The Content and In Vivo Metabolism of Gibberellin in Apple Vegetative Tissues

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ABSTRACT. Despite the demonstrated importance of gibberellins (GAs) as regulators of fruit tree stature, information on their in vivo metabolism in apple vegetative tissues is still lacking. To determine whether the GA content and metabolism differs between dwarf and standard phenotypes and the influence of rootstocks, [¹⁴C]GA₁₂, a common precursor of all GAs in higher plants, was applied to vigorously growing apple (*Malus × domestica*) shoots collected from the scion cultivar Redcort on MM.106, a growth-promoting rootstock, and dwarf and standard seedlings on their own roots from progeny 806 (a cross between a breeding selection with reduced stature and an advanced breeding selection with a standard tree form). Twenty-one metabolites were identified by high-performance liquid chromatography (HPLC) and used as tracers for the purification of endogenous GAs. The existence of endogenous and [²H]-labeled GA₁₂, GA₁₅, GA₅₃, GA₄₄, GA₁₉, GA₂₀, and GA₃ was demonstrated by gas chromatography–mass spectrometry (GC-MS); GA₂₀ was the major GA present, with slightly less GA₁₉ and GA₄₄, and with GA₃ present at approximately one-third the level of GA₂₀. Despite specific searching, neither GA₄, GA₇, GA₁, nor GA₂₉ was found, showing that [¹⁴C]GA₁₂ is metabolized mainly through the 13-hydroxylation pathway and that GA₃ is a bioactive GA in apple vegetative tissues. The invigorating rootstock led to a slow GA metabolic rate in ‘Redcort’. For self-rooted plants, the same GAs were identified in dwarf and standard seedlings from progeny 806, although standard plants metabolized at twice the speed of dwarf plants. Young branches of dwarf 806 plants treated with GA₃ were one-third longer with more nodes but similar in internode length. We conclude that the dwarf phenotype in progeny 806 is not caused by a lack of certain GAs in the GA biosynthesis pathway downstream of GA₁₂.

Apple trees with small stature are desired for high-density planting and an early transition from juvenility to production (Miller and Tworkoski, 2003). Compared with their standard counterparts, dwarf apple trees have better light penetration, which leads to more efficient photosynthesis and better fruit quality. They also require less pruning and training and are easier to harvest (El-Sharkawy et al., 2012). Commercially grown apple trees are comprised of genetically distinct parts, the scion and the rootstock. Therefore, a reduction of vigor can be achieved either by using dwarfing rootstocks or dwarf scion cultivars (genetic dwarf). Although breeding efforts focused on rootstocks for vigor and architecture control, more breeders are interested in scion breeding for the same purpose (Brown, 2012; Byrne, 2012).

Dwarf apple plants occur naturally in certain breeding populations. Alston (1976) characterized early dwarf, crinkle dwarf, and sturdy dwarf in apple breeding populations, and Steffens et al. (1989a) identified a thermo-sensitive dwarf phenotype from a hybrid population of ‘Goldspur’ Delicious × ‘Redspur’ Delicious. A breeding selection with reduced stature and reduced internodes (Selection 1, ‘Fuji’ × Co-op 18)

was identified in the breeding populations at Cornell University. Selection 1 is able to flower and set fruit, a feature rarely seen in reduced vigor scion types and crucial for genetic studies and scion breeding. A cross between Selection 1 and a parent with good fruit quality and standard tree form generated progeny 806, which had a clear segregation of dwarf plants (13%) in the first growing season.

The small stature of these dwarf plants, their reduced internode length, and dark foliage are very similar to plants with defects in GA biosynthesis or response. GAs affect many aspects of plant growth and development and are best known for their significant effects on internode elongation in dwarf and rosette species (Davies, 2010). Most mutants deficient in GA biosynthesis are characterized by shorter stature and darker green leaves in comparison with wild-type plants. Mutants impaired in GA signaling resemble GA biosynthesis mutants except that they cannot be rescued by GA application (Davies, 2010). In apple, GAs are also known to be involved in seed dormancy removal (Halińska and Lewak, 1987; Lewak, 2011; Oyama et al., 1996; Sinska and Lewak, 1970, 1977), inhibition of flower initiation and biennial bearing (Guitton et al., 2012; Kittikorn et al., 2010; Lang, 1990; Looney et al., 1985; Ramírez et al., 2004; Schmidt et al., 2010; Steffens et al., 1992; Stephan et al., 1999; Tromp, 1982) as well as fruit development (Curry, 2012; Di Lella et al., 2006). The investigation of GA content in apple often leads to varied results among different groups as

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a result of different cultivars and developmental stages examined as well as techniques used (Table 1) and evidence for GA metabolism in vegetative tissues of apple in vivo is still lacking.

Genes regulating key steps in GA synthesis have also been investigated in apple and are shown to be tissue-specific. In 'Fuji' apple, *MdGA20ox1* [GA-20oxidase (catalyzes the sequential oxidation of GA₅₃ to GA₂₀)] primarily functions in immature seeds (Kusaba et al., 2001; Zhao et al., 2010), whereas *MdGA3ox1* [GA-3oxidase (catalyzes the final step in the synthesis of bioactive GAs)] and *MdGA2ox1* [GA-2oxidase (a major catabolic enzyme that produces biologically inactive GAs)] are expressed primarily in flowers (Zhao et al., 2010). Steffens and Hedden (1992a) suggest that the enzymatic activity of GA-20oxidase is subject to temperature regulation, leading to dwarf plants with short internodes in ramped temperature regime (20–30–20 °C). *DELLA* proteins, named for the conserved order of aspartic acid (D), glutamic acid (E), leucine (L), and alanine (A) at the N-terminus, are negative regulators of the GA signaling pathway (Sponsel and Hedden, 2010). Six endogenous *DELLA* proteins were identified in apple (Foster et al., 2007) and there is a significant conservation of gene function between *DELLA* proteins from apple and arabidopsis [*Arabidopsis thaliana* (Zhu et al., 2008)]. *MdGAI* (GA-insensitive), an innate *DELLA* protein of apple, was identified in both vegetative and reproductive tissues in 'Lujia 5' (Liang et al., 2011) and its mRNA moved both upstream and downstream in grafted apple trees (Xu et al., 2010).

Although dwarfing rootstocks are used commercially to reduce the vigor of apple scion cultivars, the exact interaction between scions and rootstocks is still unclear and research results may be contradictory (Bulley et al., 2005; Costes and García-Villanueva, 2007; Costes et al., 2006; Seleznyova et al., 2003; van Hooijdonk et al., 2005). It has been suggested that the supply of root-produced GA₁₉ to shoot apices of the scion is limited by dwarfing apple rootstocks. Dwarfing interstock M.9 was also shown to limit the supply of [³H]GA₄ to the shoot tips of scion cultivar as compared with MM.115 interstock, which is non-dwarfing (Richards et al., 1986). Bulley et al. (2005) demonstrated that when the level of bioactive GAs in the scion cultivar Greensleeves was reduced by the down-regulation of GA-20oxidase, the dwarfing effect was not corrected by grafting the scion onto an invigorating rootstock MM.106 or M.25. How in vivo metabolism of GA in scion cultivars is affected by rootstocks is not known.

This study was designed to determine whether apple tree morphology could be related to the content or metabolism of gibberellins. We examined the metabolic pathway of GA in apple vegetative tissues by applying radioactive [¹⁴C]GA₁₂, a common precursor of all GAs in higher plants, to the base of vigorously growing shoot tips and following the subsequent production of downstream GA metabolites. The major metabolism pathway and endogenous GAs were identified using GC-MS. Metabolism of [¹⁴C]GA₁₂ was compared between dwarf and standard plants as well as self-rooted plants and plants on vigorous rootstocks.

Materials and Methods

PLANT MATERIAL. Vigorous apple shoots were collected in June 2011 from the cultivar Redcort on growth-promoting rootstock MM.106 in the Ithaca, NY, orchard for the examination of GA₁₂ metabolism and GA identification. Progeny 806

was generated in 2007 by crossing Selection 1 ('Fuji' × Co-op 18), which has reduced stature resulting from shorter internodes with an advanced breeding selection that has good fruit quality and a standard tree form. Seeds harvested in 2007 were stratified in the refrigerator for 90 d and planted in pots in the greenhouse in Jan. 2008 with a day temperature of 21 °C and night temperature 17 °C. Seedlings on their own roots were later transplanted into an orchard in Geneva, NY. Vigorously growing shoots were collected from both standard and dwarf phenotypes in May 2010, their third growing season, for GA biosynthesis pathway comparison.

[¹⁴C]GA₁₂ SYNTHESIS. [¹⁴C]GA₁₂ was synthesized from [¹⁴C] mevalonic acid [custom made by Amersham (now GE Life Sciences), Piscataway, NJ] using a pumpkin (*Cucurbita maxima*) endosperm extract by Halińska et al. (1989) and purified by solid phase extraction (Strata-X SPE; Phenomenex, Torrance, CA) and high-performance liquid chromatography (HPLC) (Davies et al., 1986). The [¹⁴C]GA₁₂ contained eight ¹⁴C atoms per GA₁₂ molecule (Zhu et al., 1988). The identity of the [¹⁴C]GA₁₂ fraction from HPLC was confirmed using GC-MS (data not shown).

[¹⁴C]GA₁₂ APPLICATION. Shoots ≈25 cm in length were harvested in the orchards and brought to the laboratory within 15 min with the cut ends of the stems submerged in water. They were cut under water just above the third fully expanded leaf below the apex. Samples were 5 to 10 cm in length and weighed 1.5 to 4.5 g (seedlings from progeny 806 and 'Redcort', respectively). The base of the cut stem of each sample was placed in a 1.5-mL, V-shaped-bottomed, polystyrene vial with treatment solution. For shoots from 'Redcort', each vial contained 0.5 mL of water with 7.4 GBq [¹⁴C]GA₁₂ [560 pmol, 190 ng (Zhu et al., 1988)]. Cuttings were maintained under fluorescent lamps at 77 μmol·m⁻²·s⁻¹ for 30 min, 1, 3, 6, or 48 h, with three replicates for each time period. Water in 0.5-mL aliquots was added when the vials were nearly empty. Uptake occurred at an average rate of ≈1 mL·h⁻¹, although this varied with shoot vigor. The shoots in the 48-h treatment were placed together in a 100-mL beaker with water after they had been in the vial for 12 h. For shoots from progeny 806, each vial contained 0.5 mL of water with 3.7 GBq [¹⁴C]GA₁₂ (280 pmol, 95 ng). Cuttings were left in the same light (intensity as previously described) for 15 min, 30 min, 1, 3, 6, 12, 24 or 48 h with two replicates at each time period. Water in 0.5-mL aliquots was added when the vials were nearly empty. The shoots in the 24- and 48-h treatment were placed in a 100-mL beaker with water after they had been in the vial for 12 h. After the treatments, the shoots were frozen in liquid nitrogen and stored at -80 °C until extraction. Each experiment was conducted twice for both the standard and dwarf plants.

GIBBERELLIN EXTRACTION AND PURIFICATION. All solvents used were HPLC grade. Glassware was baked at 500 °C to destroy any contaminating GAs and then silanized with siliconizing fluid (AquaSil; Pierce Biotechnology, Rockford, IL). Each frozen shoot tip was individually placed in 20 mL ice-cold 80% (v/v) MeOH and ground with a 2-cm head (Polytron; Brinkmann Instruments, Westbury, NY), which was rinsed twice with 80% (v/v) MeOH. The rinses were combined with the extract. The homogenate was left overnight at 4 °C before vacuum filtration through filter paper (Whatman, Piscataway, NJ) with a pad of filter aid (Highflo Super Cel; Sigma-Aldrich, St. Louis, MO) followed by a three-pad volume rinse of 80% (v/v) MeOH. The filtrate volume was reduced on a rotary evaporator at 36 °C to partially remove the MeOH. One milliliter NH₄OH

Table 1. Gibberellins (GAs) previously identified in apple.

Citation	GAs purportedly identified	Plant materials	Cultivar	Techniques used ^z
<i>Fruit and seeds</i>				
Halińska and Lewak (1987)	GA ₄ , GA ₇ , GA ₉	Seeds (partially stratified)	Antonovka	GC
Steffens et al. (1992)	GA ₁ , GA ₃ , GA ₄ , GA ₇ , GA ₈ , GA ₉ , GA ₁₉ , GA ₂₀ , GA ₅₃	Immature seeds (mid-June)	Spartan (own-rooted)	HPLC, GC-MS
Oyama et al. (1996)	GA ₄ , GA ₇ , GA ₉ , GA ₁₇ , GA ₃₅ , GA ₅₄ , GA ₆₂ , GA ₈₀ , GA ₈₄	Seeds 10 weeks after full bloom	McIntosh	GC-MS
Oyama et al. (1996)	GA ₁₇ , GA ₂₅ , GA ₄₅ , GA ₆₂ , GA ₆₃ , GA ₈₀ , GA ₈₄	Seeds 14 weeks after full bloom	McIntosh	Bioassay on dwarf rice (<i>Oryza sativa</i>), ^y GC-MS
Stephan et al. (1997)	GA ₁ , GA ₃ , GA ₄ , GA ₇ , GA ₂₀ , GA ₃₄	Developing apple fruit	Jonagold	LC-MS
Stephan et al. (1997)	GA ₁ , GA ₃ , GA ₄ , GA ₇ , GA ₂₀ , GA ₃₄	Immature seeds	Jonagold	LC-MS
Stephan et al. (1999)	GA ₁ , GA ₃ , GA ₄ , GA ₇ , GA ₂₀ , GA ₃₄	Fruit exudates at the time of flower induction (June)	Elstar, Golden Delicious, Jonica, Spencer Seedless	LC-MS
<i>Shoots</i>				
Koshioka et al. (1985)	GA ₁₉ , GA ₂₀ and trace amount of GA ₁ and GA ₉	Leaf and buds	McIntosh, Jonathan	Si gel partition column chromatography–bioassay–HPLC + GC-MS
Steffens and Hedden (1992a)	GA ₁₉ , GA ₂₀ , GA ₁ , GA ₈ , GA ₂₉	Shoot tips	Seedlings from a sib-cross obtained from Redspur Delicious × Goldspur Golden Delicious	HPLC + GC-MS
Motosugi et al. (1996)	GA ₁₅ , GA ₁₇ , GA ₁₈ , GA ₁₉ , GA ₂₃ , GA ₄₄ , GA ₅₃	Xylem exudates	3-year-old Fuji on Marubakaidou and M.26 rootstocks	HPLC, modified bioassay on dwarf rice ^y
Kittikorn et al. (2010)	GA ₁ , GA ₄	1-year-old shoots	32-year-old Fuji on <i>Malus prunitoria</i> rootstock	GC-MS

^zOnly gas chromatography–mass spectroscopy (GC-MS) or liquid chromatography–MS (LC-MS) provides unequivocal evaluation.

^yHigh-performance liquid chromatography (HPLC) and bioassay alone provides only an indication of hydrophobicity of the GA. Because GA₁₇ is not biologically active, it cannot be found by bioassay.

and 20 mL hexanes were then added to each sample and the mix was shaken vigorously to partition the chlorophyll into the hexanes. Then evaporation was resumed to precipitate chlorophyll on the removal of the hexanes and then until all the MeOH was removed and the volume was reduced to ≈20 mL. The sample was acidified to pH 3 to 3.5 with acetic acid and vacuum-filtered through filter aid with a two-pad volume rinse of acidified water [0.2% acetic acid (v/v), pH 3.5].

A 6-mL Strata-X SPE cartridge was washed with 5 mL of 100% MeOH and 10 mL of acidified H₂O. The sample was loaded through the reservoir on top of the cartridge. The cartridge was first washed with flask rinse and 2 mL of acidified water with the eluate discarded and then with 4 mL 100% MeOH. The eluate was collected in a 4.5-mL polystyrene tube and stored in the freezer until further use.

To remove phlorizin, which caused major problems because of precipitation, especially in the HPLC injector and columns, a combination of reverse phase/anion exchange solid phase extraction cartridges (Strata-X-A and Strata-XL-A; Phenomenex) was used. These cartridges were washed with 1 mL MeOH and equilibrated with 1 mL water. Samples were diluted

to less than 20% (v/v) MeOH and buffered to pH 6 to 7. Then they were loaded to the reservoir on top of the Strata-XL-A cartridge and drawn through by vacuum. The eluate was then loaded to Strata-X-A and vacuum filtered through to ensure the capture of all the wanted GAs. Both columns were washed with 25% (v/v) ammonium acetate followed by 100% MeOH; the eluate, containing the phlorizin, was discarded. Lastly, both cartridges were washed with 5% (v/v) formic acid and then 4 mL 100% MeOH. The MeOH eluate, containing the GAs, was combined and stored at –20 °C until further use.

HPLC PURIFICATION. Each sample was evaporated to dryness under N₂ at 37 °C and the container was rinsed with 0.1 mL MeOH and transferred to a centrifugal nylon membrane filter tube (0.45-μm; Corning Spin-x, Lowell, MA) followed by two further rinses of 0.3 mL of H₂O with 0.2% (v/v) acetic acid. The solution was then filtered by centrifuging at 3500 g_n for 5 min. Each sample of ≈0.7 mL was loaded onto an analytical C18 HPLC column (Synergi 4u Hydro-RP 80A; Phenomenex) using a 1-mL injection loop and run at 1 mL·min^{–1} in a H₂O [A (containing 2 mL·L^{–1} glacial acetic acid)] to acetonitrile (B) gradient. The gradient used (all by volume) was: 27% B for 2

min, 27% to 33% B over 5 min, 33% to 35% B over 4 min, 35% to 70% B over 15 min, 75% to 100% B over 5 min, and holding at 100% B. The column eluate passed through an in-line radioactivity monitor (Trace 7140; Packard, Downers Grove, IL) equipped with a flow cell packed with insoluble scintillator beads (170 μ L void volume). The efficiency of the monitor was $\approx$ 10% (v/v) for ^{14}C . An automatic peak-detection circuit controlled a fraction collector (Ultrarac; LKB, Bromma, Sweden) and collected each individual peak for all the samples.

IDENTIFICATION OF GC RETENTION TIME (RT) FOR GIBBERELLINS WITH ^2H STANDARDS. ^2H]GA standards (GA₁, GA₃, GA₄, GA₇, GA₈, GA₉, GA₁₂, GA₁₅, GA₁₉, GA₂₀, GA₂₉, GA₃₄, GA₄₄, and GA₅₃) were obtained from L. Mander [Australian National University, Canberra, Australia (synthesized by T. Herlt)]. GA aliquots were methylated with a surplus of freshly synthesized ethereal diazomethane. The mixture was then dried using N_2 and re-dissolved in MeOH to reach a stock concentration of 1 ng μL^{-1} for each GA. Fifty microliters (50 ng of each GA) was transferred to 1-mL tapered glass vials (Chromcol; Fisher, Sun SRI, Rockwood, TN). It was then thoroughly redried with a stream of N_2 and dissolved in 2 μL pyridine and 10 μL bis-trimethylsilyltrifluoroacetamide containing 1% (v/v) trimethylchlorosilane (Supelco, Bellefonte, PA). After 40 min in an oven at 80 $^\circ\text{C}$, GC-MS analyses were performed with gas chromatograph (5890A; Hewlett-Packard, Palo Alto, CA) connected to a 5970B Mass Selective Detector (Hewlett-Packard). Samples (1 μL) were injected without splitting onto a 20 m $\times$ 0.18 mm $\times$ 0.18- μm column (Zebtron ZB-5MS, 7FD-G010-08; Phenomenex). The temperature program for the GC was injection at 60 $^\circ\text{C}$, increasing to 240 $^\circ\text{C}$ at 30 $^\circ\text{C}\cdot\text{min}^{-1}$, to 275 $^\circ\text{C}$ at 4 $^\circ\text{C}\cdot\text{min}^{-1}$, and finally to 325 $^\circ\text{C}$ at 30 $^\circ\text{C}\cdot\text{min}^{-1}$. The injection of the mixture was analyzed in selected ion monitoring (SIM) mode to monitor the most abundant ion (at +2 mass units from the base peak for ^{12}C]GA) for each GA (Gaskin and MacMillan, 1991). When needed for confirmation, six ions per GA were monitored. Retention time obtained for each GA standard was used to adjust the GC program to monitor specific GAs at specific time windows to increase sensitivity. The standard mixture was run at the start of each day before the analysis of apple samples.

IDENTIFICATION OF ENDOGENOUS GIBBERELLINS USING GC-MS. The same GA HPLC peaks from different apple samples were bulked together and methylated with a surplus of ethereal diazomethane. The methylated samples were re-chromatographed using the analytical column and the same gradient (described previously). This provided an extra purification, because the retention time of the methylated compounds was later than the non-methylated GAs.

Samples were dried and re-dissolved in MeOH before transfer to 1-mL tapered glass vials and derivatized as described before. Each sample (1 μL) was injected on to GC-MS without splitting and run under the temperature program as the standard mixture. The first injection of each sample was under the SIM mode to monitor the most abundant native ion for each GA and the ion at plus-16 $m\cdot z^{-1}$ (allowing for the eight ^{14}C atoms per GA derived from the fed molecule) at the specific time window for each of the GAs for which we had standards (as listed previously). After preliminary results were obtained, a second injection monitoring more native ions from the GA spectrum was used to confirm the identity of the GA.

GA₃ TREATMENT. Fifteen control branches and 11 treatment branches were selected from 806 dwarf plants for a GA₃

application. Control and treatment solutions were applied to the same plant on different comparable branches. Solutions containing less than 1% ethanol and 0.1% (v/v) Tween-20 with or without GA₃ (Sigma-Aldrich) were brushed on both sides of newly grown leaves of the uppermost shoots until they were fully covered with a thin film. Applications were made weekly for 5 consecutive weeks starting on 29 July 2010.

Stem elongation above the node originally located immediately below the terminal bud of each branch was measured on the day of the third and fifth applications and 2 weeks after the fifth application. Node number of elongated sections was determined. Data were submitted to Student's *t* test for comparison at $\alpha = 0.05$, $df = 24$ with JMP9[®] (SAS Institute, Cary, NC).

Results

METABOLITES OF ^{14}C]GA₁₂ IN 'REDCORT'/MM.106. Sixteen metabolites of ^{14}C]GA₁₂ were identified in 'Redcort'/MM.106. Generally, GA metabolism produces compounds of increasing polarity and as the metabolism progresses more polar compounds are produced and elute from the HPLC (Davies et al., 1986). The feeding material ^{14}C]GA₁₂ was the last compound to elute. For convenience, major metabolites were designated with the letters A to U, corresponding to increasing polarity (Fig. 1).

Fig. 1. High-performance liquid chromatographs of metabolites of ^{14}C]GA₁₂ in cultivar Redcort/MM.106 apple shoots at 30 min (A) and 48 h (B) after feeding with ^{14}C]GA₁₂ through the base of the shoots. The detector response, indicating the amount of radioactivity, is in volts. Peaks are labeled with retention time and the gibberellins identified by gas chromatography-mass spectroscopy.

Fifty percent of the [^{14}C]GA $_{12}$ feeding material (peak A, GA $_{12}$) was metabolized quickly into peak B (GA $_{15}$) at 30 min (Figs. 1A and 2A). Other early metabolites started to be synthesized at 1 h. At 48 h, 13 of the 16 metabolites detected in 'Redcort' were evident in the HPLC chromatograph (Figs. 1B and 2A) and the major metabolites included peaks L (GA $_{19}$), G (GA $_{53}$), I (GA $_{20}$), A (GA $_{12}$), and B (GA $_{15}$). The initial appearance and trend of certain metabolites may have been missed, because samples were not collected between 6 and 48 h.

Fig. 2. Metabolites of [^{14}C]GA $_{12}$ measured using high-performance liquid chromatography (HPLC) in shoots of apple cultivar Redcort/MM.106 (A), 806 standard plants (B), and 806 dwarf plants (C) at different times after the start of feeding the [^{14}C]GA $_{12}$ to the base of the shoots; only those metabolites identified as containing gibberellin using gas chromatography–mass spectroscopy are shown. Radioactivity was calculated by HPLC peak area.

A brown oily fraction appeared at the bottom of the containers on sample concentration or sometimes instead a heavy yellow precipitate formed on lowering the methanol content of the sample. This was identified as phlorizin from its appearance, solubility, and ethyl acetate partitioning characteristics (Gmelin, 1849; Gosch et al., 2010). The supernatant of the concentrated sample was labeled Sample 1, and the bottom fraction that went through phlorizin removal using reverse phase/anion exchange solid phase extraction cartridges was labeled Sample 2. Metabolites O, P, Q, R (GA $_{3}$), and S were all missing in Sample 2 (Fig. 3). Peak T, detected in individual 48-h HPLC samples, was not found in either sample, possibly as a result of its small quantity. Peaks C [GA $_{44}$, retention time (Rt) 27.92], E (Rt 24.5), and H (Rt 20.00) were only detected in Sample 2 but not in Sample 1 nor were they detected in individual samples collected from each time point.

These two samples were methylated and further analyzed by HPLC. Twelve methylated metabolites were detected in Sample 1 and nine in Sample 2. Because the difference between the two protocols is the removal of non-acidic samples using Strata-XA in the preparation of Sample 2, it is likely that the additional compounds in Sample 1 represented GA conjugates with Sample 2 containing only the free GAs; it is unknown why GA $_{3}$ was lost in Sample 2.

IDENTIFICATION OF ENDOGENOUS GIBBERELLINS WITH GC-MS.

The endogenous GAs were copurified with the [^{14}C]GAs by following the radioactive peaks. Detection was then achieved by GC-MS and searching for the appropriate ions of the endogenous and [^{14}C]GAs at the retention times of each [^2H]GA standard. Endogenous GA $_{12}$, GA $_{15}$, GA $_{53}$, GA $_{44}$, GA $_{19}$, GA $_{20}$, and GA $_{3}$ (in that metabolic order) [Fig. 4 (molecular structures in Supplementary Fig. 1)] were present in 'Redcort' apple shoots (Tables 2 and 3). The relative HPLC elution times of these GAs were consistent with results from Koshioka et al. (1983). The

Fig. 3. Overlay high-performance liquid chromatography of metabolites of [^{14}C]GA $_{12}$ in cultivar Redcort/MM.106 apple shoots showing the results of the removal of neutral compounds using mixed-function reverse-phase/anion-exchange solid-phase-extraction cartridges (Strata-X-A; Phenomenex, Torrance, CA): total (yellow) and after removal of neutral compounds (magenta). The detector response, indicating the amount of radioactivity, is in volts. Samples collected at different times of [^{14}C]GA $_{12}$ feeding experiment were bulked together. The multiple appearing peaks on the three tallest peaks to the right are the result of detector scale reset and represent only a single peak.

native and ^{14}C mass ions for all the GAs for which $[^2\text{H}]$ GAs were available were checked in every appropriate HPLC fraction. Despite their expected appearance, GAs 34, 9, 1, 29, and 8 were not detected, and GA_3 (Fig. 5) was the only bioactive GA found in our study. There was no trace of GAs 4 and 7, the most common GAs in apple seeds and fruit.

GA_{12} and GA_{15} followed similar metabolic trends during the study. GA_{12} was consumed to produce GA_{15} and GA_{53} , whereas the latter was metabolized further to produce the bioactive GA_3 . Based on the existence of GA_{20} and GA_3 , GA_5 should also exist in ‘Redcort’ shoots (Sponsel and Hedden, 2010). GA_{53} and GA_{19} were both detected at 1 h, whereas GA_{20} was not detected until 6 h, an indication that GA_{19} to GA_{20} is a rate-limiting step. GA_3 was first detected at 48 h, but because no sample was collected between 6 and 48 h, the initial appearance of GA_3 could not be determined nor whether GA_{20} to GA_3 was a fast or slow metabolic step. GA_{44} , the intermediate between GA_{53} and GA_{19} , was only detected in bulked Sample 2 of ‘Redcort’ but not in any samples from individual time periods, perhaps

Fig. 4. The metabolic pathway of the gibberellins identified in cultivar Redcort/MM.106 apple shoots after feeding with $[^{14}\text{C}]\text{GA}_{12}$. Gibberellins in parenthesis were not detected in this study.

a combined result of fast metabolic rate and an insufficient amount in each individual sample.

$[^{14}\text{C}]\text{GA}_{12}$ METABOLISM IN DWARF AND STANDARD APPLE SEEDLINGS FROM PROGENY 806. Eighteen $[^{14}\text{C}]\text{GA}_{12}$ metabolites were identified by HPLC (Table 4). Sibling dwarf and standard progeny in 806 demonstrated similar metabolic trends (Fig. 2B–C) with more polar metabolites produced later in the time course. However, in the standard plants, GA metabolism was faster than in the dwarf plants (Fig. 6). For individual metabolites, peak N (identity unknown) (Fig. 7), which transiently existed as a principal compound in the 3-h HPLC chromatograph of standard plants, was not found at any time point in dwarf plants. Most other metabolites existed in both dwarf and standard plants but often followed different metabolic trends and existed for different durations (Fig. 8). The metabolite O was the dominant peak after 12 h in 806 standard plants. In contrast, peak Q, G (GA_{53}), and L (GA_{19}) were all major metabolites for 806 dwarf seedlings at the second half of the time course.

Plants in 806 had a much faster metabolic rate compared with that of ‘Redcort’/MM.106 (Fig. 9). For 806, GA_{12} and GA_{15} were mostly metabolized between 1 and 6 h. However, in ‘Redcort’, these two compounds were consumed at a more gradual speed with downstream metabolites also appearing later (1 h vs. 15 min in progeny 806). At the end of the study, ‘Redcort’ peak A and B still had significant amounts (20.5% and 14.5%, respectively), yet less than 5% was present in both 806 dwarf and standard seedlings. It is possible that these differences result from the much larger size of the ‘Redcort’ shoots (averaged 3.2 g) because they were taken from fully grown apple trees rather than seedlings (sample averaged 2.0 g). For 806 standard, GA_{53} peaked at 3 h, where GA_{12} and GA_{15} were at the end of their sharp decrease. After 3 h, the decrease of GA_{12} and GA_{15} slowed, where GA_{53} was quickly metabolized. The same occurred in 806 dwarf plants, just at a slower speed. However, in ‘Redcort’, the amount of GA_{53} rose from 1 to 48 h, suggesting that GA_{53} always was produced faster than it was consumed.

GA_{19} and GA_{20} appeared at the same time in standard and dwarf apple progeny, which suggests GA_{19} was metabolized immediately to GA_{20} . Because more GA_{19} was produced than consumed for GA_{20} , the conversion from GA_{19} to GA_{20} is suggested to be regulated tightly. In ‘Redcort’, GA_{20} appeared 5 h later after the initial appearance of GA_{19} at 1 h. From 6 to 48 h, GA_{19} increased $\approx 15\%$, whereas GA_{20} increased only $\approx 4\%$.

Table 2. Gas chromatography–mass spectroscopy (GC-MS) identification of endogenous gibberellins (GAs) coincident on high-performance liquid chromatography (HPLC) with peaks resulting from the metabolism of $[^{14}\text{C}]\text{GA}_{12}$ in apple shoots from cultivar Redcort/MM.106.

Peak	GA	HPLC Rt (min) ^z		Ions monitored in GC-MS	GC-MS Rt	Reported KRI ^y
		Non-methylated	Methylated			
A	GA_{12}	31.7	37.9	300/316, 328, 285, 241	10.897	2333
B	GA_{15}	29.1	35.5	239/255, 284, 312, 344	14.257	2605
G	GA_{53}	21.1	33.8	448/464, 416, 389, 373	12.082	2497
C	GA_{44}	27.9	34.42	432/448, 373, 238, 417	15.759	2786
L	GA_{19}	14.8	33.2	434/450, 402, 374, 345	13.217	2569
I	GA_{20}	18.4	24.9	418/434, 403, 375, 359	12.064	2482
R	GA_3	6.9	12.3	504/520, 475, 445, 311	13.921	2692

^zAfter methylation, each peak may separate to multiple methylated peaks. The retention time (Rt) for methylated peak is for the peak that was identified as a GA.

^yKovats retention index: the relative elution Rt of the GA in question compared with that of paraffins of differing lengths of carbon chain represented by the first two digits of the number, as reported in Gaskin and MacMillan (1991).

Table 3. Relative amounts of endogenous gibberellins (GAs) in apple cultivar Redcort/MM.106 shoots derived from the relative intensities of base peak ions of ¹⁴C:¹²C in the mass spectra of fed [¹⁴C]GA₁₂ and GAs extracted from the shoots.

Gibberellin	¹⁴ C ion mass	¹² C ion mass	Amount of ¹² C compared with the proportion of ¹² C to ¹⁴ C in the fed GA ₁₂
Fed GA ₁₂	316	300 ^z	×1.00
Extracted GA ₁₂	316	300	×1.36
Extracted GA ₁₅	255	239	×1.05 ^y
Extracted GA ₅₃	464	448	×2.83
Extracted GA ₄₄	448	432	×84.9
Extracted GA ₁₉	450	434	×95.6
Extracted GA ₂₀	434	418	×106.7
Extracted GA ₃	520	504	×34.8

^zAmount of ¹²C ion compared with ¹⁴C ion in fed GA₁₂ = × 0.97.
^yValue corrected for the fact that the ¹⁴C base peak ion at mass 255 also occurs in the ¹²C spectrum at 7.4%.

Fig. 5. Selected ion monitoring profile of extracted [¹²C/¹⁴C]GA₃ derived from [¹⁴C]GA₁₂ fed to apple cultivar Redcort/MM.106 apple shoots after separation and detection using gas chromatography–mass spectroscopy, monitoring native ¹²C ions at mass/charge (m/z) 370, 445, 475, 504, and ¹⁴C ion 520. All ions peaked together (not shown).

Table 4. [¹⁴C]GA₁₂ metabolites identified in apple shoots of apple cultivar Redcort/MM.106 (Sample 1, total; sample 2, after the removal of neutral compounds) and progeny 806 (standard and dwarf phenotypes).^z

Cultivar	Peak																			
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
	Gibberellin identity																			
	12	15	44			53		20		19									3	
Redcort 1	X	X		X	X	X		X		X					X	X	X	X	X	
Redcort 2	X	X	X		X	X	X	X	X		X									
806 standard	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
806 dwarf	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

^z“X” indicates the presence of the peak on high-performance liquid chromatography.

GA₃ appeared in 806 dwarf plants at 3 h and in 806 standard plants at 12 h. Samples were not collected between 6 and 48 h in ‘Redcort’, so the first appearance of GA₃ could be earlier than the 48 h recorded.

GIBBERELLIN TREATMENT. Based on the measurements taken 2 weeks after the fifth application, the new growth of shoots of dwarf 806 plants treated with GA₃ solution was one-third longer than control branches ($P < 0.027$, Student’s t test) with more nodes ($P < 0.007$, Student’s t test), but was similar in internode length (Table 5).

Discussion

Seven GAs, both endogenous and as metabolites of the applied [¹⁴C]GA₁₂, including GA₁₂, GA₁₅, GA₅₃, GA₄₄, GA₁₉, GA₂₀, and GA₃, were identified in apple vegetative tissues after the application of radioactive [¹⁴C]GA₁₂ to the base of isolated shoot tips. Based on the dilution of the applied [¹⁴C]GA₁₂ by endogenous GAs as metabolism proceeded (Table 3), GA₂₀ was the major GA present with slightly less GA₁₉ and GA₄₄ and with GA₃ present at approximately one-third the level of GA₂₀. GA₁₂ is the common precursor for all GAs in higher plants and is at a branch point of the GA biosynthesis pathway, undergoing either oxidation at C-20 to form GA₁₅ or hydroxylation at C-13 to produce GA₅₃ before further oxidation at C-20. GA₁₂ and GA₅₃ are precursors of the non-13-hydroxylation and the 13-hydroxylation pathways, respectively (Sponsel and Hedden, 2010). In shoots from the G2 line of a dwarf pea (*Pisum sativum*) lacking the dominant *Le* allele that controls the conversion from GA₂₀ to GA₁, GA₁₂-aldehyde (an immediate precursor of GA₁₂) was metabolized quickly into a variety of compounds including GAs 53, 44, 19, 20, and 17 under both long-day and short-day conditions (Davies et al., 1986). In potato (*Solanum tuberosum* spp. *andigena*) shoots, 20 metabolites were detected from the metabolism of [¹⁴C]GA₁₂, including GAs 53, 44, 19, 20, 29, 1, and 8 (van den Berg et al., 1995). In our study, the identification of most of the members in 13-hydroxylation pathway (along with the expected existence of GA₅) strongly suggests that this is the major metabolic pathway in vigorously growing apple shoots. This result is consistent with GA metabolism in vegetative tissues of potato (van den Berg et al., 1995) and most other plant species (Sponsel and Hedden, 2010) but different from that in apple seeds, which favors the production of GA₄ and GA₇ by the non-13-hydroxylation pathway. However, neither GA₁ nor GA₂₉ was identified, indicating the likely predominance of the shift to GA₃ following GA₂₀ in the metabolic pathway. The identification of GA₁₅, an upstream compound that leads to bioactive GA₄, may indicate the existence of the non-hydroxylation pathway in apple vegetative tissues. However, neither GA₄ nor its precursors GA₂₄ and GA₉ were identified in this study, suggesting that instead of going through this pathway, GA₁₅ could be metabolized into GA₄₄ to produce GA₃. Putative GA₁₅ was also detected by Motosugi et al. (1996), although without MS identification, together with putative GA₅₃, GA₄₄, and GA₁₉ in the xylem exudates of ‘Fuji’ apple on ‘Marubakaido’ and M.26 rootstocks. The authors suggested this was an indication that both the early hydroxylation and non-hydroxylation pathways functioned in rootstocks. The

existence of both pathways in apple was also speculated by Zhao et al. (2010), because *MdGA20ox1*, *MdGA3ox1*, and *MdGA2ox1* were able to catalyze GAs of both the 13-hydroxylation and non-hydroxylation pathways in vitro. Indeed, both 13-hydroxylated GAs (GA_1 , GA_3 , GA_8 , GA_{19} , and GA_{20}) and non-13-hydroxylated GAs (GA_4 , GA_7 , and GA_9) were identified in seeds of ‘Spartan’ apple (Steffens et al., 1992). However, it is not clear whether these two pathways function to the same capacity in vegetative tissues.

GA_3 was the only bioactive GA detected in our study and has been identified in many other plant species, although is generally not as common as GA_1 (Davies, 2010). The GA_3

Fig. 6. Overlay high-performance liquid chromatography of metabolites from dwarf (yellow) and standard (magenta) apple shoots of progeny 806 supplied with $[^{14}C]GA_{12}$ for 3 h through the base of the shoots with the feeding peaks aligned to similar scale. In dwarf seedlings, the feeding peaks are still the most prominent compounds, whereas in standard plants, they are already metabolized into a group of compounds residing in the center of the profile. The detector response, indicating the amount of radioactivity, is in volts.

Fig. 7. High-performance liquid chromatograph of metabolites of $[^{14}C]GA_{12}$ in apple shoots of 806 standard plants after feeding for 3 h through the base of the shoots. The detector response, indicating the amount of radioactivity, is in volts. Peak N was not found at any time spot of 806 dwarf plants.

was not a contamination because it contained both the native and the $[^{14}C]GA_3$ MS ions, which can only have been produced from the applied $[^{14}C]GA_{12}$. Low levels of GA_3 were also detected by Steffens and Hedden (1992a, 1992b) in the shoot tips of both standard and dwarf phenotypes. Although shoot vigor increased from 806 dwarf to 806 standard to ‘Redcort’/MM.106, the initial appearance (time) of GA_3 appeared to be negatively correlated with shoot vigor. The late appearance of GA_3 in 806 standard plants and in ‘Redcort’ can be justified on the basis of feedback regulation (Ross et al., 1999). It could be hypothesized that there were already sufficient bioactive GAs in the shoots, whereas 806 dwarf plants were in a GA-deficient situation, so that GA_3 was generated soon after the availability of its upstream precursors, GA_{19} and GA_{20} . Other bioactive GAs detected in apple by others include GA_1 , GA_4 , and GA_7 (Table 1).

Because we failed to detect GA_4 or GA_7 , we conclude that either these compounds were not produced from the applied $[^{14}C]GA_{12}$ or that they were present below our level of detection ($\approx 1 \text{ ng} \cdot \text{g}^{-1}$), and if present as a radioactive metabolite, their presence was transitory because of rapid further metabolism.

Some of the detected metabolites were neutral rather than acidic, so were likely GA conjugates. Conjugation regulates bioactive GA concentrations, and conjugation to glucose is most common. The presence of GA conjugates was suggested in the metabolites of GA_{12} -aldehyde in pea shoots from the G2 line (Davies et al., 1986) and in the metabolites of GA_{12} from potato shoots (van den Berg et al., 1995). To confirm whether these metabolites are GA conjugates, base hydrolysis to yield free GAs would be needed with further purification on HPLC for identification by GC-MS (Koshioka et al., 1983).

$[^{14}C]GA_{12}$ metabolism varied in different plant materials used in this study both in compounds produced and metabolic rates. Although discussion in previous paragraphs suggests that the 3 β -hydroxylation is regulated by the bioactive GAs present in the plant materials, the different rates of GA_{19} metabolism and accumulation of GA_{53} in ‘Redcort’ shoots demonstrate that the three-step 20-oxidation is possibly under feedback control

as well. These differences could possibly be attributed to the greater amount of radioactive material applied to the ‘Redcort’ shoots ($7.4 \text{ GBq/shoot} = 560 \text{ pmol}$, 190 ng/shoot) as compared with that of 806 shoots ($3.7 \text{ GBq/shoot} = 280 \text{ pmol}$, 95 ng/shoot), but the mass of the ‘Redcort’ shoots (4.0 g) was more than double the mass of the 806 shoots (1.7 g). Another piece of evidence supporting this is that GA_{19} and GA_{20} are the major components previously characterized in vegetative apple tissues with bioactive GAs (GA_1 and GA_3) at a much lower level (Koshioka et al., 1985; Steffens and Hedden, 1992a). In pea, both the 3 β -hydroxylation and the last step of 20-oxidation (from GA_{19} to GA_{20}) were feedback-regulated by GA_1 (Ross et al., 1999). The fact that in ‘Redcort’, GA_{19} increased to 15% of the total radioactivity, whereas GA_{20} only increased to 4% between 6 and 48 h suggests that the last step of 20-oxidation is more strongly feedback-regulated than is 3 β -hydroxylation. Faust (1989) also suggested that GA_{19} is the major GA in apple

Fig. 8. Comparison of metabolic trends of high-performance liquid chromatography peaks M, N, Q, R (GA_3), T, and U from ^{14}C GA $_{12}$ -fed dwarf (Dw) and standard (Std) apple seedlings from progeny 806.

Fig. 9. Comparison of metabolic trends of identified gibberellins in the ^{14}C GA $_{12}$ -fed apple shoots from apple cultivar Redcort/MM.106 and 806 standard (Std) and dwarf (Dw) phenotypes.

Table 5. New growth of shoots of dwarf 806 apple plants treated with gibberellin (GA_3) and control solutions.^z

	Branches (no.)	Total length (cm)	Nodes (no.)	Internode length (cm)
Control	15	11.47 $\pm$ 1.27 a ^y	11.6 $\pm$ 0.80 a	0.94 $\pm$ 0.06 a
Treatment	11	15.25 $\pm$ 1.48 b	14.7 $\pm$ 1.00 b	1.07 $\pm$ 0.07 a

^zMeasurements were taken 2 weeks after the fifth application.

^yMeans followed by different letters are significantly different by Student's *t* test at $\alpha = 0.05$, $\text{df} = 24$.

vegetative tissues. Therefore, in vigorous-growing apple shoots, the homeostasis and optimal level of bioactive GAs may be partly achieved by the regulation of enzymatic activity.

Another difference for the plant materials used in the study was that 'Redcort' was on vigorous rootstock MM.106, whereas plants from 806 were on their own roots. Transport of GAs is expected between scion and rootstock; although most research results suggest that the major compounds transported are non-bioactive GAs, the direction of the transport remains a matter of a debate (Bulley et al., 2005; Lochard and Schneider, 1981; Motosugi et al., 1996). In both pea and potato plants, GA_{20} is the major transported GA (Prat, 2010; Proebsting et al., 1992). However, GA_{20} was not identified in the xylem exudates from 'Fuji' apple, whereas GA_{19} was identified (Motosugi et al., 1996). The concentration of GA_{19} was also shown to increase with increasing rootstock vigor in the xylem sap of grafted apples in the growing season (van Hooijdonk et al., 2011). Thus, there seems to be a variation in which of the intermediate GAs is the transported GA in different species with GA_{19} being the major transported GA in apple. This would support earlier discussions that the last step of 20-oxidation is more strictly regulated than 3- β hydroxylation. A study of the transport of radiolabeled GAs in grafted apples would aid in this clarification.

In progeny 806, the same GAs were identified in dwarf and standard phenotypes, consistent with the findings of Steffens and Hedden (1992a), although GA_1 , GA_{29} , and GA_8 were not detected in our study. Peak N, which was not identified as to GA, is the only metabolite detected in standard plants but not in dwarf plants. N elutes earlier than GA_{19} , so it is more polar and may be a bioactive GA downstream of GA_{19} . However, its transient presence (3 to 6 h) suggests it is more likely to be a rapidly metabolized intermediate.

Despite similar metabolic trends, 806 dwarf plants metabolized GA_{12} at approximately half the speed of that of standard plants, perhaps as a result of the low bioactivity of certain enzymes in the GA biosynthesis pathway or may be the result of the low vigor. Looney et al. (1988) also reported that the bioactivity of polar GAs in shoot tips from compact growth strains of 'McIntosh' was significantly lower than from the normal strains. The dioxygenases GA-20ox , GA-3ox , and GA-2ox are often encoded by multigene families (Sponsel and Hedden, 2010), so defects of certain enzymes in dwarf plants may be covered by other gene family members, which may have overlapping expression or can be transported from other parts of the plant and result in leaky mutants. The differences in metabolism between 'Redcort' and progeny 806 could also be attributed to cultivar differences as well as physiological status of the plant materials such as flowering and fruiting ('Redcort') vs. juvenile stage (non-flowering) (806).

We conclude that dwarf phenotype in 806 is not caused by lack of certain GAs in the biosynthesis pathway downstream of

GA₁₂, although this does not exclude the possibility of impairment before GA₁₂. In contrast to Steffens et al. (1989b), where dwarf apple plants were not rescued by exogenous GA₃, GA application in our study did increase internode number but had no effect on internode length. Because GAs can have effects both on cell division and cell elongation (Davies, 2010), it remains a possibility that insufficient GAs are produced in dwarf plants, although clearly that is not the main cause of dwarfism. Alternatively, dwarfism can be caused by a blockage in the GA signaling pathway or by defects in other plant hormones that regulate plant form such as brassinosteroids or strigolactone (Pereira-Lorenzo et al., 2009; Rameau, 2010).

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Supplemental Fig. 1. Structures of gibberellins found in apple shoots by gas chromatography–mass spectroscopy.