Rootstocks Modify Scion Architecture, Endogenous Hormones, and Root Growth of Newly Grafted ‘Royal Gala’ Apple Trees

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ABSTRACT. ‘Royal Gala’ apple scions (Malus × domestica) were grafted onto 1-year-old rootstock stools of ‘M.9’ (M.9), ‘M.M.106’ (MM.106), ‘Merton 793’ (M.793), and ‘Royal Gala’ [R.G (control)] to elucidate how the dwarfing apple rootstock (M.9) modified scion architecture, the time from grafting when this started, and whether changes in scion architecture were explained by some endogenous hormones present within the scion. At the end of the first season of growth (April), the final length and node number of the primary shoot were similar for scions on M.9 and R.G. However, M.9 appeared to limit the number of secondary shoots formed on the primary shoot during summer. In addition, the proportion of secondary shoots that were actively extending in fall was lower for M.9; consequently, the final mean length of the secondary shoots was slightly shorter for M.9 compared with R.G. Collectively, these subtle effects of M.9 significantly reduced the final total shoot length of the scion compared with R.G. The final dry weight of the scion and root system was also lower for M.9 than MM.106, M.793, and R.G. The mean rate of indole-3-acetic acid diffusing from the apex of the primary shoot progressively declined from February onward irrespective of rootstock, whereas the mean concentration of zeatin riboside (ZR) in the xylem sap increased during the same period, and these events appeared to coincide with cumulative increases in the number of axillary growing points formed on the scion. Despite this general trend, M.9 had a greater concentration of ZR in the xylem sap during February compared with R.G, but the primary shoot on M.9 did not develop more axillary growing points, indicating that other endogenous hormonal signals were also involved in regulating scion branching. By March, M.9 lowered gibberellin A19 (GA19) concentration in the xylem sap of the scion significantly compared with R.G. We conclude that dwarfing apple rootstocks may limit root-produced GA19 supplied to shoot apices of the scion, where GA19 may be a precursor of bioactive gibberellin A1 required for shoot extension growth.

Elucidating how dwarfing apple rootstocks modify scion architecture and the precise time after propagation when this first starts is essential to identify clearly those processes that are the first physiological causes of rootstock-induced scion dwarfing from those that are subsequent developmental effects. In the first year of growth after propagation of the composite tree, M.9 modified scion architecture by reducing the mean length (Cannon, 1941; Rao and Berry, 1940) and node number (Costes et al., 2001; van Hooijdonk et al., 2010) of the primary shoot. In addition, fewer axillary buds on the primary shoot tended to grow out; thus, fewer secondary shoots formed (Jaumien et al., 1993; van Hooijdonk et al., 2010). In contrast, others reported that, irrespective of rootstock vigor, the final mean length of the primary shoot was similar at the end of the first year of growth after propagation of the composite tree (Seleznyova et al., 2008; Tukey and Brase, 1941). Subsequently, scion vigor was first reduced by the dwarfing apple rootstock in Year 2, after it increased flowering of the scion (Seleznyova et al., 2008). Thus, dwarfing apple rootstocks appear to initially modify scion architecture in either the first or early in the second year of growth after propagation of the composite tree.

Lockard and Schneider (1981) hypothesized that dwarfing apple rootstocks control scion vigor by reducing the basipetal transport of indole-3-acetic acid (IAA) from scion to root that limits root growth and/or cytokinin biosynthesis and consequently the amount of root-produced cytokinin supplied to the scion in the xylem vasculature. In support of this hypothesis, cytokinins were identified in the xylem sap of apple trees (Jones, 1973; Kamboj et al., 1999a), and ‘Fiesta’ scions on M.9 contained a lower total concentration of zeatin (Z) plus ZR in their xylem sap than scions on MM.106 (Kamboj et al., 1999a). Atkinson et al. (2003) reported that the graft union of a composite apple tree may also contribute to rootstock-induced scion dwarfing by limiting the movement of water, nutrients, and hormones into the scion. However, the total concentration of cytokinins in the xylem sap of composite trees on a dwarfing apple rootstock was similar above (i.e., in scion xylem sap) or below (i.e., in root exudate) the graft union (Kamboj et al., 1999a). Thus, it appears the root system rather than the graft union is the primary part of the M.9 rootstock responsible for limiting the supply of cytokinins to the scion.

A lower amount of root-produced cytokinins supplied to the scion on a dwarfing apple rootstock was postulated to limit shoot extension growth of the scion (Jones, 1973; Kamboj et al., 1999a). Despite this general trend, M.9 had a greater concentration of ZR in the xylem sap during February compared with R.G, but the primary shoot on M.9 did not develop more axillary growing points, indicating that other endogenous hormonal signals were also involved in regulating scion branching. By March, M.9 lowered gibberellin A19 (GA19) concentration in the xylem sap of the scion significantly compared with R.G. We conclude that dwarfing apple rootstocks may limit root-produced GA19 supplied to shoot apices of the scion, where GA19 may be a precursor of bioactive gibberellin A1 required for shoot extension growth.
Indeed, Jones (1973) showed that cytokinin stimulated the outgrowth of rootless apple shoots in vitro. Kamboj et al. (1999a) also reported that increased rates of shoot extension growth for composite apple trees on vigorous rootstocks may be explained because of a higher total concentration of cytokinins in the xylem sap of their scion; however, their study presented no shoot growth data to support this hypothesis. If endogenous cytokinins do regulate shoot extension growth of the scion, it would be reasonable to expect exogenous cytokinins to stimulate the development of longer annual shoots. When applied to the scion of young composite apple trees, benzylaminopurine (BAP) did not stimulate primary growth (Wertheim and Estabrooks, 1994) or secondary shoots (Popenoe and Barritt, 1988). Rather, BAP stimulated axillary buds along the primary shoot to break and form secondary shoots (Kender and Carpenter, 1972; van Hooijdonk et al., 2010; Williams and Stahly, 1968). Thus, a lower endogenous concentration of cytokinins in the xylem sap of the scion on M.9 (Kamboj et al., 1999a) may contribute to scion dwarfing by limiting the total number of axillary growing points (i.e., spurs + shoots) that form on the primary shoot.

Dwarfing apple rootstocks are also reported to limit scion vigor by increasing the proportion of annual shoots that end extension growth early during the growing season (Robitaille and Carlson, 1976; van Hooijdonk et al., 2010). Unlike cytokinins, gibberellin(s) applied to apple scions stimulated shoot extension growth (Robitaille and Carlson, 1976; Sironval et al., 1962; van Hooijdonk et al., 2010) and increased the proportion of annual shoots that grew late in the season (Lluckwill and Silva, 1979). Gibberellins were found in the xylem sap of apple trees (Jones and Lacey, 1968; Motosugi et al., 1996), indicating that apple roots also synthesize and supply gibberellins to the scion. Compared with vigorous rootstocks, dwarfing rootstocks had lower endogenous concentrations of gibberellin-like substances within the root (Yadava and Lockard, 1977), xylem sap (Ibrahim and Dana, 1971), and leaves or shoots (Fontana-Degradi and Visai, 1978). Listed in order of most to least abundant, the xylem sap of apple trees was reported to contain gibberellin A19, gibberellin A53 (G A53), gibberellin A23 (G A23), gibberellin A44 (G A44), gibberellin A15 (G A15), gibberellin A17 (G A17), and gibberellin A18 (G A18) (Motosugi et al., 1996). These gibberellins are biologically inactive, but forms like GA19 concentration within the xylem sap of the scion. Rates of IAA diffusing from the apex of 'Royal Gala' primary shoots on different size-controlling rootstocks were also measured to further understand possible shoot–root relationships between shoot-produced IAA and root-produced cytokinins present in the xylem sap.

Materials and Methods

SITE, TREE MATERIAL, AND CULTURAL MANAGEMENT. An experiment was conducted during the 2005–2006 growing season at the Plant Growth Unit, Massey University, Palmerston North, New Zealand. On 1 Sept. 2005, 'Royal Gala' scions were cleft-grafted at a height of 350 mm onto 30 1-year-old rootstock stools of M.9 [clone ‘NZ9’ (dwarf)], MM.106 (semi-dwarf), M.793 (vigorous), and R.G (very vigorous, self-rooted control). The root systems of newly grafted trees were bedded into moist sawdust immediately after grafting. Trees were subsequently planted into 50-L black polyethene bags on 20 Sept., placed outside on a weed mat, and arranged as a single row. Planting height was standardized for each tree to leave 150 mm of rootstock stem above the surface of the growing medium. The growing medium was a mixture of 1 bark:1 pumice (by volume) containing 3 g L⁻¹ 14-month slow-release fertilizer [15N-4P-7.5K (Osmocote; Scotts, Marysville, OH)]. Supplementary fertilizer [10N–13P–17K (Peters Professional; Scotts)] was dissolved in water (2 g L⁻¹ H₂O), and 1 L of this solution was applied manually to the growing medium of each tree every 14 d. The irrigation system consisted of a 19-mm polytube line to which pressure-compensating minisprinklers were attached (one 4-L-h⁻¹ minisprinkler per tree). Irrigation was scheduled daily for 1 h at dawn and dusk using an automated time controller (Smart Valve Controller; Hunter, San Marcos, CA). In mid-October, scions were debudded to a single shoot and thereafter received no pruning.

MEASUREMENTS OF TREE GROWTH AND SAMPLE COLLECTION. Six trees per rootstock were randomly selected for destructive harvest on 12 Dec., 9 Jan., 9 Feb., 6 Mar., and 6 Apr. At each harvest, the diameter (D) of the primary shoot was measured 20 mm from its base with digital calipers, and shoot cross-sectional area (SCA) was calculated as πD²/4. Measurements of shoot lengths and node numbers began at the shoot base and ended at the first un unfurled leaf at the shoot apex. The number of spurs (less than 25 mm with minimal internode extension) and secondary shoots (25 mm or greater with internode extension) arising from axillary buds on the primary shoot were also counted. The term “axillary growing points” is used to describe the total number of both spurs and secondary shoots formed per scion. At the March and April harvests, the percentage of secondary shoots that had ended extension growth was calculated for each tree replicate by dividing its number of secondary shoots that had terminated in a terminal bud by its total number of secondary shoots × 100.

To measure the diffusion of IAA, the apex of each primary shoot was excised 20 mm beneath its tip to leave two small unfurled leaves per apex. The basal 5 mm of the shoot apex was placed immediately into 700 µL 2-(N-morpholino)ethanesulfonic acid (M3671; Sigma-Aldrich, St. Louis, MO) buffer solution [0.05 mol L⁻¹, pH 6.2 (Li and Bangerth, 1999)] contained in a 1.5-mL polypylene microcentrifuge tube (with the lid remaining open). Samples were then placed in a dark room with an air conditioning unit operating to maintain a constant air temperature of 25 °C. An automated misting unit (505;
Defensor, Zurich, Switzerland) was used to maintain high humidity. After 10 h of incubation, the base of each apex was recut with a sharp scalpel before being placed into fresh buffer solution. The bases of shoot apices were recut at 10 h to expose fresh phloem tissue, thereby sustaining IAA diffusion into the buffer by minimizing any phloem blockage that may have resulted from wound healing responses. At 20 h, apices were stored at –20 °C until analysis. The root system of each tree was washed to remove the growing medium. To obtain the total root length of each root system, fine (less than 2 mm in diameter) and coarse roots (greater than 2 mm in diameter) were measured using either a root length scanner (Commonwealth Aircraft Corp., Melbourne, Australia) or a ruler, respectively. Roots, leaves, and stems were oven-dried at 70 °C to a constant weight.

**Quantification of indole-3-acetic acid.** Sep-Pak® C18 columns (Waters, Milford, MA) were each preconditioned sequentially with 5 mL 100%, 50%, 15%, and 0% solutions (v/v) of methanol and 0.1 mol·L⁻¹ formic acid. Each sample of IAA diffusate was spiked with 250 Bq carboxyl-labeled IAA methyl ester (³¹C-IAA-Me) as an internal standard before adsorption onto a preconditioned C18 column. The ³¹C-IAA-Me internal standard was synthesized by methylating carboxyl-labeled IAA (Sigma-Aldrich) with ethereal diazomethane. Each C18 column was eluted with 5 mL 15% methanol before IAA and ³¹C-IAA-Me was removed from the C18 in 5 mL methanol. Methanol was evaporated to dryness under vacuum, and samples were resuspended in starting solvent [20% acetonitrile:80% 0.04 mol·L⁻¹ acetic acid solution (v/v) adjusted to pH 3.38 with triethylamine (TEA)] before high-performance liquid chromatography (HPLC).

HPLC of hormones was made using an octadecyl silica (ODS) column (220 × 4.6 mm i.d.) in line with an ODS guard column (3 × 4.6 mm i.d.) (Brownlee Sphero-5; Applied Biosystems, Foster City, CA). Solvent was delivered to the column at 1 mL·min⁻¹ using a Waters 501 and a Waters 510 HPLC pump controlled by an automated gradient controller. Solvents were HPLC-grade acetonitrile and TEA; their elution gradient was: 20% acetonitrile from 0 to 14 min, 40% acetonitrile from 15 to 20 min, and 98% acetonitrile from 22 to 25 min returning to 20% acetonitrile at 30 min.

During HPLC of diffusate samples, a scanning florescence detector (Waters 474) was used to quantify IAA (Crozier et al., 1980) at a retention time of 14.26 min. The limit of quantitation for IAA measured by florescence detection was 1 ng; the standard curve produced (10 to 100 ng) was linear (r² = 0.99). Before HPLC of diffusate samples, the retention time of ³¹C-IAA-Me was established (24.30 min) using a flow-through radioactivity detector (βRam; In/Us systems, Tampa, FL). During HPLC of samples, ³¹C-IAA-Me within each diffusate sample was collected based on its known retention time. Because the radioactivity detector was not used during HPLC of diffusate samples, collected fractions of ³¹C-IAA-Me were taken to a liquid scintillation counter (Tri-Carb 2900TR; PerkinElmer, Boston, MA) to count their radioactivity. The recovery of radioactivity within each sample was used to correct for losses of endogenous IAA incurred during purification. Average recovery of ³¹C-IAA-Me was 70%.

**Quantification of cytokinins and gibberellins.** Sep-Pak® C18 columns were each preconditioned sequentially with 5 mL 100%, 50%, 10%, and 0% solutions of methanol and 0.1 mol·L⁻¹ formic acid (v/v). Before adsorption onto a C18 column, xylem sap (≤5 mL) was spiked with 333 Bq tritiated dialcohols of ZR ([³²H]-ZR-dialcohol) and isopentenyladenosine ([³²H]-IPA-dialcohol) (synthesized by Currie, 1997) and 50 ng deuterium-labeled GA₁₉ ([³²H]₂GA₁₉) (purchased from Olchemim Ltd., Olomouc, Czech Republic) as internal standards. Each column was eluted with 5 mL 0.1 mol·L⁻¹ formic acid before cytokinins and the gibberellin were recovered from the C₁₈ in 5 mL methanol.

For cytokinin ribotides, the 5 mL of 0.1 mol·L⁻¹ formic acid used to wash the C₁₈ after loading of the sap sample was collected and incubated with phosphatase to convert ZR-5-monophosphate and IPA-5-monophosphate to ZR and IPA, respectively. In short, the 5 mL of 0.1 mol·L⁻¹ formic acid was spiked with 333 Bq [³²H]-ZR-dialcohol and [³²H]-IPA-dialcohol, dried in a vacuum freeze drier, resuspended in 1 mL 20 mmol·L⁻¹ Tris buffer (pH 9.8) containing 5 units alkaline phosphatase (P552; Sigma-Aldrich) and incubated at 37 °C for 4 h in a water bath. After incubation, each sample was adsorbed onto a preconditioned C₁₈ column, the C₁₈ was eluted with 5 mL 0.1 mol·L⁻¹ formic acid, and ZR and IPA were recovered from C₁₈ in 5 mL methanol.

Each fraction of methanol was dried under vacuum, resuspended in HPLC starting solvent (6% acetonitrile), and separated with the aforementioned HPLC and solvent system described for IAA. The solvent gradient for cytokinins was: 9% acetonitrile from 0 to 15 min, 27% acetonitrile from 20 to 30 min, and 95% acetonitrile from 35 to 40 min returning to 9% acetonitrile at 45 min. Endogenous Z, ZR, IPA, or isopentenyladenine (2iP) were collected from the HPLC between 11 to 14 min, 18 to 21 min, 27 to 28 min, or 30 to 33 min, respectively. These elution times for endogenous cytokinins were established previously using authentic cytokinin standards (Sigma-Aldrich), the aforementioned HPLC and solvent gradient, and a programmable ultraviolet detector (Waters 490E) set at 268 nm. The [³²H]-ZR-dialcohol and [³²H]-IPA-dialcohol internal standards were also collected during HPLC as separate fractions between 9 to 11 min and 21 to 23 min, respectively. To determine the losses of cytokinins that occurred during chromatography, fractions of [³²H]-ZR-dialcohol and [³²H]-IPA-dialcohol were taken to a liquid scintillation counter and counted for radioactivity. Average recovery of the [³²H]-ZR-dialcohol and [³²H]-IPA-dialcohol internal standard was 70% and 71%, respectively.

HPLC fractions containing endogenous Z, ZR, IPA, or 2iP were quantified by radioimmunoassay (RIA) using the methodology described by MacDonald et al. (1981) and Weiler (1980). RIA of Z and ZR or IPA and 2iP used polyclonal antisera to ZR or IPA, respectively. The antisera, [³²H]-ZR-dialcohol, and [³²H]-IPA-dialcohol, used in the RIA were synthesized at Massey University by Currie (1997). The RIA was most sensitive between 0.03 pmol and 60 pmol of Z, ZR, IPA or 2iP; the standard curves generated were linear (r² = 0.99) following logit transformation (Currie, 1997). For each sap sample, the concentration of endogenous Z and ZR quantified by the RIA was corrected for losses estimated from the recovery of the [³²H]-ZR-dialcohol standard in that sample; the recovery
of $[^1\text{H}]$-IPA-dialcohol was used to correct IPA and 2iP concentrations.

To determine the retention time of gibberellin, 500 ng GA$_{19}$ (Olchemim Ltd.) was injected into the same HPLC and solvent gradient described for cytokinins, and 1-min fractions were collected. Fractions were dried under vacuum and then dissolved in a 1:1 solution (v/v) of acetonitrile and 0.01 mol L$^{-1}$ ammonium acetate. Each fraction was then manually injected into a single quadrupole mass spectrometer with an electrospray interface (MicroMass; Waters). Flow rate of sample into the mass spectrometer was 100 µL min$^{-1}$. Cone and capillary voltage were +30 V and +3 kV, respectively. Source and dissolution temperatures were 100 and 250 °C, respectively. Nitrogen was used as both dissolution gas (400 L h$^{-1}$) and cone gas (50 L h$^{-1}$). Each fraction was scanned in negative ionization mode to determine the presence of the ion for the GA$_{19}$ standard (m/z 361). The GA$_{19}$ standard was present in fractions collected between 28 to 30 min; hence, endogenous GA$_{19}$ in the xylem sap samples was collected from our HPLC between these times. HPLC fractions containing $[^1\text{H}]$GA$_{19}$ and endogenous GA$_{19}$ were dried of solvent, methylated with diazomethane, and then silylated with N-methyl-N-(trimethylsilyl) trifluoroacetamide (M7891; Sigma-Aldrich) to produce gibberelin methyl esters trimethylsilyl ethers ($[^1\text{H}]$GA$_{19}$-MeTMSi and GA$_{19}$-MeTMSi) (Croker et al., 1990). Gibberellin-MeTMSi was injected (2 µL) into a gas chromatograph (GC-2010; Shimadzu, Kyoto, Japan) connected to a mass spectrometer (GC-MSQP2010; Shimadzu). The carrier gas was helium at a flow of 1.3 mL min$^{-1}$. Sample separation was made on a capillary column [30 m, 0.25 mm i.d., 0.25 µm, film thickness (ZB-5; Phenomenex, Torrance, CA)]. The injection temperature was 270 °C, and the column start temperature was 40 °C. The injection splitter (10:1) was closed for 1 min. After 1 min, the oven temperature was increased to 180 °C at 10 °C min$^{-1}$ and then to 290 °C at 6 °C min$^{-1}$. The interface temperature, source temperature, and ionization voltage of the mass spectrometer were 200 °C, 270 °C, and 70 eV, respectively. Ions m/z 436 (retention time 24.68 min) and m/z 434 (retention time 24.70 min) of $[^1\text{H}]$GA$_{19}$-MeTMSi and GA$_{19}$-MeTMSi, respectively, were quantified by single ion monitoring. The concentration of endogenous GA$_{19}$ in each sample was determined from a calibration curve of the peak area ratios between m/z 434 and m/z 436 plotted against the ratio of GA$_{19}$-MeTMSi concentration to $[^1\text{H}]$GA$_{19}$-MeTMSi concentration. The limit of quantitation for GA$_{19}$ was 0.05 ng; the calibration curve constructed (0.05 ng to 10 ng GA$_{19}$) was linear ($r^2 = 0.99$). Average recovery of $[^1\text{H}]$GA$_{19}$-MeTMSi was 58%.

**Statistics.** The experiment was a completely randomized design with four rootstock treatments ['Royal Gala' scions on M.9, MM.106, M.793, and R.G (control) rootstocks]. Six replicate trees per rootstock were destructively measured at each of the five harvest dates. Samples from two replicate trees were pooled before hormone analysis; hence, there were only three replicates per rootstock and harvest date for all hormone data. For each harvest, data were subjected to analysis of variance (ANOVA) using the GLM procedure of SAS (Version 9.1; SAS Institute, Cary, NC). Mean separation of data with a significant ANOVA F-test ($P \leq 0.05$) was made using the least significant difference at $P = 0.05$. Allometric relationships between natural log (ln) root dry weight and ln scion dry weight were investigated using major axis regression (War ton et al., 2006) to estimate the slope of the regression lines over two 3-month periods: December to February and February to April. The regression slopes of rootstocks were compared using the method of Warton et al. (2006).

**Results**

**Scion growth.** Rootstocks did not affect the mean length or node number of the primary shoot by April (Fig. 1A–B). However, the final SCA of the primary shoot was lower on M.9 and M.793 (Fig. 1C), which reduced the final dry weight of the primary shoot stem compared with R.G (Table 1). These final differences in SCA, particularly for M.9, occurred between March and April (Fig. 1C). Rootstocks did not significantly affect the final mean internode length of the primary shoot or the number of primary shoots that ended growth early (data not shown). During January and February, there were trends ($P = 0.08$) that R.G had developed more secondary shoots than M.9, MM.106, or M.793 (Fig. 1F). By March and April, rootstocks did not significantly affect secondary shoot numbers; however, scions on M.9 had developed approximately half the number of secondary shoots than the other rootstocks (Fig. 1F). Consequently, the final mean total length and node number of the secondary shoots tended to be lower on M.9 (Fig. 1D–E). In March, 90%, 84%, 82%, and 74% ($P = 0.70$) of secondary shoots had ended extension growth for the scion on M.9, MM.106, M.793, and R.G, respectively. By April, M.9 had a lower proportion of long secondary shoots present on its scion, thereby reducing the final mean length and node number of these shoots slightly compared with R.G (data not shown). The final mean total shoot length (Fig. 1G) and dry weight of stems (Fig. 1I) was also lower for scions on M.9 than R.G. There was also a trend ($P = 0.09$) that the final total node number of the scion was lower on M.9 (Fig. 1H).

**Root growth.** From December to February, rootstocks did not differ in the mean total length or dry weight of the root system (Fig. 2A–B). However, root systems of invigorating rootstocks grew strongly from March to April, whereas the total length and dry weight of the M.9 root system did not greatly increase during this period (Fig. 2A–B). Consequently, mean dry weight and total length of the M.9 root system were smaller in April compared with R.G (Fig. 2A–B). The mean total dry weight of the M.793 root system was greater than the other rootstocks (Fig. 2B). For each rootstock, specific root weight (i.e., total root dry weight/total root length) steadily increased from January onward (Fig. 2C). By April, M.9 had a lower specific root weight than MM.106, M.793, and R.G (Fig. 2C). In contrast, specific root weight of M.793 was greater than the other rootstocks (Fig. 2C).

**Total growth per tree.** By April, the effect of M.9 was to limit dry matter accumulation in the root, rootstock stem, and stem of the primary shoot compared with R.G, collectively lowering the mean total dry weight of the tree (Table 1). M.9 also lowered the root:scion dry weight ratio by April compared with R.G, and differences between these rootstocks were apparent after March (Fig. 2D). MM.106 did not affect the final scion dry weight ratio compared with R.G (Fig. 2D), but it had a lower total tree dry weight with less dry matter allocated to the leaves of the primary shoot and the rootstock stem (Table 1). Although M.793 did not affect total dry weight of the tree compared with R.G, final dry matter allocation into the root system was greater (Table 1). However, less dry matter had accumulated in the rootstock stem of M.793 and in the leaves and stem of the primary shoot (Table 1). Increased dry
weight of the root system and decreased dry weight of the primary shoot imposed by M.793 (Table 1) increased its final root:scion dry weight ratio compared with R.G (Fig. 2D). **Allometry between Root and Scion.** The allometric coefficients ($k$) or slopes of fitted major axis regression lines (Fig. 3) were similar among rootstocks in either the December to
February ($\chi^2 = 2.5, df = 3, P = 0.48$) or in the February to April period ($\chi^2 = 2.2, df = 3, P = 0.52$) and could be estimated by a common slope in each period (Table 2). For each rootstock, $k$ was greater in February to April compared with the December to February period (Fig. 3; Table 2).

**Indole-3-acetic acid, cytokinins, and branching.** Rootstocks did not significantly affect the rate of IAA diffusion from the primary shoot apex throughout the growing season (data not shown). The rate of IAA diffusing from the primary shoot apex declined from January onward (Fig. 4). In December and January, volumes of xylem sap extracted from the primary shoot were insufficient for reliable detection and quantification of cytokinins or gibberellins. Therefore, these hormone data are reported only for February, March, and April. In order of most to least abundant, putative cytokinins identified within the xylem sap included ZR, IPA, 2iP, Z, 2iP ribotide, Z ribotide, and a novel isopentenyl-type compound recovered as 2iP after incubation of the sap with alkaline phosphatase. Concentrations of Z, cytokinin ribotides, and the novel isopentenyl-type cytokinin were very low, and rootstocks did not significantly affect the concentration of Z, cytokinin ribotides, the February than the other rootstocks (Fig. 5A), but the scion on M.9 had developed a similar number of axillary growing points (i.e., spurs + secondary shoots) to MM.106, M.793, and R.G (Fig. 6D).

**Gibberellins and shoot termination.** During February, GA$_{19}$ concentration in the xylem sap was unaffected by rootstock (Fig. 5B). In March, GA$_{19}$ concentration was lower for M.9, MM.106, and M.793 compared with R.G (Fig. 5B). There were also trends in March that mean GA$_{19}$ concentration increased with increasing rootstock vigor (Fig. 5B), and higher GA$_{19}$ concentration appeared to correspond with lower mean proportions of secondary shoots on the scion that had ended extension growth (90%, 84%, 82%, and 74% for M.9, MM.106, M.793, and R.G, respectively). However, this effect of rootstock on shoot termination was not statistically significant ($P = 0.70$). In April, xylem sap from M.9 contained a greater concentration of ZR in isopentenyl-type compound (data not shown), IPA, or 2iP at any harvest date. In February, the primary shoot on M.9 contained a greater concentration of ZR in its xylem sap than MM.106, M.793, or R.G (Fig. 5A). In March and April, however, ZR concentration was not different among rootstocks; although in April, ZR concentration appeared to increase with increasing rootstock vigor (Fig. 5A). Interestingly, mean ZR concentration in the xylem sap of the primary shoot increased for each rootstock from February to April (Fig. 5A), whereas mean rates of IAA diffusion from the apex of the primary shoot declined during this same period (Fig. 4). Hence, a putative relationship existed whereby higher mean rates of IAA diffusion from the shoot apex appeared to correspond to lower mean concentrations of ZR in the xylem sap (Fig. 6B). In contrast, concentrations of IPA or 2iP declined in the xylem sap from February to April; thus, higher mean rates of IAA diffusion from the primary shoot apex appeared to correspond with higher mean concentrations of these cytokinin forms (Figs. 6A and 6C). From February to April, increases in the mean concentration of ZR in the xylem sap appeared to correspond with cumulative increases in the mean number of axillary growing points formed on the primary shoot (Fig. 6D). However, xylem sap from M.9 contained a greater concentration of ZR in

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**Fig. 2.** Effect of rootstocks [M.9, MM.106, M.793, and ‘Royal Gala’ control (R.G)] on (A) the mean total length of the root system, (B) total dry weight (DW) of the root system, (C) specific weight of the root system, and (D) DW ratio between root system and the scion of composite ‘Royal Gala’ apple trees during their first growing season (2005–2006) after spring grafting (1 Sept. 2005). For (D), scion DW includes stems and leaves; root DW excludes the rootstock stem. An asterisk denotes a significant analysis of variance F-test ($P = 0.05$) among the rootstock treatment means for that month; the corresponding vertical bar is the least significant difference at $P = 0.05$. 

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Discussion

Mean total shoot length and total dry weight of the scion were significantly lower on M.9 compared with the R.G rootstock control by growth cessation in April (Fig. 1G; Table 1). Hence, scion dwarfing was imposed by M.9 during the first year of growth after grafting of the composite tree. Total shoot growth of the scion was limited by M.9 (Fig. 1G) mostly because fewer secondary shoots formed compared with R.G (Fig. 1F), and the mean length of the secondary shoots was slightly shorter because of a tendency for a slightly higher proportion of secondary shoots to end extension growth early. Similarly, fewer secondary shoots formed for other scion cultivars grown on M.9 (Jaumien et al., 1993), and this rootstock increased the proportion of shoots on the scion that ended extension growth early (Robitaille and Carlson, 1976). M.9 did not affect the length of the primary shoot during the first year of growth after tree grafting (Fig. 1A), and similar results were reported by Seleznyova et al. (2008) and Tukey and Brase (1941). However, others have reported that the final length of the primary shoot was shorter on M.9 by the end of the first year of growth after tree grafting (Cannon, 1941; Costes et al., 2001; Rao and Berry, 1940; van Hooijdonk et al., 2010), indicating there is a degree of plasticity concerning the year when dwarfing of the primary shoot is first expressed. When tree height is unaffected by M.9 in the first year of growth after grafting (Fig. 1A), rootstock-imposed dwarfing of the primary shoot is likely to be expressed in the second growing season after increased flowering of the scion on M.9 (Seleznyova et al., 2008).

In root:shoot allometry, a $k$ greater than one indicates that dry matter accumulation is prioritized into root rather than shoot growth (Atwell et al., 1999). From February to April, $k$ greatly increased for each rootstock (Table 2; Fig. 3) because the priority of dry matter allocation increasingly favored the root system over the scion. This increase in $k$ from February to April is due to a higher proportion of shoots ending extension growth early.

Table 2. Estimates of major-axis regression slopes ($k$) and 95% confidence intervals for the allometric relationships of Figure 2 between natural log (ln) root dry weight and ln scion dry weight during the December to February and February to April growing periods for newly grafted ‘Royal Gala’ apple trees on M.9, MM.106, M.793, and ‘Royal Gala’ [R.G (control)] rootstocks.

| Rootstock | $k$ December to February | $k$ February to April |
|-----------|--------------------------|-----------------------|
| M.9       | 0.97 (0.78, 1.21)         | 1.75 (1.46–2.14)      |
| MM.106    | 0.97 (0.86, 1.08)         | 1.57 (1.30–1.91)      |
| M.793     | 1.07 (0.94, 1.22)         | 1.86 (1.62–2.15)      |
| R.G       | 0.92 (0.76, 1.10)         | 1.78 (1.56–2.05)      |
| Common slope | 0.99 (0.92, 1.06)   | 1.76 (1.63–1.91)      |

*Data in parentheses are 95% confidence intervals of the major axis regression slopes (Fig. 2).
The physiological mechanisms that increased ‘Spartan’ on M.9 or MM.106 in their second year of growth predominated from late summer onward for trees of Webster (1989) reported that root rather than shoot extension from early March onward (Fig. 1G). Similarly, Abod and growth, which became apparent for M.9, MM.106, and R.G (Table 2; Fig. 3) coincided with the slowing of shoot extension (Waring, 1970). Putative signaling mechanisms may exist (Fig. 3) are likely to involve signaling by endogenous hormones controlling rootstocks and whether rootstocks preferentially deliver to the scion. In this study, M.9 had a lower concentration of GA19 in the xylem sap of different size-controlling rootstocks and whether rootstocks preferentially transport different gibberellins. In addition, a limitation of our study was that it did not take into account potential rootstock differences in shoot termination among the rootstocks were not significant (P = 0.70).

In a previous study, we found that scions on M.9 had a higher proportion of shoots that ended extension growth early compared with more invigorating rootstocks, and this effect of M.9 was largely prevented by applying gibberellins to the scion (van Hooijdonk et al., 2010). Similar to M.9, restricting basipetal IAA transport of vigorous rootstocks, by applying 1-N-naphthylphthalamic acid (NPA) to the rootstock stem, caused shoots on the scion to end extension growth; however, shoot growth could be reinstated by applying gibberellin to the scion (van Hooijdonk et al., 2010). Therefore, decreased basipetal transport of IAA imposed by NPA, and possibly by the stem tissue of M.9 (Kamboj et al., 1997), may limit the amount of root-produced gibberellins that is synthesized and supplied to the scion for shoot extension growth. To confirm this hypothesis, it would be necessary to demonstrate that the M.9 rootstock limits the basipetal transport of radiolabeled IAA to the root, that a limited IAA supply at the root lowers the amount of root-produced gibberellins supplied to shoot apices of the scion in the xylem sap, and that these events coincide with earlier cessation of shoot extension growth for the scion on M.9. Although this study provides new evidence that dwarfinh rootstocks can lower the concentration of root-produced GA19 in the xylem sap of the scion (Fig. 5B), in further studies, it will be important to ascertain whether other biologically important gibberellin forms are present in the xylem sap of different size-controlling rootstocks and whether rootstocks preferentially transport different gibberellins. In addition, a limitation of our hormone data (measured as nanograms per milliliter of xylem sap) was that it did not take into account potential rootstock differences in xylem sap flow and thus rates of hormone delivery to the scion.

It has been known for some time that ABA concentration within the xylem sap of the scion is higher for trees on dwarfing rootstocks (Kamboj et al., 1999b). Injection of abscisic acid (ABA) into the xylem sap of composite apple trees on M.9, M.7, or MM.111 caused the primary shoot to cease growth, although trees on the more dwarfing rootstocks ended shoot extension growth sooner after ABA treatment. However, injection of GA3 into the xylem sap increased shoot extension growth markedly more as rootstock vigor decreased (Robitaille and Carlson, 1971). A balance of both root-produced gibberellin (promoter) and abscisic acid (inhibitor) could therefore interact to impose earlier shoot termination of the scion on M.9. Lockard and Schneider (1981) hypothesized that reduced basipetal transport of IAA within the phloem of the rootstock stem to the root system may limit the synthesis of root-produced cytokinins and their consequent transport to the scion. However, restricting basipetal transport of IAA from shoot to root, by using shoot decapitation and/or NPA, increased cytokinin

(Tables 2; Fig. 3) coincided with the slowing of shoot extension growth, which became apparent for M.9, MM.106, and R.G from early March onward (Fig. 1G). Similarly, Abod and Webster (1989) reported that root rather than shoot extension growth predominated from late summer onward for trees of ‘Spartan’ on M.9 or MM.106 in their second year of growth. The physiological mechanisms that increased k from February (Fig. 3) are likely to involve signaling by endogenous hormones (Waring, 1970). Putative signaling mechanisms may exist whereby IAA transport is decreased from the shoot apex (Fig. 4) and/or in the rootstock stem during the summer (Kamboj et al., 1997), leading to increased root synthesis of some cytokinin forms including ZR (Figs. 5A and 6B). Increased ZR synthesis by the root may change the dynamics of assimilate partitioning within the composite apple tree. Evidence for this was reported for grapevines (Vitis vinifera) in which BAP applied to the root increased partitioning of 14C-photosynthate into the root at the expense of the shoot (Shindy et al., 1973). For apple, BAP applied to the root increased the root:shoot ratio compared with untreated seedlings (Richards, 1980). Elevated endogenous concentrations of gibberellins may also influence allometry because gibberellins applied to apple scions stimulated shoot extension growth (Luckwill and Silva, 1979) and lowered the root:shoot dry weight ratio of young ‘M.26’ trees (Guak et al., 2001).

The ability of exogenous gibberellins to stimulate shoot extension growth of apple shoots (Robitaille and Carlson, 1976; Sironval et al., 1962; van Hooijdonk et al., 2010) indicates endogenous gibberellins may have a role in rootstock-induced scion dwarfing. We quantified GA19 because it appeared to be an important transport form present in the xylem sap of apple (Motosugi et al., 1996) that could be converted to bioactive GA1 (Yamaguchi, 2008) by shoot apices of the scion. In this study, M.9 had a lower concentration of GA19 in the xylem sap during March compared with the vigorous R.G rootstock control, and there were general trends that GA19 concentration increased with increasing rootstock vigor (Fig. 5B). Furthermore, the lower concentrations of GA19 measured for the more dwarfinh rootstocks in March (Fig. 5B) appeared to coincide with the termination of a slightly higher percentage of secondary shoots on their scion (90%, 84%, 82%, and 74% for M.9, MM.106, M.793, and R.G, respectively). These differences in shoot termination among the rootstocks were not significant (P = 0.70).

Fig. 5. Mean concentration of (A) zeatin riboside (ZR) and (B) gibberellin A19 (GA19) in xylem sap extracted from ‘Royal Gala’ primary shoots on M.9, MM.106, M.793, and ‘Royal Gala’ [R.G (control)] rootstocks during February, March, and April of the first growing season (2005-2006) after spring grafting (1 Sept. 2005). An asterisk denotes a significant analysis of variance F-test (P ≤ 0.05) among the rootstock treatment means for that month; the corresponding vertical bar is the least significant difference at P = 0.05.
concentration in the xylem sap (Bangerth et al., 2000; Currie, 1997). Similarly, we found that ZR increased in the xylem sap of each rootstock treatment from February to April, and this increase in ZR appeared to coincide with decreasing rates of IAA diffusion from the apex of the primary shoot (Fig. 6B). Although IAA diffusion from the shoot apex does not represent the amount of IAA transported basipetally within the rootstock stem to the root, these data (Fig. 6B) indicate that decreased IAA transport from the scion may increase root-synthesized ZR. In contrast, reduced basipetal transport of IAA from the shoot apex appeared to coincide with declining concentrations of IPA and 2iP in the xylem sap (Figs. 6A and 6C). Thus, these cytokinins interacted with IAA in the manner hypothesized by Lockard and Schneider (1981). We propose a refined hypothesis whereby limited transport of IAA from scion to root of a composite apple tree may differentially affect the synthesis of different root-derived cytokinin forms. Additional studies to ours would be required to confirm this hypothesis.

Others have also identified Z (Kamboj et al., 1999a; Tromp and Ovaa, 1994), ZR (Jones, 1973; Kamboj et al., 1999a; Tromp and Ovaa, 1994), IPA, 2iP (Tromp and Ovaa, 1994), and Z ribotide (Jones, 1973) in the xylem sap of apple trees. Within the composite apple tree, it is likely that endogenous cytokinins are involved in regulating scion branching, especially because BAP applied to young apple scions stimulated axillary buds along the primary shoot to break and form secondary shoots (van Hooijdonk et al., 2010; Williams and Stahly, 1968). Interestingly, there was a trend ($P = 0.08$) that fewer secondary shoots had formed for the scion on M.9 in January (Fig. 1F), suggesting that endogenous cytokinins were limiting axillary bud outgrowth. Unfortunately, it could not be ascertained whether M.9 lowered cytokinin concentration in the xylem sap at this critical time of branch formation because insufficient sap was extractable from the scion. Nevertheless, there were trends from February onward for the mean concentration of ZR to increase in the xylem sap of each rootstock treatment, which appeared to coincide with cumulative increases in the mean number of axillary growing points formed on the primary shoot (Fig. 6D). However, xylem sap from M.9 contained a significantly higher concentration of ZR in February compared with R.G (Fig. 5A), but the primary shoot on M.9 had developed a similar number of axillary growing points (Fig. 6D). Thus, the development of axillary growing points for M.9 was not explained satisfactorily by ZR concentration alone and may have involved other hormonal substances transported in the xylem sap.

The novel isopentenyl-type compound measured in this study was also present in kiwifruit (Actinidia deliciosa) (Woolley and Currie, 2006); its presence in apple suggests there might be important cytokinin forms present in xylem sap of apple trees that are yet to be identified. Hydroxylated derivatives of benzyladenine (topolins) could be important candidates because the meta-topolin form has high bioactivity (Mok et al., 2005). Identification of strigolactones in pea (Pisum sativum), and demonstration of their role as an inhibitor of axillary bud outgrowth (Gomez-Roldan et al., 2008), also opens new possibilities that branching of apple trees is not regulated solely by interactions between shoot-derived IAA and root-produced cytokinins.

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**Fig. 6.** Relationships during the February to March growing period between the mean concentration of indole-3-acetic acid (IAA) diffusing from the shoot apex of ‘Royal Gala’ primary shoots and the mean concentration of (A) isopentenyladenine (2iP), (B) zeatin riboside (ZR), and (C) isopentenyldenosine (IPA) in the xylem sap of the primary shoot; and (D) the mean concentration of ZR in the xylem sap of the primary shoot and the mean total number of axillary growing points (GP) formed per scion during its first growing season (2005–2006) after spring grafting (1 Sept. 2005) onto M.9, MM.106, M.793, and ‘Royal Gala’ [R.G (control)] rootstocks. GP are defined as the total number of spurs plus secondary shoots formed on the primary shoot.

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