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Hormone Profiling by LC-QToF-MS/MS in Dormant *Macadamia integrifolia*: Correlations with Abnormal Vertical Growth

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Abstract A method for analyzing multiple plant hormone groups in small samples with a complex matrix was developed to initiate a study of the physiology of abnormal vertical growth (AVG) in *Macadamia integrifolia* (cv. HAES344). Cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA), and auxins were detected in xylem sap and apical and lateral buds using high-performance liquid chromatography quadrupole time-of-flight tandem mass spectrometry (LC-QToF-MS/MS). The extraction method separated compounds with high sensitivity in positive (CKs) and negative (ABA, auxins, GAs) modes of QToF-MS/MS. CK profiles differed in xylem sap and apical and lateral buds irrespective of AVG symptoms. *Trans*-zeatin riboside (*t*-ZR) was dominant in sap of normal and AVG trees ($\sim$4 and 6 pmol g^{-1} FW, respectively). In apical buds isopentenyl adenine (iP) ($\sim$30 pmol g^{-1} FW) was the most abundant CK, and in lateral buds *trans*-zeatin (*t*-Z) (22–24 pmol g^{-1} FW) and iP (24–30 pmol g^{-1} FW) were the most abundant. *t*-Z levels of AVG trees were higher in apical buds (13.88 vs. 6.6 pmol g^{-1} FW, $p < 0.05$) and lower in sap (0.16 vs. 0.51 pmol ml^{-1}, $p < 0.005$) compared to normal trees. ABA in lateral buds was 1.9 times higher ($p < 0.001$) in AVG. IAA was below quantification, whereas indole-3-butyric acid (IBA) was consistently present. GA_7 was the dominant GA in apical and lateral buds of all trees (100–150 pmol g^{-1} FW). $GA_{3,\ 4,\ \&\ 9}$ were consistently present at low concentrations (<12 pmol g^{-1} FW) in buds. $GAs_{1,\ 3,\ \&\ 9}$ were detected in xylem sap at low concentrations (<0.5 pmol g^{-1} FW). Differences in sap amino acids (AA) were also assessed. In sap from AVG trees, asparagine and glutamine increased significantly ($p < 0.05$) in their contribution to total AA. Potential AVG hormone correlations are discussed.

Keywords Cytokinins · Auxins · Gibberellins · ABA · Amino acids · Apical · Lateral · Bud · Xylem sap

Introduction

Macadamia integrifolia (cv. HAES344) is a subtropical evergreen rainforest tree cultivated for its high quality kernel. A growth disorder affecting yield has been increasingly reported in orchards throughout Australia. This disorder is named abnormal vertical growth (AVG) because visual symptoms include a more vertical growth form with increased tree height and reduced lateral branching. AVG in *Macadamia* shares visual traits with coppice regrowth and juvenility in other tree species such as strong vertical growth, enhanced elongation, reduced/absent flowering, increased stem diameter and simultaneous outgrowth of many vegetative buds from particular nodes. Similar morphologic traits have been correlated with endogenous hormone characteristics in a range of tree species (Bertling and Bangerth 1995; Chen and others 1996; Day and others 1995; Doumas and Zaerr 1988; Imbault and others 1988; Rinne and Saarelainen 1994; Stern and others 2003).

Correlating the levels of specific hormone groups or hormone ratios with specific aspects of growth and development has improved the understanding of tree hormone physiology. For example, endogenous *t*-ZR concentrations in apical and lateral buds of spruce were positively correlated with bud vigor during the following growth cycle (Chen and others 1996). Initiation and growth of coppice

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shoots was associated with increased riboside-type CK export from the roots of birch (Rinne 1994; Rinne and Saarelainen 1994) without changes in auxin. Abnormal plagiotropy (horizontal growth habit) in leading shoots of blue spruce was characterized by a lower CK-to-auxin ratio resulting from reduced CK and increased auxin levels (Matschke 1993). Bending of vertical shoots decreased polar auxin transport and CK levels while increasing ethylene production and inducing flower bud formation (Sanyal and Bangerth 1998).

The biological activity of GAs differs widely, but most commonly affects growth vigor (Pharis and others 1991) and flowering (Ogata and others 1996). Elongated vegetative growth correlated with altered GA content (Hisamatsu and others 2004). GAs with florigenic properties independent of stem elongation (Evans and others 1994; Pharis and others 1987) or with only a moderate effect on elongation have also been identified (Evans and others 1990; King and others 2001). Other GAs may be candidates for root-produced floral inhibitors (Wareing and Frydman 1976). Juvenile and mature growth phases of woody species were associated with qualitative and quantitative differences in endogenous GA status (Blake and others 2000; Moritz and others 1989). Changes in GA metabolism concurred with flower bud differentiation in spruce (Oden and others 1994, 1995). Furthermore, columnar growth of apple, morphologically similar to AVG, correlated with an altered profile of GA groups (Watanabe and others 2004, 2006).

Because the visual symptoms of AVG in *Macadamia* combine morphologic traits previously associated with hormone characteristics, it is plausible to hypothesize that multiple hormonal factors/interactions contribute to the expression of AVG. Clarifying any hormonal basis for morphologic differences in AVG requires analysis of CK, auxin, GA, and ABA profiles of symptomatic and symptomless trees. To date there are no published reports on hormone analysis in *Macadamia*. The small size of lateral and apical buds and high levels of interference associated with the complex tissue matrix substantially increase the difficulty of hormone analyses in *Macadamia*. Therefore, the purpose of this study was (1) to develop methods to analyze multiple hormone groups from the same sample and (2) to generate hypotheses for future investigations through improved understanding of hormone interactions underlying AVG symptoms in *Macadamia.*

Material and Methods

Plant Material

In *Macadamia* vegetative flushing occurs through 9 months of the year with varying intensity while the crop develops over 6–10 months (Stephenson and Cull 1986) creating a complex asynchronous phenological cycle. For the purpose of this pilot study collection took place during the period of minimal vegetative growth when physiologic parameters were expected to be most uniform. Samples were taken from a commercial orchard at Beenleigh, Australia (153.2°E, 27.7°S) on 30 April 2004 and 5 May 2004. All samples were taken from branches 1–2.5 m above ground level on 15-year-old trees growing in close proximity (<50 m). Branches were cut from the tree immediately below the oldest attached leaf.

Sap and Bud Collection

Sap and bud samples were collected in triplicate from AVG and normal trees. Xylem sap was collected by a mild vacuum (–677 to –840 mbar) from branches 10–15 mm in diameter. All leaves were removed immediately to eliminate transpiration. The branches were cut back sequentially from the distal end to ensure continued sap flow. Approximately 15–20 ml of sap was collected per sample. This required pooling of sap collected from at least five individual branches. Apical buds were collected by hand from northern oriented branches, placed into liquid N, and stored at –80°C prior to weighing and hormone extraction. Lateral buds were removed from branches used for sap collection using scalpels and stored in the same manner as apical buds. Between 1100–1600 lateral and 300–500 apical buds were needed to provide 1 g fresh weight (FW).

Sample Preparation

Extraction and cleanup were performed according to Mader and others (2003a) with modifications extending the method to include GAs. Compounds were separated by liquid chromatography (Agilent 1100 series HPLC) and detected by quadrupole time-of-flight tandem mass spectrometry (LC-QToF-MS/MS; Applied Biosystems, MDS SCIEX, API QSTAR pulsar i LC-MS/MS system). An LC-MS/MS method involving positive and negative ionization mass spectroscopy was derived from profiling methods of Chiwocha and others (2003) and a report by Voigt and others (1977) on superior sensitivity of detection of GAs in negative mode. Selected-ion monitoring (Chiwocha and others 2003) was replaced with full-spectrum monitoring as used by Pirttilä and others (2004).

Internal standards were added to bud samples prior to ball mill grinding with acid-washed sand at low temperature. Standards added were deuterated CKs [*t*-Z, *t*-ZR, *t*-Z7G, *t*-Z9G, DHZ, DHZR, iP, iPA, iP9G, *t*-ZOG and *t*-ZROG 20ng each (Apex Organics, Honiton, Devon, UK)],

[$^2H_6$]ABA (200 ng, gift from CSIRO Plant Industry, Adelaide, Australia), deuterated $GAs_{(1, 4, 9, 20)}$ (5 ng, gift of C. Turnbull Imperial College, London, UK), and [$^{13}C_6$]IAA (200 ng, Cambridge Isotope Laboratories UK). Extraction was by repeated washing with (4 × 2 ml) 80% methanol (MeOH) at –20°C. Compounds without internal standards were quantified by establishment of ratios with other internal standards (for example, IBA, GA_3, and GA_7).

Extracts were washed through C-18 SepPak cartridges (Waters 051910) conditioned with 85% MeOH. The eluent (8 ml) and 2 ml 85% MeOH flush was dried to air dryness under vacuum. Samples were resuspended in 1 ml 0.1mM ammonium acetate (pH 7–8). A pH not above 8 minimizes degradation of some GAs that are sensitive to alkaline conditions (Kirkwood and others 1980). Alkaline phosphatase (5 units, Sigma P-0280) was added to each sample and incubated for 2 h at room temperature. Phosphatase activity at pH 7–8 was confirmed by dephosphorylation of *t*-zeatin riboside-5-phosphate and detection of *t*-ZR by HPLC/UV (absorption at 235 nm). Conversion was between 98 and 99%.

Positive Mode Sample Preparation

Phosphatized samples were diluted with 15 ml acetic acid (0.2 N) and adjusted if necessary to pH 1.5–2. Acidified samples were loaded onto cation exchange PRS columns (Varian Bond elute #12102094) in series with a C-18 SepPak. Columns were preconditioned with 10 ml 100% MeOH followed by 10 ml acetic acid (0.1 N). CKs were eluted from the PRS column with 7 ml of ammonium hydroxide (2 N in 50% MeOH and 20% ethanol). Samples were dried under vacuum then resuspended in 5 µl MeOH using sonication (3 min at 40 kHz) (FXP-10D, Unisonics, Australia). Sample volume was increased to 100 µl with 10 mM ammonium acetate (pH 7) and the sample was filtered (0.22-µm pore size) and analyzed by LC-QToF-MS/MS in positive mode.

Negative Mode Sample Preparation

Auxins, GAs, and ABA were eluted from the C-18 with 7 ml 90% MeOH and evaporated to dryness. Further cleanup involved resuspension in 200 µl 0.2 M acetic acid and extraction three times with 400 µl diethyl ether saturated with 0.2 N acetic acid. The colorless diethyl ether fraction was evaporated to dryness. Samples were resuspended in methanol by the same method as CK fraction. Sample volume was increased to 100 µl using 0.02% acetic acid (pH 3). Auxins, GAs, and ABA were analyzed by LC-QToF-MS/MS in negative mode.

Instrumental

A C-18 column was used for separation (C-18, 3 µm, 20 mm × 2.1 mm i.d. LCMS column, part No. 43803, Alltech Associates, Australia) fitted with a guard column (C-18, 5 µm, 4 mm long × 2.0 mm i.d., Phenomenex AJO-4286). Flow rate in all cases was 0.1 ml/min.

CKs, including the *cis*- and *trans*- isomers of ZR, were base line separated at neutral pH using a binary solvent gradient [Solvent A: 10 mM ammonium acetate; Solvent B: 350 ml MeOH, 100 ml acetonitrile (ACN), 50 ml 10 mM ammonium acetate]. The solvent gradient consisted of linear increases in solvent B as follows: initial conditions 5%, 3 min 10%, 27 min 43%, 30 min 80%, 33 min 100%, followed by wash at 100% for 15 min.

The fraction containing auxins, ABA, and GAs was separated under acidic conditions using the same column. All compounds were base line separated using a binary solvent gradient (Solvent A: 0.02% acetic acid; Solvent B: 80:20 MeOH:ACN). Solvent gradient changes were linear for all steps as follows: initial conditions 10% B, 20 min 45%, isocratic 22 min, 30 min 80%, isocratic 35 min, followed by 100% at 40 min; 15 min 100% B wash regenerated the column.

CKs were detected using positive mode with the following settings: ion source gas (GS1), 16; curtain gas, 20; ion spray voltage, 4500 V; declustering potential 1, 71.0 V; focusing potential, 230 V; declustering potential 2, 11 V; and detector MCP (CEM), 2200 V. Extracted parent and diagnostic transition ions are presented in Table 1.

ABA, auxins, and GAs were detected using negative mode with the following settings: ion source gas (GS1), 16; curtain gas, 20; ion spray voltage, –4500 V; declustering potential 1, –71.0 V; focusing potential, –230 V; declustering potential 2, –11 V; and detector MCP, 2200 V. Extracted parent ions are presented in Table 2.

Sap Amino Acids

AAs were measured by HPLC as phenylthiocarbamyl derivatives according to Rosenlund (1990). HPLC separation was based on column T-4 from Vasanits and Molnar Perl (1999). Quantified amino acids were aspartic and glutamic acid, serine, glycine, asparagine, glutamine, γ-aminobutyric acid, histidine, threonine, alanine, arginine, proline, valine, methionine, tyrosine, cysteine, iso-leucine, leucine, phenylalanine, tryptophan, and lysine.

Statistical Analysis

Significance was determined by Student's *t* test using Microsoft Excel.

Table 1 Endogenous CK ions and corresponding deuterated standard ions extracted from the 100–900-mass unit spectrum

Compound	Ion $[M+H]^+$	
	Endogenous	Deuterated
Zeatin (Z)	220 [136]	225 [137]
Zeatin riboside (ZR)	352 [220]	357 [225]
Dihydrozeatin (DHZ)	222 [136]	225 [137]
Dihydrozeatin riboside (DHZR)	354 [222]	357 [225]
Zeatin glucosides (ZOG, Z7G, Z9G)	382 [220]	387 [225]
Zeatin riboside-O-glucoside (ZROG)	514 [382]	519 [387]
Dihydrozeatin glucosides (DHZOG, DHZ7G, DHZ9G)	384 [222]	
Dihydrozeatin riboside-O-glucoside (DHZROG)	516 [384]	
Isopentenyl adenine (iP)	204 [136]	210 [137]
Isopentenyl adenosine (iPA)	336 [204]	342 [210]
Isopentenyl glucosides (iP7G, iP9G)	366 [204]	372 [210]

The diagnostic breakdown fragments are listed in square brackets for each compound

Table 2 Masses of compounds detected in negative mode using LC/QToF MS/MS analysis

Compound	Ion $[M]^-$	
	Endogenous	d_2-standard
ABA	263	269
IAA	174	180
IBA	202	
GA_1	347	349
GA_3	345	347
GA_4	331	333
GA_7	329	
GA_8	363	365
GA_9	315	317
GA_{19}	361	363
GA_{20}		333
GA_{29}		349
GA_{34}		349
GA_{37}	345	
GA_{44}	345	
GA_{51}	331	
GA_{55}	363	
GA_{57}	363	
GA_{14}	347	
GA_{53}	347	

Results

Hormone Analyses (Table 3)

t-Z increased twofold ($p < 0.05$) in apical buds in AVG-affected trees compared to normal trees but was similar in lateral buds. *t*-ZROG was easily quantifiable in apices in normal trees but at the limit of detection in AVG trees. There was significantly less GA_9 ($p < 0.0005$) in apices of AVG trees compared to normal. In lateral buds iP and *t*-Z were the dominant CKs. AVG correlated with significantly decreased iP content in lateral buds ($p < 0.05$) relative to normal trees. Lateral bud *t*-ZR concentration compared closely with sap concentrations. Elevated ABA concentrations (1.9-fold, $p < 0.001$) characterized lateral buds of AVG trees. In xylem sap of all trees *t*-ZR was the dominant CK. AVG trees differed from normal trees in having sap CK levels higher in *t*-ZR ($p < 0.05$) but lower in *t*-Z ($p < 0.005$) and iP ($p < 0.05$). *t*-ZROG was quantifiable in AVG sap but below detection in normal sap. In contrast to buds, *c*-ZR concentration was lower in AVG sap ($p < 0.05$). GA_9 levels in AVG sap were one third that of normal sap ($p < 0.05$).

This pilot study provided the first GA profile in *Macadamia*. In xylem sap at dormancy GAs were low and inconsistently detected apart from GA_9. GA_1 was detected only in sap of normal trees. GA_3 and GA_4 were not detected in sap. In buds GA_7 was most and GA_9 least abundant and GA_3 and GA_4 were intermediate.

At the present level of replication, only GA_9 showed statistical significance for differences between AVG and normal growth form at dormancy. Although other GAs varied more between replicate trees, there were trends that will potentially gain significance with more replication and could be important for the interpretation of AVG. On average GA_3 was higher in apical buds and xylem sap of AVG trees, and GA_4 and GA_7 in buds of normal trees.

IAA recoveries were low. Free indole-3-butyric acid (IBA) was readily detectable. In all samples there was a consistent trend to elevated IBA in AVG, but none were significant ($p < 0.05$).

Despite dormancy, the levels of free ABA were dynamic and changed considerably between sampling dates. The temporal variation in ABA reduced statistical significance at current levels of replication to lateral buds of AVG that

Table 3 Hormone concentrations (pmol g^{-1} FW for bud tissue, pmol ml^{-1} for xylem sap) in apical and lateral bud tissues and xylem sap

Hormone	Apical buds			Lateral buds			Xylem sap		
	AVG	Normal	*p*	AVG	Normal	*p*	AVG	Normal	*p*
***t*-Z**	13.88 ± 3.68	6.60 ± 2.41	<0.05	24.55 ± 2.31	22.14 ± 5.52		0.16 ± 0.08	0.51 ± 0.07	<0.005
***t*-ZR**	9.49 ± 2.88	13.39 ± 5.14		6.19 ± 1.19	4.27 ± 1.39		6.43 ± 0.09	4.39 ± 1.39	<0.05
***c*-ZR**	7.57 ± 2.04	6.80 ± 3.63		2.61 ± 0.63	2.54 ± 0.40		0.15	0.27 ± 0.09	<0.05
***t*-Z0G**	7.74 ± 4.92	5.87 ± 4.12		11.93 ± 2.07	12.55 ± 2.16		n.d.	n.d.	
***t*-ZROG**	0.53	12.21 ± 4.37	<0.005	9.70	8.14		2.61	n.d.	
iP	32.72 ± 5.44	30.29 ± 11.08		24.75 ± 1.55	30.04 ± 3.46	<0.05	1.43 ± 0.79	2.44 ± 0.14	<0.05
iPA	4.83 ± 0.78	4.27 ± 1.20		5.96 ± 1.50	5.28 ± 0.37		0.96 ± 0.29	0.53 ± 0.21	
DHZR	3.59 ± 0.73	3.41 ± 0.80		4.70 ± 2.71	n.d.		n.d.	n.d.	
ABA	4844.09 ± 3453.05	3310.38 ± 1430.74		3649.91 ± 293.49	1804.65 ± 252.38	<0.001	206.30 ± 119.38	144.43 ± 78.03	
IBA	178.88 ± 58.30	157.83 ± 36.09		192.75 ± 74.02	139.66 ± 52.15		2.72 ± 1.46	1.75 ± 0.71	
GA_1	n.d.	n.d.		n.d.	n.d.		n.d.	0.17 ± 0.02	
GA_3	11.48 ± 2.20	7.45 ± 3.43		3.15 ± 0.79	5.32 ± 2.75		0.35	0.11	
GA_4	8.07 ± 8.17	10.38 ± 2.90		7.38 ± 2.87	8.24 ± 2.86		n.d.	n.d.	
GA_7	103.26 ± 76.71	151.47 ± 63.06		130.65 ± 46.57	114.78 ± 34.42		n.d.	n.d.	
GA_9	1.51 ± 0.13	2.45 ± 0.13	<0.0005	1.88 ± 0.23	1.76 ± 0.39		0.02 ± 0.01	0.06 ± 0.02	<0.05

Data shown are average concentrations ± standard deviation for three replicate AVG and normal trees. Significance determined by Student's *t* test shown where $p < 0.05$. n.d. = not detected. Values without standard deviation indicate that the compound was not detected in all replicates

were twofold higher than normal trees. However, the level of free ABA in AVG was consistently higher in other tissues, 1.4 times in xylem sap and 1.5 times in apical buds compared to normal trees.

Sap Amino Acid Analyses

Aspartate, glutamate, asparagine, and glutamine combined represented 71–74% of the total AA detected in xylem sap. In AVG symptomatic trees compared with normal trees the proportion of aspartic acid ($p < 0.0005$) and glutamic acid ($p < 0.005$) as a percentage of total AA was approximately halved, 4% vs. 11% and 4% vs. 9%, respectively. AVG symptoms were also accompanied by a significant increase in asparagine and glutamine ($p < 0.05$) as a proportion of total AA content.

Discussion

Method Development

Advancing current knowledge of plant physiology requires an increasingly comprehensive analysis of structurally diverse plant hormones and metabolites from small individual samples. For trace analysis the simultaneous detection of native state compounds maximizes information gained from samples of limited size.

Chiwocha and others (2003) developed a profiling method to screen for selected compounds in dark germinated lettuce seedlings. To achieve adequate sensitivity for compounds, the LC-MS/MS run was separated into time windows of positive and negative detection modes (positive mode for CKs; negative mode for GAs, auxin, and ABA). This method required time separation of compounds detected in negative and positive modes, although simultaneous recording of positive and negative modes was employed in a run section where elution windows overlapped. Overlap of retention times becomes a problem when analyzing increasing numbers of structurally different analytes (Chiwocha and others 2003). Therefore, the aim of our project was to accommodate the different chemical properties of these compounds while maintaining the selectivity and sensitivity required for quantification.

Compound quantification is not only limited by chromatographic overlap, but also by the choice of MS detection technology. Triple-quadrupole LC-MS/MS, as previously used by Mader and others (2003a) and Chiwocha and others (2003), is a powerful technique that is suitable for many applications of trace-level analysis. However, there are inherent characteristics that make triple-quadrupole MS less suitable for wide-range hormone

profiling. For this kind of application QToF-MS provides particular advantages: higher mass accuracy and better resolution are achieved while sensitivity remains acceptable. This difference in performance of triple-quadrupole MS and QToF-MS is due to the different acquisition procedures used: scanning a specific mass transition versus acquiring a complete spectrum (QToF-MS).

Although triple-quadrupole instruments achieve a higher level of sensitivity, this advantage can be applied to monitoring only a relatively small number of compounds. Because only one type of ion is recorded at any given time, sensitivity is inversely related to the number of monitored ions. In contrast, time-of-flight detectors (ToF) record all ions in parallel, providing full spectra with better signal-to-noise ratios than comparable spectra obtained with a scanned quadrupole (Marchese and others 2003). In principle the number of detectable compounds is unlimited with simultaneous ToF detection.

Another advantage of QToF mass spectrometers over triple-quadrupole mass spectrometers is their high resolution and accuracy. By differentiating fragment ions that differ in mass by 250 ppm, interferences of ions with similar mass are minimized (Steen and others 2001). Furthermore, because quadrupole scanning filters out most ions, it results in the loss of a large amount of qualitative information compared to time-of-flight MS that simultaneously provides qualitative and quantitative information. Thus, QToF is a very powerful tool not only for quantification purposes, as demonstrated in this study, but also when studying metabolic routes and degradation pathways. Acquisition of accurate complete product ion mass spectra provides unequivocal information on compound structure and allows sample spectra to be screened for additional compounds such as hormone precursors and metabolites.

In conclusion, the use of LC-QToF-MS technology maximizes the information gained without needing to split small samples for different analyses. It provided the tool to improve the methods developed in earlier studies (Mader and others 2003a) using triple-quadrupole MS detection. With no compromise between the numbers of compounds quantified per injection and sensitivity, QToF improved time and cost efficiency per compound analyzed. Detection of native state auxins, ABA, and GAs saved preparation time, reduced the chance of compound loss, and eliminated hazards like the synthesis of diazomethane used in auxin derivatization. Although processing time remained essentially unchanged, this method of detection produced more data. By separating positive mode and negative mode compounds during sample cleanup, we also reduced interfering compounds in each run and gained access to the complete range of compounds by optimizing separation gradients for each mode. A method resulted that handled the complex sample matrix of *Macadamia* tissues and reliably detected and quantified a broader compound spectrum in small samples.

There is potential for this method to be further refined by expanding the number of GAs quantified. Limited resources of this study did not allow further exploration of the range of detectable GAs (Table 2). Although partitioning with a range of solvents of different pH is widely used in extract purification (Blake and others 1993), method development has to consider the possibility of pH-related artifacts in the GA profile (Kirkwood and others 1980; Mander 2003) arising from extraction conditions. Blake and others (1993) reported differential partitioning of GA-like characteristics following multiple partitioning steps. Therefore, when expanding the range of GAs quantified in the absence of specific internal standards, recovery ratios in relation to available internal standards need to be established.

Because it was beyond the scope of this initial study to analyze CK phosphates separately, extraction of CKs from phosphatized samples with solid-phase cation exchange was used (Mader and others 2003a). However, the physiology of CK phosphates is likely to receive more interest in the future becausse CK phosphates are an integral part of CK metabolism (Mader and others 2003b; Zhang and others 2003). CK phosphates are not retained by cation exchange and remain in the same fraction as GAs, auxins, and ABA. Direct detection in negative mode together would be desired to avoid the necessity of additional lengthy procedures [phosphatase treatment at this stage of sample preparation followed by cation separation of the dephosphorylated CKs and a second positive mode run (Mader and others 2003a)].

Methanol-only extraction of IAA is commonly used (Croker and Hedden 2000) but could be inadequate for *Macadamia* tissues, considering that the recovery was lower in samples than standards. Oxidative degradation of IAA during the extraction and purification process may have contributed to the low recoveries of IAA. Antioxidant addition may improve IAA detection (Iino and others 1980) and has been recently used (Jackson and others 2002; Taylor and Cowan 2001). In future work on *Macadamia* or other tree species based on this method, IAA recovery might be improved by antioxidant addition during hormone extraction.

AVG and Hormones

AVG in *Macadamia* has two key aspects, the change to a vertical growth habit and the loss of flowering. The change to a more vertical tree form raises the question of intensified apical dominance (AD), or more importantly apical control (AC). Crown shape is not determined solely by

which buds on a branch grow out (AD) but also by the extent and angle of further growth of lateral branches (AC).

AVG: Apical Dominance/Control A large body of literature has been accumulated on apical dominance (AD) in herbaceous plants through studies of exogenous hormone treatments and transgenic and mutant plants (Beveridge 2006). In contrast, the mechanisms of AD in trees are poorly understood due to the structural complexity of trees. Suitable mutants and transgenic plants are rare but are becoming available (Busov and others 2006). The change to vertical growth in AVG provides an opportunity to investigate interaction of endogenous hormone physiology and crown architecture.

Previous evidence suggested a role of CKs and in some cases of auxin in regulating verticality of growth and branch angle. Bending vertical shoots decreased CK levels and polar auxin transport (Sanyal and Bangerth 1998a). Abnormal plagiotropy in leading shoots of blue spruce was characterized by a lower CK-to-auxin ratio resulting from reduced CK and increased auxin levels (Matschke 1993). Furthermore, orthotropic shoots formed after decapitation were repressed by auxin replacement (Wilson 1986, 2000; House and others 1998) suggesting that the loss of the apical auxin source shifts the hormone balance toward CKs. In AVG there is no loss of the auxin source, but an increase in CKs could result in a hormone shift similar to decapitation.

The evidence most relevant to AVG comes from apple, the only other tree system where it is possible to compare vertical and normal growth forms. The columnar growth habit of some apple trees correlated with increases of ZR in shoots (Looney and others 1988; Watanabe and others 2004, 2006) and is consistent with the elevated ZR levels in xylem sap and lateral buds of AVG symptomatic trees.

Although the role of CKs in both AD and AC is considered conclusive, the inhibitory role for auxin holds only for AD and remains inconclusive for AC (reviewed in Beveridge 2006). In apple trees no significant auxin difference was found between normal and columnar growth forms during the entire season (Watanabe and others 2004). Cline and Sadeski (2002) found no support for the role of auxin as a repressor signal for apical control in *Ipomoea* and concluded that the auxin response of AD and AC differs. There is also evidence that gibberellins may reinforce the control of auxin on bud outgrowth (Davenport and others 2001).

Free IAA follows a seasonal pattern, with low levels from autumn through winter (Rodriguez and others 1991; Rodriguez and Sanchez 1986; Sandberg and Ericsson 1987). This study, possibly because of oxidative loss at dormancy, failed to consistently detect IAA, but IBA was present in all samples. IBA can be metabolized to IAA (Ludwig-Müller 2000; Woodward and Bartel 2005) and has been shown to be able to reinstate AD in woody species (reviewed in Cline 2000; House and others 1998). It is also possible that IBA exerts some effect on growth independent of conversion to IAA (Ludwig-Müller 2000; Poupart and Waddell 2000). There was a trend toward elevated IBA in lateral buds of AVG trees ($p = 0.07$). The data at this stage are insufficient to support IBA participation in the expression of vertical growth in AVG. Profiling of a wider range of auxin-related compounds, including precursors and conjugates, will help to elucidate the role of auxin in AVG.

The role of ABA in bud dormancy, apical dominance, and branching control is less studied. In particular, the interactions with other hormones are not fully understood. The influence of ABA on bud outgrowth and flowering was found to be affected by the composition of other hormones (Takeno and Maeda 1996; Wijayanti and others 1997; Rounala and others 2006). However, based on results presented in the literature, an inhibitive role is likely for ABA. Reduced lateral branching in AVG associated with a twofold increase in ABA ($p < 0.001$) suggested an inhibitory role for ABA in lateral bud outgrowth which is consistent with current knowledge. ABA content correlated with growth inhibition in lupin branch buds (Emery and others 1998) and onset of dormancy with a 2.5-fold accumulation of ABA in buds of cedar (Piola and others 1998). Furthermore, induction of lateral bud outgrowth by decapitation resulted in significant ABA decreases (Gocal and others 1991; Mader and others 2003a; Destefano-Beltrán and others 2006). Transgenic birch rendered insensitive to ethylene showed a loss of apical dominance and reduction in apical meristem ABA levels compared to wild-type trees (Rounala and others 2006). The source of elevated ABA in AVG buds could be the roots (reviewed in Davies and others 2005) as indicated by consistently increased xylem sap concentrations. This would also be consistent with reports that basally applied ABA partially restored AD in *Ipomoea* and *Solanum* and additively repressed bud growth when combined with apical auxin (Cline and Oh 2006). The involvement of ABA in AC and tree architecture deserves further consideration due to the consistently increased ABA concentrations measured in all samples of AVG trees.

AVG: Vigor vs. Flowering Visual identification of AVG in *Macadamia* is based on assessment of increased vegetative growth that is strongly vertical and a large reduction in flowering and nut production. Apical dominance established by IAA was reinforced by GA_3 (Davenport and others 2001) and/or ABA (Cline and Oh 2006). A trend ($p = 0.099$) toward elevated GA_3 in apical buds of AVG symptomatic trees may support this hypothesis. Elevated endogenous GA levels stimulated secondary growth by increasing number and length of xylem fibers in transgenic

aspen (Eriksson and others 2000) and resulted in earlier lignin production and increased vegetative biomass accumulation in tobacco (Biemelt and others 2004). Furthermore, short-term GA_3 application increased lignin formation as well as cambium activity (Biemelt and others 2004). Although this pilot study did not quantify sap flux rates, greater quantities of xylem sap were obtained from branches of AVG symptomatic trees compared to normal trees. Trees removed from *Macadamia* orchards are generally processed to mulch. AVG symptomatic trees are reported to be more difficult to shred suggesting increased lignification (Andrew Pearce, personal communication). Microscopy and sap flux measurements may confirm increased xylem formation and lignification in AVG.

In spruce exhibiting differential flowering ability due to clonal properties (Mortiz and others 1990b) or environmental conditions (Moritz and Oden 1990), poor flowering was associated with gibberellin synthesis directed toward GA_1 and GA_3. GA_1 was not detected in buds of *Macadamia* at the time of sampling. GA_3, however, showed a trend ($p = 0.099$) to higher levels in apical buds of AVG trees. A single application of GA_3 was sufficient to reduce flowering in *Macadamia* by 93% (Nagao and Sakai 1990) and bloom density by 92% in *Prunus avium* (Lenahan and others 2006).

In contrast to GA_1 and GA_3, GA_7 and GA_4 were often associated with promoted flowering, especially when applied exogenously in conifers (Ross 1992; Cecich and others 1994). In the current study, GA_7 was the most abundant GA in buds but below detection in xylem sap. Differences between AVG and normal trees were not significant at the present level of replication.

In spruce a higher capacity for GA_9 and GA_4 synthesis was associated with precocious clones and flower bud–inducing conditions (Moritz and Oden 1990; Moritz and others 1990). Consistent with this, GA_9 occurred at significantly higher concentrations in apical buds and xylem sap of normal trees capable of flowering compared to AVG trees. GA_9 is metabolized to the physiologically effective GA_4 (Moritz and others 1989) and a tendency for GA_4 to be higher in apices of normal trees would be consistent with a function in flower bud induction in *Macadamia*. Although the *Macadamia* trees were visibly dormant at the time of sampling, it was likely that flower bud induction was occurring because buds become noticeably differentiated in May (Nagao and Hirae 1992). Similarities in alternate cropping trees support this hypothesis. A lack of GA_4 correlated with biennial bearing habit in apple (Stephan and others 1999). In olives (Ulger and others 2004) GA_3 increased in the cropping year and GA_4 in the noncropping year, associating GA_3 with inhibition and GA_4 with promotion of flowering in the following season.

In conclusion, differences in GA_9 and GA_4 in *Macadamia* may correlate with the ability to form flower buds and therefore serve as markers for normal tree development. More detailed studies are required to confirm the effectiveness of GAs as markers and to determine the optimal time for testing. Higher replication and sampling throughout the year will clarify whether the trends observed in the more variable GA_3, GA_7, and GA_4 are significant. Because AVG symptoms range along a continuum in *Macadamia*, variation in hormone contents between individual trees is not surprising.

AVG and AA in xylem sap Changes in nitrogen metabolism are interconnected with the carbohydrate status and/or respiration rates (Morcuende and others 1998). In the AA profile of *Macadamia* the proportion of aspartate and glutamate were halved in the xylem sap of AVG trees suggesting altered metabolism. The cause of these metabolic changes is unknown. Nevertheless, the magnitude of the reduction of aspartate and glutamate in the AA profile in xylem sap of AVG symptomatic trees raises the question of whether AA could be used as a marker for AVG.

AVG: A Matter of Root-Shoot Imbalance? This study provided results implicating altered root/scion relations in the AVG growth disorder. *Macadamia* root growth is highest during autumn (March-May) (Firth and others 2003). There are also anecdotal reports of a deeper more vertical root system in AVG trees (Patrick O'Farrell, personal communication) that differs from the shallow fibrous system of normal *Macadamia* described by Firth and others (2003). Roots are accepted as a major source of CKs. Significantly increased *t*-ZR and iP levels in shoot xylem sap suggest elevated root export in AVG trees, which is likely to alter root/shoot interactions. This conclusion is supported by the correlation of increased root export of ZR-type CKs with coppice shoot characteristics and increased vigor in birch (Rinne 1994; Rinne and Saarelainen 1994). The impact of AVG symptoms on water use and corresponding xylem flow rates requires further study. It was noticeable that AVG branches yielded higher sap volumes. If higher flow rates were confirmed, CK delivery to the AVG shoots would be even higher than concluded from the concentrations measured in this study.

In addition to changes in root CK export, there were also indications of altered CK metabolism in the shoot. The concentration of *t*-Z was doubled in apical buds of AVG compared to normal trees despite sap *t*-Z being reduced by 68%. Likewise, the *t*-ZR level of AVG apical buds did not reflect the twofold increase in *t*-ZR in AVG xylem sap. Combined, these changes in sap *t*-ZR and *t*-Z in AVG resulted in a fivefold reduction of the *t*-Z:*t*-ZR ratio ($p < 0.05$, Table 4), which suggests that the *t*-Z:*t*-ZR ratio in shoot xylem sap could be an indicator of vegetative vigor in *Macadamia*. This corresponds well with findings

Table 4 Significant differences in hormone ratios of AVG trees compared to normal trees

Tissue	Compound ratio	AVG % normal	*p* value
Apical	iP:GA_7	183.72	<0.05
	t-Z:iPA	189.18	<0.05
	IBA:GA_9	181.73	<0.05
	ABA:GA_7	211.93	<0.05
	DHZR:GA_9	170.13	<0.05
Lateral	ABA:GA_3	365.50	<0.05
	c-ZR:GA_1	249.78	<0.05
	iP:ABA	42.33	<0.05
	iPA:GA_3	325.37	<0.01
	t-ZR:GA_3	227.69	<0.01
Sap	iP:iPA	28.30	<0.05
	iP:ABA	35.75	<0.05
	t-Z:iPA	16.30	<0.05
	t-Z:*t*-ZR	19.80	<0.05
	t-Z:IBA	19.15	<0.05
	IBA:GA_9	402.41	<0.05
	t-ZR:GA_9	411.38	<0.05

Data are the mean AVG ratio (n = 3) as a percent of normal (n = 3)

in apple where CKs in the xylem sap of vigorous rootstocks were dominated by *t*-ZR in contrast to *t*-Z in dwarfing rootstocks (Kamboj and others 1999). The dominance of *t*-ZR in the xylem sap of all trees is consistent with the current selection of *Macadamia* seedling rootstocks to provide vigorous grafted trees. Selection of clonal rootstocks inducing a higher *t*-Z:*t*-ZR in the shoot could be a way to reduce vegetative vigor in *Macadamia* and susceptibility to AVG. In particular, root zone conditions causing high turnover of fine roots, a major source of both CKs and ABA, deserve consideration when investigating the causality of AVG.

The altered *t*-Z:*t*-ZR ratio discussed above was not the only statistically significant change in hormone ratios in dormant *Macadamia*. A set of statistically significant changes in ratios between particular hormones resulted from the distinct differences in hormone profiles of AVG and normal branches (Table 4). Currently it is not possible to interpret the physiological significance of most of them because of a lack of other studies analyzing hormones in similar detail and correlating ratios with specific physiological processes. With increased application of LC-QToF MS technology, the significance of these ratios will emerge and some may prove to be useful markers for AVG or indicate potential for susceptibility to AVG.

In conclusion, the presented method advances analytical capacity by simultaneously detecting a broad number of compounds and being suited to applications where sample size is limited, hormone abundance low, and sample matrix complex. Characterizing compounds from four hormone groups was the first step taken toward exploring the physiology underlying AVG in *Macadamia* and demonstrated the complexity of plant architecture control and the fragmentary nature of the current understanding.

Method development in this study was conducted during the period of limited vegetative and reproductive activity which allowed collection of relatively uniform material from individually selected trees. Hormone levels are generally lowest during dormancy so sampling at this time was most suitable for testing limits of sample size. The identification of hormonal changes associated with the expression of AVG in this study has provided direction for future work. Sampling throughout the vegetative and reproductive cycle of the tree will be required to determine the most reliable markers (specific hormones or hormone ratios) and the optimal time for testing. In the absence of mechanical pruning *Macadamia* trees have a highly asynchronous flushing pattern (Olesen 2005). Flushing occurs to some extent throughout the year (Stephenson and Cull 1986) while the crop is retained on the tree for up to nine months. Seasonal hormone profiling will have to accommodate this complexity by careful selection of material at equivalent morphologic stages. Predicting and counteracting AVG susceptibility in trees will require more comprehensive study, especially in relation to rootstock scion compatibility.

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