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Reversible sequestration and transport of [8-^{14}C]6-(benzylamino) purine and [8-^{14}C]ribosyl 6-(benzylamino) purine in carnation flowers

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Abstract. Ribosyl 6-(benzylamino) purine, ([9R]BA), was identified as an early metabolite in stem and receptacle tissues of carnation flowers following pulsing with the free base cytokinin, 6-(benzylamino) purine. Incubation of cut flowers with [8-^{14}C]-[9R]BA provided evidence for the occurrence of reversible sequestration between the free base and its riboside within the flower components. Such reversible sequestration provides a means of regulating specific cytokinin activity levels in carnation flowers. When applied as the riboside transport to the receptacles, ovaries and petals occurred to a much greater degree than was the case with the free base. The free base was largely retained within the stems.

Keywords: 6-(Benzylamino) purine; Carnation; Cytokinin; Metabolism; Ribosyl 6-(benzylamino) purine.

Abbreviations: ADE, adenine; **BA**, 6-(benzylamino) purine; **Bq**, Becquerel; **DMSO**, dimethyl sulphoxide; **GC-MS**, gas chromatography mass spectroscopy; **HPLC**, high performance liquid chromatography; **TLC**, thin layer chromatography; **[7G]BA**, 7-β-D-glucopyranosyl 6-(benzylamino) purine; **[9R]BA**, ribosyl 6-(benzylamino) purine; **[9R-MP]BA**, ribosyl 6-(benzylamino) purine monophosphate.

Introduction

Research into cytokinin metabolism, particularly with the cytokinin 6-(benzylamino) purine, suggests that the riboside is often the first metabolite produced following application of the free base to a number of different tissues (Laloue and Pethe, 1982; Forsyth and Van Staden, 1987; Van Staden and Mallett, 1988; Van Staden et al., 1990; 1992). It has been postulated that the ribosides are transport forms of the cytokinin (Letham and Palni, 1983). In carnation flowers, the rapid ribosylation of 6-(benzylamino) purine indicates conversion of the free base to its riboside (Upfold and Van Staden, 1992). Evidence from work concerning cell division in tobacco cultures suggests that the free base is most likely the biologically active form of cytokinin (Laloue and Pethe, 1982). An active role may also be attributed to the ribosides, as [9R]BA was detected as a major metabolite in carnation stem tissue long after this cytokinin had reached the flower components and its role as a transport agent was no longer required (Upfold and Van Staden, 1992). In addition, [9R]BA was as active as BA in delaying senescence of whole flowers and isolated petals (Van Staden et al., 1990). It is possible that the rate of ribosylation and the occurrence of reversible sequestration between cytokinin ribosides and the free bases may control both cytokinin transport and "biological action" in carnation flowers.

It is necessary therefore, to establish conclusively that in the cut carnation flower the riboside is one of the first-formed metabolites in the cytokinin metabolic pathway. If this is so, it is of significance to follow its transport and metabolism within the carnation flower to obtain more information on its role or function in flower senescence. In earlier work using TLC and HPLC, it was tentatively shown that [9R]BA is produced in both the stem and receptacle tissues of cut carnation flowers pulsed with [8-^{14}C]BA (Upfold and Van Staden, 1992).

Materials and Methods

Various techniques exist for the estimation and tentative identification of cytokinins (Horgan, 1992). These include HPLC and TLC fractionation (Van der Krieken et al., 1988; Van Staden and Mallett, 1988), bioassays (Dumbroff and Walker, 1979; Hofman et al., 1986; Lough and Jameson, 1990; Van Staden and Drewes, 1991) and various acid and enzyme hydrolysis tests (Van Staden and Mallett, 1988). The development of GC-MS techniques (Summons et al., 1979a and b; Blakesley et

al., 1991; Horgan, 1992) have resulted in the identification of many cytokinin metabolites.

GC-MS identification of [9R]BA

Carnation flowers were pulsed with cold BA (1 mg ml^{-1}) for 3 h, after which the flowers were placed in distilled water and left for a further 12 h. The tissues were then treated as described previously (Upfold and Van Staden, 1992). Stem and receptacle tissues were isolated, ground in liquid nitrogen, and extracted in 80% ethanol. Extracts were filtered, concentrated, and refiltered before being fractionated by HPLC (Lee et al., 1985). Fractions which co-eluted with authentic [9R]BA were combined, and concentrated to dryness. These samples were prepared for GC-MS analysis.

Permethylation, as described by Hakamori (1964), was carried out using the modified method for cytokinins (Morris, 1977; Young, 1977). Potassium t-butoxide was added to dry dimethyl sulphoxide (DMSO) (30 mg ml^{-1}) under nitrogen and left for 1 h at 45 °C in order to generate the required methyl sulphinyl anion ($DMSO^-$) (Horgan and Scott, 1991). This was then added to the dry sample. An excess of $DMSO^-$ was ensured by the appearance of red coloration following the addition of a trace amount of triphenylmethane. The vial was left for 30 min at 45 °C. Excess methyl iodide was added and the sample left at room temperature for 1 h, and then 500 μl water was added to quench the reaction. The permethylated derivatives were extracted with 500 μl chloroform, after which an equal volume of water was added to remove excess base and $DMSO^-$. The samples were then dried under a stream of nitrogen and resuspended in dichloromethane before being injected onto the GC-MS. GC-MS analysis was performed using a Finnigan MAT ITS40 instrument fitted with a fused silica capillary column (DB-5) (30m x 0.25mm I. D., J & W Scientific Inc.). Oven temperature was initially held at 200 °C for 2 min and then increased to 320 °C at 5 °C min^{-1}. The column was then held at this temperature for 25 min.

Production and extraction of [8-^{14}C]-[9R]BA

Once [9R]BA had been identified by GC-MS as being produced from BA in cut flowers, the flowers were treated with [8- ^{14}C]BA in order to produce labelled riboside. Carnation stem and receptacle components of whole flowers pulsed with 8500 Bq [8-^{14}C]BA (specific activity = 55 μCi $mmol^{-1}$) over 3 h and incubated for a further 12 h were filtered, concentrated and fractionated by HPLC as described previously (Upfold and Van Staden, 1992). Labelled peaks of radioactivity co-eluting with authentic [9R]BA were combined and concentrated for later use.

Stem pulsing of carnation flowers with [8-^{14}C]-[9R]BA

Flowers were treated with the labelled [9R]BA (1650 Bq), which was pulsed into the stem over 3 h. Then the flowers were placed in distilled water to incubate for a further 3 h. For comparison, a batch of flowers was also pulsed with 8500 Bq of [8-^{14}C]BA for the same time periods. Flowers were divided into stem plus calyx, ovary, receptacle, and petal tissues; these were flash frozen in liquid nitrogen. The flower components were ground in liquid nitrogen and extracted in 80% ethanol overnight as described previously (Upfold and Van Staden, 1992). The extracts were filtered, concentrated, and refiltered before being resuspended in 300 μl 80% HPLC-methanol and fractionated by HPLC. Ninety one-milliliter fractions were collected and four millilitres of Beckman Ready-Value was added to each. Radioactivity was determined using a Beckman LS 3800 Scintillation Counter.

All separations were performed twice.

Results

GC-MS identification of [9R]BA

Permethylated authentic [9R]BA was separated by GC-MS analysis and found to have a retention time of 22.4 min. HPLC separated fractions of extracts of stem and receptacle components of carnations treated with BA yielded a peak with a retention time similar to that of [9R]BA. The mass spectra of the authentic [9R]BA and the BA metabolite that co-eluted with [9R]BA were very similar; each had a base ion of m/e = 240 (Figures 1A and B).

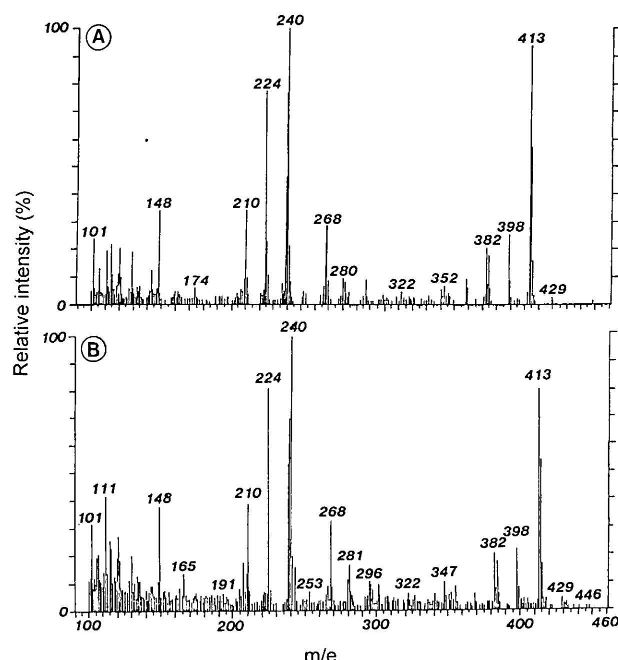

Figure 1. Mass spectra of authentic permethylated [9R]BA (**A**) and permethylated HPLC fractions, from stem and receptacle extracts of carnation flowers, that co-eluted with [9R]BA 12 h after incubation with BA (**B**).

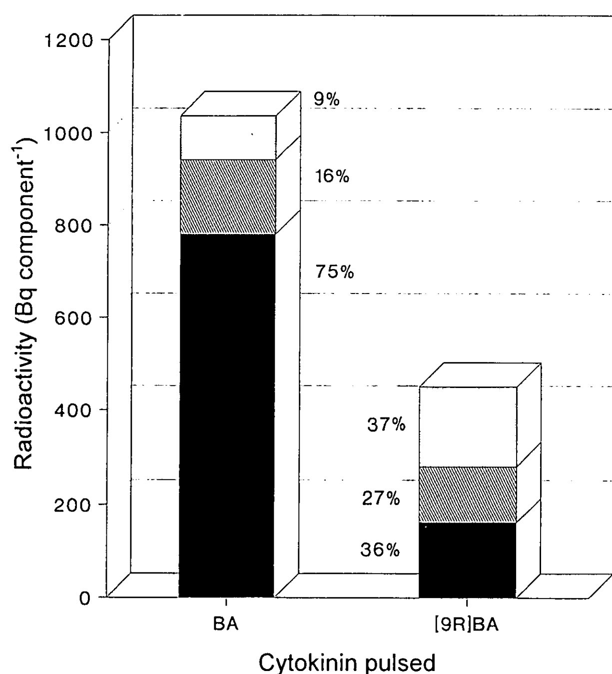

Figure 2. Relative radioactivity (Bq) in extracts of stem (solid), receptacle (stripes), and petal and ovary (dots) tissues of carnation flowers 3 h after having been pulsed with [8-^{14}C]BA or [8-^{14}C]-[9R]BA. (Percentage radioactivity of that recovered per component is indicated).

Transport and metabolism of [9R]BA

The labelled riboside which had been extracted from flowers treated with radioactive BA was applied to whole flowers. Only the receptacle and stem tissues of these flowers are considered separately, as these two tissues were major sinks for cytokinin movement in the pre-senescent flower. As was expected, the overall radioactivity detected in flowers treated with [8-^{14}C]BA was much higher than in those treated with the labelled [8-^{14}C]-[9R]BA. The percentage radioactivity extracted from the stem relative to that extracted from the the receptacle tissues of flowers treated with the two cytokinins varied (Figure 2). More than four times the amount of radioactivity was detected in stems (75%) than in receptacle (16%) tissue of BA-treated flowers. In [8-^{14}C]-[9R]BA-treated flowers, however, the relative percentage of radioactivity recovered from the stems was only slightly higher (36%) than that recovered from receptacle tissue (27%). Whereas only 9% of the radioactivity reached the petals and ovaries of [8-^{14}C]BA-treated flowers, this value increased to 36% with the application of [8-^{14}C]-[9R]BA to the stems.

In the stems of flowers treated with [8-^{14}C]BA, the free base was largely metabolized to [9R]BA (Figure 3A). Only 16% of the recovered radioactivity was still associated with the applied BA. Low levels of labelled compounds co-eluting with [7G]BA, [9R-MP]BA, and

Ⓐ [9R-MP]BA 11% BA 16% [7G]BA 9% POLAR (4–5 min) 8% [9R]BA 56%

Ⓑ BA 34% POLAR (4–5 min) 19% [9R]BA 47%

Ⓒ [9R-MP]BA 11% [7G]BA 9% [9G]BA 8% ADE 3% POLAR (4–5 min) 10% BA 24% [9R]BA 36%

Ⓓ POLAR (4–5 min) 27% BA 40% [9R]BA 33%

Figure 3. Pie charts showing the percentage radioactivity co-eluting with cytokinin standards after HPLC fractionation of carnation flower extracts obtained from stem (**A** and **B**) and receptacle (**C** and **D**) tissue 3 h after pulsing the stems of the flowers with [8-^{14}C]BA (**A** and **C**) and [8-^{14}C]-[9R]BA (**B** and **D**), respectively.

an unknown polar compound (s) with a retention time of 4–5 min were detected. Applied [8-^{14}C]-[9R]BA was partially metabolized to BA in the stems of carnation flowers (Figure 3B). No radioactivity was found coincident with [7G]BA and BA-nucleotide, but the polar unknown peak of radioactivity was present. After 3 h the riboside was still present as the major radioactive compound (Figure 3B). Metabolism was more extensive in the receptacle tissue of the BA-treated flowers. In addition to the metabolites detected in the stems, two additional peaks that co-eluted with ADE and [9G]BA were detected in the receptacles after 3 h (Figure 3C). A considerable amount (24%) of the recovered radioactivity still co-chromatographed with BA. When [8-^{14}C]-[9R]BA was applied, most (40%) of the recovered radioactivity co-eluted with BA (Figure 3D). The radioactivity profile for the receptacle and stem tissues were similar, only three peaks of radioactivity were detected when the riboside was applied to the carnation stems.

Discussion

Positive identification by GC-MS analysis shows that ribosylation of BA to produce [9R]BA in carnation stem and receptacle tissue is an early metabolic step. When [8-^{14}C]BA was applied to whole flowers it was largely retained in the stem, with little (25%) movement into the rest of the flower components. Flowers pulsed with labelled [9R]BA, however, showed a higher percentage (64%) of radioactivity associated with the receptacles, ovaries, and petals over the same time period. In the stem tissue, most of the detected radioactivity was associated with the riboside irrespective of whether labelled BA or [9R]BA was applied. In the case of BA-treated flowers, the delay in transport of the cytokinin into the rest of the flower tissue may be due to the necessity to metabolize it to [9R]BA prior to transport. This finding is in agreement with the proposal by Letham and Palni (1983), that [9R]BA serves as a transport form of the cytokinin. The presence of both BA and [9R]BA in stem and receptacle tissues indicates that there is a continuous interconversion or reversible sequestration between these two derivatives. Thus, both derivatives may have a role in the "biological" action of cytokinins. In the stem extracts of flowers pulsed with the free base, radioactivity was associated with other derivatives of BA, including the nucleotide [9R-MP]BA. It was expected that at least trace amounts of the nucleotide would be present in the flower tissues pulsed with labelled [9R]BA, if the riboside is an intermediate form of the cytokinin required for the conversion of free base to its nucleotide by the enzyme adenosine kinase (Chen and Eckert, 1977). The apparent production of [9R-MP]BA in flowers treated with [8-^{14}C]BA, and the failure to detect it in tissues of riboside-treated flowers suggests that the free base may give rise directly to the nucleotide through the action of adenine phosphoribosyltransferase (Doree and Guern, 1973; Chen et al., 1982; Griggs et al., 1989). It also appears that cytokinin transport, and possibly initial action in cut carnations, is regulated by the degree of interconversion between the free base and the riboside, and that production of the nucleotide, which may fulfil a different physiological role, occurs directly from the free base rather than via its riboside.

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[8-^{14}C]6-(benzylamino) purine和 [8-^{14}C]ribosyl 6-(benzylamino) purine 在康乃馨交互變化及傳送的機制

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Ribosyl 6-(benzylamino) purine, ([9R]BA) 聚集在花托及莖是康乃馨浸漬在 6-(benzylamino) purine (BA) 的早期代謝產物。利用切花浸漬在 [8-^{14}C]-[9R]BA 可証明這上述兩種化學物質在花中可互相轉換。如此互相轉換的機制，可調節康乃馨花中 cytokinin 活性。這實驗顯示 [9R]BA 比 BA 容易傳送到花托、子房及花瓣等組織，BA 大部份停留在莖中。

關鍵詞：6-(Benzylamino) purine；康乃馨； Cytokinin；代謝；Ribosyl 6-(benzylamino) purine。