Apple dwarfing rootstocks exhibit an imbalance in carbohydrate allocation and reduced cell growth and metabolism

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Apple dwarfing rootstocks cause earlier shoot termination and reduced root and shoot mass. To identify physiological factors responsible for rootstock-induced growth restriction, we compared vascular-enriched gene expression between two dwarfing rootstocks (‘M27’ and ‘M9’) and the vigorous rootstock ‘M793’ using RNA sequencing and quantitative reverse transcriptase PCR. Differentially expressed genes common to both dwarfing rootstocks belonged to five main biological processes: (1) primary metabolism, (2) cell wall synthesis and modification, (3) secondary metabolism, (4) hormone signalling and response and (5) redox homeostasis. Genes promoting the biosynthesis of amino acids, lipids and cell walls were downregulated in dwarfing rootstocks, whereas genes promoting the breakdown of these compounds were upregulated. The only exception to this trend was the upregulation of starch synthesis genes in dwarfing rootstocks. Non-structural carbohydrate analysis demonstrated that starch concentrations in ‘M9’ roots, stems and grafted ‘Royal Gala’ (RG) scions were double that of equivalent tissues from ‘RG’ homografted trees (‘RG/RG’). Fructose and glucose concentrations were much lower in all three tissues of the ‘RG/M9’ trees. Together, these data indicate that dwarfing rootstocks are in a state of sugar depletion and reduced cellular activity despite having large starch reserves. Another significant finding was the over-accumulation of flavonoids and the downregulation of auxin influx transporters MdAUX1 and MdLAX2 in dwarfing rootstocks. We propose that both factors reduce polar auxin transport. The results of this study contribute novel information about the physiological state of dwarfing rootstocks.

INTRODUCTION

Dwarfing rootstocks are widely used in commercial apple production to reduce scion vigour, allowing high-density plantings and increased yield index. Despite their utility and a century of research, the underlying mechanism of rootstock-induced dwarfing is still unknown. The effects of dwarfing rootstocks are apparent within the first year after grafting and include reduced root growth,4–3 a decrease in the length and node number of the primary axis,5–6 a decrease in the number and length of sylleptic shoots3,8 and/or an increase in the proportion of floral buds along the primary axis.9–10

The most widely used and best-characterised dwarffing apple rootstock is ‘Malling 9’ (‘M9’), which was first phenotyped in the early twentieth century.11 The ‘Malling’ series have been used to breed new rootstocks that confer a range of vigour control, including the strongly dwarfing ‘M27’. Rootstock-induced dwarfing is conferred by two major quantitative trait loci.12–15 Most of the known dwarfing rootstocks carry markers linked to one or both loci, suggesting that there is one source of dwarfing rootstocks and they reduce scion vigour by the same underlying physiological mechanism.14,15

Many of the hypotheses to explain how dwarfing rootstocks affect scion growth include altered levels, transport and signalling of hormones between scion and rootstock. Perhaps, the best-supported model proposes that dwarfing rootstocks reduce basipetal auxin transport, thereby limiting root growth and the amount of root-synthesised cytokinin supplied to the scion.16 Stem segments of ‘M9’ transport radiolabelled IAA (auxin) at a lower rate than that of vigorous genotypes.17,18 Levels of free IAA and Zeatin (cytokinin) in cambial sap are lower in ‘M9’ than vigorous rootstocks.19 Other studies have shown an inverse relationship between the rate of IAA diffusion and xylem concentration of cytokinin.4 Van Hooijdonk et al.20 demonstrated that application of the polar auxin inhibitor 1-N-naphthylphthalamic acid to the stem of an invigorating rootstock had the same effect on scion growth as that of the ‘M9’ rootstock. Application of cytokinin and 1-N-naphthylphthalamic acid restored the number of secondary shoots to levels typical of scions on vigorous rootstocks. Likewise, application of cytokinin to scions grafted onto ‘M9’ increased the number of secondary shoots.20 Other hormones may also have a role in rootstock-induced dwarfing. The concentration of gibberellin acid in the xylem is lower in ‘M9’ relative to vigorous rootstocks.20 Application of gibberelic acid to 1-N-naphthylphthalamic acid-treated vigorous rootstocks or scions on ‘M9’ increased node number of both the primary axis and secondary shoots.20 Abscisic acid (ABA) concentrations are higher in dwarfing than in vigorous rootstocks.22,23

Anatomical factors have also been implicated as causal factors in rootstock-induced dwarfing. The roots and stems of dwarfing rootstocks have a higher proportion of bark, which consists of
phloem and cortex, and a reduction in the number and diameter of xylem cells relative to that of vigorous rootstocks.\textsuperscript{24–26} Vasculature at the graft junction between ‘M.9’ and the scion is disorganised, suggesting auxin accumulation in the region, but no evidence of graft incompatibility was found.\textsuperscript{25,26} Some researchers have suggested that a smaller root system and reduced amount of conducting tissues observed in dwarfing rootstocks were limiting the transport of water and metabolites to the scion.\textsuperscript{27,28} The effect of root restriction on shoot growth is the basis of bonsai cultivation and has been shown to have the same effect on apple scion growth as that of an dwarfing rootstock.\textsuperscript{3} The rate of hydraulic conductivity is lower in roots and stems of dwarfing rootstocks,\textsuperscript{23,29} however, this does not take into effect the smaller ‘M9’ root mass.\textsuperscript{30}

The relative importance of the root versus the stem in rootstock-induced dwarfing has been the subject of numerous studies. Beakbane and Rogers\textsuperscript{31,32} demonstrated that scions grafted directly onto dwarfing roots were reduced in vigour and concluded that the roots alone were able to exert some effect, although the presence of a dwarfing stem enhanced this effect. Other researchers have shown that a segment of stem, or ‘interstock’, of dwarfing tissue inserted between vigorous roots and scion reduces scion vigour, with larger stem segments having a greater effect.\textsuperscript{10,16,31,32} Bark implants from dwarfing genotypes or even an inverted ring of bark from a vigorous genotype have been shown to dwarf the scion, providing strong evidence that the mechanism of rootstock-induced dwarfing is likely to involve the vasculature and/or bark.\textsuperscript{23} Hormones, soluble sugars, metabolites and nitrogen are translocated via the vascular system and have all been implicated in rootstock-induced dwarfing. Phenols affect many biological processes including auxin levels, and are all been implicated in rootstock-induced dwarfing. Previous work has shown that the scion bud type affects both scion and rootstock growth,\textsuperscript{3} therefore, all RNAs were collected from compound trees with a monopodial shoot (originating from a vegetative scion bud). The outer bark was removed with a razor blade and vascular-enriched tissue was collected from the rootstock stem 2–10 cm below the graft junction and immediately snap-frozen in liquid nitrogen. Tissue was harvested between 4 and 5 h after sunrise for all time points. Total RNA was isolated using previously described\textsuperscript{30} procedures. The quantity and concentration of the RNA samples were assessed using a Fragment Analyzer (Advanced Analytical, Ankeny, IA, USA). Only samples with a RNA integrity number value of 8 or higher were further analysed by sequencing or quantitative reverse transcriptase PCR (qRT-PCR).

RNA sequencing, data processing and analysis
RNA from the 60 DABB (November) collection was sent to Axeg/Macrogen for library preparation and sequencing using an Illumina Hiseq 2000 (San Diego, CA, USA) instrument. RNA from six individuals of each genotype were made into separate libraries. The 18 libraries were run as a multiplexed sample on one lane to produce 100 nucleotide paired end sequence reads. The first 13 bases of all RNA sequencing (RNAseq) reads were trimmed using an in-house perl script. Adapters were removed using fastq-mcf from the ea-utils package.\textsuperscript{37} Quality score analysis was performed using fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) both before and after trimming. Trimmed reads with a minimum length of 30 bp and an average quality score greater than 20 were mapped to the Apple Genome V1.0-predicted coding DNA sequence (CDS) sequences (https://www.rosaceae.org/species/malus/malus_x_domestica/genome_v1.0) using bowtie2 v2.2.5 (ref. 38) using the following settings: end-to-end mapping in sensitive mode with a maximum of one mismatch per alignment. A count table was generated for each predicted CDS across all the libraries by querying for the best alignment for each sequence using samtools v1.2.\textsuperscript{39} Raw read counts and reads per kilobase per million values were extracted from BAM files using the multicov option of bedtools\textsuperscript{40} and either an in-house R script or cufflinks.\textsuperscript{41} The RNAseq data can be found in NCBI project PRJNA338443. Pairwise comparisons were made between ‘M9’ and ‘M793’ and ‘M27’ and ‘M793’. Differentially expressed genes (DEGs) were selected using the DEseq\textsuperscript{2} package\textsuperscript{2} in BioConductor. Significant DEGs were selected using an adjusted P value of <0.05 and [log2 fold change] >1. Significantly over-represented gene categories in the DEGs were identified using Fisher’s exact test and visualised in Pageman using MapMan 3.5.0 and mappings for Malus domestica.\textsuperscript{43} Metabolic pathways were visualised using Kyoto Encyclopedia of Genes and Genomes (KEGG) gene ontologies.\textsuperscript{44} Arabidopsis orthologues were determined by BLAST against the TAIR database. Chromosome and position were determined by an in-house database. Venn diagrams were generated by Venny 1.0 (http://bioinfgp.cn.bsc.es/tools/venny/index.html).

Gene expression by qRT-PCR
First strand complementary DNA was synthesised from 1.0 µg total RNA using oligo dT primer and Primerscript Reverse Transcriptase (TaKaRa, Clontech, Mountain View, CA, USA). qRT-PCR was performed with KAPA Sybrfast qRT-PCR mastermix on a Roche 480 Light Cycler (Basel, Switzerland). For the qRT-PCR reactions, 2 µL complementary DNA (1:20 dilution) was used as a template in a reaction volume of 7 µL. For each analysis, there were four to six biological replicates of each genotype and four technical replicates of each sample. Complementary DNAs were loaded into a 384-well plate by a Biomeck liquid handling robot (Biomeck, Waltham, MA, USA) to minimise pipetting errors. PCR cycles are as follows: initial denaturation at 95 °C for 5 min, followed by 45 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 10 s and a final melt curve analysis to determine whether a single product was amplified. Primers were designed by Primer 3 to span an intron (if possible) and to amplify products of 100–120 bp pair (Supplementary Table 1). For each analysis, a no complementary DNA template was included as a negative control. Actin (MDP0000752428) and MDP0001730252 were used as reference genes and gave similar results.\textsuperscript{45} All qRT-PCR results are shown as expression relative to actin, except for MDP0000264875. Primer efficiencies and relative expression were calculated using the Roche 480 Light Cycler software (version SW1.5).

Dry weight measurements
After tissue was collected for RNA extractions (60, 120, 180 and 300 DABB), four to six trees of each rootstock genotype were harvested, severed at the graft junction, and then oven-dried at 60 °C to a constant mass before
Non-structural carbohydrate quantification

After extension growth had ceased (250 DABB), final architectural measurements were made of six ‘RG’/’M9’ and six ‘RG’/’RG’ grafted trees. A segment of stem tissue was collected from 20 cm above the graft junction (scion), 5 cm below the graft junction (rootstock stem) and roots. Tissue was snap-frozen in liquid nitrogen, dried in a freeze-dryer, and then ground to a fine powder. A 0.05 g subsample was extracted with 80% ethanol with Adonitol added as the internal standard and then incubated for 1 h at 60 °C. Extracted samples were centrifuged and the supernatant decanted off. The residue was re-suspended in 80% ethanol re-spun and supernatants combined. The insoluble residue was transferred into Erlenmeyer flasks and analysed for starch as per Smith et al. 10 A sub-sample of the supernatant was taken and dried using a centrifugal concentrator; samples were then re-dissolved in ultrapure water. The sugars were analysed using Dionex ICS-5000 Reagent-Free IC (RFIC; Thermo Fisher Scientific, Waltham, MA, USA) system with a CarboPac MA1 column with electrochemical detection.

Secondary metabolite analysis

The ‘RG’ and ‘M9’ stem samples used for analysis by liquid chromatography–high resolution accurate mass–mass spectrometry (LC–HRAM–MS) were the same as those used for the carbohydrate analysis. There were six biological replicates and each sample was run in duplicate. The LC–HRAM–MS system was composed of a Dionex Ultimate 3000 Rapid Separation LC and a microOTOF QII high resolution mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray ion source. Metabolite separation by LC was achieved using a Luna Omega Polar 2.1 × 100 mm, 1.6 μm (Phenomenex, Auckland, New Zealand) maintained at 40 °C. The flow was 350 μL min⁻¹. The solvents were A = 0.2% formic acid and B = 100% acetonitrile. The solvent gradient was: 10% A 90% B 0–0.5 min; linear gradient to 50% A 50% B, 0.5–12 min; linear gradient to 5% A 95% B, 12–15 min; composition held at 5% A 95% B, 15–17 min; linear gradient to 10% A, 90% B, 17–17.2 min; to return to the initial conditions before another sample injection at 20 min. The injection volume for samples and standards was 1 μL. The microOTOF QII parameters for polyphenolic analysis were: temperature 225 °C; drying N₂ flow 6 L min⁻¹; nebuliser N₂ 1.5 bar, endplate offset – 500 V, mass range 100–1500 Da, acquired were acquired at 5 scans per s. Negative ion electrospray was used with a capillary voltage of +3500 V. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

The molecular features present in each sample were found using the find-molecular-feature-algorithm in the DataAnalysis (Bruker Daltonics). The find-molecular-feature process combines mass spectral signals that are related to each other (isotope clusters and molecular adducts) into single molecular features. The molecular features for each analysis were combined into a single data table using ProfileAnalysis (Bruker Daltonics). The intensity values were normalised by ‘the sum of all intensities in an analysis’. To assess the distribution of metabolites in the ‘RG’ and ‘M9’ sample sets, the data table was analysed using both principal component analysis and t-tests using ProfileAnalysis. Metabolites that showed a [fold change] >1.2 between ‘M9’ and ‘RG’, and a P value < 0.05, were selected for further analysis. The chemical identity of each metabolite was based on accurate mass and chemical formula calculations and authentic standards when available.

RESULTS

Rootstock-induced dwarfing first manifests late in first season of growth

Primary shoots began growth shortly after bud break and sylleptic shoots began to extend about 80 DABB. Primary and sylleptic shoot growth slowed around 200 DABB, with trees on the dwarfing rootstocks terminating earlier, consistent with earlier studies. 7 All trees terminated extension growth by 250 DABB. No significant difference was detected in either scion or rootstock dry weight between any of the rootstock treatments until 300 DABB (Figure 1). Trees on the dwarfing rootstocks gained very little dry weight after 180 DABB, whereas trees on ‘M793’ had a constant increase in dry weight from 120 to 300 DABB.

Genes differentially expressed between dwarfing and vigorous rootstocks

To identify differences in rootstock gene expression before any phenotypic changes to ‘RG’ scion growth were detected, we undertook a global transcriptomic analysis at 60 DABB. Vascular-enriched rootstock RNA was also extracted at time points corresponding to floral bud initiation (120 DABB) and slowing of scion growth (180 DABB). Read counts of 10 063 621 to 17 934 007 million were obtained. Using an adjusted P value of < 0.05 as a cutoff, we identified 8880 DEGs between ‘M9’ and ‘M793’, and 7827 DEG between ‘M27’ and ‘M793’. Genes with a [log2 fold change] >1 were selected for further analysis (Figure 2). We reasoned that DEGs common to both dwarfing rootstocks could yield information about biological processes that are essential for rootstock-induced dwarfing. Compared with ‘M793’, 1576 genes were upregulated and 1760 were downregulated in both dwarfing rootstocks (Figures 2a and b).

The 3336 DEGs common to both ‘M9’ and ‘M27’ were analysed by Fisher’s exact test using the Mapman ontology. 43 This revealed...
functional categories that were over-represented in both upregulated and downregulated DEGs (Figure 3). Throughout this paper, gene expression is presented as relative to the vigorous 'M793'. Gene ontology categories that were over-represented in upregulated DEGs were involved with carbohydrate metabolism, cell wall degradation, redox and post-translational modification. DEGs involved with amino acid and protein synthesis, secondary metabolism and hormone response were over-represented in
downregulated genes. Stress response and kinase signalling genes were found in both up- and downregulated DEGs.

Dwarfing rootstocks contain more starch and less glucose and fructose. Of the DEG involved in primary metabolism, amino acid and lipid metabolism showed a similar trend; genes promoting biosynthesis were downregulated in dwarfing rootstocks, while those promoting degradation were upregulated (Table 1). We used the KEGG and gene ontologies to visualise metabolic pathways identified as having over-represented DEGs. Genes in the KEGG pathway of fatty-acid synthesis were particularly downregulated in dwarfing rootstocks (Supplementary Figure S1).

In contrast, DEG in the starch and sucrose metabolism pathway showed the opposite trend. *MdStarch synthesis* genes (*MdBSS2*) were upregulated in dwarfing rootstocks, whereas starch breakdown genes *MdBeta-amylase* (*MdBAM1*) were downregulated (Table 1). Sucrose synthase (SUS) reversibly hydrolyses sucrose into fructose and UDP-glucose. *MdBSS2* genes were downregulated in dwarfing genotypes (Table 1). We compared the expression of starch and sucrose metabolism genes over three time points during the year. Both *MdBSS2* genes (MDP0000842179 etc.) had a similar trend; genes promoting biosynthesis were downregulated in dwarfing rootstocks, while those promoting degradation were upregulated (Table 1).

### Table 1. Selection of DEG common to both 'M9' and 'M27' relative to 'M793'

| Matrix Gene ID | Matrix Gene Name | Position (bp) | Annotable | Genes | Annotative | Genes Annotate | log2(M9/M793) | p-value | log2(M27/M793) | p-value | GO biological process |
|---------------|-----------------|---------------|-----------|-------|-----------|----------------|--------------|---------|-----------------|---------|-------------------|
| MDP0000842179 | *MdBSS2* | 34922465 | 163 | *COSY3| | | 1.51 | 0.003 | 0.22 | 0.28 | Starch biosynthesis |
Reduced cell wall synthesis in dwarffootstocks

Genes promoting cell wall biosynthesis and modification were downregulated in dwarffootstocks, whereas genes promoting cell wall hydrolysis and catabolism were upregulated. Multiple cellulose synthase genes were downregulated in dwarffootstocks, whereas cellulases, pectin lyase and glycosyl hydrolases were upregulated (Table 1). To investigate this further, gene expression was measured by qRT-PCR over a time course. MdCellulose synthase A (MdcCES A, MDP0000313995), which encodes a key enzyme in secondary cell wall synthesis, was expressed higher in ‘M793’ throughout the year (Figure 5a). In contrast, genes promoting cell wall degradation were upregulated in dwarffootstocks (Table 1). MdBeta-D-xylosidase 7 (MdBXL7, MDP0000156045) encodes a glycoside hydrolase involved with the degradation and reorganisation of the cell polysaccharides. MdBXL7 was expressed much higher in the dwarffootstocks throughout the year (Figure 5b).

DEG shows flux towards flavonoid and away from lignin biosynthesis in dwarffootstocks

Many of the secondary metabolism genes were downregulated in dwarffootstocks relative to ‘M793’ (Table 1). 4-coumaroyl CoA is a key branch point at which compounds divert to either the flavonoid or lignin synthesis pathway. The KEGG pathway for phenylpropanoid biosynthesis shows the upregulation of the lignin synthesis pathway in vigorous rootstock tissue (Figure 6a). MdCaffeic acid O-methyltransferase (MdCAOMT, MDP0000656929), which encodes a key enzyme in lignin biosynthesis, was expressed at much lower levels in the dwarffootstocks throughout the year (Figure 6b). Much of the lignin produced is incorporated into secondarily thickened cell walls, especially that of xylem cells. MdFlavonoid 3’ hydroxylases (MdF3’H, MDP0000616265, MDP0000190489) were upregulated in dwarffootstocks (Figure 6c and Table 1).
The upregulation of MdF3H in dwarfing rootstocks suggests an increase in flavonoid biosynthesis (Supplementary Figure S4). To identify any differences in secondary metabolite concentration between dwarfing and non-dwarfing rootstocks, we performed LC–HRAM–MS on stem tissue from the 'RG'/M9 and 'RG'/RG trees used for carbohydrate analysis. The LC–HRAM–MS workflow produced a metabolite data table containing intensity values for 200 metabolites with each metabolite labelled by accurate mass m/z and liquid chromatography retention time. Forty-four metabolites that differed in concentration between 'RG' and 'M9' were selected for further analysis (Supplementary Figure S5). The majority of the compounds that could be tentatively identified were flavonoids, 19 were found in 'M9' and 3 in 'RG' (Table 2). Different isoforms of phloretin coumarylglucoside over-accumulated in both 'M9' and 'RG'. There was a tenfold higher concentration of the amino-acid arginine in 'M9' relative to 'RG'.

Figure 4. Dwarfing rootstocks accumulate more starch and less glucose and fructose. Relative expression levels of (a) Mdstarch synthase 2 (MdSS2) and (b) Mdsucrose synthase 4 (MdSUS4). Symbols are averages of four to six biological replicates, bars are s.e. Average values of (c) starch, (d) sorbitol, (e) glucose, (f) fructose and (g) myo-inositol in the roots, the rootstock stem and scion stem of 'RG'/M9 and 'RG'/RG grafted trees; bars indicate standard error. Values were compared by analysis of variance (ANOVA); significant differences between rootstock treatments are indicated by asterisks, *P value < 0.05, **P value < 0.01.
Figure 5. Reduced cell wall synthesis in dwarfining rootstocks. Relative expression of (a) MdCellulose synthase A (MdCES A), a key cell wall synthesis gene, and (b) MdBeta-D-xylosidase 7 (MdBXL7), which promotes cell wall degradation. Symbols are averages of four to six biological replicates; bars are s.e.

Figure 6. Differentially expressed gene (DEG) in phenylpropanoid biosynthesis pathways. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways showing genes that promote phenylpropanoid biosynthesis. The left side of each box represents the relative expression of 'M27'/ 'M793', the right is 'M9'/ 'M793'; red is upregulated, green is downregulated. Relative expression of (c) MdCaffeic acid O-methyltransferase (MdCAOMT) and (d) MdFlavonoid 3' hydroxylase (MdF3'H). Symbols are averages of four to six biological replicates; bars are s.e.
Most of the DEGs involved with hormone synthesis and response were downregulated in dwarfing rootstocks (Table 1). Abscisic acid synthesis and response genes were highly downregulated in dwarfing rootstocks. Both copies of the auxin influx transporter, LIKE AUXIN RESISTANT 2 (MdLAX2, MDP0000020317 and MDP0000155074) and two Small Auxin Upregulated (SAUR) genes (MDP0000737171 and MDP0000148780) were downregulated in dwarfing rootstocks. A Grechen Hagen 3.6 gene (MdGH3.6, MDP0000402444), involved with auxin homeostasis, was upregulated. 

Redox status, signalling kinases and stress response

Most of the DEG genes regulating cellular redox homeostasis were upregulated in dwarfing rootstocks. Both copies of Dehydroascorbate reductase, central to ascorbate and glutathione metabolism, were expressed at much higher levels in dwarfing rootstocks (Table 1). Three peroxidase genes were highly upregulated in dwarfing rootstocks, indicating response to oxidative stress. The only redox genes that were downregulated were three encoding 20G-Fe (II) oxygenase proteins.

Genes encoding leucine-rich repeat transmembrane kinases were upregulated in both 'M9' and in the dwarfing rootstocks (Supplementary Table S3). Type VIII leucine-rich repeats were upregulated in dwarfing rootstocks, whereas type XII were predominately upregulated in 'M793'. The only functional category that was upregulated only in 'M9' and 'M27' were the Wall-associated kinases (WAK), which bind pectin and are often associated with response to biotic stress. All five of the WAK genes were expressed at significantly higher levels in the dwarfing rootstocks. This result was confirmed by qPCR (Supplementary Figure S6). Biotic stress response genes were upregulated in both 'M793' and the dwarfing rootstocks.
ment accordingly. Sensing nutrient status and coordinating growth and development not just as nutrients, but also as signalling molecules capable of meristem outgrowth in rose, pea and sorghum. The low very low levels of the glucose and fructose relative to accumulated large amounts of starch in roots and stem, yet had metabolic pathways or glycolysis is limiting. We found that Normally, starch reserves in roots are catabolized when carbon for before any visible effects are apparent in either rootstock or scion. Amino acid, lipid and cell wall biosynthesis pathways are in a general state of reduced cell growth and metabolism long before any visible effects are apparent in either rootstock or scion. Reduced biogenesis in dwar... Reduced root mass and hydraulic conductance observed in and modi... cell wall synthesis pathways are highly downregulated in these observations and our findings, we propose that apple dwarfing rootstocks act as super sinks, holding excess starch reserves at the expense of both root and scion growth. Competition between sink tissues is a well-documented phenomenon in fruiting trees. Guitton et al. described molecular signatures of carbon starvation and oxidative stress in apple buds from heavily flowering trees with a high propensity for biennial bearing. They suggest that competition for carbohydrates between developing fruit (strong sinks) and nearby apical buds (weaker sinks) leads to a local carbon depletion and reduced cellular activity in the vegetative meristems, thus blocking the onset of floral development.

New insight into reduced auxin transport in dwarfing rootstocks Researchers have long speculated that rootstock-induced dwarfing involved reduced auxin transport. Reduced auxin transport in ‘M9’ was finally proven late last century, but few advances have been made since towards identifying the underlying concentration of glucose, fructose and myo-inositol in trees with ‘M9’ rootstocks would have a significant impact on the physiology of both rootstock and scion. Colby used double-grafted apple trees to demonstrate that interstocks (stem segments) of ‘M9’ grafted onto seedling roots retained high concentrations of starch in the roots and reduced growth of scion, whereas interstocks of an invigorating genotype depleted all starch from ‘M9’ roots and led to vigorous growth of the scion. Higher starch concentrations in citrus roots have also been correlated with reduced scion growth. On the basis of these observations and our findings, we propose that apple dwarfing rootstocks are impaired in sensing and/or maintaining the balance between starch reserves, cellulose and hemicelluloses for glycolysis and cell metabolism. Because of this defect, dwarfing rootstocks act as ‘super sinks’, holding excess starch reserves at the expense of both root and scion growth.

Imbalanced carbon allocation influences growth and development Normally, starch reserves in roots are catabolized when carbon for metabolic pathways or glycolysis is limiting. We found that ‘M9’ accumulated large amounts of starch in roots and stem, yet had very low levels of the glucose and fructose relative to ‘RG’. Even ‘RG’/‘M9’ scions accumulated more starch and less glucose and fructose than scions in the homo-grafted trees, indicating a non-autonomous effect of ‘M9’ on carbohydrate allocation. Myo-inositol concentrations were also significantly lower in ‘M9’ roots and scions on ‘M9’ rootstocks. Myo-inositol is commonly used in tissue culture media and has been shown to promote apple and pear root and shoot growth in a dosage-sensitive manner. A number of reviews have illustrated the role of sugars not just as nutrients, but also as signalling molecules capable of sensing nutrient status and coordinating growth and development accordingly. For example, sugar promotes lateral meristem outgrowth in rose, pea and sorghum. The low

Figure 7. Decreased auxin and cytokinin signal transduction in dwarfing rootstocks. Relative expression of (a) the auxin efflux transporter MdAuxin Resistant 1 (MdAux1), auxin response genes (b) MdSmall Auxin Upregulated 32 (MdSAUR32), (c) Gretchen Hagen 3.6 (GH3.6) and (d) Cytokinin dehydrogenase 7 (MdCKX7). Symbols are averages of four to six biological replicates; bars are s.e.

DISCUSSION

Reduced biogenesis in dwarfing rootstocks

The results of this study indicate that apple dwarfing rootstocks are in a general state of reduced cell growth and metabolism long before any visible effects are apparent in either rootstock or scion. Amino acid, lipid and cell wall biosynthesis pathways are downregulated in dwarfing rootstocks, whereas the degradation pathways of these compounds are upregulated. Lignin and cellulose biosynthesis pathways are highly downregulated in dwarfing rootstocks, consistent with reduced cell wall synthesis and modification, and possibly a contributing factor to the reduced root mass and hydraulic conductance observed in dwarfing rootstocks.
mechanism responsible. The results of our RNAseq analysis indicated that the phenylpropanoid pathway showed flux towards flavonoid biosynthesis and away from lignin biosynthesis in dwarfing rootstocks. LC–HRAM—MS data confirmed the over-accumulation of flavonoids in ‘M9’ relative to ‘RG’. Both genetic and metabolomic analyses have provided strong evidence that flavonoids inhibit polar auxin transport.⁶¹–⁶⁸ Plants that over-accumulate flavonoids have reduced auxin transport and dwarfed phenotypes,⁶⁷,⁶⁸ and those that are blocked in flavonoid synthesis or glycosylation (CHS) have increased auxin transport and also show abnormal root and shoot phenotypes.⁶²–⁶⁶ Chalcone synthase catalyses the first committed step in flavonoid biosynthesis. Silencing of the apple CHS gene completely removed many of the same flavonoid compounds that over-accumulated in ‘M9’.⁶⁶ Future research is needed to explore the role of flavonoids in rootstock-induced dwarfing. The reduced expression of MdAUX1 and MdLAX2 observed in the dwarfing rootstocks may also contribute to reduced polar auxin transport.

Interactions between sugar and hormone signalling
Auxin and cytokinin act antagonistically to regulate root and shoot growth, the outgrowth of axillary meristems and the synthesis and transport of one another.⁶⁹ Both have been implicated in the mode of rootstock-induced dwarfing.¹⁶ Downregulation of SAUR genes in dwarfing rootstocks indicates a reduced auxin response. Expression of MdAUX1 and the auxin conjugating MdGH3.6 was variable over the season, which likely reflects the fact that auxin signalling is tightly regulated by feedback, feedforward and cross-talk with other signalling pathways. Cytokinin signal transduction genes were downregulated, which is consistent with previous reports of lower cytokinin concentrations in ‘M9’ and ‘M27’ xylem sap.⁷⁰ MdCKX7, which encodes a cytokinin-degrading enzyme, was upregulated in dwarfing rootstocks. Overexpression of CKX genes in Arabidopsis and tobacco results in slow-growing, dwarfed shoots and a reduction in soluble sugars.⁷¹–⁷³ Transgenic manipulation of cytokinin activity has implicated cytokinin in regulating sink strength in storage organs.⁷⁴–⁷⁷

The xylem sap of ‘M9’ and ‘M27’ has higher ABA concentrations than that of vigorous rootstocks.²² In contrast, we found that multiple ABA biosynthesis and response genes were downregulated in dwarfing rootstocks. Glucose increases expression of the ABA biosynthetic genes (ABA1-ABA3), which were also identified as glucose insensitive (gin) mutants.⁷⁶–⁷⁸ Numerous studies have demonstrated a clear connection between sugar-sensing pathways and hormone metabolism and signalling.⁷⁰,⁷⁹–⁸³ Sugar promotes auxin biosynthesis⁸³–⁸⁵ and polar auxin transport.⁸⁶,⁸⁷ Glucose and auxin transcriptionally regulate many of the same genes and appear to act synergistically in plant development.⁸⁸ Cytokinin and glucose also share many transcriptional targets.⁸⁹ The gin2 mutant, which is defective in glucose sensing, is hypersensitive to cytokinin and insensitive to auxin.⁹⁰ It is unclear whether the reduced glucose concentrations in dwarfing rootstocks are the cause or result of altered hormone levels or signalling, but changes to either would have a profound impact on growth and development of roots and shoots.

A recent publication comparing the transcriptomes of genetically identical Malus roots that had been grafted to different scion genotypes also found that hormone signal transduction and sugar metabolism genes were highly represented in the DEGs.⁹⁰ The authors concluded that the scion genotype can affect root phenotype by altering sugar metabolism, and auxin and cytokinin signalling. Our results indicate that the rootstock genotype influences scion growth by the same signalling pathways.

Genetic variation between ‘M9’ and ‘M27’ may influence gene expression
The aim of this study was to identify DEGs common to two different dwarfing rootstocks. However, it is worth noting that there were a large number of DEGs that were unique to ‘M9’ or ‘M27’, and even DEGs that were common to both did not always show the same degree of differential expression. ‘M9’ is a parent of ‘M27’,¹¹ and while both contain the dwarfing loci Dw1 and Dw2, the non-dwarfing alleles of these loci, as well as many other unlinked loci differ between these two genotypes.¹²–¹⁵ Harrison et al.¹⁵ have reported that the specific allelic combinations at Dw1, Dw2 and a region of LG13 influence the expression of rootstock-induced dwarfing. Allelic variation at these or other loci could be responsible for the differences in gene expression observed between ‘M9’ and ‘M27’. We could not identify any obvious candidate genes that would affect carbohydrate metabolism or flavonoid biosynthesis within either the Dw1 or Dw2 mapping intervals. Further research is needed to identify the genetic basis for the transcriptional changes identified in this study.

CONCLUSIONS
The most consistent manifestation of apple dwarfing rootstocks is earlier termination of primary axis and sylleptic shoot growth and reduced biomass accumulation in both roots and shoots. This generally manifests within the first year of growth after grafting and becomes more pronounced with successive growth seasons and fruiting. Our results indicate that dwarfing rootstocks do not respond to sugar depletion and eventually run out of carbon to support growth in both roots and scion. By holding excess starch reserves, apple dwarfing rootstocks would be unable to provide carbon to the scion in early spring, before the scion becomes a source of fixed carbon. Once the tree began fruiting, the developing fruit would create strong carbon sinks, further decreasing competition for carbon by vegetative meristems. On the basis of our findings, we propose that excess flavonoids and reduced MdAUX1 and MdLAX2 expression contribute to the reduced auxin transport observed in dwarfing rootstocks.

CONFICT OF INTEREST
The authors declare no conflict of interest.

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