14C-adenine incorporation into cytokinin in light and dark germinating Zea mays embryos

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Abstract

This study was aimed at testing the biosynthetic capacity of free cytokinins in the embryos of dark germinating Zea mays seeds, supplied with 14C-adenine and incubated under darkness and white light of 24 hrs, respectively. By using HPLC and TLC methods, eight metabolites including Ade, Ado, 5'-AMP, [9R-5'P]iP, [9R-5'P]IZ, [9R]Z, and [9R-5'P]DHZ were recovered from both treatment of dark incubation and white light irradiation. Besides these, three more metabolites [9R]iP, iP and DHZ were only found in white light treatment. White light also induces a higher level of Ado, 5'-AMP, [9R]Z and Z.

Keywords: White light; Darkness; 14C-adenine incorporation; Cytokinins; Germinating Zea mays embryos.

Abbreviations

Ade=adenine; Ado=adenosine; 5'-AMP=adenosine-5'-monophosphate; [9R-5'P]iP=isopentenyladenine nucleotide; [9R]iP=isopentenyladenine; [9R-5'P]Z=zeatin nucleotide; [9R]Z=zeatin nucleoside; Z=trans-zeatin; [9R-5'P]DHZ=dihydrozeatin nucleotide; [9R]DHZ=dihydrozeatin nucleoside; DHZ=dihydrozeatin; HPLC=high performance liquid chromatography; TLC=thin layer chromatography; BA=benzyladenine; [9R]BA=BA ribonucleoside; IMP=inosine-5'-monophosphate; CMP=cytidine-5'-monophosphate; GMP=guanosine-5'-monophosphate; UMP=uridine-5'-monophosphate; IPP=isopentenyl pyrophosphate.

1. Introduction


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step for cytokinin biosynthesis was not known until Taya et al. (1978) and Chen and Melitz, (1979) found that cell free preparation from slime mold Dictyostelium discoides and cytokinin-autonomous tobacco callus could catalyze the formation of [9R-5'-PiP] and isopentenyl pyrophosphate. Up to date schemes essential for the pathways of this hormonal biosynthesis have already been proposed (Letham and Pain, 1983; Chen, 1982), but parts of the pathway have not been resolved and substantiated.

In this study, dark germinating corn embryos were treated with $^{14}$C-adenine solution and incubated under darkness and white light to see if there is any difference of the biosynthetic capability of free cytokinins between these two conditions. Not only different types of cytokinins were found between these treatments but eight normal cytokinins were recovered at a time in white light-receiving embryos. Results found in this study may provide a more complete information for a better understanding of the pathway of free cytokinin biosynthesis in plants.

2. Materials and methods

2.1 Plant material

Corn seeds (Zea mays L. CV. Honey jean No. 2) were sterilized with 10% sodium hypochlorite for 10 min, then rinsed with a few times of distilled water and germinated on wetted filter papers contained in petri dishes in darkness at 25°C. Dark germinating seedlings at 2 day and 22-hour-old were used as experimental materials. Seedling endosperms were carefully cut away under safe green light in a dark room and embryos were then immediately weighed and used for $^{14}$C-adenine treatment.

2.2 Preparation of $^{14}$C-adenine solution and corn embryo incubation in $^{14}$C-adenine solution

[8-$^{14}$C]adenine solution (1.85 M Bq/ml), purchased from Amersham International, was diluted into 200 ml (1.85 x 10$^4$ Bq/ml) with distilled water. Dark germinating corn embryos were partially immersed in $^{14}$C-adenine solution (5 ml and 20 ml were used for dark and white light treatment, respectively) and incubated under darkness and white light (white fluorescent lamps with an intensity of 20 μmol m$^{-2}$ s$^{-1}$) at 25°C for 24 hrs, respectively.

2.3 Homogenation and purification

After dark and white light treatment, embryos were quickly frozen in liquid nitrogen and macerated with 10 to 15 ml of 80% ethanol in a pestle-mortar (for dark treatment this step was done under safe green light). Ethanol samples were then kept in a refrigerator at 4°C for at least 24 hrs, then centrifuged at 1153 g for 20 min to clean the sample and filtered through a Waters sep-pak C18 cartridge (Millipore Corp., USA). The filtrate in a small flask was then evaporated to dryness by a rotatory evaporator at 40°C prior to being used for HPLC and TLC analysis.

2.4 HPLC analysis of cytokinin standards

Chemicals including Ade, Ado, 5'-AMP, [9R-5'-PiP], [9R]PiP, IP, [9R-5'-PiZ], [9R]Z, [9R-5'-PiDHZ], [9R]DHZ and DHZ, purchased from Sigma (USA) and Apex Organics (U.K.), were made a mixture of solution (10$^{-3}$ M each). 100 μl was injected into a Spherisorb HPLC column (ODS2, 5 μm, 250 x 4.6 mm i.d.) fitted to a Hitachi L-7100 HPLC pump connected with an UV detector and a Hitachi D-2500 Chromato-Integrator at a flow rate of 1 ml min$^{-1}$. The buffer system used was
0.2M acetic acid buffered to pH 3.5 with triethylamine: methanol (5% to 50% methanol over 90 mins). After being run by HPLC, each chemical gave a peak at a specific retention time (min or mins). Fig 1a which was done for the first experiment included 5'-AMP at 4, Ade at 4-6, Ado at 9-11, [9R-5'P]Z at 20-22, Z at 27-28, DHZ at 29-31, [9R]Z at 39-41, [9R-5'P]iP at 69-71, iP at 70-71 and [9R]iP at 73-74; Fig 1b which was done for the second experiment had 5'-AMP at 4, Ade at 5-6, Ado at 9-11, [9R-5'P]Z at 21-24, Z at 28-29, [9R-5']IDHZ at 28-29, DHZ at 30-32, [9R]Z at 40, [9R]DHZ at 42, [9R-5']iP at 69-70, iP at 71-72 and [9R]iP at 73-74. Each standard's retention time changed slightly, especially the column had been continuously used for a longer period. Thus the retention time of each standard should be run and determined by HPLC each time before the sample was fractionated by HPLC. This retention time (min or mins) of each standard chemical would set as a guidance of 'specific fraction (or s)' for HPLC fractionation of 14C-adenine-treated samples.

2.5 HPLC analysis of 14C-adenine-treated sample

A dried sample in the small flask was dissolved in a small amount (500 to 600 µl) of 80% HPLC-grade methanol and a part of this methanol-sample was injected into HPLC column. Conditions for HPLC process were the same as those described above. In the first experiment, fractions collected from both dark and white light-treated samples were in accordance with the retention time of each chemical standard listed in Fig 1a: 4(5'-AMP), 6(Ade), 11(Ado), 20(9R-5'P)Z and 69(9R-5'P)iP). In the second experiment, fractions collected form both dark and white light-treated samples were in accordance with the retention time of each chemical standard listed in Fig 1b: 28(9R-5'P)DHZ, 29(Z), 31(DHZ), 40(9R)Z, 42(9R)DHZ, 71(iP) and 74([9R]iP). Besides these fractions, other fractions also tested in the dark-treated sample included 27(Z), 30(DHZ), 32(DHZ), 72(iP) and 73(9R)IP, 9R-5'P)DHZ.

2.6 Radioactivity analysis of TLC Rf values

In this part, standard chemicals Ade, Ado, 5'-AMP, [9R-5'P]IP, [9R]IP, iP, [9R-5'P]Z, [9R]Z, [9R-5'P]DHZ, [9R]DHZ and DHZ should be first determined their each Rf value on the strip (3 cm width) of Polygram Sil G/uv 254 TLC plate (No. 605183, Macherley, Nagel, Germany) under UV light after being developed in a specific solvent system. Table 1 listed each TLC Rf value of these twelve chemicals including four solvent systems.

Those designed fractions collected from a sample each was dried in the small distilled flask, dissolved in about 70 µl of 80% methanol, then applied to a strip (3 cm width) of TLC plate (Z1227-7, Sigma-Aldrich) and developed in a specific solvent system depending on the specific type of cytokinin. After the solvent system reached the top line of TLC strip, the strip was dried and made 10 Rf values with a TLC spotting guied and Rf reader (Sigma). Silica gel of each Rf value was scraped off and transferred to a scintillation counting vial, to which 1 ml methanol and 4 ml counting cocktail (PRIC, USA) were added in order and mixed well before being read by a Pharmacia scintillation counter to get a dpm value.
\(^{14}\)C-adenine incorporation into cytokinin in light and dark germinating Zea mays embryos.

Fig. 1a. First HPLC analysis of standard chemicals for specific retention times: 5'-AMP at 4; Ade at 4-6; Ado at 9-11; [9R-5']Z at 20-22; Z at 27-28; DHZ at 29-31; [9R]Z at 39-41; [9R-5']iP at 69-70; iP at 71-72; [9R]iP at 73-74.

Fig. 1b. Second HPLC analysis of standard chemicals for specific retention times: 5'-AMP at 4; Ade at 5-6; Ado at 9-11; [9R-5']Z at 21-24; [9R-5']DHZ at 28-29; Z at 23-29; DHZ at 30-32; [9R]Z at 40; [9R]DHZ at 42; [9R-5']iP at 69-70; iP at 71-72; [9R]iP at 73-34.
The radioactivity (Bq g⁻¹Fw) of each Rf value was calculated, based on the dpm value, Bq value (1 Bq = 59.4 dpm), volume of the sample in 80% methanol before HPLC and fresh weight of the tissue.

Table 1. Solvent systems and TLC Rf value of standard chemicals

<table>
<thead>
<tr>
<th>Standard chemicals</th>
<th>Solvent system*</th>
<th>The range of Rf value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ade</td>
<td>2</td>
<td>0.56 – 0.58</td>
</tr>
<tr>
<td>Ado</td>
<td>2</td>
<td>0.42 – 0.44</td>
</tr>
<tr>
<td>5’-AMP</td>
<td>2</td>
<td>0.00 – 0.01</td>
</tr>
<tr>
<td>[9R-5’P]iP</td>
<td>3</td>
<td>0.29 – 0.31</td>
</tr>
<tr>
<td>[9R]iP</td>
<td>1</td>
<td>0.56 – 0.58</td>
</tr>
<tr>
<td>iP</td>
<td>1</td>
<td>0.69 – 0.72</td>
</tr>
<tr>
<td>[9R-5’P]Z</td>
<td>4</td>
<td>0.85 – 0.87</td>
</tr>
<tr>
<td>[9R]Z</td>
<td>1</td>
<td>0.07 – 0.09</td>
</tr>
<tr>
<td>Z</td>
<td>1</td>
<td>0.06 – 0.08</td>
</tr>
<tr>
<td>[9R-5’P]DHZ</td>
<td>4</td>
<td>0.85 – 0.87</td>
</tr>
<tr>
<td>[9R]DHZ</td>
<td>1</td>
<td>0.58 – 0.60</td>
</tr>
<tr>
<td>DHZ</td>
<td>1</td>
<td>0.60 – 0.62</td>
</tr>
</tbody>
</table>

* (1) Butanol : 25% NH₄OH : Water (6:1:2)
(2) Butanol : NH₄OH : Water (6:1:2)
(3) Butanol : Formate : Water (20:5:8)
(4) 0.03M Borate (pH 8.8) + 10% Acetone

** Each standard was applied to a TLC strip (3cm width) about 2 mm band due to a reason that the sample of HPLC fraction was also applied in a similar way of about 2 mm narrow band.

*** Butanol, product (L. C. Grade) of Alps Chem. Co., Ltd. (Taiwan).

3. Results and Discussion

After TLC and radioactivity analysis of necessary HPLC fractions in dark germinating corn embryos treated with ¹⁴C-adenine, eight metabolites Ade, Ado, 5’-AMP, [9R-5’P]iP, [9R-5’P]Z, [9R]Z, Z and [9R-5’P]DHZ were recovered from both treatments of dark incubation and white light irradiation. Other three metabolites [9R]iP, iP and DHZ were also found only in white light treatment (Fig.2 and Fig.3). ¹⁴C-adenine was first incorporated into Ado and 5’AMP in dark and white light-treated samples. But the level of Ado and 5’AMP in white light treatment was much higher than in dark one (Fig.2 and Fig.3).

[9R-5’P]iP, which was identified as the first product of the biosynthesis of cytokinins (Taya et al., 1978; Chen et al., 1979), appeared in both treatment of darkness and white light in corn embryos (Fig.2 and Fig.3). Then [9R-5’P]iP was transformed into [9R-
Fig. 2. Radioactivity (Bq g⁻¹FW) of TLC Rf values detected in specific HPLC fraction numbers of the extract of dark germinating Zea mays embryos after having been treated in ¹⁴C-adenine solution and incubated in darkness of 24 hrs. TLC radioactivity at a certain Rf value (−) in each fraction number was identified as the location of a specific authentic standard, based on the TLC specific Rf value of each chemical standard listed in Table 1.
Fig. 3. Radioactivity (Bq G⁻¹ Fw) of TLC Rf values detected in specific HPLC fraction numbers of the extract of dark germinating Zea mays embryos after having been treated in ¹⁴C-adenine solution and incubated under white light of 24 hrs. TLC radioactivity at a certain Rf value (—) in each fraction number was identified as a location of the specific authentic standard, based on the TLC specific Rf value of each chemical standard listed in Table 1.
$5'PZ$ through transhydroxylation and then $9R-5'PZ$ into $9R-5'PZH$ by a reductase. These processes also occurred in dark and white light treatment (Fig.2 and Fig.3). Thus the first steps of the biosynthetic pathway of cytokinins in corn embryos would be $\text{Ade} \rightarrow \text{Ado} \rightarrow 5'\text{-AMP} \rightarrow [9R-5'P]iP \rightarrow [9R-5'PZ] \rightarrow [9R-5'PZH]$. Then metabolites $[9R]Z$ and $Z$ were also found in dark and white light treatment (Fig.2 and Fig.3). $[9R]Z$ was possible from $[9R-5'PZ]$ by a $5'$-nucleotidase. Although no evidence has been given to $[9R-5'PZ]$ as the substrate of this enzyme before, some workers have shown $5'$-nucleotidase could use $[9R-5'P]iP$, IMP, GMP, $5'$-AMP and CMP as its substrate (Chen and Kristopeit, 1981; Gibson and Drammond, 1972; Shiio and Ozaki, 1978). These results suggest $5'$-nucleotidase is highly non-specific. It is interestingly noted that $[9R]Z$ level in white light treatment was much higher than in dark one (Fig.3 and Fig.2). This increased level of $[9R]Z$ by white light could be from $[9R-5'P]iP$ and $[9R]iP$. $[9R]Z$ from $[9R]iP$ through transhydroxylation has been demonstrated from the endosperm of young corn kernel and microsomal fraction of fresh cauliflower (Miura and Hall, 1973; Chen and Leisner, 1984). But in the present study of corn embryos, it is not known the source of this white light-increased $[9R]Z$ level is from $[9R]iP$ or $[9R-5'P]iP$ or from both.

Then $Z$ was highly from $[9R]Z$ through a $5'$-nucleosidase. Again no evidence has been reported on the pathway of $[9R]Z \rightarrow Z$ so far. But enzymes adenosine nucleosidase and cytokinin nucleosidase were shown to catalyze $iP$ formation from $[9R]iP$ (Chen and Kristopeit, 1981; Roole and Chism III, 1989). Adenosine nucleosidase was suggested to be non-specific (Bruch and Stuchbury, 1987; Van Staden and Crouch, 1996). Additionally, A level in white light treatment was much higher than in dark on (Fig.3 and Fig.2). This increased $Z$ level by light could be from $[9R]Z$ and $iP$. But judged from a very small value of $iP$ measured (Fig.3), this white light-induced $Z$ level is highly from $[9R]Z$ whose value was very high in white light-treated sample. (Fig.3)

Other three metabolites $[9R]iP$, $iP$ and $DHZ$ were only found in white light treatment (Fig.3). The origin of $[9R]iP$ and $iP$ could be from $[9R-5'P]iP$ and $[9R]iP$ by $5'$-nucleotidase and adenosine nucleosidase, respectively (Chen and Kristopeit, 1981; 1981). Naturally, these enzymes are newly formed by light for the process of these pathways. As mentioned above, $[9R]Z$ and $Z$ level in white light treatment was higher than in dark one. So white light-induced $5'$-nucleotidase and adenosine nucleosidase could trigger the formation of $[9R]iP$, $iP$, $[9R]Z$ and $Z$. However, actual isoforms of these enzymes are unknown. DHZ is highly form $Z$ through a reductase activated by light. In Phaseolus embryos and lupine seeling roots, DHZ was found to be formed from $Z$ (Sondheim and Tzou, 1971; Martin et al., 1989; Parker et al., 1978).

In conclusion, white light, besides inducing three more metabolites $[9R]iP$, $iP$ and $DHZ$, has a capacity of causing a higher level of Ado, $5'AMP$, $[9R]Z$ and $Z$.

From the results of this study, a scheme is proposed and suggested for the metabolic process of these products produced by $^{14}C$-adenine transformation in germinating corn embryos (Fig.4).
Fig. 4. A scheme was suggested to be cytokinin biosynthetic pathways in dark germinating corn embryos treated with $^{14}$C-adenine and grown under white light and darkness for 24 hrs, respectively: solid lines (pathways proceed under both darkness and white light); dotted lines (pathways go on only under white light).

**Literature cited**


14C-adenine 拼入 free cytokinins 在白光與黑暗下正發芽的玉米胚

董敏生 陳柏衣 徐希世 楊其暉

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摘要：本文研究的目地在探討玉米胚發芽時，在光及暗的條件下合成的 cytokinin 的能力。暗室生長的玉米胚被置於14C-adenine 溶液中，培養於暗室與白光下各 24 小時。應用 HPLC 與 TLC 方法分析其 cytokinins 的代謝物，結果得知在暗室與白光下培養的玉米胚其代謝物有八種：Ade、Ado、5'-AMP、[9R-5'P]iP、[9R-5'P]Z、[9R]Z、Z 與 [9R-5'P]DHZ。另外，有三種代謝物 [9R]iP、iP 與 DHZ 只被發現在白光下的處理。又白光可誘導較多量的 Ado、5'-AMP、[9R]Z 與 Z。

關鍵詞：白光、暗光、14C-adenine 的拼入、cytokinins、正發芽的玉米胚。