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Short Communication

Identification of Cytokinins from Xylem Exudate of *Phaseolus vulgaris* L.

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ABSTRACT

The principal biologically active cytokinins in xylem exudate of young *Phaseolus vulgaris* L. plants were identified by bioassay, high-performance liquid chromatography, enzymic degradation and combined gas chromatography-mass spectrometry (selected ion monitoring) a zeatin riboside, zeatin nucleotide, dihydrozeatin riboside, dihydrozeatin nucleotide, *O*-glucosyl zeatin, *O*-glycosyl dihydrozeatin, *O*-glucosyl dihydrozeatin riboside, and *O*-glucosyl dihydrozeatin nucleotide. Trace amounts of *O*-glucosyl zeatin riboside and *O*-glucosyl zeatin nucleotide were also detected.

The major cytokinins in leaves, stems and roots of *Phaseolus vulgaris* L. have been rigorously identified by physicochemical methods (10, 14, 21, 22), and there exists a wealth of information on cytokinin metabolism in this species as a result of studies using quantitative MS (9), bioassay (*e.g.* 1, 4, 7, 19), and radiolabeled cytokinins (*e.g.* 10, 11, 15). The nature and distribution of cytokinin-active ribonucleosides in *P. vulgaris* tRNA has been determined (2, 3) and this species has also been used as a model system to study cytokinin biosynthesis (8). Although the design and interpretation of these and other physiological experiments would benefit greatly from knowing the identity of translocated bean cytokinins, to date such analyses have provided only limited bioassay data (4, 7, 19). The present study seeks to identify the biologically active cytokinins in bean xylem exudate through high resolution chromatography and mass spectroscopic analysis.

MATERIALS AND METHODS

Bean plants (*P. vulgaris* cv Hawkesbury Wonder) were grown in 2-L plastic pots containing sterilized garden soil, in a glasshouse, under full sunlight, 16 h days, and a temperature range of 18 to 32°C. Every 2 d, starting 1 week after germination, each plant received 100 ml of Hewitt's complete nutrient solution containing 12 mM nitrate (6). Plants were harvested 20 d after germination, at a stage of development which has been correlated with high root system conductance (5), by decapitating just above the cotyledonary node. Exudate was removed continually for 1 h from the cut surfaces using a microsyringe, and transferred to a beaker containing 50 ml extraction solvent (methanol/chloroform/formic acid/water, 12:5:1:2), chilled to −5°C. A total of 24.6 ml exudate was collected from 120 plants.

Exudate was stored in extraction solvent overnight at −5°C, then purified as described previously (10), by cation-exchange on cellulose phosphate, followed by alkaline phosphatase treatment of the acidic/neutral fraction, and partitioning of both the basic fraction (containing cytokinin bases and glycosides) and phosphatased fraction (containing nucleotide-derived cytokinins) against butan-1-ol at pH 8.0. The two butanol-soluble fractions were then purified by reversed-phase HPLC, as described previously (17). Initially, a Waters μBondapak C-18 column (7.8 mm × 30 cm) was used to effect a preparative separation of cytokinin bases, ribosides, and glucosides (Fig. 1; Table I). All HPLC solvents contained a constant concentration (0.1 M) of acetic acid. Samples were injected in 10% methanol (0.5 ml) and eluted with a concave gradient (Waters Gradient 9) of 10 to 30% methanol for 10 min, followed by 30% methanol isocratically for 20 min, at 2 ml min^{-1}. The first 20 ml was discarded, and the next 40 ml collected as ten 4 ml fractions (Fig. 1). One-eighth of each fraction was removed and tested for cytokinin activity using the soybean cotyledon callus assay (12).

The four fractions showing biological activity were then purified separately by analytical reversed phase HPLC and analyzed by GC-MS. To improve recovery and assist in identification, 2.0 μg of an appropriate pentadeuterated internal standard was added to each biologically active fraction before analytical HPLC. Standards used were: ($^2H_5$)ZR,[1] ($^2H_5$)DZR, and ($^2H_5$)OGDZ synthesized as described by Summons *et al.* (16). Since these cytokinin analogs are also biologically active, they were not added to the extract until after the bioassay.

The cytokinin glucoside-like fractions 1 and 3 (Fig. 1) were each diluted with ($^2H_5$)OGDZ and analyzed using a Waters C_8 radial compression column (8 × 100 mm), eluted with a linear gradient of 10 to 40% methanol in 15 min, at 3 ml min^{-1} (system A). The cytokinin riboside-like fractions 2 and 4 (Fig. 1) were each diluted with both ($^2H_5$)ZR and ($^2H_5$)DZR and analyzed on the same column, using a linear gradient of 30 to 40% methanol in 20 min, at 3 ml min^{-1} (system B). The resolution of systems A and B is shown in Table I. Fractions were collected every 30 s, or according to peaks of UV absorbance (280 nm). Glucoside-like fractions were first treated with β-glucosidase, as described previously (12), then purified as their aglycones on HPLC system B before derivatization.

The TMS derivatives of cytokinin ribosides (12), and tBuDMS derivatives of cytokinin bases (13) were prepared as described previously. Chemical ionization GC-MS was performed on a Finnigan 4500 instrument, interfaced to a Nova 4 computer,

[1] Abbreviations: ZR, zeatin ribosides; DZ, dihydrozeatin; DZR, dihydrozeatin riboside; OGDZ, *O*-glucosyl dihydrozeatin; OGDZR, *O*-glucosyl dihydrozeatin riboside; OGZ, *O*-glucosyl zeatin; OGZR, *O*-glucosyl zeatin riboside; tBuDMS, tertiary-butyl dimethylsilyl; TMS, per-trimethylsilyl; Z, zeatin; SIM, selected ion monitoring.

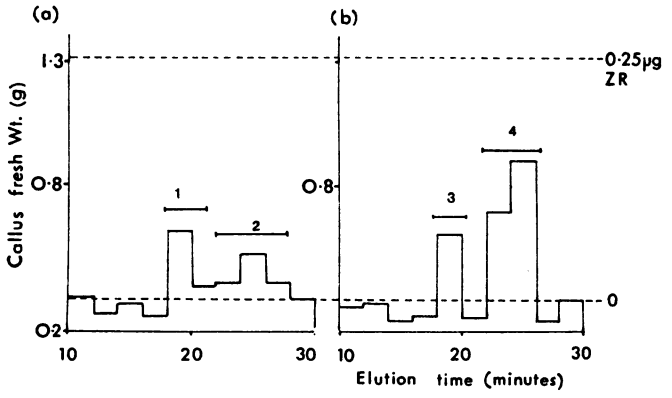

FIG. 1. Soybean callus bioassay of preparative HPLC-purified extracts of bean xylem exudate. Fractions in (a) were derived from the basic eluate after cation-exchange, and could contain cytokinin bases and/or glycosides, occurring as such in the crude extract. Those in (b) were isolated from the phosphatase-treated acidic/neutral eluate, and comprise cytokinin glycosides, derived from nucleotides in the crude extract. Pentadeuterated internal standards were added to the putative glucoside (1 and 3) and riboside (2 and 4) fractions, which were then purified by analytical HPLC and analyzed by GC-MS.

Table I. *Chromatographic Resolution of Cytokinins*

GC data are retention times of TMS (ZR, DZR) or tBuDMS (Z, DZ) derivatives.

Cytokinin	Retention Time			
	Preparative HPLC	Analytical HPLC system A	Analytical HPLC system B	GC
		min		
OGZ	15.7	7.5		
OGDZ	16.8	9.8		
OGZR	17.3	12.6		
OGDZR	18.3	15.1		
Z	18.5	18.0	18.8	6.3
DZ	19.5	21.2	22.8	5.3
ZR	20.1		8.2	6.5
DZR	21.4		10.5	5.5

and SIM was done using the Incos data system. Samples were introduced via a Hewlett Packard high performance fused silica capillary column (25 m × 2 mm), operated isothermally at 280°C. Mass spectra were recorded in ammonia reagent gas at 1 torr, 140 ev, and a source temperature of 120°C. Each sample was scanned for the presence and intensity of the following diagnostic (MH^+) ion masses along the GC elution profile: 448 (tBuDMS-Z), 450 (tBuDMS-DZ), 455 (tBuDMS)-($^2H_5$)DZ), 640 (TMS-ZR), 642 (TMS-DZR), 645 (TMS-($^2H_5$)ZR), 647 (TMS-($^2H_5$)DZR).

RESULTS AND DISCUSSION

Bioassay of samples following preparative HPLC revealed cytokinin activity in four broad fractions. Comparison of bioassay profiles (Fig. 1) with HPLC retention times of authentic standards (Table I) indicated that both basic and nucleotide-derived extracts contained a mixture of cytokinin ribosides, bases, and/or glucosides. Further purification of these fractions by analytical HPLC showed large peaks of UV-absorption co-chromatographing with ZR, DZR, and OGDZ (attributable to a combination of deuterated internal standards and endogenous compounds), and smaller peaks co-chromatographing with OGZ,

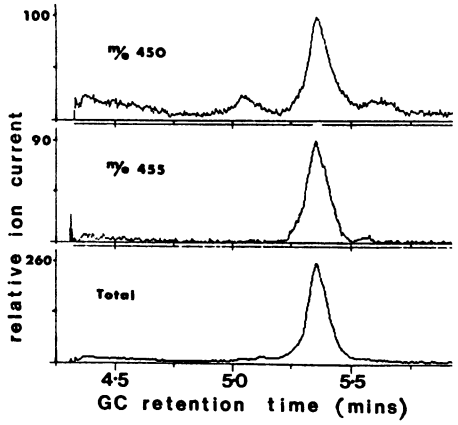

FIG. 2. Ion-current traces resulting from GC-MS analysis of putative tBuDMS-DZ containing tBuDMS-($^2H_5$)-DZ internal standard. The endogenous compound was derived from a cytokinin-active fraction co-chromatographing with OGDZ on HPLC. After addition of ($^2H_5$)-OGDZ as an internal standard, the unknown fraction was treated with β-glucosidase and purified as the putative aglycone, DZ, by HPLC. The major UV-absorbing fraction, assumed to be a mixture of deuterated and nondeuterated DHZ, was collected, derivatized, and analyzed by GC-MS and SIM as a mixture of tBuDMS derivatives. Final identification of the aglycone of the endogenous cytokinin glucoside as DZ was based on the coincidence of ion-current peaks due to m/e 450 (MH^+ for tBuDMS-DZ) and m/e 455 (MH^+ for tBuDMS-($^2H_5$)-DZ) at the GC retention time of authentic tBuDMS-DZ (see also Table I).

Table II. *Endogenous Cytokinins of P. vulgaris L.*

Plant Part	Cytokinins Present	Reference
Primary leaves	DZR,[a] OGDZ	21, 22
Stem	ZR, Z-nucleotide, DZR, DZ-nucleotide, OGDZ,[a] OGZ-nucleotide[a]	10
Xylem sap	ZR, Z-nucleotide, DZR, DZ-nucleotide, OGZ, OGDZ, OGDZR, OGDZ-nucleotide, OGZR[b], OGZ-nucleotide[a]	This study[b]
Roots	OGZ, Z,[a] ZR,[a] OGZR[a]	14

[a] Minor components. [b] Identification based on co-chromatography with authentic standards in two (C_8 and C_{18}) reversed phase HPLC systems (see Table I), followed by GCMS analysis using SIM and deuterated internal standards (see Fig. 2 for example). Glucosides were analyzed indirectly by GC-MS identification of the aglycones following enzymic hydrolysis of glucoside-like HPLC fractions.

OGZR, and OGDZR. Neither basic nor nucleotide-derived fractions contained UV-absorbing material which co-chromatographed with Z or DZ. After treatment with β-glucosidase, the majority of UV-absorbing compounds co-chromatographed either Z, DZ, ZR, or DZR.

Analysis by GC-MS of samples tentatively identified by HPLC indicated the presence of high-intensity molecular ions for the following derivatized cytokinins, all at the exact GC retention times of authentic standards: ZR, DZR and their deuterated analogs, from both basic and nucleotide-derived fractions; Z, DZ, DZR and deuterated DZ, derived from basic glucosides; and DZR derived from an acidic glucoside fraction. Low-intensity molecular ions were detected for derivatized ZR, from both basic and acidic glucoside fractions. An example of such an analysis is shown in Figure 2.

Thus, eight major cytokinins were identified from bean exudate: ZR, DZR, OGZ, OGDZ, OGDZR and the nucleotides of Z, DZ, and OGDZ. In addition OGZR and OGZ nucelotide were identified as minor components. In relation to known patterns of cytokinin metabolism in *P. vulgaris* (9–11, 14, 21, 22), it seems reasonable to assume that the glucosides of ZR, DZR, Z-nucleotide, and DZ-nucleotide isolated from bean xylem exudate are *O*-β-D-glucosyl side-chain conjugates. However, in a recent analysis of cytokinins from *Pinus radiata* buds (18), a novel ZR glycoside was isolated and tentatively identified by GC-MS as 9-(glucosyl-β-D-ribofuranosyl) zeatin. This compound has not been isolated from *P. vulgaris*, but it should be noted that if it does occur here, and as in the present study, the cytokinin glucoside fractions are analyzed indirectly after enzymic hydrolysis, it may well be indistinguishable from OGZR.

In this experiment, to avoid interference in the bioassay, deuterated cytokinin standards were added to samples only after thorough purification by ion-exchange, solvent partition, and HPLC. Although data such as those shown in Figure 2 permit the quantification of cytokinins present in certain HPLC fractions, they do not take into account the losses that may have occurred prior to HPLC. Limited evidence from bioassay, UV-absorbance, and relative ion intensities indicated that the most abundant cytokinins were Z- and DZ-nucleotides, but it is not possible to calculate actual concentrations of cytokinins in the exudate other than to state that their levels appear to fall within the range 10 to 100 $ng \cdot ml^{-1}$. A summary of these results, and those from other analyses of the same species is given in Table II.

The distribution of endogenous cytokinins in *P. vulgaris* (Table II), and in particular the remarkable accumulation of very high levels of OGDZ in the primary leaves of decapitated plants (9), has been suggested to be due to the translocation of ZR and Z-nucleotide from the roots, partial or complete metabolism of these compounds to DZR and DZ-nucleotide (more stable forms) in the stem, and finally glucosylation to OGDZ, apparently the most stable bean cytokinin, in the leaves (10). Results of the present study appear to support the hypothesis that DHZ-derivatives originate in shoot tissues, since these compounds were present in exudate from the base of the stem, whereas they are reported to be absent from root tissues (14). However, with the demonstration in this study of the presence of cytokinin *O*-glucosides in xylem exudate, it now seems likely that although bean leaf tissue is known to be able to glucosylate Z and DZ (11), OGDZ accumulation in the leaves could also be a result of the import of the glucoside *per se* (Table II).

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