost bud were removed with a knife.

From 2004 to 2007, to assess rooting under constant conditions, we evaluated the rooting of hardwood cuttings in a phytotron at an air temperature of 18 °C and a soil temperature of 20 °C (phytotron experiment). Owing to space limitations, tests were conducted in three batches between April and June of each year. The base of each cutting was dipped in 1000 ppm IBA solution and immediately planted in vermiculite. After 1 month, all of the cuttings were dug up, and the number of cuttings that formed roots was recorded. The number of roots on each cutting was scored on a five-point scale (Table 1, Online Resource 1). Data were pooled over the 4 years, and the RR of each individual was calculated as the number of rooted cuttings divided by the number of planted cuttings. The root quantity (RQ) was also calculated as the sum of the scores of all planted cuttings divided by the number of rooted cuttings. The RQ of individuals that did not root was regarded as 0. The number of planted cuttings per offspring ranged from 2 to 135 and averaged 47.5±27.5 (mean±SD). Five offspring (#20, #21, #35, #123, and #125) were removed from the analysis because they did not have enough cuttings (≤5).

From 2010 to 2011, to assess rooting under field conditions, we evaluated rooting in the orchard at the NARO Institute of Fruit Tree Science (outdoor experiment). In early April, the volcanic ash soil in the propagation beds was tilled and immediately tamped, and then overlaid with a black

Table 1 Categorical scores and criteria for the evaluation of rooting from hardwood cuttings

Experiment	Score	Criterion number of roots formed
Phytotron	1.00	>20
	0.75	11–20
	0.50	6–10
	0.25	1–5
	0.00	0
Outdoor	1.0	≥5 long, thick roots with many thin roots
	0.8	≥5 long, thick roots with few thin roots
	0.6	<5 long, thick roots with few thin roots
	0.4	only thin roots
	0.2	only short, thin roots
	0.0	0

Examples of each category are shown in Online Resource 1

polyethylene film. In mid-April, the base of each cutting was dipped in 1000 ppm IBA solution and immediately planted in the soil. At the end of November, all of the cuttings were dug up, and the number of cuttings that formed roots was recorded. The number of roots on each cutting was scored on a six-point scale (Table 1, Online Resource 1). Data were pooled over the 2 years, and RR and RQ were calculated as in the phytotron experiment. The number of planted cuttings per offspring ranged from 9 to 55 and averaged 34.6±8.2 (mean±SD). Three offspring (#81, #82, and #125) were removed from the analysis because they did not have enough cuttings (≤5).

Estimation of broad-sense heritability and proportion of genetic variance explained by QTLs

We chose 69 offspring that had 30 or more cuttings tested in each of the two experiments. Subsequently, we calculated RRs using 30 randomly-selected cuttings from the raw data on each offspring. Then, the RR in each of the two experiments were arcsine-transformed to obtain uniform variances, denoted as ARR and subjected to the following statistical analysis:

(1) Analysis of variance (ANOVA) for the phenotypic data in the two experiments.

We assumed an ANOVA model with a two-way classification as follows:

$$P_{ij} = \mu_1 + G_i + T_j + E_{ij},$$

where P_{ij} is the ARR in the ith offspring (genotype) of the jth experiment, μ_1 is the grand mean, G_i is the effect of the ith offspring (genotype), T_j is the effect of the jth experiment, and E_{ij} is residual in the ith offspring (genotype) of the jth experiment.

$$i = 1\text{–}69, j = 1\text{–}2.$$

G_i and E_{ij} are assumed as random effects, and T_j is the fixed effect.

The variances of the ARR were not significantly different between the two experiments (P=0.05); therefore, we assumed that the model was applicable to the data. The ANOVA provided the genetic variance component due to the effect of G_i (σ_g^2), the error variance component due to the effect of E_{ij} (σ_e^2), and ΣT_j^2, which we describe as the variance component due to the effect of T_j (κ_t^2). The broad-sense heritability (h_B^2) for the mean ARR in the two experiments on each offspring was estimated using the formula: $h_B^2=\sigma_g^2/[\sigma_g^2+(\sigma_e^2/2)]$.

(2) The ANOVA for the two groups (classified based on QTL alleles) of phenotypic data from each offspring with the mean value in the two experiments.

The mean ARR in the two experiments for each offspring were classified into two groups, 37 offspring having the QTL marker (248-bp allele of simple sequence repeat (SSR) marker MEST020) and 32 offspring did not having the QTL marker (278-bp allele).

The data were subjected to an ANOVA with the following model:

$$Y_{ij} = \mu_2 + Q_i + R_{ij},$$

where Y_{ij} is the mean ARR of the two experiments in the jth offspring of the ith QTL grouping, μ_2 is the grand mean, Q_i is the effect of the ith QTL grouping, and R_{ij} is the residual in the jth offspring of the ith QTL grouping. The offspring with 248-bp and 278-bp alleles were assigned i=1 and i=2, respectively. R_{ij} is assumed as the random effect and Q_i is the fixed effect.

$$i = 1\text{–}2, j = 1\text{–}37 \textit{ for } i = 1, \textit{ and } j = 1\text{–}32 \textit{ for } i = 2.$$

The variance among Y_{1j} was not significantly different from the variance among Y_{2j} (P=0.05); therefore, we assumed that the model was applicable to the data. The ANOVA provided the residual variance component due to the effects of R_{ij} (σ_r^2) and ΣQ_i^2, which we describe as the variance component due to the effect of Q_i (κ_q^2).

Genetic linkage maps and QTL detection

QTL analysis based on the genetic linkage maps of 'JM7' (17 linkage groups (LGs), 415 loci, 998.0 cM) and 'Sanashi 63' (17 LGs, 310 loci, and 981.8 cM) (Moriya et al. 2012a) using the ARR in the MapQTL 6 software (van Ooijen 2009). For

RR, interval mapping was performed first. The logarithm of odds (LOD) threshold for the significance of QTLs was calculated using 5,000 permutations. LOD peaks larger than a LOD score with a 95 % confidence were identified as QTLs. We performed a restricted multiple QTL model (rMQM) procedure using the nearest markers to the QTLs identified by interval mapping as cofactors. When an additional LOD peak that was greater than the LOD threshold was found, the nearest marker was set as an additional cofactor and the rMQM analysis was performed again with the new set of cofactors.

For RQ, the Kruskal–Wallis test was performed. QTLs were identified when the K^* statistic was significant at $P<0.005$, according to van Ooijen (2009).

Testing additional SSR loci in LG 17

To obtain graphical genotypes of the plant materials, we tested 22 additional SSR loci in the middle of LG 17 in all offspring and their relatives (JM rootstocks, 'M.9', and 'Sanashi 63'). Seven of these SSRs (AJ001681-SSR, CH02g04, SAmsEE663640, SAmsAU301254, SAmsCO414947, SAmsCN492417, and SAmsCN490324) had been published previously (Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006; van Dyk et al. 2010). We identified their physical locations of using a BLASTN search of the 'Golden Delicious' genome version 1.0 contigs available on the genome database for Rosaceae (GDR; http://www.rosaceae.org/) (Velasco et al. 2010). We designed the other 15 SSRs (Mdo.chr17.1–15) based on the 'Golden Delicious' genome (Table 2, Online Resource 2). SSR-PCR and the detection of these markers were performed using the method of Moriya et al. (2012b).

Database search for candidate genes in the identified QTL region

We downloaded coding sequences of predicted genes from the 'Golden Delicious' genome between two SSR markers (Mdo.chr17.5 and SAmsCN490324) flanking the candidate region of the QTL identified by the relationship between the phenotype and the graphical genotype (see Results). Then, we performed a BLASTX search of the *Arabidopsis* protein database (The Arabidopsis Information Resource: http://www.arabidopsis.org/) using these predicted genes as queries.

For *ARRO-1* (AJ225045), we performed a BLASTN search of the 'Golden Delicious' putative coding sequences dataset. We also performed a BLASTN search of the 'Golden Delicious' contigs dataset for SSR markers used in this study.

Results

Phenotype data

Although we evaluated RR and RQ as different traits, they were highly correlated: $r=0.78$ in the phytotron experiment and $r=0.72$ in the outdoor experiment (Fig. 1). Therefore, we considered that RR could be used as a representative index of rooting capability. The RR of the segregating population under both experimental conditions showed a continuous distribution (Fig. 2). In the phytotron experiment, RR ranged from 0.05 to 1.00, with a population mean of 0.64±0.02. In the outdoor experiment, RR ranged from 0.00 to 1.00, with a population mean of 0.42±0.03. Values of RR were significantly correlated between conditions ($r=0.76$, $P<0.01$; data not shown). Rooted cuttings of 'JM7' had RR values of 0.56 in the phytotron experiment and 0.91 in the outdoor experiment (Fig. 2). 'Sanashi 63' was not tested in these experiments, but in tests conducted in previous years, it did not form any roots from hardwood cuttings, so we assigned it a phenotypic value of RR=0.

QTL analysis

First, we confirmed the normality of the ARR distribution using the Kolmogorov–Smirnov test ($P>0.05$). The initial QTL analysis by interval mapping suggested a QTL in LG 17 of 'JM7', which had LOD scores of 23.7 and 13.3 in the phytotron and the outdoor experiments, respectively (data not shown). The subsequent rMQM analysis identified a QTL in LG 17 of 'JM7', which had LOD scores of 25.5 and 13.3 in the phytotron and the outdoor experiments, respectively, explaining 57.1 % and 40.8 % of the phenotypic variances, respectively (Fig. 3, Table 3). In addition, it identified a minor QTL in LG 13 with a LOD score of 3.6, explaining 5.1 % of the phenotypic variance in the phytotron experiment. The QTL in LG 17 of 'JM7' was confirmed by QTL analyses of the single-year data (Online Resource 3). The close match between the map positions in LG 17 under the two experimental conditions indicates that this QTL is a major genetic factor for rooting from hardwood cuttings in this population. Furthermore, the Kruskal–Wallis test of the RQ from each experiment identified QTLs at 14.6 cM where the SSR marker MEST020 was located (Fig. 3). The K* statistics were highly significant ($P<0.0001$) at 70.6 and 61.8 in the phytotron outdoor experiments, respectively.

To analyze how this major QTL contributed to the RR, we genotyped the offspring at the SSR marker MEST020 as an indicator of the root-promoting allele of the major QTL. Since the offspring that had the 248-bp allele showed higher RR values than offspring that had the 278-bp allele, the putative root-promoting allele of the major QTL was linked with the 248-bp allele of

Table 2 Simple sequence repeat (SSR) markers mapped on the genetic map of LG 17 of 'JM7'

SSR name	Source contig/apple genome coordinate	Motif of SSR	Forward primer (5′–3′) Reverse primer (5′–3′)	Expected product size (bp)
Mdo.chr17.1	MDC004598.473 chr17:6592434..6613368 (+ strand)	(at)12	TGCTCTCCAGGTTCATTTGCT TCTGGATAAAACCTGGATGTGGT	211
Mdo.chr17.4	MDC009603.335 chr17:8091895..8121740 (− strand)	(ca)16	TACTCAAGGGATGTGGTGCG ACACACATCTCTTGCCCCAG	85
Mdo.chr17.5	MDC010937.192 chr17:8598130..8632795 (− strand)	(at)11	ACACAGCACCAAGATCATCGT AGGAATGCAAGGAATTAGGACA	207
Mdo.chr17.11	MDC018595.226 chr17:8426516.0.8454619 (+ strand)	(ga)11	TCACAAACTTTTCTTCTGGAG GTCTTTGTTTTTGAGCAGGTA	229
Mdo.chr17.13	MDC019411.279 chr17:8749656..8765583 (+ strand)	(ac)18	GGATATCACAAGAAAATGTGG ACAACCAGACGACTCGTATTA	201
Mdo.chr17.15	MDC008494.124 chr17:8996398..9012592 (− strand)	(tc)11	TTAGCATTGAACAAGGTGAAC TACGAGGGAATACACAGAGAA	233

A full list of the newly developed SSR markers is presented in Online Resource 2

MEST020 (Fig. 4). In both experiments, the RR distributions of the offspring that had the root-promoting allele and those that did not overlapped.

Graphical genotypes of the region around the major QTL

To locate the major QTL more precisely, we tested seven previously published SSR markers expected to be near markers MEST197 (11.1 cM in LG 17 of 'JM7') and MEST020 (14.6 cM in LG 17 of 'JM7') using a linkage map comparison in the progeny and their relatives. Four of these (AJ001681-SSR, CH02g04, SAmsCN492417, and SAmsCN490324) were mapped to LG 17 of 'JM7' (Fig. 5). To identify the physical locations of these markers, and markers already mapped on the 'Golden Delicious' genome, we searched the apple genome for the nucleotide sequences of these markers using a BLASTN search. Specific contigs were identified for four markers (CH02g04, CH01h01, SAmsCN490324, and MEST020; Fig. 5). Of these markers, CH02g04 (9.7 cM, 6015 kbp; MDC013381.253) and SAmsCN490324 (14.6 cM, 9143 kbp; MDC013709.214) appeared to be nearest to, and on either side of, the major QTL.

To further narrow down the major QTL, we downloaded DNA sequences of contigs located between CH02g04 and SAmsCN490324 and designed 15 SSR markers based on them (Online Resource 2). Six mapped in LG 17 (Table 2, Fig. 5), with five (Mdo.chr17.4, Mdo.chr17.5, Mdo.chr17.11, Mdo.chr17.13, and Mdo.chr17.15) located between CH02g04 and SAmsCN490324.

Graphical genotypes were constructed for the progeny and their relatives (Fig. 6). The graphical genotype between SAmsCN492417 and SAmsCN490324 revealed one informative rootstock ('JM7') and three recombinants (#35, #65, and #110). 'JM7', which is the source of the root-inducing allele of the QTL, inherited a chromosome from its parent 'Morioka Seishi' with a recombination event between Mdo.chr17.11 and Mdo.chr17.5. The three recombinants showed a range of RR values (Fig. 6). Recombinant #110 seemed to have the root-inducing allele because of its relatively high RR

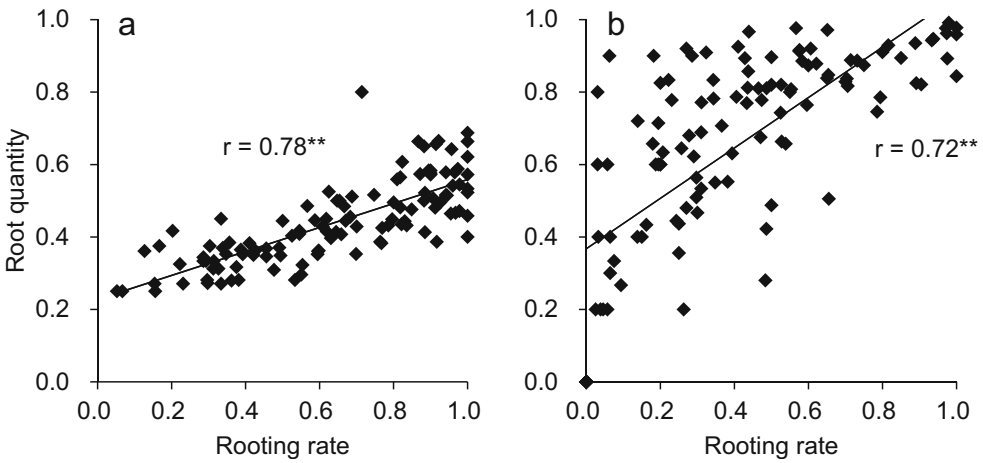

Fig. 1 Scatter plots for root quantity and rooting rate of an F_1 population derived from 'JM7' × *Malus sieboldii* 'Sanashi 63' in the **a** phytotron and **b** outdoor experiments. $^{**}P<0.01$

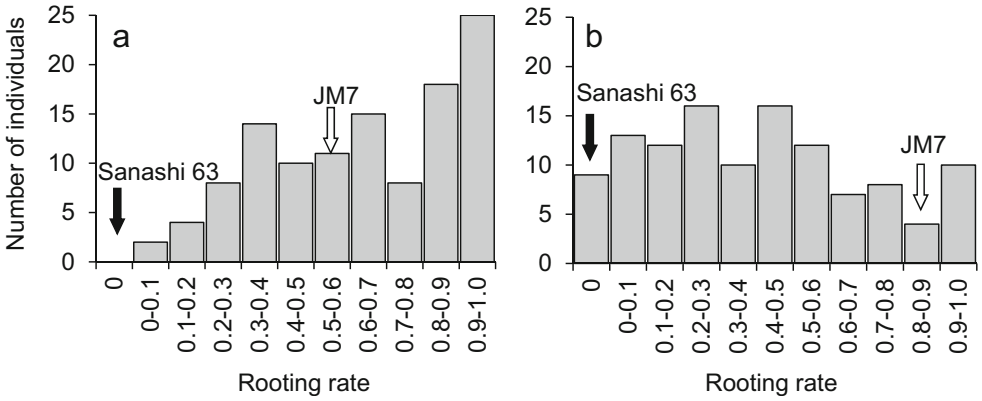

Fig. 2 Distributions of rooting rate of an F_1 population derived from 'JM7' × *Malus sieboldii* 'Sanashi 63' in the **a** phytotron (N=115) and **b** outdoor (N=117) experiments. Arrows mark parental values

(0.75) in the outdoor experiment. To evaluate the phenotypes of #35 and #65 more precisely, we grew them outdoors for another 2 years, and six of nine cuttings of #35 (RR=0.67) and 11 of 57 cuttings of #65 (RR=0.19) formed roots (Fig. 6). Mdo.chr17.13 explained the relationship between the genotype and phenotype well. Offspring (#35 and #110) with relatively higher RR values had the 226-bp allele and those (#65) with relatively lower RR values had the 228-bp allele.

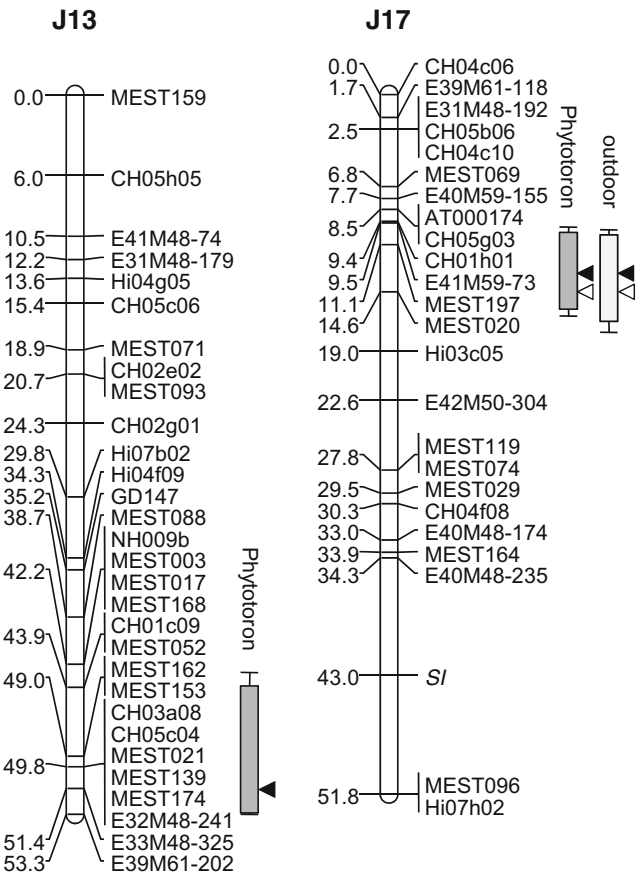

Fig. 3 Genomic positions of the significant QTLs identified on the genetic map of 'JM7'. J13, linkage group 13; J17, linkage group 17. Genetic distance is indicated in cM. *Boxes* and *range lines* indicate 1- and 1.5-LOD support intervals, respectively. *Black arrowheads* indicate the position of the maximum LOD scores using the rMQM analysis of arcsine-transformed rooting rates. *White arrowheads* indicate the positions of maximum K^* values by Kruskal Wallis analysis of root quantity

Broad-sense heritability and proportion of genetic variance explained by the major QTL

To assess the genetic contribution of the major QTL statistically, we estimated the broad-sense heritability and the proportion of genetic variance it explained. The results of a two-way ANOVA showed variance components for ARR (Table 4, Online Resource 4), allowing us to estimate its broad-sense heritability as h_B^2=0.85 for the mean value in the two experiments. The result of a one-way ANOVA with genetic values showed the variance contributed by the major QTL (Table 5, Online Resource 5). The proportion of genetic variance explained was estimated as κ_q^2/σ_g^2=66 %. The proportion of phenotypic variance explained was estimated as $\kappa_q^2/[\sigma_g^2+(\sigma_e^2/2)]$=57 %.

Predicted genes in the major QTL region

To identify the gene involved in the rooting capability of cuttings, we searched the *A. thaliana* protein database for matches to the coding sequences of predicted genes in the 'Golden Delicious' genome sequence between markers Mdo.chr17.5 and SAmsCN490324. Of the 86 predicted genes, 82 matched genes in *A. thaliana*, including two related to auxin response, one related to ethylene response, and one encoding WRKY TF (Table 6, Online Resource 6).

A BLASTN search for *ARRO-1* identified a high sequence similarity with chr (equivalent to LG) 2 and 9, but not with chr 17. We identified a high sequence similarity between the 1-LOD confidence interval of the LG 17 QTL and the *ARRO-1* flanking region of LG 9 using a BLAST search of SSR markers to the genome of 'Golden Delicious' (Online Resources 7).

Table 3 Significant quantitative trait loci (QTLs) for rooting rate detected by restricted multiple QTL model (rMQM) analysis

Experiment	Linkage group	Position (cM)	LOD	Percent[a]	Flanking markers
Phytotron	J17	13.1	25.5	57.1	MEST197–MEST020
	J13	51.4	3.62	5.1	E33M48-325[b]
Outdoor	J17	13.1	13.3	40.8	MEST197–MEST020

[a] Percentage of phenotypic variance explained by the QTL

[b] LOD score was at the peak on the position of E33M48-325

Discussion

QTL for adventitious rooting

We identified a QTL for rooting from hardwood cuttings in LG 17 (Table 3, Fig. 3). It was detected at the same position under two experimental conditions, and its contribution to genetic variance was high, indicating that it is a major genetic factor for the rooting capability of hardwood cuttings in the experimental cross population. The root-inducing allele of this QTL appears to have originated in *M. prunifolia*.

The earliest attempts to detect QTLs for rooting from hardwood cuttings used forest tree species, mainly *Eucalyptus* (Grattapaglia et al. 1995; Marques et al. 1999; Thumma et al. 2010). Since JM rootstocks have become important in the Japanese apple rootstock breeding program, it has become desirable to detect QTLs for rooting capability in those to select individuals with high rooting capabilities.

Experimental populations of interspecific origin often allow the detection of QTLs that have a high contribution to phenotypic variance. In *Corymbia*, an analysis of an F_2 population of *C. torelliana* × *C. citriodora* identified a QTL that explained 66 % of the phenotypic variance in rooting (Shepherd et al. 2008). In *Pinus*, an analysis of an F_2 population of *P. elliottii* × *P. caribaea* identified a QTL that explained 40 % of phenotypic variance in rooting (Shepherd et al. 2006). Shepherd et al. (2006, 2008) suggested that these strong QTLs reflect species effects on rooting. Our study similarly identified one major QTL that accounted for nearly half of the phenotypic variance. Further study using materials having different genetic background is necessary to conclude if species effect results our strong QTL.

The detection of another QTL in LG 13 of 'JM7' only in the phytotron experiment suggests that the effect of this QTL depends on environment. Similarly, the analysis of an intergeneric F_1 population of *Citrus sunki* × *Poncirus trifoliata* identified multiple QTLs, including two that explained 20.9 % and 15.8 % of the phenotypic variance in *P. trifoliata* (Siviero et al. 2003). Those relatively low contributions might be due to the limited accuracy of the phenotypic evaluation (a single test with 16 cuttings per genotype) and the low marker density on the linkage maps used.

All of these studies detected QTLs in parents that showed high rooting capabilities. These results suggest that a few strong or several moderate QTLs are responsible for the high rooting capability in these woody species. To our knowledge, ours is the first report of the QTL for adventitious rooting in *Malus* and its estimated contribution to genetic variance.

The major QTL contributed more to phenotypic variance in the phytotron experiment than in the outdoor experiment, and the population mean RR was higher in the phytotron experiment. One explanation for the difference between experiments

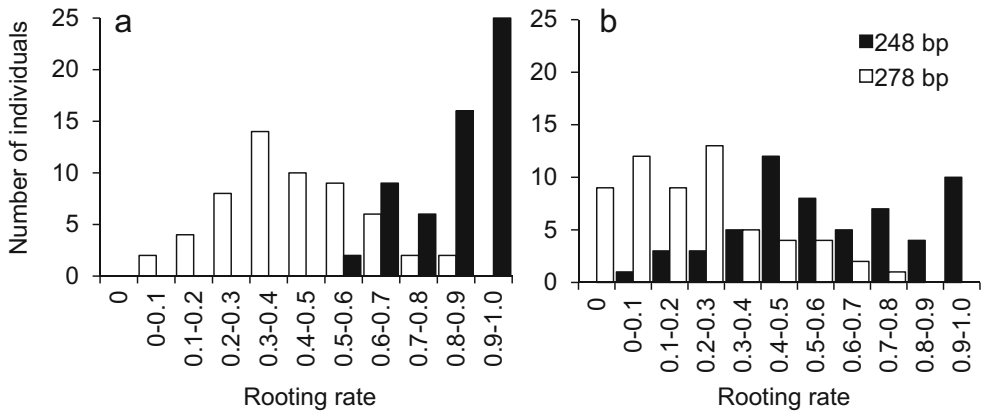

Fig. 4 Distribution of rooting rates of an F_1 population derived from 'JM7' × *Malus sieboldii* 'Sanashi 63' in the **a** phytotron and **b** outdoor experiments. Individuals were grouped according to the genotype at marker MEST020. The 248-bp allele was linked with the root-inducing allele of the major QTL on LG 17 (N=58 for both experiments). The 278-bp allele was linked with the non-rooting allele (N=57 and 59 for the phytotron and outdoor experiments, respectively)

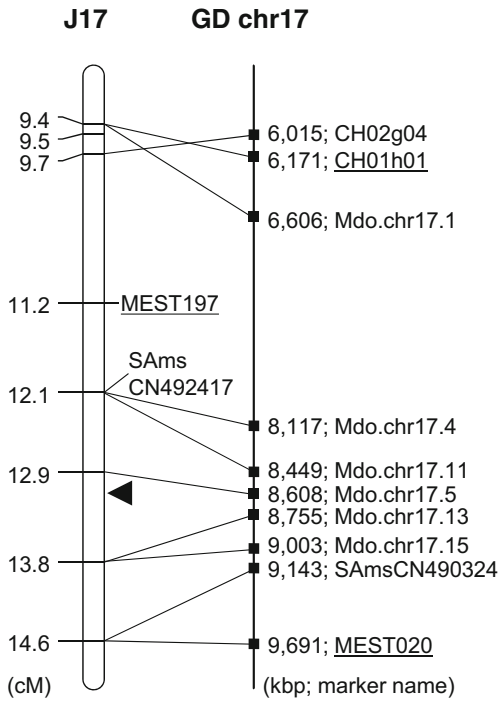

Fig. 5 Relationship between the locations of DNA markers on the enriched genetic map of 'JM7' LG 17 (J17) and the physical location on 'Golden Delicious' chromosome 17 (GD chr17). The *arrowhead* indicates the position of the maximum LOD scores by rMQM analysis. *Underlined markers* are also shown in Fig. 3. AJ001681-SSR, mapped at 8.4 cM, is not shown in this figure. Markers shown only in J17 could not have their physical locations identified in GD chr17

is the greater suitability of the phytotron conditions for root formation by plants containing the root-promoting allele. Another possible explanation is the more advanced maturity of the stock materials (sourced from original trees) in the phytotron experiment than those (sourced from grafted trees) in the outdoor experiment although we did not observe signs of maturation, like flowering. However, maturity is negatively correlated with adventitious rooting success (Legue et al. 2014).

We observed a large variance in RQ on low RR individuals in the outdoor experiment. Long term experiment may affect variances in RQ on low RR individuals; namely, genetic factors other than the LG 17 QTL may stimulate root growth after rooting and generate the variance. Although such variance existed, the high correlation between RR and RQ in both experiments and the co-location of the QTLs for RR and RQ suggested that the root-inducing allele of this QTL could act under both environmental conditions and could exert pleiotropy on root formation and root elongation. In *Corymbia*, such pleiotropy is also suggested by a strong positive correlation between RR and root quality (Shepherd et al. 2007) and by the co-location of QTLs for RR and other root quality traits (Shepherd et al. 2008).

Graphical genotypes of key recombinants (Fig. 6) allowed us to narrow down the QTL location. Four of the five JM rootstocks (JM1, JM2, JM5, and JM8) which can root well from cuttings inherited an identical chromosome from 'Morioka Seishi'. The other, 'JM7', inherited a chromosome that was identical to that in other JM rootstocks proximal to the marker Mdo.chr17.5 but reciprocal to that distal to Mdo.chr17.11 possibly because of a recombination event. As the rooting capability is broadly distributed in the JM population derived from 'Morioka Seishi' × 'M.9' (Yoshida, unpublished), and JM rootstocks were selected for their high rooting capabilities, the root-inducing allele of the QTL is likely to be present among the JM rootstocks. Recombinants #35 and #110, with relatively high rooting rates (Fig. 6), are likely to have inherited the root-inducing allele. However, #65, with a relatively low RR, especially during the additional 2-year outdoor assessment, probably does not to carry it. Thus, the QTL location could be narrowed down to between markers Mdo.chr17.13 and Mdo.chr17.15, corresponding to the region between Mdo.chr17.5 and SAmsCN490324 of the 'Golden Delicious' genome, which spans approximately 535 kbp.

The SSR markers developed here will be useful for MAS to develop rootstocks that are easily propagated from hardwood cuttings. However, it will be necessary to perform phenotypic selection after MAS because rooting capability varied even among individuals with the root-promoting allele. It will be important to unravel how such variations occur among individuals with the same allelic composition. Therefore, the genetic cause of adventitious rooting of cuttings in apple should be studied more deeply.

Candidate genes for adventitious rooting

In adventitious root formation, auxins play an essential role, interacting with most other phytohormones and acting at the biosynthesis, transport, and signaling levels (Pacurar et al. 2014), although the detailed mechanisms are not fully understood yet. The *ARRO-1* gene is involved in adventitious rooting in *Malus* (Smolka et al. 2009). Although it was upregulated under indole-3-acetic acid treatment in comparison with control stem discs, indole-3-acetic acid treatment failed to induce any adventitious roots from two rootstock materials, suggesting that it is responsible for auxin homeostasis but not for rooting (Li et al. 2012). The *ARRO-1* gene was associated with either chromosome 2 or chromosome 9 by in silico analysis (Online Resource 7; Li et al. 2012). We identified sequence similarities between the QTL region of LG 17 (chromosome 17) and the *ARRO-1* region of chromosome 9. This is very interesting in the context of an ancient duplication of the *Malus* genome (Velasco et al. 2010). It can be hypothesized that an *ARRO-1* homolog exists in the QTL of LG 17 in *M. prunifolia* but not in 'Golden Delicious' genome assembly and possibly *M.* × *domestica*. Therefore, *ARRO-1* is recognized as a candidate gene of QTL.

Generation: Grandparents

SSR marker	Genetic position (cM)	Physical position (kbp)	SSR genotype (bp) *Morioka Seishi*		M.9	
SAmsCN492417	12.1	No hit	143	147	141	143
Mdo.chr17.4	12.1	8,117	95	114	108	110
Mdo.chr17.11	12.1	8,449	252	null	273	273
Mdo.chr17.5	12.9	8,608	227	220	247	249
Mdo.chr17.13	13.8	8,755	226	212	228	228
Mdo.chr17.15	13.8	9,003	null	null	260	260
SAmsCN490324	14.6	9,143	260	239	243	243

Generation: Parents

SSR marker	SSR genotype (bp) JM rootstocks: *Morioka Seishi* × M.9 *JM7*		*JM1*, *JM8*		*JM2*, *JM5*		Sanashi 63	
SAmsCN492417	147	141	143	143	143	141	137	139
Mdo.chr17.4	114	108	95	110	95	108	93	114
Mdo.chr17.11	null	273	252	273	252	273	266	247
Mdo.chr17.5	227	249	227	247	227	249	246	219
Mdo.chr17.13	226	228	226	228	226	228	null	230
Mdo.chr17.15	null	260	null	260	null	260	null	null
SAmsCN490324	260	243	260	243	260	243	238	238

Experiment	Rooting rate (No. of rooted cuttings/total no. of cuttings) *JM7*	*JM1*	*JM8*	*JM2*	*JM5*	Sanashi 63
Phytotron	0.56 (146/260)	-	-	-	-	0 (0/18)
Outdoor	0.91 (60/66)	0.85[a]	0.74[a]	0.97[a]	0.82[a]	-
Additional outdoor	0.92 (203/220)	-	-	-	-	0 (0/36)

[a] Soejima et al. (2010, 2013)

Generation: Progeny

SSR marker	SSR genotype (bp) Experimental F_1 population: *JM7* × Sanashi 63 *#35*		#65		*#110*	
SAmsCN492417	141	137	147	137	147	137
Mdo.chr17.4	108	93	114	93	114	93
Mdo.chr17.11	273	266	null	266	null	266
Mdo.chr17.5	249	246	249	246	227	246
Mdo.chr17.13	226	null	228	null	226	null
Mdo.chr17.15	null	null	260	null	null	null
SAmsCN490324	260	238	243	238	243	238

Experiment	Rooting rate *#35*	#65	*#110*
Phytotron	0.67 (2/3)	0.60 (49/82)	0.62 (44/71)
Outdoor	-	0.45 (14/31)	0.75 (27/36)
Additional outdoor	0.67 (6/9)	0.19 (11/57)	-

Fig. 6 Graphical genotypes of the major QTL region in LG 17 and rooting rates of the experimental population and its progenitors. Materials named in *italics* have high rooting capabilities. Genetic and physical positions of SSR markers refer to Fig. 5. *Shading* indicates parental chromosomes

We identified two auxin-related genes (*MDP0000232135* and *MDP0000232281*) in the presumed region of the major QTL (Table 6), which makes them putative candidates for the QTL itself. *MDP0000232135* shares sequence similarity with indole-3-butyric acid-response5 (*IBR5*; At2g04550) of *A. thaliana*, which encodes a dual-specificity mitogen-activated protein kinase phosphatase (Monroe-Augustus et al. 2003), but no meaningful studies could be found for *MDP0000232281*. IBR5 inactivates one of the negative regulators of auxin signaling (Lee et al. 2009). A mutation in *IBR5*

Table 4 Variance components estimated by ANOVA of arcsine-transformed rooting rate of an F_1 population derived from 'JM7' × *Malus sieboldii* 'Sanashi 63' in the phytotron and outdoor experiments

Variance component	Estimate	Percentage explained (%)
σ_g^2 (genotype)	298.9	56
κ_t^2 (experiment)	131.4	25
σ_e^2 (error)	101.8	19

Table 5 Variance components estimated by ANOVA of expected genetic value for arcsine-transformed rooting rate in an F_1 population derived from 'JM7' × *Malus sieboldii* 'Sanashi 63'

Variance component	Estimate
κ_q^2 (QTL)	197.8
σ_r^2 (error)	152.0

Table 6 Predicted candidate genes in the major QTL region in the LG 17 of 'JM7'

Predicted gene in 'Golden Delicious' genome	Physical position in 'Golden Delicious' genome	Best match protein in Arabidopsis	E-value	Description
MDP0000839828	chr17:8,613,282..8,613,965 (− strand)	AT1G50640.1	3.00E-25	ERF3, ATERF3 | ethylene responsive element binding
MDP0000232135	chr17:8,696,622..8,699,707 (− strand)	AT2G04550.1	1.00E-85	IBR5, DSPTP1E | indole-3-butyric acid response 5
MDP0000232281	chr17:8,990,737..8,992,489 (− strand)	AT2G04850.1	E-161	Auxin-responsive family protein
MDP0000260803	chr17:9,013,295..9,015,664 (+ strand)	AT2G04880.2	9.00E-70	ZAP1, ATWRKY1, WRKY1 | zinc-dependent activator protein-1

A complete gene list is presented in Online Resource 6

conferred reduced sensitivity to auxin in *Arabidopsis* roots (Monroe-Augustus et al. 2003). An *IBR5-GUS* fusion gene was expressed throughout the plant (including root tips and root vasculature) but was not expressed in lateral root primordia and newly emerged lateral roots; however, signal transduction to these tissues was confirmed (Monroe-Augustus et al. 2003). Thus, *MDP0000232135* is a strong candidate for the gene encoding the apple's adventitious rooting capability from hardwood cuttings. Additionally, *MDP0000232281* is also a candidate because its function remains unknown.

We also found other candidate genes, including *MDP0000839828*, a homolog of an ethylene response factor/APETALA 2 (ERF/AP2) TF and *MDP0000260803*, with sequence similarity to a WRKY TF. *ERF/AP2* TFs are involved in plant stress responses and the control of development (Licausi et al. 2013).

WRKY TFs are involved in many plant processes, including both biotic and abiotic stress responses (Rushton et al. 2010). Thus, these genes also remain candidates.

To identify which of these genes underlies the QTL for adventitious rooting from hardwood cuttings in apple, further graphical genotype and gene expression analyses will be necessary. However, the candidate genes have to be examined carefully because differences in genome composition have been suggested even among *M.* × *domestica* cultivars (Velasco et al. 2010). Because we used *M. prunifolia* as the source of the QTL, the genetic differences between the two apple species are likely to be great. Therefore, differences in genome composition between 'Golden Delicious' (the source of the genome sequence) and *M. prunifolia* should be studied carefully. In addition, an anatomical approach may be effective. Fukuda et al (1988) found root primordia in the cortex around the bud in *M. prunifolia* accession 'Mo 84' and observed rooting from these primordia of hardwood cuttings. However, they were not able to find any root primordia in the same place in 'M.9' and 'M.26'. They showed the ability to form these primordia could be inherited by the offspring. Therefore, the relationships among QTL, rooting behavior, and formation of root primordia should be studied.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Data archiving statement Genetic linkage maps of 'JM7' and 'Sanashi 63' will be submitted to the genome database for Rosaceae (www.rosaceae.org) after the final acceptance.

References

Antanaviciute L, Fernandez-Fernandez F, Jansen J, Banchi E, Evans KM, Viola R, Velasco R, Dunwell JM, Troggio M, Sargent DJ (2012) Development of a dense SNP-based linkage map of an apple rootstock progeny using the *Malus* Infinium whole genome genotyping array. BMC Genomics 13:203

Arakawa O, Komori S (2006) Apple. In: The Japanese Society for Horticultural Science (ed) Horticulture in Japan 2006. Shoukadoh Publication, Kyoto, pp 34–42

Bus VGM, Chagne D, Bassett HCM, Bowatte D, Calenge F, Celton JM, Durel CE, Malone MT, Patocchi A, Ranatunga AC et al (2008) Genome mapping of three major resistance genes to woolly apple aphid (*Eriosoma lanigerum* Hausm.). Tree Genet Genomes 4: 223–236

Butler ED, Gallagher TF (1999) Isolation and characterization of a cDNA encoding a novel 2-oxoacid-dependent dioxygenase which is up-regulated during adventitious root formation in apple (*Malus domestica* 'Jork 9') stem discs. J Exp Bot 50: 551–552

Celton JM, Tustin DS, Chagne D, Gardiner SE (2009) Construction of a dense genetic linkage map for apple rootstocks using SSRs developed from *Malus* ESTs and *Pyrus* genomic sequences. Tree Genet Genomes 5:93–107

Fazio G, Wan YZ, Kviklys D, Romero L, Adams R, Strickland D, Robinson T (2014) *Dw2*, a new dwarfing locus in apple rootstocks and its relationship to induction of early bearing in apple scions. J Am Soc Hort Sci 139:87–98

Foster TM, Celton JM, Chagné D, Tustin DS, Gardiner SE (2015) Two quantitative trait loci, *Dw1* and *Dw2*, are primarily responsible for rootstock-induced dwarfing in apple. Hort Res 2:15001

Fukuda H, Ohnuma K, Nishiyama Y (1988) Rooting behavior of hard wood cuttings of several apple rootstocks in relation to root primordia. Bull Fruit Tree Res Stn C 15:29–40

Grattapaglia D, Bertolucci FL, Sederoff RR (1995) Genetic-mapping of QTLs controlling vegetative propagation in *Eucalyptus grandis* and *E. urophylla* using a pseudo-testcross strategy and RAPD markers. Theor Appl Genet 90:933–947

Howard BH (1968) Influence of 4(indolyl-3) butyric acid and basal temperature on rooting of apple rootstock hardwood cuttings. J Hortic Sci 43:23–31

Lee JS, Wang S, Sritubtim S, Chen J-G, Ellis BE (2009) Arabidopsis mitogen-activated protein kinase MPK12 interacts with the MAPK phosphatase IBR5 and regulates auxin signaling. Plant J 57: 975–985

Legue V, Rigal A, Bhalerao RP (2014) Adventitious root formation in tree species: involvement of transcription factors. Physiol Plant 151: 192–198

Li TY, Wang Y, Zhang XZ, Han ZH (2012) Isolation and characterization of *ARRO-1* genes from apple rootstocks in response to auxin treatment. Plant Mol Biol Report 30:1408–1414

Licausi F, Ohme-Takagi M, Perata P (2013) APETALA2/ethylene responsive factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. New Phytol 199:639–649

Liebhard R, Gianfranceschi L, Koller B, Ryder CD, Tarchini R, Van de Weg E, Gessler C (2002) Development and characterisation of 140 new microsatellites in apple (*Malus* × *domestica* Borkh.). Mol Breed 10:217–241

Marques CM, Vasquez-Kool J, Carocha VJ, Ferreira JG, O'Malley DM, Liu BH, Sederoff R (1999) Genetic dissection of vegetative propagation traits in *Eucalyptus tereticornis* and *E. globulus*. Theor Appl Genet 99:936–946

Monroe-Augustus M, Zolman BK, Bartel B (2003) IBR5, a dual-specificity phosphatase-like protein modulating auxin and abscisic acid responsiveness in *Arabidopsis*. Plant Cell 15:2979–2991

Moriya S, Iwanami H, Takahashi S, Kotoda N, Suzaki K, Abe K (2008) Evaluation and inheritance of crown gall resistance in apple rootstocks. J Jpn Soc Hortic Sci 77:236–241

Moriya S, Iwanami H, Takahashi S, Kotoda N, Suzaki K, Yamamoto T, Abe K (2010) Genetic mapping of the crown gall resistance gene of the wild apple *Malus sieboldii*. Tree Genet Genomes 6:195–203

Moriya S, Iwanami H, Kotoda N, Haji T, Okada K, Terakami S, Mimida N, Yamamoto T, Abe K (2012a) Aligned genetic linkage maps of apple rootstock cultivar 'JM7' and *Malus sieboldii* 'Sanashi 63' constructed with novel EST-SSRs. Tree Genet Genomes 8:709–723

Moriya S, Okada K, Haji T, Yamamoto T, Abe K (2012b) Fine mapping of *Co*, a gene controlling columnar growth habit located on apple (*Malus* × *domestica* Borkh.) linkage group 10. Plant Breed 131: 641–647

Nekoduka S, Kawamura T, Nakatani F, Sasaki H, Onoda K (2001) Occurrence of crown gall on apple rootstock 'JM' strain. Annu Rep Plant Prot North Jpn 52:105–108

Pacurar DI, Perrone I, Bellini C (2014) Auxin is a central player in the hormone cross-talks that control adventitious rooting. Physiol Plant 151:83–96

Pilcher RLR, Celton JM, Gardiner SE, Tustin DS (2008) Genetic markers linked to the dwarfing trait of apple rootstock 'Malling 9'. J Am Soc Hortic Sci 133:100–106

Rushton PJ, Somssich IE, Ringler P, Shen QJ (2010) WRKY transcription factors. Trends Plant Sci 15:247–258

Shepherd M, Huang S, Eggler P, Cross M, Dale G, Dieters M, Henry R (2006) Congruence in QTL for adventitious rooting in *Pinus elliottii* × *Pinus caribaea* hybrids resolves between and within-species effects. Mol Breed 18:11–28

Shepherd M, Pomroy P, Dieters M, Lee D (2007) Genetic control of propagation traits in a single *Corymbia torelliana* × *Corymbia variegata* family. Can J For Res 37:2563–2574

Shepherd M, Kasem S, Lee DJ, Henry R (2008) Mapping species differences for adventitious rooting in a *Corymbia torelliana* × *Corymbia citriodora* subspecies *variegata* hybrid. Tree Genet Genomes 4: 715–725

Silfverberg-Dilworth E, Matasci CL, van de Weg WE, van Kaauwen MPW, Walser M, Kodde LP, Soglio V, Gianfranceschi L, Durel CE, Costa F et al (2006) Microsatellite markers spanning the apple (*Malus* × *domestica* Borkh.) genome. Tree Genet Genomes 2: 202–224

Siviero A, Cristofani M, Machado MA (2003) QTL mapping associated with rooting stem cuttings from *Citrus sunki vs. Poncirus trifoliata* hybrids. Crop Breed Appl Biotechnol 3:83–88

Smolka A, Welander M, Olsson P, Holefors A, Zhu LH (2009) Involvement of the *ARRO-1* gene in adventitious root formation in apple. Plant Sci 177:710–715

Soejima J, Abe K, Kotoda N, Kato H, Komori S, Bessho H, Ito Y (2002) Analysis of factors related to the results obtained by hardwood cuttings of JM series apple dwarfing rootstock cultivars. J Jpn Soc Hortic Sci 71(Suppl 1):201

Soejima J, Takahashi S, Iwanami H, Kotoda N, Abe K (2004) Effect of compaction of bed soil in hardwood cuttings of apple dwarfing rootstocks, JM1 and JM7. J Jpn Soc Hortic Sci 73(Suppl 2):114

Soejima J, Yoshida Y, Haniuda T, Bessho H, Tsuchiya S, Masuda T, Komori S, Sanada T, Ito Y, Sadamori S, Kashimura Y (2010) New dwarfing apple rootstocks 'JM1', 'JM7' and 'JM8'. Bull Natl Inst Fruit Tree Sci 11:1–16

Soejima J, Yoshida Y, Haniuda T, Bessho H, Tsuchiya S, Masuda T, Komori S, Sanada T, Ito Y, Sadamori S et al (2013) New semi- and extremely dwarfing apple rootstocks 'JM2' and 'JM5'. Bull Natl Inst Fruit Tree Sci 16:19–36

Thumma BR, Baltunis BS, Bell JC, Emebiri LC, Moran GF, Southerton SG (2010) Quantitative trait locus (QTL) analysis of growth and vegetative propagation traits in *Eucalyptus nitens* full-sib families. Tree Genet Genomes 6:877–889

van Dyk MM, Soeker MK, Labuschagne IF, Rees DJG (2010) Identification of a major QTL for time of initial vegetative budbreak in apple (*Malus* × *domestica* Borkh.). Tree Genet Genomes 6:489–502

van Ooijen JW (2009) MapQTL 6 Software for the mapping of quantitative trait loci in experimental populations of diploid species. Kyazma B.V., Wageningen, the Netherlands

Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, Fontana P, Bhatnagar SK, Troggio M, Pruss D et al (2010) The genome of the domesticated apple (*Malus* × *domestica* Borkh.). Nat Genet 42:833–839

Webster AD (1995) Temperate fruit tree rootstock propagation. NZ J Crop Hortic Sci 23:355–372

Wertheim SJ, Webster AD (2003) Propagation and nursery tree quality. In: Ferree DC, Warrington IJ (eds) Apples: botany, production and uses. CABI Publishing, Cambridge, pp 125–151