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Hormone-like product of vascular tissue stimulating starch accumulation in pith explants of kale and endogenous cytokinins

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Abstract

When stem explants of kale (*Brassica oleracea* L. var. *medullosa*), containing pith parenchyma and a strip of vascular tissue, were cultured on simple sucrose medium, a hormone-like factor was transported from the vascular tissue to the adjacent pith, where it stimulated accumulation of starch. Similarly, up to a sevenfold increase of starch content in explants could be induced by cytokinins added to the culture medium. The relative stimulatory effect of several cytokinins (5×10^{-6} M) and hormone-like product of vascular tissue (HPVT) in a typical experiment were: control (1.0), trans-zeatin (6.7), HPVT (6.2), N^6-[2-isopentenyl]adenine (5.4), trans-zeatin riboside (5.2), N^6-[2-isopentenyl]adenosine (5.4), kinetin (3.6), 6-benzylaminopurine (3.5), and adenine (2.1). Concentration of endogenous cytokinins was determined using ELISA (trans-zeatin, N^6-[2-isopentenyl]adenine and their ribosides) and *Amaranthus* bioassay (total cytokinins). No effect of vascular tissue on the level of endogenous cytokinins in explants was found. The results support the conclusions of previous experiments that the HPVT stimulating starch accumulation is not a cytokinin.

Introduction

Experiments with stem explants of kale cultured on simple sucrose medium showed that the presence of vascular tissue in explants stimulated starch and soluble protein accumulation in adjacent pith (Luštinec *et al.* 1984a,b, Luštinec 1990). When pith explants were saturated with extract from explants containing vascular tissue and cultured on medium with saccharose, starch accumulation was induced as well. Vascular tissue supplied with sucrose was thought to produce a hormone-like starch-inducing factor(s). Starch accumulation in pith explants of kale could also be

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enhanced by exogenous supply of cytokinins and auxins. However, the effect of these phytohormones was small or absent when they were applied to explants derived from plants cultured in the greenhouse during winter. At the same time, the effect of vascular tissue on starch synthesis was significant. Conversely, abscisic acid, 24-epi-brassinolide and gibberellic acid showed an inhibitory effect (Luštinec *et al.* 1985). The extract from explants containing vascular tissue was fractionated chromatographically and several fractions with starch-inducing activity (SA) were obtained. There was no cytokinin activity in fractions with the highest SA, as determined by *Amaranthus* bioassay (Luštinec *et al.* 1985). Thus, on the basis of bioassay data it was concluded that the starch synthesis-inducing factors of vascular tissue were not cytokinins.

The principal aim of the present study was to obtain information about cytokinin levels in explants with and without vascular tissue.

Materials and methods

Plant material: Kale (*Brassica oleracea* L. var. *medullosa* cv. Krasa) stem explants were cultivated as described previously (Luštinec *et al.* 1984a, 1985, 1988). Two types of explants were cut from kale stems (Fig.1): simple explants (C, blocks consisting of pith only, approximately 10 × 10 × 4 mm) and complex explants (AB, blocks consisting of pith with adjacent vascular tissue and a part of cortex). These were cultured for 7 d in darkness at 24 °C in 100 cm^3 Erlenmeyer flasks on agar medium containing 10 % sucrose with cytokinins as indicated below. Complex explants were cut at the end of the cultivation period and their pith (A) was compared with that of the control (C). Explants used for cytokinin analysis were usually larger and irregularly shaped. Three kale plants were used for preparation of explants in each experiment.

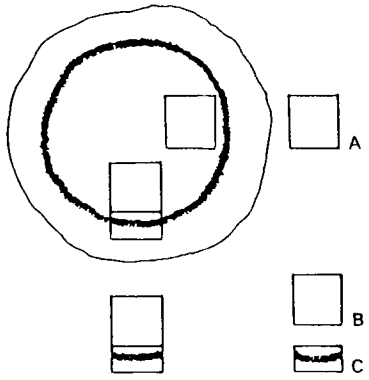

Fig.1. A scheme of explant preparation and comparison. C, blocks consisting of pith only (control); AB, blocks consisting of pith with adjacent vascular tissue and a part of cortex.

Cytokinin analysis: Cytokinins were extracted from explants and purified by the method of Morris *et al.* (1982) as modified by Vaňková *et al.* (1987). The explants (50 g fresh mass) were frozen in liquid nitrogen, homogenized in Waring blendor and

extracted with 200 cm^3 of 80 % methanol and 400 μg g^{-1}(f.m.) of sodium diethyldithiocarbamate at 0 °C. Benzylaminopurine (^{14}C) was added as a tracer. The homogenate was centrifuged (10 000 *g*, 20 min.) and an aliquot of the supernatant corresponding to 3 g of explant fresh mass was removed and concentrated *in vacuo* at 40 °C (the sediment was dried and used for starch determination). Samples were dissolved in ammonium acetate buffer (40 mM, pH 4.8) and cytokinin 5'-phosphates were converted to free nucleosides by treatment with acid phosphatase [1 mg g^{-1}(f.m.), 27 °C, 30 min). The samples were adjusted to pH 3 and applied to a 20 cm^3 cellulose phosphate column. After washing (ammonium acetate buffer, 40 mM, pH 3), the column was eluted with 1 M ammonium hydroxide (Horgan and Scott 1987). The eluate (pH adjusted to 6.5) was then percolated through a column of DEAE-cellulose (20 cm^3 bed volume) (Morris *et al.* 1982) after which the cytokinins were adsorbed onto a reverse-phase C_{18} cartridges (*Silica-Cart Tessek*). After washing with water, the cytokinins were eluted with 15 cm^3 of 80 % methanol. Eluates were concentrated under vacuum and stored at -15 °C. Samples were fractionated by HPLC (4 × 250 mm, *Separon SGX* C_{18}, 7 μm) using a gradient of methanol in water (40 - 80 % v/v). Cytokinin levels in the HPLC fractions were estimated by ELISA, using the method of Weiler *et al.* (1981) as modified by Vaňková *et al.* (1987).

Bioassay: The *Amaranthus* betacyanin bioassay (Biddington and Thomas 1973) was used to estimate total cytokinin activity. The explants (25 g fresh mass) were extracted and cytokinins purified as described above with following difference: only DEAE-cellulose (50 cm^3), not cellulose phosphate column was used before two Sep-paks C_{18} .

Quantitative determination of starch: Starch content in explants and in the sediment of the methanol homogenate (of explants used for cytokinins analysis) was estimated by the method of Luštinec *et al.* (1983), as modified by Hájek and Luštinec (1993).

Reproducibility of results: The effects of cytokinins and HPVT were compared in a number of experiments performed during several successive years. Results of cytokinin analyses in three different experiments are presented.

Results and discussion

The ability of cytokinins (kinetin, trans-zeatin) and HPVT to stimulate starch synthesis and other processes in pith explants of kale has been demonstrated (*cf.* Luštinec *et al.* 1984 a,b). Similar effects were seen in the present study in which seven cytokinins were compared with HPVT (Tables 1 and 3). The efficiency of individual cytokinins depended to some extent on genetical and physiological state of original plants and on cytokinin level, but trans-zeatin was usually the most active cytokinin. The stimulatory effect of HPVT was usually 3 - 6 times that of the control value and was comparable to that of the most active cytokinin.

The possible participation of endogenous cytokinins in the action of HPVT has

Table 1. Starch content in pith explants (C) cultured on simple sucrose medium (control) or on this medium supplemented with cytokinin (5×10^{-6} M), and in the pith part (A) of complex explants cultured on simple sucrose medium.

Type of explant	Cytokinin tested	Starch [μg explant^{-1}]*	Starch [%]
C	-	34 ± 6.7	100
C	trans-zeatin	226 ± 18.7	670
C	trans-zeatin riboside	178 ± 30.0	520
C	N^6-[2-isopentenyl] adenine	184 ± 63.7	540
C	N^6-[2-isopentenyl] adenosine	136 ± 20.7	400
C	kinetin	122 ± 26.0	360
C	6-benzylaminopurine	118 ± 17.3	350
C	adenine	72 ± 6.0	210
A	-	210 ± 15.3	620

*mean ± S.E.

remained an open question despite indirect evidence suggesting that cytokinins were not responsible for the stimulatory effect of vascular tissue (see Introduction). This question can be answered by measuring cytokinin levels in pith part of the complex explants influenced by adjacent vascular tissue. The results of cytokinin

Table 2. Content of four cytokinins [10^{-10} mol g^{-1}(f.m.)] in simple pith explants (C) and in pith part of complex explants (A) after cultivation on simple sucrose medium in three different experiments.

Cytokinin	A	C	A/C
trans-zeatin	8.8	4.6	1.91
	1.0	1.9	0.53
	2.4	1.5	0.63
trans-zeatin riboside	2.9	2.5	1.16
	0.5	1.6	0.31
	1.5	0.9	1.67
N^6-[2-isopentenyl] adenine	16.2	32.0	0.51
	1.2	0.8	1.50
	4.5	3.6	1.25
N^6-[2-isopentenyl] adenosine	5.8	9.2	0.63
	2.1	3.1	0.68
	2.1	2.1	1.00

determinations can be seen in Table 2. The content of trans-zeatin, N^6-[2-isopentenyl]adenine and their ribosides in explants varied from one experiment to the next. This variability can be attributed to properties of the experimental material and to the analytical method itself. Mean values of A/C were one or close to it, which means that cytokinin levels were not significantly influenced by the presence of vascular tissue. In another set of experiments the ratio A/C for total cytokinin activity

was determined using *Amaranthus* bioassay. The values obtained were 1.00, 1.22 and 1.93 (mean 1.38). In this case too, there was no significant enhancement of explant cytokinin content by vascular tissue.

Table 3. Starch content [mg g^{-1}(d.m.)] in sediments of methanol homogenate from explants used for cytokinin analysis. Cn, non-cultured (fresh) simple pith explants, A and C as in Table 2.

Cn	A	C	A/C
3.0	88.5	18.3	4.8
4.0	148.5	42.4	3.5
2.0	60.6	17.1	3.5

In one experiment cytokinin content was determined in both cultured and non cultured (fresh) explants. As shown in Table 4, the levels of three of the four analysed cytokinins increased considerably. In a further experiment still higher increases (C/Cn = 9.2) were seen in total cytokinin activity. There are at least two possible explanations for increased cytokinin levels in simple explants: (a) cell wounding, (b) stimulation by exogenous sucrose. Conrad (1968, 1975, 1977) found that the concentration of cytokinins and auxins in kohlrabi and potato tissues rose after wounding to a level sufficient to induce cell division. This concentration would be sufficient to stimulate starch accumulation in our system. However, the effect of wounding was limited to the explant surface cell layers where marked accumulation of starch granules can be observed. Starch accumulation predominantly occurs inside the explants (*cf.* Kutík and Beneš 1979) where presumably it was induced by increasing concentration of sucrose (due to its uptake from the medium). Exogenous sucrose might intensify different metabolic processes including synthesis of cytokinins. On the basis of our results, we conclude that HPVT and cytokinins stimulate starch synthesis in kale explants independently.

Table 4. Cytokinin content [10^{-10} mol g^{-1}(f.m.)] in fresh, non cultured (Cn) and cultured (C) pith explants.

Cytokinin	Cn	C	C/Cn
trans-zeatin	0.8	1.9	2.4
trans-zeatin riboside	0.5	2.5	5.0
N^6-[2-isopentenyl] adenine	32.1	32.0	1.0
N^6-[2-isopentenyl] adenosine	2.5	9.2	3.7

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