Bot. Bull. Acad. Sin. (1994) 35: 81-85

Metabolism of 6-(benzylamino)purine in shoots, shoot-derived callus, and shoot cell-suspension cultures of *Erythrina caffra*

S. J. Upfold, J. van Staden, and H. J. Meyer

NU Research Unit for Plant Growth and Development, Department of Botany, University of Natal, P.O. Box 375, Pietermaritzburg 3200, South Africa

(Received June 10, 1993; Accepted February 18, 1994)

Abstract. The metabolism of the synthetic cytokinin 6-(benzylamino) purine was investigated in three tissue systems derived from the shoots of *Erythrina caffra*. Shoot explants, shoot-derived callus, and shoot cell suspension cultures provided a means of studying cytokinin metabolism in different tissues from the same source. Uptake and metabolism of [8-^{14}C]6-(benzylamino) purine (BA) varied to some degree within the three systems. Subsequent to BA application, the major peak of radioactivity in all plant material consistently co-chromatographed with the free base, BA. Ribosylation of the applied free base to ribosyl 6-(benzylamino) purine ([9R]BA) appeared to be the primary metabolic route in callus and shoot tissues. Ribosyl 6-(benzylamino) purine monophosphate ([9R-MP]BA) was the major product of BA metabolism in cell suspensions. It appears that cytokinin metabolism is regulated at the subcellular level.

Keywords: 6-(Benzylamino) purine; Cytokinins; *Erythrina caffra*; Metabolism.

Abbreviations: Ade, adenine; **Ado**, adenosine; **BA**, 6-(benzylamino) purine; **[9R]BA**, ribosyl 6-(benzylamino) purine; **[9R-MP]BA**, ribosyl 6-(benzylamino) purine monophosphate; **[9G]BA**, 9-β-D glucopyranosyl 6-(benzylamino) purine; **Bq**, Bequerels.

Introduction

The transport and metabolism of 6-(benzylamino) purine (BA) has been investigated in many plants (McCalla et al., 1962; Wilson et al., 1974; Letham et al., 1975; Tao et al., 1983; Van Staden et al., 1990) and tissue culture systems (Deleuze et al., 1972; Dyson et al., 1972; Doree and Guern, 1973; Gawer et al., 1977; Van der Krieken et al., 1988,1990; Blakesley et al., 1991). From these reports, it appears that the metabolic route of applied BA within plants varies among species. Variation of the metabolic pathway of BA within different tissues of the same plant has also been reported (Upfold and Van Staden, 1992). Variation in the nature of metabolites produced after application of the free base to material derived from one plant component, however, had not received adequate attention. It was necessary to determine whether the pathway of BA metabolism varies within uniform meristematic cells derived from the same plant component, which might indicate whether, as suggested (Fusseder and Ziegler, 1988; Fusseder et al., 1989), metabolic control is regulated at the subcellular level. Shoots, callus, and cell suspension cultures derived from shoot explants provide such a system and were used in this investigation.

Materials and Methods

All work was carried out under sterile conditions. Shoot internodes (1 cm long) obtained from 3-month-old seedlings of *Erythrina caffra* (a deciduous, leguminous tree) in early summer, and taken from the apical part of the shoot, were sterilized in 0.2% $HgCl_2$ for 10 min. The shoots were then washed and cut into 2-mm sections. An equal number of shoot internodes were cut and immediately placed into four 100-ml bottles with 40 ml of modified Murashige and Skoog (1962) incubation medium. Radiolabeled [8-^{14}C]BA (10 kBq, specific activity: 1.98 MBq millimole^{-1}) was added to each bottle and the bottles were left for 24, 32, 48, and 54 h to incubate at 24±2 °C. Three replicates per incubation time were used.

Three-week-old friable callus was selected which had been produced from shoot explants on a solid Murashige and Skoog (1962) medium (2 g l^{-1} Gelrite) supplemented with 0.1 g l^{-1} myoinositol, 10 μM BA, 5 μM 2,4-D and 3% sucrose. This was suspended in 4 x 100-ml bottles in 40 ml of the liquid nutrient incubation medium. After addition of [8-^{14}C]BA (as for the shoots), the bottles were incubated at 24±2 °C for 1, 2, 4, 8, 16, and 32 h each. Three replicates per incubation time were used.

Table 1. Total radioactivity, expressed as Bq g^{-1} fresh weight, detected in the different tissues of *Erythrina caffra* treated with [8-^{14}C]BA.

Plant Material	Incubation time (h)	Radioactivity (Bq g^{-1} fwt)
Intact shoots	24	188.35
	32	342.66
	48	434.50
	54	548.57
Callus	1	273.65
	4	217.80
	8	320.55
	16	264.20
	32	487.00
Cell suspensions	24	591.08
	32	459.21
	48	201.32
	54	323.50

Suspension-cultured cells (from three-week-old callus) in an active growth phase (with 84% viability and 72% of the cells actively dividing), maintained in a modified liquid Murashige and Skoog medium (1962) supplemented with 0.1 g l^{-1} myoinositol, 1 μM BA, 0.5 μM 2,4-D, and 3% sucrose, were selected. Forty millilitres was pipetted into four 100-ml bottles. The cells were left to settle, after which the nutrient medium was poured off. The same volume of fresh nutrient medium was added and radiolabeled [8-^{14}C]BA was added as above. The bottles were incubated at 24±2 °C for 24, 32, 48, and 54 h respectively, and three replicates per incubation time were used.

At the end of the incubation period, all the treated material was filtered under vacuum through Whatman No. 1 filter paper and washed three times with 10 ml distilled water. The residue was weighed, then flash-frozen with liquid nitrogen, freeze-dried, and the dry mass recorded. The tissue was boiled in 80% methanol (80 °C) for 30 min and then filtered under vacuum. The residue was washed three times with 80% methanol. The filtrate was reduced to dryness with a flash evaporator at 35 °C, resuspended in 1 ml HPLC-grade 80% methanol and pipetted into Eppendorf tubes. After clarification of the filtrate using a Hägar microfuge, 100-μliter aliquots of the supernatant liquid were separated on a Varian HPLC fitted with a Supelcosil LC-18-DB column (25 cm x 4.6 mm) using a gradient elution program with a flow of 1 ml min^{-1}, as previously described (Lee et al., 1985). Over a 90-min period, the ratio changed from 5% methanol: 95% 0.2 M acetic acid, buffered to pH 3.5 with triethylamine, to 50% of each solvent. Ninety 1-ml fractions were collected and four millilitres of Beckman EP Ready-Value was added to each. Radioactivity levels were determined with a Beckman 3800 scintillation counter. The retention times of radioactivity peaks were compared with those of authentic BA standards separated in the same way.

Results

The levels of radioactivity incorporated into the callus tissue (expressed as Bq g^{-1} fresh weight) fluctuated, and were greatest after 32 h (Table 1). For this reason the cell suspensions and shoots were incubated for longer time periods. Total radioactivity recovered from cell suspensions decreased with increased incubation time. There was, however, an increase in the total radioactivity recovered from shoot tissue with increasing incubation time.

Table 2. Relative percentage of radioactivity associated with peaks co-chromatographing with authentic BA standards in different cultures derived from *Erythrina caffra* shoots following incubation with [8-^{14}C]BA.

Tissue Type and Incubation Time (h)	Retention Time of Standards (min)						
	Unknown 4–5	ADE 8–9	ADO 19–21	[9RMP]BA 54–57	[9G]BA 62–63	BA 77–79	[9R]BA 80–82
Shoots							
24	1.2	4.5	–	3.7	6.6	58.8	14.4
32	0.6	–	–	–	4.9	68.5	15.3
48	1.2	6.1	–	–	5.8	59.6	19.4
54	1.8	1.8	–	28.3	56.2	4.1	–
Callus							
1	–	–	–	–	–	44.4	19.8
4	–	–	–	–	–	55.9	4.7
8	2.3	4.6	4.9	2.4	1.8	40.3	20.8
16	2.1	3.7	2.2	3.2	1.6	31.2	31.2
32	8.2	9.5	8.4	3.9	3.5	29.1	12.7
Cell suspensions							
24	1.4	3.4	–	12.3	–	66.6	6.9
32	1.3	2.4	–	13.4	–	63.9	8.5
48	2.5	2.1	–	15.2	–	57.6	5.5
54	5.8	3.1	–	12.6	–	55.5	9.9

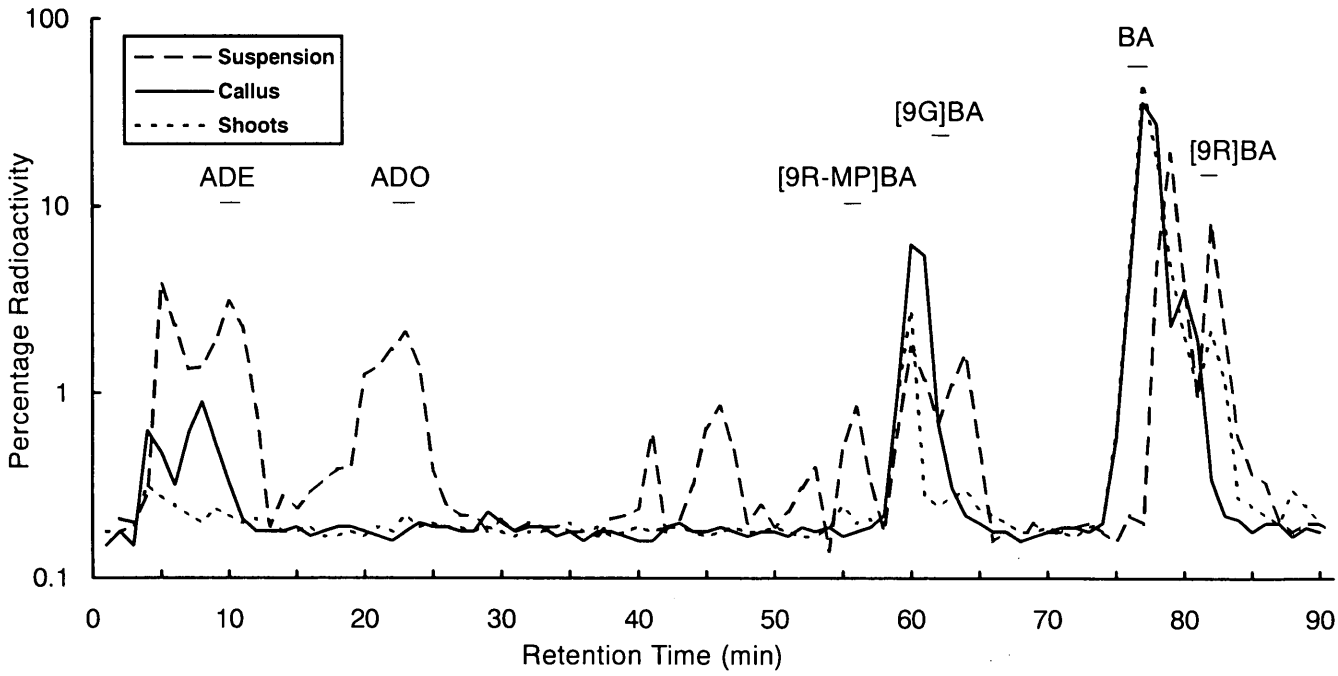

Fig. 1. Radioactivity profiles for *Erythrina caffra* shoots, callus tissue, and shoot-derived cell suspensions 32 h after the application of [8-^{14}C]BA to the material. Extracts were separated by HPLC. The retention times of co-eluting authentic standards are indicated.

Fig. 1 shows the radioactivity associated with BA metabolites within the different tissues over an incubation time of 32 h; co-chromatographing authentic standards are indicated. It is clear that BA metabolism appears to be more diverse in suspension cultures and less so in shoot tissue (Table 2). The applied free base (BA) was the major radioactive compound isolated from all tissues. The second-largest radioactive BA metabolite isolated from callus tissue and from shoots co-chromatographed with the riboside [9R]BA, but in cell suspensions, the second-largest peak of radioactivity co-chromatographed with [9R-MP]BA (Table 2).

Table 2 shows the trends in BA metabolism over time for the different tissues. Within callus tissue, partial ribosylation of BA to [9R]BA appeared to be the major metabolic route. With the exception of the 16-h treatment, the levels of radioactivity coincident with BA were consistently higher than for [9R]BA. Minor peaks of radioactivity including an unidentified polar, early eluting compound (s) (retention time = 4–5 min) as well as peaks co-chromatographing with Ade, Ado, [9R-MP]BA, and [9G]BA were detected after incubation for 8 h and longer.

After incubation times of 24 h or longer, the levels of labeled peaks co-chromatographing with [9R-MP]BA and [9G]BA were higher in shoots than in callus tissue (Table 2). After 24 h, however, labeled fractions co-eluting with BA and [9R]BA were still the largest radioactive peaks isolated from shoot tissue. With increasing incubation time, up to 48 h, these trends remained the same. Levels of radioactivity co-chromatographing with Ade decreased with time, while those associated with [9G]BA and [9R-MP]BA increased with time. After 54 h [9R]BA was no longer detectable in shoot tissue. Radioactivity associated with BA decreased, while levels of radioactivity associated with [9R-MP]BA and [9G]BA increased.

After 24 h, radioactivity associated with BA and [9R-MP]BA formed the major peaks detected in the cell suspension cultures (Table 2). Radioactivity co-chromatographing with the riboside was also detected. Increasing the incubation time did not alter these trends. With the exception of the 48-h sample, levels of radioactivity associated with [9R]BA increased as those associated with BA decreased. From 24 through to 54 h small amounts of radioactivity co-chromatographing with Ade and Ado as well as with the early eluting unidentified polar compound (s) were detected.

Discussion

It appears that the pathways of BA metabolism in these three tissues differ with respect to the ratios of metabolites produced. Radioactivity that was coincident with unmetabolized BA was always the largest peak detected in all tissues. This may well support the suggestion that it is the active form of the cytokinin (Laloue and Pethe, 1982), or it might indicate that the free base is not readily taken up or metabolized in these tissues.

It is surprising that despite the common shoot origin of the tissues, the pathways of BA metabolism in callus

tissue and in cell suspensions vary over the same incubation time. In callus tissue, ribosylation of BA appears to be the main metabolic route, with low levels of radioactivity co-chromatographing with [9R]BA. Such a system is also operative in tomato shoots (Van Staden and Bayley, 1991) and intact carnation stems (Van Staden et al., 1990). In cell suspensions, however, BA directly or indirectly metabolizes to compounds that co-chromatograph with the [9R-MP]BA. This is also the case for *Acer pseudoplatanus* cell cultures (Doree and Guern, 1973) and soybean callus cultures (Dyson et al., 1972). Low levels of the riboside were observed, indicating that ribotide production may be via [9R]BA. A comparison of the production of the ribotide in callus and cell suspensions (8 h and 24 h) to that in shoot tissues (24 h and 54 h) suggests that it may play a specific role in the process of cytokinin metabolism and utilization in these tissues. The differentiated cells of the shoot tissue are not likely to be undergoing continuous active cell division as in the callus cultures and cell suspensions. This may be reflected in the apparent production of [9G]BA, a potential storage form of BA (Letham and Palni, 1983).

It appears that cytokinin metabolism in cell cultures does not extrapolate directly to the whole plant. This is important, in that callus and cell suspension culture systems are often used to elucidate the metabolic pathways in plants. The complexities arising from uptake, transport, and compartmentation into and through differentiated tissues of plants must be taken into account. Our results suggest that control of the fate of applied BA is determined at the subcellular level. Investigation of cytokinin metabolism at this level is clearly required.

Acknowledgements. We thank the University of Natal Research Fund and the FRD Molecular Biology Programme for financial support.

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6-苯甲氨基嘌呤在海紅豆 (*Erythrina caffra*) 地上莖、其導來癒合組織及懸浮培養細胞之代謝

S. J. Upfold, J. van Staden and H. J. Meyer

NU Research Unit for Plant Growth and Development, Department of Botany, University of Natal, P. O. Box 375, Pietermaritzburg 3200, South Africa

使用海紅豆 *(Erythrina caffra)* 地上莖所得之三種組織：explant、由其誘導得之癒合組織，及懸浮培養細胞來探討合成的細胞分裂素 6-苯甲氨基嘌呤 (6-BA) 的代謝。如此可提供同一來源的不同組織中細胞分裂素的代謝。對於利用 ^{14}C 標定 6-苯甲氨基嘌呤的吸收與代謝呈現某種程度之差異性。隨著 6-BA 之加入，在所有測試組織中其放射線活性之尖峰一致地與自由之 6-BA 共同層析出。癒合組織與地上莖組織之主要代謝路徑明顯係將所加入自由之 6-BA 核糖基化成〔9R〕BA ；而在細胞懸浮中，其主要的 6-BA 代謝物卻是具單磷酸之〔9R-MP〕BA 。此結果顯示細胞分裂素之代謝可能在亞細胞層次控制。

關鍵詞：6-苯甲氨基嘌呤；細胞分裂素；海紅豆；代謝作用。