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Precolumn Derivatization and Capillary Liquid Chromatographic/Frit-Fast Atom Bombardment Mass Spectrometric Analysis of Cytokinins in *Arabidopsis thaliana*

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New cytokinin derivatives with high surface activity were developed for capillary liquid chromatography/frit-fast atom bombardment (FAB) mass spectrometry. Propionyl ester derivatives of cytokinin nucleosides and glucosides and benzylamine derivatives of cytokinin bases gave stronger $[M + H]^+$ ion currents than the underivatized compounds. In trace analysis by selective reaction monitoring, low (fmole) detection limits were found. In qualitative analysis by *B*/*E*-linked scanning, the derivatives also gave more spectral information, owing to the presence of fragment ions, diagnostic for the sugar moieties of nucleosides and glucosides, not present in the spectra of underivatized compounds. The proposed FAB method was used to identify and quantify 10 isoprenoid cytokinins in *Arabidopsis thaliana*, including free bases, nucleosides, nucleotides and glucosides. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: capillary liquid chromatography/mass spectrometry; fast atom bombardment; derivatization; cytokinins; *Arabidopsis thaliana*

INTRODUCTION

Cytokinins are an important class of growth-promoting substances, defined by their capacity to promote cell division in plant tissue culture in the presence of auxins.[1,2] They affect a wide array of biological processes in plant development, such as lateral bud growth, delay of leaf senescence and cell division.[3] Chemical species containing adenine substituted at N-6 with an isoprenoid side-chain form the most intensively studied group of compounds with cytokinin activity found in plants.[4] They mainly occur as free bases, nucleosides, nucleotides and glucosides, often present in very low concentrations, i.e. 1–100 pmol g^{-1} fresh mass. Consequently, studies of cytokinin physiology require very sensitive analytical tools. As a plant extract is a complex multicomponent mixture, accurate cytokinin determinations can only be obtained with highly purified samples and selective detection methods, e.g. immunoassays,[5] and mass spectrometry (MS).[6]

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Although immunoassays often show high sensitivity, their accuracy can be compromised by cross-reactivity of the antibodies and interference with impurities originating from the plant. However, even higher sample purity is needed when using non-specific methods of detection such as UV absorbance. A common approach has therefore been to use immunoaffinity chromatography in sample preparation prior to analysis by HPLC/PDA[7], but this method is limited by the comparatively low sensitivity of PDA detectors. High levels of purification have also been needed, to date, for cytokinin analysis of plant extracts by mass spectrometry, but the method can give significantly greater accuracy than immunoassays, especially when heavy isotope-labelled internal standards are used.[8]

For a number of other plant hormones, such as indoleacetic acid (IAA) and gibberellins (GAs), coupled capillary gas chromatography/mass spectrometry (GC/MS) has been used with success. Prior to analysis, samples are often derivatized by methylation or trimethylsilylation and a quadrupole mass spectrometer provides a simple means of analysis. Magnetic sector instruments give high sensitivity, with practical detection limits in the low femtomole range, which makes analysis of IAA and GA contents in milligram samples of plant tissue possible.[9,10]

GC/MS has also been used for cytokinin analysis. Chemical modification of hydrogen binding functional groups is a prerequisite for converting these compounds into volatile derivatives suitable for GC. A range of different derivatization methods have been tested, e.g. tri-

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methylsilylation (TMS),[11,12] *tert*-butyldimethylsilylation (t-BuDMS),[13] permethylation,[14,15] trifluoroacetylation (TFA)[16] and acetylation.[17]

These methods are often associated with a number of technical problems. High temperatures (>250 °C) are needed to elute the permethyl or TMS derivatives of cytokinin ribosides and glucosides and interaction of the basic cytokinins with active sites of the support silica material leads to loss of sample and pronounced peak tailing.[18]

The TMS and TFA derivatives are also extremely sensitive to moisture and the use of t-BuDMS is restricted to free bases. The derivatization involved is also generally performed with extremely water-sensitive reagents and the preparation is time consuming and inconvenient in the case of permethylation. The acetyl derivatives are, in contrast, stable and easy to prepare but their volatility is low. To circumvent the problems associated with GC, attention has been focused on LC/MS. Imbault *et al.*[19] showed that frit-fast atom bombardment interface MS (LC/frit-FAB-MS) could be used in the analysis of underivatized cytokinins. In FAB, it is well known that there is a positive correlation between analyte surface activity and ion signal.[20–22] Hence LC/frit-FAB-MS analysis of cytokinins results in higher sensitivity for the cytokinin nucleoside iPA than for the more hydrophilic nucleoside ZR. It is possible, however, to increase the sensitivity of the analyses by acetylation of appropriate compounds such as cytokinin ribosides and glucosides.[23] Acetylation has also been used to quantify ribosides in tobacco.[24]

Yang *et al.*[25] have reported on the use of an atmospheric pressure ionization (API) interface and Prinsen *et al.*[26] showed that electrospray tandem mass spectrometry (ESI-MS/MS) could be used in the quantitative analysis of cytokinins. In the MS/MS approach a detection limit of 1 pmol of ZR was reported. However, no chromatographic separation was achieved in this system and suppression effects are expected when extracts, although highly purified, are applied.[27]

The MS-based methods mentioned above have been used to identify and quantify isoprenoid cytokinins in a number of plant species such as tobacco,[24] rice,[28] bean[29] and Scots pine.[30] However, although *Arabidopsis thaliana* has become one of the major genetic models for plant research, studies of cytokinins in this species have been limited, possibly because current analytical techniques demand large samples, and the plant produces small amounts of tissue. So far, quantitative analyses of cytokinins in *Arabidopsis* have been performed by HPLC/PDA of samples purified by immunoaffinity chromatography.[31,32] In order to obtain more accurate analysis of cytokinins in *Arabidopsis*, mass spectrometric methods have to be developed to meet the new demands set by the *Arabidopsis* model system. Based on the characteristics of the frit-FAB ionization technique, we belive that sensitivities superior to those of all other cytokinin mass spectrometric methods used can be achieved after appropriate chemical modification of the surface activity. The derivatization has, for practical reasons, to be based on highly reliable reactions and must lack the problems associated with GC/MS derivatization procedures. A major disadvantage with FAB, due to the glycerol matrix used, is the high background noise observed in analysis by single ion monitoring (SIM) or normal scanning. The increased selectivity of a double-focusing magnetic sector instrument, operated to detect fragment ions formed in the first field-free region, limits the matrix effect. Using *B*/*E*-linked scanning[33] for qualitative analysis and selective reaction monitoring (SRM)[34] for quantitative analysis, the larger size of parent ions, obtained by appropriate derivatization, would also contribute to a reduction in matrix-derived background. Our ultimate aim is to identify and quantify isoprenoid cytokinins in *Arabidopsis thaliana* grown in a range of conditions, for purposes such as identifying and characterizing hormone metabolism mutants. In this work we report the improved sensitivity obtained for cytokinin analysis by derivatization and assess the suitability of the LC/frit-FAB-MS method for analysing cytokinins in *Arabidopsis thaliana*.

EXPERIMENTAL

Plant material

Arabidopsis thaliana ecotype Colombia plants were grown in soil at 22 °C under short day conditions of 9 h photoperiod, with a photon density of 130 μE m^{-2} s^{-1}. After 7 weeks the leaf rosettes (0.5–0.8 g) of single plants were harvested and immediately frozen in liquid nitrogen.

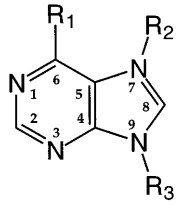
Chemicals

Unlabelled and stable isotope-labelled cytokinins were purchased from Apex Organics (Devon, UK). Using a high-sensitivity analytical balance (Mettler AT 20, resolution 2 μg), stock solutions of cytokinins in dimethylformamide (DMF) were prepared. The compounds and their structures are listed in Table 1. Tritium-labelled Z, iP and iPA (specific activities 0.9, 1.6 and 1.6 TBq $mmol^{-1}$, respectively) were purchased from the Institute of experimental botany (Academy of Sciences of the Czech Republic, Prague, Czech Republic) and tritium-labelled AMP (0.7 Tbq $mmol^{-1}$) from Amersham (Buckinghamshire, UK). Benzyl bromide, propionic anhydride, butyric anhydride, isobutyric anhydride, caproic anhydride and 2-ethylbutyric anhydride (purum) were purchased from Fluka (Buchs, Switzerland). Merck (Darmstadt, Germany) supplied *N*-methylimidazole (synthesis grade) and potassium carbonate (p.a.). Except for DMF (glass distilled, Fluka), MeOH (HPLC grade, Baker, Deventer, The Netherlands), acetonitrile (HPLC grade, Labscan, Ireland) and water (Milli-Q), the solvents used were all of standard p.a. quality.

Derivatization of cytokinins

Hydroxyl groups were esterified with a method modified from Connors and Pandit.[35] The samples were derivatized in DMF, *N*-methylimidazole and a carbox-

Table 1. Cytokinins analysed in this study[a]



Compound	R_1	R_2	R_3
Zeatin(Z)	*trans*-$NHCH_2CHC(CH_3)CH_2OH$	—	H
[$^2H_5$]Zeatin(D_5-Z)	*trans*-$NHCH_2CHC(CD_3)CD_2OH$	—	H
Zeatin riboside(ZR)	*trans*-$NHCH_2CHC(CH_3)CH_2OH$	—	β-Rib
[$^2H_5$]Zeatin riboside(D_5-ZR)	*trans*-$NHCH_2CHC(CD_3)CD_2OH$	—	β-Rib
Zeatin-7-glucoside(Z7G)	*trans*-$NHCH_2CHC(CH_3)CH_2OH$	β-Gluc	—
[$^2H_5$]Zeatin-7-glucoside(D_5-Z7G)	*trans*-$NHCH_2CHC(CD_3)CD_2OH$	β-Gluc	—
Zeatin-9-glucoside(Z9G)	*trans*-$NHCH_2CHC(CH_3)CH_2OH$	—	β-Gluc
[$^2H_5$]Zeatin-9-glucoside(D_5-Z9G)	*trans*-$NHCH_2CHC(CD_3)CD_2OH$	—	β-Gluc
Zeatin-*O*-glucoside(ZOG)	*trans*-$NHCH_2CHC(CH_3)CH_2O$-β-Gluc	—	H
[$^2H_5$]Zeatin-*O*-glucoside(D_5-ZOG)	*trans*-$NHCH_2CHC(CD_3)CD_2O$-β-Gluc	—	H
Zeatin riboside-*O*-glucoside(ZROG)	*trans*-$NHCH_2CHC(CH_3)CH_2O$-β-Gluc	—	β-Rib
[$^2H_5$]Zeatin riboside-*O*-glucoside(D_5-ZROG)	*trans*-$NHCH_2CHC(CD_3)CD_2O$-β-Gluc	—	β-Rib
Zeatin riboside monophosphate(ZR 5′-MP)	*trans*-$NHCH_2CHC(CH_3)CH_2OH$	—	β-Rib 5′-MP
[^{15}N,$^2H_5$]Zeatin riboside monophosphate(^{15}N,D_5-ZR 5′-MP)	*trans*-$^{15}NHCH_2CHC(CD_3)CD_2OH$	—	β-Rib 5′-MP
Dihydrozeatin(DHZ)	$NHCH_2CH_2CH(CH_3)CH_2OH$	—	H
Dihydrozeatin riboside(DHZR)	$NHCH_2CH_2CH(CH_3)CH_2OH$	—	β-Rib
Isopentenyladenine(iP)	$NHCH_2CHC(CH_3)_2$	—	H
[$^2H_6$]Isopentenyladenine(D_6-iP)	$NHCH_2CHC(CD_3)_2$	—	H
Isopentenyladenosine(iPA)	$NHCH_2CHC(CH_3)_2$	—	β-Rib
[$^2H_6$]Isopentenyladenosine(D_6-iPA)	$NHCH_2CHC(CD_3)_2$	—	β-Rib
Isopentenyladenosine monophosphate(iPA 5′-MP)	$NHCH_2CHC(CH_3)_2$	—	β-Rib 5′-MP
[^{15}N,$^2H_5$]Isopentenyladenosine monophosphate(^{15}N,D_5-iPA 5′-MP)	$^{15}NHCH_2CHC(CD_3)_2$	—	β-Rib 5′-MP

[a] β-Rib: β-D-ribofuranosyl; β-Rib 5′-MP: β-D-ribofuranosyl 5′monophosphate; β-Gluc: β-D-glucopyranosyl. The numbering system of the purine ring is illustrated

ylic acid anhydride (5:3:1, v/v). The carboxylic acid anhydrides used for cytokinin nucleosides and glucosides were propionic and butyric anhydride. For Z and DHZ; propionic, butyric, isobutyric, caproic and 2-ethylbutyric anhydride were used. The samples were heated at 37 °C for 30 min, after which the solvents and reagents were evaporated *in vacuo*. To increase the rate of evaporation, acetonitrile could be used instead of DMF for ribosides and benzylated zeatin and dihydrozeatin. The caproic and 2-ethylbutyric esters were purified after dilution in water by reversed-phase separation on a C_{18} cartridge (100 mg Bond Elut) (Varian, Harbor City, CA, USA). Monobenzylation of purine NH groups on the cytokinin bases was performed by dissolving the samples in acetone and benzyl bromide (4:1, v/v), followed by heating at 37 °C for 30 min. To achieve multiple benzylation, finely ground potassium carbonate (1–5 mg in 20 μl) was added to the vial and the samples were heated at 70 °C for 30 min. After the treatment, the volume of the reaction mixture was reduced *in vacuo*. The carbonate was removed by partitioning between butan-1-ol and water. The side-chain hydroxyl groups of Z and DHZ were subsequently esterified as described above. The derivatizations were confirmed by UV-absorption analysis of the reaction products following separation by HPLC using a Waters (Milford, MA, USA) system consisting of a Model 600 pump, a Model 717 autosampler and a Model 996 PDA detector. Addition of ^3H-labelled cytokinins to the derivatization mixture enabled reaction yields to be checked by radiocounting fractions collected from the HPLC eluate.

Liquid chromatography/mass spectrometry

The capillary LC system consisted of a Model 680 gradient controller and two Model 510 pumps equipped with microflow pump heads (Waters). In a precolumn split setting, a final flow-rate of 4 μl min^{-1} (injection phase; 10 μl min^{-1}) was established by splitting the major part of the flow through a 120 × 2.1 mm i.d. HPLC balance column. The other outlet of the splitting tee (Valco, Houston, TX, USA) was connected via a pneumatic switching valve (Waters) to a 150 × 0.3 mm i.d. capillary LC column (LC Packings, Amsterdam, The Netherlands) packed with Symmetry ODS packing material (Waters). A Model 717 autosampler (Waters) was connected to the pneumatic valve. In the injection phase, a 10 μl min^{-1} flow of a mobile phase containing 20% solvent B was pumped through the autosampler (sample injections 20 μl). After 10 min the derivatized cytokinins were bound to the head of the capillary column. The valve was then switched, cutting off the autosampler from the flow, so the subsequent mobile phase gradient (flow-rate 4 μl min^{-1}) passed only through the capillary column. The cytokinins were collected by gradient elution: 10–12 min, 20–50% B; 12–40 min, isocratic 50% B. A third Model 501 pump

was used for washing the autosampler (while off-line) with a mobile phase containing 20% B. Solvent A contained 98% water, 1% formic acid and 1% glycerol and solvent B contained 97% acetonitrile, 1% water, 1% formic acid and 1% glycerol.

The capillary HPLC column was connected, via a continuous-flow frit-FAB LC/MS interface (Jeol, Tokyo, Japan) to the ion source of a double-focusing JMS-SX102 mass spectrometer (Jeol). The ion source temperature was 55 °C. Ions were generated with a beam of 3 kV xenon atoms at an emission current of 10 mA. Normal scan analysis was performed by scanning the magnet at a rate of 5 s per scan for a mass range of 40–800 or 40–1000 u. Fragment ion spectra were obtained by *B*/*E* linked scanning[33] at a rate of 5 s per scan for a mass range of 0–800 or 0–1000 u. Spectra were background subtracted. Trace amounts were quantified in the selective reaction monitoring (SRM) mode,[34] detecting diagnostic transitions of the quasi-molecular ion to an abundant daughter ion. The daughter ion resolution was 1000 and the dwell time was 200 ms. Data were processed by a Jeol MD-7010 data system.

Cytokinin extraction and sample preparation

The frozen plant material was ground in liquid nitrogen and 500 mg of homogenized tissue were dropped into 5 ml of pre-cooled methanol–chloroform–formic acid–water (12:5:2:1, v/v)[36] and stable isotope-labelled cytokinins were added as internal standards. The samples were extracted overnight at −20 °C. To stabilize the pellet, 1.5 ml of methanol–formic acid–water (25:25:1, v/v) was added and the cell debris was removed by centrifugation (2000 *g*, 15 min). The cytokinins were extracted from the supernatant by the use of a 500 mg strong cation-exchange cartridge (SCX) (Varian, Harbor City, CA, USA), pre-equilibrated with methanol water–formic acid (25:24:1, v/v). After washing the cartridge with 4 ml of extraction buffer and 4 ml of MeOH, the cytokinins were eluted with 4 ml of MeOH–4 M aqueous NH_3 (3:2, v/v) and then the eluate was reduced under vacuum to aqueous phase.

The extracts were diluted with 10 mM ammonium formate to a volume of 4 ml and further purified on a 0.5 ml DEAE-Sephadex anion exchanger (Pharmacia, Uppsala, Sweden), in series with a 100 mg C_{18} cartridge (Varian). After washing with 8 ml of 10 mM ammonium formate, the columns were separated. The nucleotides were eluted from the anion exchanger with 12 ml of 1 M ammonium formate and bases, nucleosides and glucosides were eluted from the C_{18} cartridge with 4 ml of MeOH–water–acetic acid (80:20:1, v/v). Nucleotides were converted into nucleosides by alkaline phosphatase treatment (P-5931 from *Escherichia coli*, supplied by Sigma, St Louis, MO, USA) in 50 mM ammonium acetate buffer (37 °C, 30 min) and purified by reversed-phase separation on a 100 mg C_{18} cartridge (Varian). The samples were then analysed by LC/MS as described above.

Bases, nucleosides and glucosides were separated into five groups of compounds on a reversed-phase-column (Symmetry 150 × 4.6 mm i.d.; Waters). The HPLC system (Waters) consisted of a Model 600 pump, a Model 717 Autosampler and a Model 996 PDA detector connected to an ISCO Retriever II fraction collector. The cytokinins were separated by gradient elution. With the multiple solvent pump, four different solvents were used. Solvent A consisted of 98% water and 2% acetic acid, solvent B consisted of 98% methanol and 2% acetic acid, solvent C consisted of 1 mM ammonium acetate and solvent D consisted of methanol. The following gradient was used: 0–4 min, 80% C–20% D; 4–10 min, a linear gradient to 75% A–25% B; 10–15 min, a linear gradient to 50% A–50% B; 15–18 min, a linear gradient to 100% B. Five fractions were collected: 4–12 min (containing Z and DHZ-glucosides), 12–14 min (Z and DHZ), 14–19 min (ZR, DHZR, ZROG and DHZROG), 19–21 min (iP) and 21–23 min (iPA). Radiolabelling in 50 µl portions of the fractions was measured using an LS 6500 scintillation counter (Beckman, Fullerton, CA, USA) to verify that cytokinin retention times in plant samples conformed to those of standard samples.

The fractions containing the cytokinin bases were evaporated to dryness *in vacuo*, dissolved in 50 µl of 70% ethanol, diluted with 950 µl of PBS (25 mM NaH_2PO_4, 15 mM NaCl, pH 7.2) and applied to an immunoaffinity column (IAC).[37] The IAC contained 0.4 ml of affinity purified polyclonal anti-cytokinin antibodies bound to Affi-gel 10 (Bio-Rad, Richmond, CA, USA).[37] HPLC fractions were passed through five times. The IAC was then washed with 5 ml of PBS and 10 ml of H_2O and eluted with 3 ml of ice-cold methanol.[38]

Cytokinin identification in *Arabidopsis thaliana* samples

For identification of endogenous cytokinins, the purification method was scaled up to process 10 g of plant tissue samples. In this case, no stable isotope-labelled internal standards were added to the extract. Full-scan LC/MS analyses were performed on derivatized samples originating from 10 g of plant material. If the quasi-molecular ion and some of the larger fragments were found to be present in the full-scan analysis, *B*/*E*-linked scanning[33] was subsequently used to identify the compound of interest in samples from three pooled 10 g extracts.

Cytokinin quantification in *Arabidopsis thaliana* samples

Endogenous cytokinins were quantified by the isotope dilution method,[39] with stable isotope-labelled cytokinins as internal standards (Table 1) added to the extracts prior to extraction. Mass spectrometric analyses were performed by selective reaction monitoring (SRM),[34] using the diagnostic transitions as displayed in Table 3. For quantification of the nucleotides, double-labelled standards, [$^2H_6$, ^{15}N]iPMP and [$^2H_5$, ^{15}N]ZMP were used. The possible hydrolysis of nucleotides during extraction and purification was monitored using the approach of Horgan,[18] whereby the activity of endogenous plant phosphatases would be detected as an enrichment in the $[M + H + 7]^+$ channel for iPA.

Calibration curves were prepared by mixing labelled and unlabelled cytokinins prior to derivatization. In the case of nucleotides, the mixed monophosphates were treated with phosphatase and purified on a C_{18} cartridge prior to derivatization.

RESULTS AND DISCUSSION

New ester derivatives of cytokinin nucleosides and glucosides for FAB-MS

The obvious targets for derivatization of nucleosides and glucosides are the hydroxyl groups. These functional groups are converted into esters if the compounds are heated with a carboxylic acid anhydride together with a basic catalyst.[40] The propionic and butyric esters of ZR were prepared (Fig. 1). The yields of the reactions were found to be >90% (data not shown). The $[M + H]^+$ ion current for a propionylated ZR sample was 20-fold stronger compared with the underivatized sample (data not shown). This can be explained by the analyte accumulation in the surface layer of the FAB-probe glycerol droplet due to increased surface activity of the derivatives.[20–22] No further increase in the quasi-molecular ion current intensity was found for the butyric ester.

Propionyl derivatives were chosen for subsequent analysis of nucleosides and glucosides and the tabulated positive daughter ion spectra are shown Table 2. The primary effect of the propionylation is to increase the mass of the derivatives and its fragments by 56 mass units per ester group present. As a basis for the interpretation, published FAB mass spectra of underivatized cytokinins are used (static FAB[41]; cf. FAB[19]). The gas-

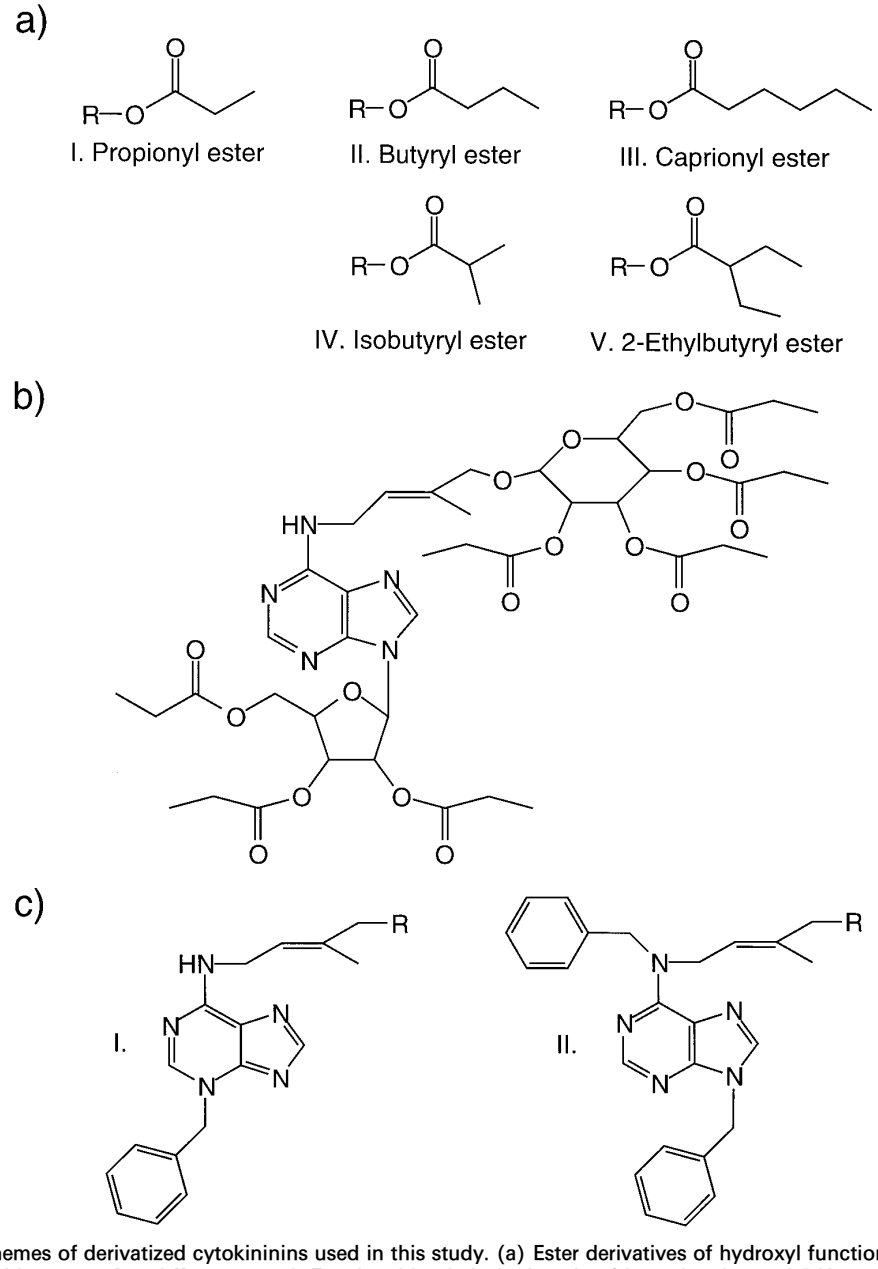


Figure 1. Structural themes of derivatized cytokininins used in this study. (a) Ester derivatives of hydroxyl functional groups. For cytokinin nucleosides and glucosides esters **I** and **II** were used. For the side-chain hydroxyls of benzylated Z and DHZ, esters II–V were used. (b) Propionylated cytokinin exemplified by pro-ZROG. (c) Monobenzyl (**I**) and dibenzyl (**II**) derivatives of amine groups in cytokinin bases.

Table 2. Tabulated positive-ion FAB daughter ion mass spectra of derivatized cytokinin standards, with spectra normalized to the most abundant daughter ion (bold entries)

Compound	Parent ion $[M+H]^+$	Diagnostic fragment ion: *m/z* (relative intensity, %)
Cap-MB-Z	408	364(35), 308(21), **292(100)**, 238(12), 226(34), 225(39), 197(13), 91(23)
EtBut-MB-Z	408	379(21), 364(16), **292(100)**, 238(10), 226(27), 225(21), 197(7), 91(20)
But-MB-Z	380	308(46), **292(100)**, 238(18), 226(27), 225(28), 91(9)
IsoBut-MB-Z	380	308(16), **292(100)**, 238(9), 226(16), 225(22), 91(9)
IsoBut-MB-DHZ	382	310(64), **294(100)**, 238(34), 226(23), 225(28), 91(9)
IsoBut-MB-D_5-Z	385	313(10), **297(100)**, 238(12), 227(18), 226(10), 225(24), 91(14)
Pro-DB-Z	456	382(60), 366(47), 316(87), **315(100)**, 292(50), 238(20), 224(28), 91(70)
Pro-DB-DHZ	458	384(64), **368(100)**, 328(39), 315(28), 294(40), 238(15), 224(22), 91(94)
Pro-DB-D_5-Z	461	387(82), 371(65), 316(81), **315(100)**, 297(62), 238(23)), 224(35), 91(74)
DB-iP	384	328(8), **316(100)**, 292(25), 238(7), 224(6), 91(6)
DB-D_6-iP	390	328(7), **317(100)**, 298(23), 238(6), 224(3), 91(11)
Pro-ZR	576	502(43), 436(25), 304(19), 301(41), **276(100)**, 202(33), 153(29), 136(6), 97(9)
Pro-D_5-ZR	281	507(48), 436(27), 309(15), 301(38), **281(100)**, 207(25), 153(20), 136(4), 97(5)
Pro-DHZR	278	504(26), 306(12), 301(24), **278(100)**, 204(6), 153(17), 97(4)
Pro-iPA	504	436(15), 301(53), 232(19), **204(100)**, 153(46), 136(15), 97(14)
Pro-D_6-iPA	510	437(12), 301(55), 238(19), **210(100)**, 153(43), 137(12), 97(15)
Pro-ZOG	606	218(8), **202(100)**, 183(14), 136(20), 109(7)
Pro-D_5-ZOG	611	223(10), **207(100)**, 183(15), 137(18), 109(10)
Pro-Z7G	662	**588(100)**, 448(8), 387(41), 304(13), 276(58), 202(21), 183(52), 136(7), 109(14)
Pro-D_5-Z7G	667	**593(100)**. 448(6), 387(33), 309(12), 281(48), 207(17), 183(46), 136(4), 109(13)
Pro-Z9G	662	588(80), 387(25), 304(19), **276(100)**, 202(31), 183(41), 136(5), 109(12)
Pro-D_5-Z9G	667	593(85), 387(24), 309(17), **281(100)**, 207(34), 183(38), 136(5), 109(13)
Pro-ZROG	906	832(27), **606(100)**, 502(26), 301(18), 202(23), 183(9), 153(17), 136(5), 97(7)
Pro-D_5-ZROG	911	837(37), **610(100)**, 507(27), 301(16), 207(21), 183(9), 153(17), 137(5), 97(13)

phase unimolecular chemistry of protonated nucleosides and glucosides is characterized by the preferential release of the sugar portion as a neutral species. This feature remained after derivatization and ions at *m/z* 202, 204, 276, 278 and 606 (Table 2) were the most abundant fragments of the nucleoside and glucoside spectra, except for the pro-Z7G, which had a dominant fragment ion at *m/z* 588 through the neutral loss of propionic acid from the side-chain. The ions at *m/z* 502 for pro-ZR and at *m/z* 504 for pro-DHZR display the same loss of propionic acid. The loss of the sugar moiety combined with the acid loss produced the ion at *m/z* 202 and further fragmentation with the neutral loss of the side-chain resulted in the ion at *m/z* 136, corresponding to charged adenine. *N*-linked conjugation to a sugar is indicated by the fragmentation leaving a formyl group at the purine nitrogen, resulting in the fragment ion at *m/z* 304 of pro-ZR and pro-Z7G.

Another important effect of propionylation was the increased proton affinity of the sugar moieties of nucleosides and glucosides. This is indicated by the presence of sugar daughter ions through the neutral loss of the purine base (glucose, *m/z* 387; ribose, *m/z* 301; Table 2). Furthermore, the loss of two propionic acids and a ketene from the propionylated glucose formed the resonance-stabilized ion at *m/z* 183 and the additional loss of the proximal acid formed the ion at *m/z* 109. These two fragments could probably be used to distinguish between cytokinins conjugated to different hexoses, as differences in probability for the equivalent reactions in acetylated glucose and galactose has been used to differentiate between the compounds in FAB-MS/MS.[42] Similarly, in the charged, propionylated ribose, the loss of two acids yields the *m/z* 153 fragment and additional loss of a ketene results in the fragment at *m/z* 97.

Propionylation also made it possible to distinguish between the ZOG and ZNG through the resulting difference in mass of quasi-molecular ions, *m/z* 606 and *m/z* 662, respectively, due to the difference in number of stable ester groups. The fragmentation patterns also differ between the 7-and 9-substituted pro-ZNG. In pro-Z7G, the fragment at *m/z* 588 was the most abundant fragment ion, while in the latter *m/z* 276 was the dominant fragment. In the propionylated ZROG, with $[M+H]^+$ at *m/z* 906, the preferential reaction was the release of the glucoside moiety as the neutral loss, resulting in the fragment ion at *m/z* 606. Here, as in pro-ZOG, resonance stabilization of the side-chain cation formed by the adjacent double bond is likely to direct the fragmentation pattern (see discussion for free bases below).

New benzyl derivatives of cytokinin bases for FAB-MS

Acetylation of the cytokinin base iP yields an N-9 derivative.[23] However, the compound is rapidly hydrolysed and is not suitable for LC. In contrast, permethylated bases are stable in water[15] but the preparation procedure is laborious. In this study, using a benzylation approach, compounds of equal stability were produced, without the need for the strong bases used in permethylation. The aromatic π-electron system of benzyl halides stabilizes the transition state in the nucleophilic substitution and lowers the activation

energy. Formation of mono-*N*-benzyl derivatives of cytokinin bases in a phase-transfer system has been described earlier by Letham *et al.*[43] In the present study, this reaction was carried out in a polar, aprotic solvent with an insoluble base (K_2CO_3) as catalytic surface, resulting in di-*N*-benzyl derivatives of iP, Z and DHZ (Fig. 1) with a recovery of >90% for iP and 60–70% for Z and DHZ. After dibenzylation, the side-chain hydroxyl groups of Z and DHZ were propionylated. The absorbance maximum in UV spectroscopy was 269 nm at pH 2.5 and is comparable to the maxima reported for N^6,N^9-permethylated cytokinins (270 nm, pH 2.5)[15]. The protonated dibenzyladenine (*m/z* 316), an important fragment ion of the dibenzyl derivatives, was formed through the neutral loss of the side-chains. The abundance of these fragment ions formed from the dibenzyl derivatives of iP and Z shows a direct effect of the electron structure at the exocyclic nitrogen, thus verifying that the compounds are N^6,N^9-substituted derivatives. The equivalent fragment of [$^2H_6$]dibenzyl-iP contains one deuterium atom (*m/z* 317), supporting the hypothesis suggested by Greco *et al.*[41] that the aza-Cope mechanism (Fig. 2(a)) is involved in this reaction. The exact reaction mechanism for the Z-derivative is unknown, however, as no incorporation of deuterium can be observed in the [$^2H_5$]propionyldibenzyl-Z (Table 2). Other important fragments are the ions at *m/z* 292 for dibenzyl-iP and *m/z* 366 for propionyldibenzyl-Z, resulting from the neutral loss of a benzyl group. The latter shows also a fragment at *m/z* 382, due to the loss of propionic acid from the side-chain. The resonance-stabilized benzyl cation (*m/z* 91) is also detected in the spectra of both cytokinins.

Adenine gives 3-benzyladenine when treated with benzyl halide in polar aprotic solvents without a base.[44,45] The equivalent reactions of Z and DHZ gave monobenzylated analogues in high yield (>90%). The UV absorbtion maxima are identical with that of N^3-substituted cytokinins (287 nm, pH 2.5).[15] After subsequent esterification of the side-chain hydroxyl group, elimination of the corresponding acid was the most abundant unimolecular transition (Table 2). The suggested mechanism, shown in Fig. 2(b), would require less energy for the transition than the corresponding loss of water in the underivatized Z,[19] resulting in more abundant daughter ion formation. In order to increase selectivity and sensitivity in SRM analysis, the larger esters of butyric, isobutyric, caproic and 2-ethylbutyric acid were tested. The former three esters were formed in high yield (>85%) but the branched ester of 2-ethylbutyric anhydride gave only a 50% recovery. The highest sensitivity in SRM analysis was found for the branched ester derivatives (Table 3) and it was also indicated by the differences in the mass spectra (Table 2). In the mass spectra of the branched derivatives, the dominance of the fragment ion at *m/z* 292 was greater than in the linear derivatives. Both neutral loss stability and proton affinity (Field's rule) are expected to favour loss of branched acids and would explain the differences observed.

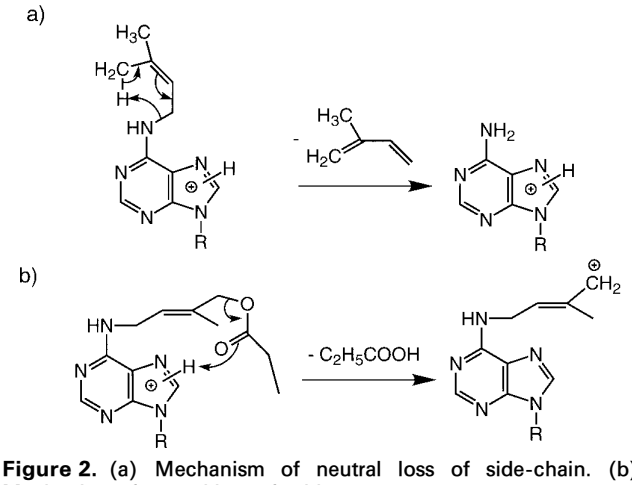


Figure 2. (a) Mechanism of neutral loss of side-chain. (b) Mechanism of neutral loss of acid.

Advantages of the new cytokinin derivatives for LC/frit-FAB-MS analysis

For further analysis, the propionyl esters of nucleosides and glucosides were chosen, as these derivatives combined high sensitivity with ease of preparation. For iP, the *N*-dibenzyl derivative possesses the same properties. In the case of Z and DHZ, a number of derivatives are available (Fig. 1). The *N*-dibenzyl derivative of DHZ

Table 3. Diagnostic transitions and detection limits for analysis of derivatized cytokinins by selective reaction monitoring (SRM), with bold entries indicating transitions used for the quantification in *Arabidopsis*

Compound	Diagnostic transition	Detection limit (fmol)[a]
IB-MB-Z	**380 → 292**	**20**
IB-MB-D_5-Z	**385 → 297**	
But-MB-Z	380 → 292	100
EtBut-MB-Z	408 → 292	20
Cap-MB-Z	408 → 292	80
IB-MB-DHZ	**382 → 294**	**40**
Pro-DB-Z	456 → 316	40
DB-iP	**384 → 316**	**20**
DB-[$^2H_6$]iP	**390 → 317**	
Pro-ZR	**576 → 276**	**30**
Pro-[$^2H_5$]ZR	**581 → 281**	
Pro-[^{15}N,$^2H_5$]ZR	**582 → 282**	
Pro-DHZR	**578 → 278**	**10**
Pro-iPA	**504 → 204**	**10**
Pro-[$^2H_6$]iPA	**510 → 210**	
Pro-[^{15}N,$^2H_6$]iPA	**511 → 211**	
Pro-ZOG	**606 → 202**	**140**
Pro-[$^2H_5$]ZOG	**611 → 207**	
Pro-Z7G	**662 → 276**	**170**
Pro-[$^2H_5$]Z7G	**667 → 281**	
Pro-Z9G	**662 → 276**	**80**
Pro-[$^2H_5$]Z9G	**667 → 281**	
Pro-ZROG	**906 → 502**	**100**
Pro-[$^2H_5$]ZROG	**911 → 507**	

[a] Detection limit defined as peak height > 3 × noise.

Table 4. Identification of cytokinins in *Arabidopsis thaliana*: tabulated positive-ion FAB daughter ion mass spectra of derivatized samples with spectra normalized to the most abundant daughter ion (bold entries)

Compound	Parent ion $[M+H]^+$	Diagnostic fragment ions: *m/z* (relative intensity, %)
EtBut-MB-Z	408	379(31), 364(7), **292(100)**, 238(31), 226(22), 225(36), 197(20), 91(24)
DB-iP	384	328(15), **316(100)**, 292(46), 91(69)
Pro-ZR	576	502(22), 304(4), 301(26), **276(100)**, 202(26), 153(17), 136(4), 97(22)
Pro-iPA	504	301(80), **204(100)**, 153(60)
Pro-ZOG	606	218(29), **202(100)**, 183(47), 136(32), 109(29)
Pro-Z7G	662	**588(100)**, 448(7), 387(26), 304(12), 276(63), 202(24), 183(72), 136(7), 109(28)
Pro-Z9G	662	588(66), 387(10), 304(17), **276(100)**, 202(38), 183(40), 109(19)
Pro-ZROG	906	832(14), **606(100)**, 502(28), 301(13), 202(28), 183(12), 153(20), 136(6), 109(5), 97(3)
Pro-ZR derived from ZR 5′-P	576	502(31), 301(40), **276(100)**, 202(21), 153(15)
Pro-iPA derived from iPA 5′-P	504	301(60), **204(100)**, 153(21)

was not used since it did not provide any abundant products with neutral losses suitable for SRM analysis. The *N*-monobenzyl-*O*-isobutyryl derivatives of Z and DHZ showed the highest sensitivity in quantitative analysis. For qualitative analysis, large parent ion size tended to reduce the background noise (data not shown), so the *N*-monobenzyl-*O*-caprionyl derivatives were chosen for qualitative analysis of Z and DHZ.

The low flow-rates of capillary LC are needed in continuous-flow FAB to maintain stability of the FAB ionization. With flow-rates of 2–10 μl min^{-1}, common injection volumes (0.2–1 μl) may cause poor peak symmetry if trace enrichment procedures cannot be used. The hydrophobic character of all the new derivatives enables large sample injections (20 μl) to be made in a relatively hydrophobic mobile phase. This allows the injection of 80% of the total sample volume, on-column, from a standard HPLC autosampler. Furthermore, all of the cytokinins analysed were separated with the capillary HPLC system used. In the case of the isomeric *N*-glucosides, this fundamental property is essential, as it is a prerequisite for the analysis of these compounds (see Fig. 4(c)). Chromatographic separation also minimizes suppression effects in biological extracts.[21] The use of *B*/*E*-linked mass spectrometric modes greatly reduced the chemical background originating from the extract and the FAB glycerol matrix. Quantitative analysis, performed by SRM with diagnostic transitions shown in Table 3 (bold entries), resulted in a sensitivity in the femtomole range (Table 3). The improved sensitivity as a result of the derivatization and the possibility of injecting 80% of the sample on-column and therefore into the ion source, make trace analysis of cytokinins in plant tissues feasible.

Purification, identification and quantification of isoprenoid cytokinins in *Arabidopsis thaliana*

One of the major problems with analysing compounds present in low concentration in plants is that purification methods have to be developed for each type of plant material. A purification method developed for a specific type of material might not give satisfactory results (in terms of yield or freedom from interfering substances, for instance) if applied to other tissues or species. In the present study, a purification protocol was developed for *Arabidopsis* and the recoveries were found to range from 60% (for iP and iPA) to 80% (for Z7G and Z9G). By using the derivatizations developed for high-resolution analysis of cytokinins by LC/frit-FAB-MS, it was possible to identify and quantify 10 different isoprenoid cytokinins in *Arabidopsis thaliana* (Table 4, Fig. 3, Table 5). Full-scan analysis revealed the presence of the potential quasi-molecular ions and reconstructed chromatograms were used to confirm that the ions observed did not result from fragmentation of compounds of higher molecular mass. However, as the full-scan analysis resulted in low-quality mass spectra, especially in the lower mass range, *B*/*E* linked scanning was also performed to confirm the identity of the cytokinins.

Daughter ion spectra of the identified cytokinins are given in Table 4 and the spectra for ZR and Z7G are shown in Fig. 3. The spectra contain all the major informative fragment ions observed in the spectra

Table 5. Cytokinin content quantified in individual *Arabidopsis* plants[a]

Compound	Content (pmol g^{-1})[b]	*n*
Z	1.8 ± 0.7	6
iP	1.2 ± 0.5	4
ZR	2.2 ± 0.7	5
iPA	1.0 ± 0.8	3
ZMP	31 ± 3.0	6
iPMP	15 ± 2.8	6
ZOG	12 ± 1.0	6
Z7G	67 ± 0.6	6
Z9G	20 ± 1.7	6
ZROG	5.2	1

[a] Extracts of plant material (500 mg) were purified and derivatized prior to analysis. Biological variation is indicated by the standard deviation.
[b] pmol g^{-1} fresh weight (mean ± SD).

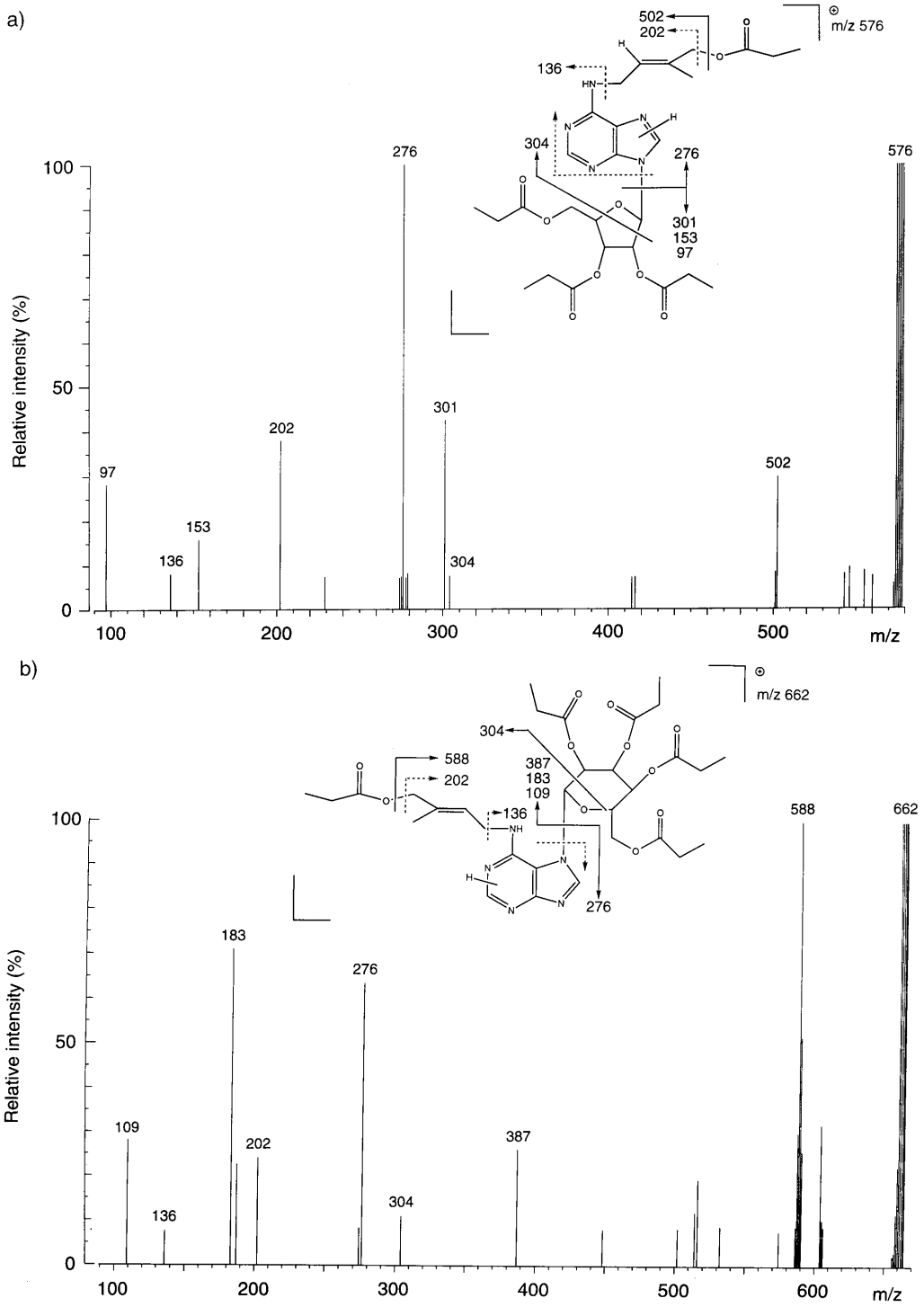


Figure 3. Daughter ion FAB mass spectra of (a) propionyl-ZR and (b), propionyl-Z7G purified from *A. thaliana* plant material and propionylated prior to analysis. The fragmentation pattern is indicated by full lines. Dotted lines correspond to ions formed by multiple fragmentations including loss of sugar moiety.

from standard cytokinins. The daughter ion spectra show satisfactory quality, with no interfering ions, commonly observed in the full-scan analysis. This is due to the selectivity of the *B*/*E*-linked scanning mode and, as a result, reduction of the background noise. The compounds identified include the bases, nucleosides and nucleotides of both the isopentenyladenine and zeatin-type cytokinins, the *O*-glucosides of Z and ZR and the N^7-and N^9-glucosides of Z. DHZ, DHZR and DHZ-glucosides were not detected by scan analysis, but some traces of DHZ and DHZR were observed by SRM analysis (data not shown).

The high sensitivity gained by the new derivatives allowed the quantification of identified cytokinins in purified extracts from 500 mg of plant tissue, originally from a single plant. The SRM chromatograms, from the quantification of iP, ZR and ZNG, are shown in Fig. 4 and the endogenous levels with the biological variations

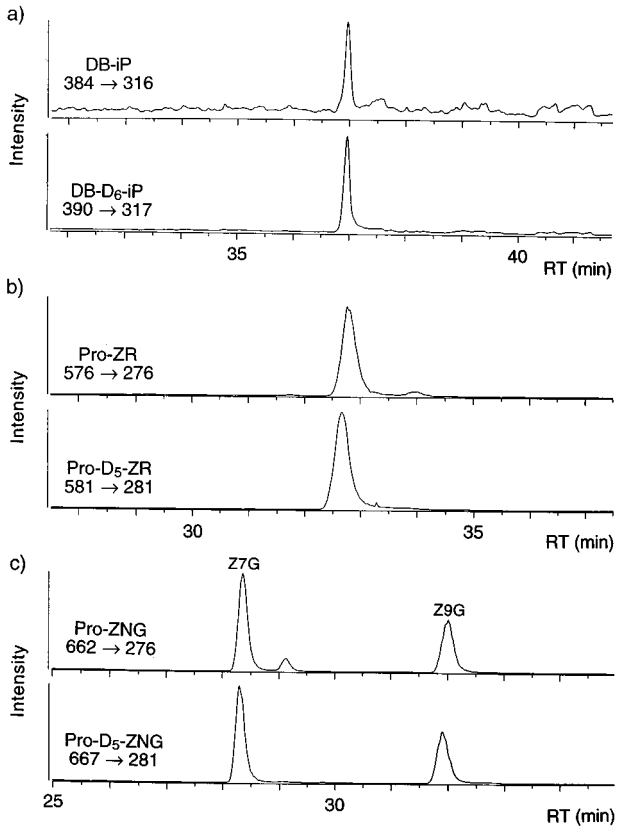


Figure 4. SRM chromatograms from the quantitative analysis of (a) dibenzyl-iP, (b) propionyl-ZR and (c) propionyl-ZNG in 500 mg of *A. thaliana* plant material. The diagnostic transitions used are shown.

are presented in Table 5. The minimum amount of plant material needed to permit quantification was estimated from the ion chromatograms. The data indicated that to quantify cytokinin bases at least 250 mg of tissue were needed but that nucleosides, nucleotides and glucosides could be quantified in less than 50 mg of tissue. The contents of Z, the ZR + ZR5′-MP pool and the iPA + iPA 5′-MP-pool found in this study resemble the levels found in young tobacco leaves except for the Z7G levels, which are fivefold higher in *Arabidopsis*.[46] Chory *et al.*[32] quantified cytokinins by HPLC/PDA in 14-day-old seedlings of ecotype Colombia and found the ZR + ZR5′-MP pool to contain 3.8 ng g^{-1} (11 pmol g^{-1}) fresh mass and an iPA + iPA5′-MP pool size of 6.6 ng g^{-1} (20 pmol g^{-1}) fresh weight (average of published data). Direct comparisions are difficult, however, owing to the differences in growth condition, age of the plant material and analytical methodology. The nucleotide contents are, as expected, higher than the nucleoside levels, resulting in a ribotide/riboside ratio close to 15, for both ZR/ZRMP and iPA/iPMP. In the iPA quantification, no enrichment was found in the additional [$^2H_6$, ^{15}N]iPA channel, indicating that no hydrolysis of iPMP by endogenous plant phosphatases took place during extraction and purification. The glucosides were separated on the capillary column and quantified. The high contents of *N*-glucosides are indicative of accumulation of these apparently inactive conjugates.[47]

CONCLUSION

We have reported a reliable method for cytokinin analysis with improved sensitivity that allows detailed analysis of cytokinins in individual compartments of *Arabidopsis thaliana* plants. This technique will now make it possible to analyse, on individual plants, a number of *Arabidopsis*-mutants believed to be impaired in the cytokinin metabolism.

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