J Plant Growth Regul (1991) 10:197–199

Journal of
Plant
Growth
Regulation

© 1991 Springer-Verlag New York Inc.

Effects of Synthetic Cytokinins on Levels of Endogenous Cytokinins and Respiration Patterns of *Beta vulgaris* Cells in Suspension

Radomira Vankova,[1] Ke-Cheng Hsiao,[2] Chris H. Bornman,[2] and Alena Gaudinova[1]

[1]Institute of Experimental Botany, Czechoslovak Academy of Sciences, Ke dvoru 15, 166 30 Prague, Czechoslovakia; and [2]Research and Development, Hilleshög AB, Box 302, S-261 23 Landskrona, Sweden

Received April 15, 1991; accepted June 20, 1991

Abstract. Respiration patterns and growth of cytokinin-dependent cell suspensions of *Beta vulgaris* L., precultured in media with or without three different synthetic cytokinins [benzyladenine (BA), kinetin (KIN), and thidiazuron (TDA)], were compared. The content of endogenous cytokinins, especially zeatin and isopentenyladenine, as well as the dry mass yield, were dependent on the kind of synthetic cytokinin present in the culture medium and decreased in the following order: thidiazuron, kinetin, benzyladenine, no cytokinin. The apparent capacity of the alternative pathway, as measured after blocking of the cytochrome pathway by cyanide, was inversely proportional to the content of endogenous cytokinins. Some synthetic cytokinins (e.g., benzyladenine), when exogenously applied, are known to inhibit selectively the alternative pathway. However, this does not necessarily imply that the mechanism of action of endogenous cytokinins on the respiration pattern is limited to a single effect on the alternative pathway. Multiple effects on oxidative processes cannot be excluded.

An inhibitory effect of cytokinin on plant respiration was reported by Dedolph et al. (1961, 1962). Later, Miller (1980) found that cytokinins selectively affect the cyanide-resistant or alternative pathway. Musgrave and Siedow (1985) reported that benzyladenine (BA) at a concentration of 20–40 μM could eliminate electron flow through the alternative pathway in intact tissue without inhibiting it per se. These authors also found that disengagement of the alternative pathway was correlated with developmental responses to cytokinin application. It has also been demonstrated that inhibitors of the alternative pathway, such as salicylhydroxamic acid and propyl gallate, exhibit cytokinin activity in two antisenescence bioassays (Musgrave et al. 1987). The alternative pathway seems to be an inherent feature of cytokinin-dependent lines (Siedow and Musgrave 1987) in higher plants.

Synthetic cytokinins are known to increase the level of endogenous cytokinins (Hansen and Meins 1987, Vankova et al. 1987). Whether the change in the level of endogenous cytokinins could affect the respiratory pattern of the cells is not known. Therefore, it is of interest to know whether a correlation exists between the concentration of various endogenous cytokinins and the capacity of the alternative pathway.

In this report the levels of different endogenous cytokinins of cell suspensions of *Beta vulgaris*, cultured in media containing BA, kinetin (KIN), or the cytokinin-like compound thidiazuron (TDA) (Mok et al. 1982), were determined. The results were then compared with data on the respiratory patterns measured on the same samples.

Materials and Methods

Cell suspensions originating from callus of *Beta vulgaris* leaf discs were grown in the medium of Schenk and Hildebrandt (1972) supplemented with 0.5 μM naphthaleneacetic acid (NAA) and 1 μM BA. The suspensions were maintained at 20 ± 2°C under a 16-h photoperiod and a photon-flux density of 10–15 μmol m^{-2} s^{-1} for the wavelength band of 400–700 nm, provided by fluorescent tubes (Osram 136 W/77).

For the experiment, cells were washed with culture medium lacking cytokinin, and then grown in this wash medium for 1 passage of 7 days before transfer to a medium containing 1 μM of either BA, KIN, or TDA. Cells from suspensions in the linear growth phase were harvested for analysis of their endogenous cytokinins and for respiration measurements.

Cytokinins were extracted and purified by a procedure modified from Morris et al. (1982). Freeze-dried cells were extracted with methanol (0.15 g with 10 ml of redistilled methanol) in the presence of 200 μg ml^{-1} of the antioxidant Na-diethyldithio-

Table 1. Endogenous cytokinin levels in cell suspensions of *Beta vulgaris* cultured in the absence of exogenous cytokinin (control) or in the presence of 1 μM each of benzyladenine (BA), kinetin (KIN), and thidiazuron (TDA).

Cytokinin (in media)	Endogenous cytokinins, pmol (g dry wt)$^{-1}$			
	Z	(9R)Z	iP	(9R)iP
None	122	64	87	74
BA	160	79	105	102
KIN	158	84	115	92
TDA	201	92	120	100

The data are mean values of two sets of duplicate experiments.

carbamate. Extracts were concentrated under vacuum at 40°C and methanol was replaced with ammonium acetate buffer (40 mM, pH 6.5). Ribotides were hydrolyzed by acid phosphatase (7 mg g^{-1} of cells, 27°C, 30 min). The samples' pH was adjusted to 3 with acetic acid. Samples were then applied to a column of phosphocellulose (5 ml). The column was washed with 10 volumes of starting buffer and the cytokinins were eluted with 0.3 M ammonium, pH 8.5. The effluent's pH was adjusted to 6.5 and the samples passed through a column of DEAE cellulose (5 ml). Cytokinins were adsorbed on reverse-phase column cartridges of Sep-Pak SiC_{18} (Waters, Milford, MA, USA), washed with water, and eluted with 8 ml 80% methanol. Eluates were concentrated under vacuum and stored at −15°C. Samples were fractionated by HPLC (Spherisorb 5 ODS, 4 × 250 mm column) using a gradient of methanol in water (10–80%, vol/vol) as a mobile phase. Content of cytokinins in HPLC fractions was estimated by ELISA.

Immunoassays were carried out as described by Weiler et al. (1981) with the following modifications: $IgG_{[9R]Z}$ or $IgG_{[9R]iP}$ (2 μg ml^{-1}) was used to coat the wells of microtiter plates, and alkaline phosphatase conjugated either to (9R)Z or (9R)iP (2 μg ml^{-1}) was used to detect the remaining free IgG-binding sites.

For respiration measurements, the cells were washed five times with 0.1 M K-phosphate buffer (pH 6.0), resuspended in the buffer, and placed on a rotary shaker for 30 min. Respiration was measured at 25°C with a Hansatech oxygen electrode (Norfolk, England). The electrode was calibrated with air-saturated distilled water. KCN and SHAM were dissolved in water and dimethylsulfoxide, respectively.

Dry mass yield of different suspensions was determined by right angle light scatter of cells suspended in a cuvette in a Shimadzu RF-5000 spectrofluorimeter (Sendai, Japan). Excitation and emission monochromators were set at 560 nm with slits of 1.5 nm on both sides. Dry weight can be calculated from the empirical linear equation $y = 0.0975 + 8.50x$ ($r^2 = 0.998$), where y is the dry weight in mg ml^{-1} and x is the intensity of scattered light. Cells in their linear growth phase were sampled for cytokinin assays and respiration measurements.

Results and Discussion

The levels of endogenous cytokinins in cell suspensions grown in media with different synthetic cytokinins are shown in Table I. All exogenous cytokinins increased the levels of endogenous cytokinins.

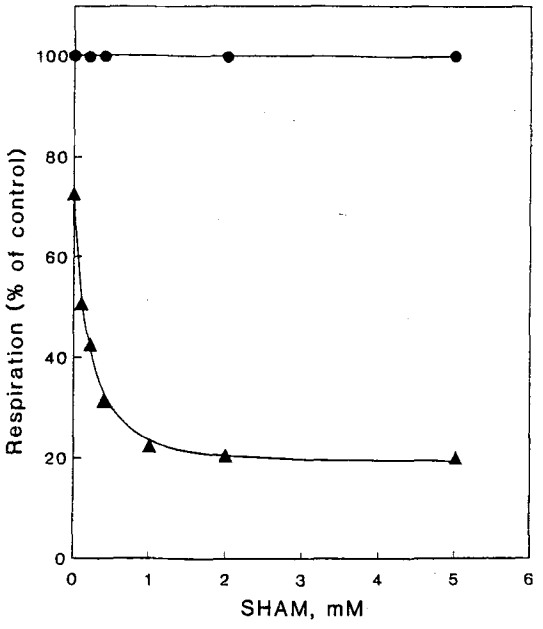

Fig. 1. SHAM titration curves in the presence (▲) and absence (●) of 1 mM KCN. Cell suspensions of *Beta vulgaris* were cultured in the medium of Schenk and Hildebrandt (1972) containing 0.5 μM NAA and 1 μM TDA. Respiration was measured at 25°C. The results are expressed as % of control with each point representing a single measurement.

The greatest increase in the endogenous cytokinin level was found in cells cultured in suspension in the presence of TDA. BA and KIN influenced the endogenous cytokinins less but to a quite similar extent. The concentrations of cytokinin bases were higher than that of ribosides in all cases.

Typical SHAM titration curves of cytokinin-dependent cell suspensions of *Beta vulgaris* are shown in Fig. 1. In this case, the cells were cultured in a medium containing TDA. The results demonstrate that for the concentration range used, SHAM has no side effects on either the cytochrome pathway or the activity of peroxidase (Bingham and Farrer 1987). Therefore, SHAM was used at 5 mM for the determination of the respiratory pattern (see below). In order to eliminate the artifacts in respiration measurements involving the application of KCN (Hsiao and Bornman 1989), we were careful to wash the cells thoroughly with buffer before measurements so as to replace the sugar-containing medium that had been sterilized by autoclaving.

The cells cultured in the presence of TDA displayed no activity of the alternative pathway in the absence of KCN (Fig. 1). This was consistently found to be the case in cells either grown in media containing the other cytokinins, BA and KIN, or in media without exogenous cytokinin (results not

Table 2. Dry mass yield and degree of inhibition of the alternative pathway by KCN or the combination of SHAM plus KCN, in cells grown in media containing different exogenous cytokinins (1 μM).

Cytokinin (in media)	% inhibition E_{max} KCN	% inhibition E_{max} SHAM + KCN	Dry wt (mg ml^{-1})
None	8.9 (2.1)	78 (2.9)	0.86 (0.03)
BA	23 (3.3)	81 (2.8)	1.21 (0.06)
KIN	26 (3.5)	80 (3.3)	1.38 (0.08)
TDA	34 (0.4)	83 (2.8)	1.52 (0.05)

BA, benzyladenine; KIN, kinetin; TDA, thidiazuron. The final concentrations of KCN and SHAM were 1.0 and 5.0 μM, respectively. Numbers in parentheses refer to SD (N = 2 or 3).

shown). The extent of inhibition by KCN applied alone was greater for cells grown in media containing cytokinin (Table 2). It suggests that for cells cultured in the presence of cytokinin, the capacity of the alternative pathway is reduced. Sequential application of SHAM and KCN results in an inhibition of about 80% in all cultures (Table 2). It shows that the apparent differences in the capacity of the alternative pathway was not due to the difference in the oxygen consumption activity of other terminal oxidases (Butt 1980).

The results (Tables 1 and 2) show that there exists an inverse relationship between the capacity of the alternative pathway and the level of endogenous cytokinins. It was demonstrated by Dizengremel et al. (1982) that SHAM and exogenous cytokinins act at different sites when they inhibit the alternative pathway in isolated mitochondria. SHAM and propyl gallate share no structural features known to be required for cytokinin activity and cytokinins share none of the structural features necessary for inhibition of the alternative pathway (Musgrave et al. 1987).

It seems possible that also synthetic and endogenous cytokinins may act at different binding sites, as quite a large structural diversity exists among them. However, besides a possible direct regulatory mechanism by site inhibition, one cannot rule out that endogenous cytokinins may exert their effect by reducing the size of the alternative pathway through other metabolic processes.

Acknowledgments. The authors thank Dr. Miroslav Strnad, Institute of Experimental Botany, Olomouc, Czechoslovakia, for the gift of antibodies and cytokinin tracers.

References

Butt VD (1980) Direct oxidase and related enzymes. In: David DD (ed) The biochemistry of plants, vol. 2: Metabolism and respiration 1980. Academic Press, New York, pp 81–123

Dedolph RR, Wittwer SH, Tuli V (1961) Senescence inhibition and respiration. Science 134:1075

Dedolph RR, Wittwer SH, Tuli V, Gilbart D (1962) Effect of N^6-benzylaminopurine on respiration and storage behavior of broccoli (*Brassica oleracea* var. italica). Plant Physiol 70:509–512

Dizengremel P, Chauveau M, Roussaux J (1982) Inhibition by adenine derivatives of the cyanide-insensitive electron transport pathway of plant mitochondria. Plant Physiol 70:585–589

Hansen CE, Meins F Jr, Aebi R (1987) Hormonal regulation of zeatin-riboside accumulation by cultured cells. Planta 172:520–525

Hsiao K-C, Bornman CH (1989) Cyanide-initiated oxygen consumption in autoclaved culture medium containing sugar. Plant Cell Rep 8:90–92

Miller CO (1980) Cytokinin inhibition of respiration in mitochondria from six plant species. Proc Natl Acad Sci USA 77:4731–4735

Miller CO (1985) Possible regulatory roles of cytokinins. NADH oxidation by peroxidase and a copper interaction. Plant Physiol 79:908–910

Mok MC, Mok DWS, Armstrong DJ, Shudo K, Isogai Y, Okamoto T (1982) Cytokinin activity of N-phenyl-N′-1,2,3-thiadiazol-5-ylurea (thidiazuron). Phytochemistry 21: 1509–1511

Morris RO, Akiyoshi DE, MacDonald EMS, Morris JW, Regier DA, Zaerr JB (1982) Cytokinin metabolism in relation to tumor induction by *Agrobacterium tumefaciens*. In: Wareing PF (ed) Plant growth substances 1982. Academic Press, London, pp 175–183

Musgrave ME, Miller CO, Siedow JN (1987) Do some plant responses to cytokinins involve the cyanide-resistant respiratory pathway? Planta 172:330–335

Musgrave ME, Siedow JN (1985) A relationship between cyanide-resistant respiration and plant responses to cytokinins. Physiol Plant 64:161–166

Siedow JN, Musgrave ME (1987) The significance of cyanide-resistant respiration to carbohydrate metabolism in higher plants. In: Moore AL, Beechey RB (eds), Proceedings of the International Meeting on plant mitochondria. pp 351–359

Vankova R, Kaminek M, Eder J, Vanek T (1987) Dynamics of production of trans-zeatin and trans-zeatin riboside by immobilized cytokinin-autonomous and cytokinin-dependent tobacco cells. J Plant Growth Regul 6:147–157

Weiler EW, Jordan PS, Conrad W (1981) Levels of indole-3-acetic acid in intact and decapitated coleoptiles as determined by a specific and highly sensitive solid-phase enzyme immunoassay. Planta 153:561–571