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Metabolism of Cytokinins by Tissue Culture Lines of Oil Palm (*Elaeis guineensis* Jacq.) Producing Normal and Abnormal Flowering Palms

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Abstract. The metabolism of cytokinins in tissue cultures of two oil palm clones previously known to regenerate palms ultimately manifesting normal and abnormal flowering was studied using radiolabeled benzyladenine and isopentenyladenosine, with particular regard to the kinetics of formation of the cytokinin 9-glucoside. Labeled products were separated by high performance liquid chromatography and identified by comparison of retention times with authentic cytokinin standards run immediately before or after the experimental sample. Using benzyladenine, which is insensitive to cytokinin oxidase, ribotide appeared rapidly and then declined. 6-Benzylaminopurine (BA) 9-glucoside quickly became the major soluble product with some formation of riboside. No other ethanol-soluble products were found. Over an incubation period of 24 h up to 30% of label appeared in the ethanol-insoluble fraction. The uptake of label was consistently faster in the normal than the abnormal clone. Dose-rate and time course experiments produced an in vivo asymptotic dose-response curve for the accumulation of BA 9-glucoside analogous to a Michaelis-Menten first-order reaction with a "V_{max}" of 3.5 nmol·g^{-1}·h^{-1} (on a fresh weight basis) and a "K_m" of 0.12 mM. There were no differences between clones in the rate of synthesis. Using isopentenyladenosine, which is susceptible to cytokinin oxidase and cannot be glucosylated without prior deribosylation, a complex pattern of metabolism was seen, with much slower production of 9-glucoside. A number of transient unidentified compounds were seen, together with adenosine and adenine. Comparison of normal and abnormal flowering clones showed striking differences in the kinetics of production of a compound thought to be [9R]Z and in a transient compound eluting at 22 min which accounted for 42% of the radioactivity after a 7-h incubation in the abnormal line. By 17 h there was no difference between normal and abnormal lines in the radioactivity in this compound. Cytokinin uptake was slower in the abnormal than in the normal flowering clone.

Key Words. Cytokinin—Oil palm—Tissue culture—Abnormal flowering

Oil palm tissues have been shown to contain significant amounts of both isoprenoid (Besse et al. 1992, Jones 1990, Jones et al. 1995) and aromatic cytokinins (Jones et al. 1996). The most abundant compounds found in the tissues are the 9-glucosides of virtually all of the cytokinin types. In tissue cultures, kinetin was shown to be metabolized primarily to 9-glucoside over the normal culture cycle of 8 weeks (Jones and Hanke 1992).

These studies have relevance to the problem of abnormal flowering in some oil palm clones derived from tissue culture since various reports have implicated cytokinins in both the induction and expression of the abnormality (Agamuthu and Ho 1992, Besse et al. 1992, Duval et al. 1995, Ho et al. 1991). Unfortunately, there is no clear consensus in these reports (Jones 1995), but the evidence suggests that high cytokinin in the medium increases the risk of abnormality in palms flowering years later (Eeuwens, personal communication, Paranjothy et al. 1995). In tissues from abnormal flowers and from embryoids of culture lines giving rise to abnormally

Abbreviations: The abbreviations for cytokinins follow the proposals of Crouch et al. (1993). HPLC, high performance liquid chromatography; iP, isopentenyladenine; [9R]iP, isopentenyladenosine; [9G]iP, 9-glucoside of iP; Z, zeatin; [9R]Z, zeatin riboside; [9G]Z, 9-glucoside of zeatin; BA, 6-benzylaminopurine; [9R]BA, benzyladenosine; [9R-MP]BA, ribotide of BA; [9G]BA, 9-glucoside of BA; MS, Murashige and Skoog (1962) tissue culture medium; 2,4-D, 2,4-dichlorophenoxyacetic acid.

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flowering clones there is some evidence of a lower level of [9R]Z compared with normal lines and of increased [9G]iP (Besse et al. 1992, Duval et al. 1995, Jones et al. 1995).

The experiments reported here were carried out over a period of several years, initially to study the metabolism of exogenous cytokinins used in tissue culture, but later to attempt to understand endogenous cytokinin metabolism. During this period the natural occurrence in oil palm of the family of aromatic cytokinins was discovered, and labeled compounds of high specific activity became available. Particular attention was given to the kinetics of inactivation of cytokinins by 9-glucosylation in oil palm tissues.

Materials and Methods

Tissue Cultures

Cultures of oil palm embryoids were obtained from Dr. C. J. Eeuwens, Unifield TC (Bedford) Ltd. (now transferred to Unilever Plantations and Plant Science Group, Maris Lane, Trumpington, Cambridge).They were sampled 3 weeks after transfer to MS medium containing sucrose (30 g·liter^{-1}), casein hydrolysate (0.1 mg·liter^{-1}), naphth-1-yl acetic acid (0.05 mg·liter^{-1}), and kinetin (0.01 mg·liter^{-1}).

Cultures from two oil palm clones were used. One, Clone 4 (90a), had been shown to give rise to abnormally flowering palms. The other, Clone 128, had produced palms bearing only normal flowers.

Radioisotopes

[8-^{14}C]Benzyladenine (2.0 GBq·mmol^{-1}) was obtained from Amersham International plc, and [U-^{3}H]isopentenyladenosine (1.1 TBq·mmol^{-1}) was kindly supplied by Dr. M. Strnad, Institute of Experimental Botany, Czech Academy of Sciences, Olomouc, Czech Republic. The purity of the isotopes was checked by HPLC of samples before use, and in both cases they were found to be better than 99% pure.

Labeling Method

The isotope was administered by shaking tissue pieces (approximately 0.5 g fresh weight) in 2 mL of sterile MS medium containing sucrose (3% w/v) and an appropriate dose of radiolabeled cytokinin, but without other added growth regulators, in 50-mL Sterilin jars. In later experiments up to 5 g of tissue was used in 40 mL of MS medium containing 3% sucrose. The isotope dilution required was calculated from the data sheet provided. Radioactivity of the diluted isotope was checked by scintillation counting, and the cytokinin concentration was determined from the UV absorption at 268 nm using a molar extinction coefficient for benzyladenine of 20,000.

After the uptake period (depending on the experiment), the tissue pieces were rinsed in sterile water and either frozen in liquid nitrogen or returned to their original agar medium or into fresh MS liquid medium containing 3% sucrose for a further period of metabolism. At sampling time each tissue piece was weighed before being frozen in liquid nitrogen.

Frozen samples were stored at −70°C until required.

Sample Extraction and Purification

Sample extraction, purification, and separation by HPLC were as described previously (Jones 1990). As an internal standard to estimate extraction losses, 50 μL of the ^{3}H-diol of isopentenyladenosine (approximately 167 Bq) (Kraigher et al. 1991) was added at the first step of grinding in liquid nitrogen before extraction with ice-cold aqueous ethanol (80% v/v). After preliminary purification on a C_{18} Sep-Pak (Waters Associates Ltd.) the cytokinins were separated by reverse-phase HPLC as described previously. A set of authentic cytokinin standards was run either immediately before or after each experimental sample, and the retention times of the radiolabelled peaks were compared with the retention times of the known standards. The standards used in the BA labeling experiments were BA, [9R]BA, [9R-MP]BA, [9G]BA, [7G]BA, Ade, and Ado. Reference compounds for the experiment with [9R]iP were the free base, ribotide, riboside, and 9-glucoside of zeatin and isopentenyladenine, together with adenine and adenosine.

HPLC fractions (1.5 mL) were collected at 1-min intervals in small scintillator vials (nominally 5 mL). Four mL of Optiphase Hi-Safe 3 scintillator fluid (LKB Ltd.) was added, and the radioactivity in the samples was counted on a Packard Tri-Carb 2000 scintillation counter. The results were converted to nmol of cytokinin equivalents from the initial specific activity and dose of isotope applied, corrected for recovery losses estimated from the recovery of ^{3}H-[9R]iP-diol. This compound elutes from the HPLC at 20–21 min, slightly later than [9G]BA and [9G]iP but earlier than BA.

The ethanol-insoluble tissue residues were digested in 5 mL of Scintran tissue solubilizer (BDH Ltd.) at 60°C for 2 h. Ten mL of Optiphase 3 was added and the radioactivity measured. The digested samples varied in color, and there was always considerable quenching, so the radioactivity may be underestimated but was always at significant levels. Counts were also made on the Sep-Pak eluates and washings. Very little radioactivity was found in these samples. The residual medium after incubation was purified on a C_{18} Sep-Pak and subjected to HPLC separation followed by scintillation counting. Attempts to estimate the amounts of radiolabel leaking into the postfeed agar medium were abandoned quickly because of the difficulties of extraction from agar. Such extracts subsequently completely clog up the Sep-Pak and HPLC columns.

Experiments and Results

For the first experiment, tissues of Clone 4 (abnormal) were incubated for 6 and 24 h following a 2-h feed with 1.73 nmol of [8-^{14}C]BA (approximately 3.7 KBq). Fig. 1 shows the distribution of radioactivity in the tissues after a further 24 h on “cold” medium. The peaks of radioactivity correspond to the elution times of [9R-MP]BA, [9G]BA, BA, and [9R]BA. There was little indication of any other radiolabeled metabolites except a very small peak at 13 min, when [7G]BA would elute. The residual incubation medium contained 99% BA with a trace of [9G]BA. This result was consistent with analytical results from radioimmunoassay and confirmed the expectation that the major metabolic product was the 9-glucoside, with little or no 7- or 3-glucosylation. In the period between 6 and 24 h the concentration of [9G]BA rose from 12% to 20% of the total radiolabel, the [BA] declined, and the riboside and ribotide peaks remained roughly constant at about 5% and 3%, respectively.

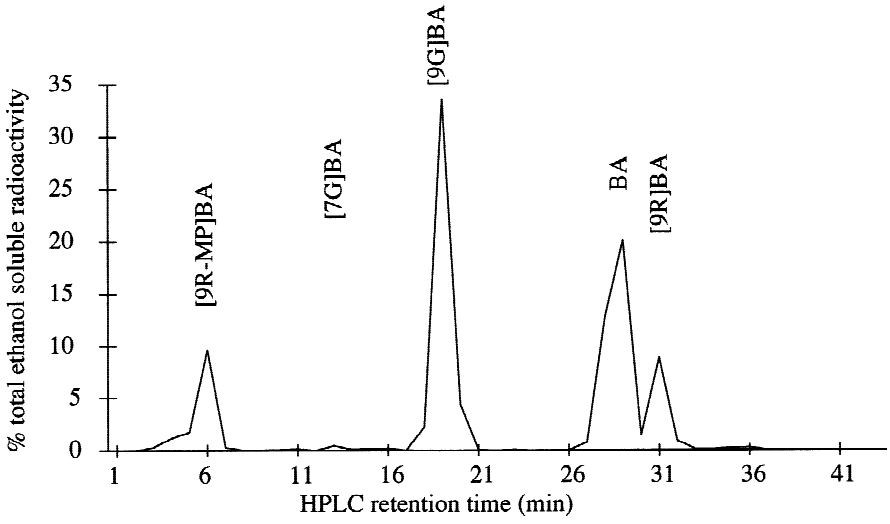

Fig. 1. Distribution of radioactivity in HPLC fractions of ethanol-soluble extract of embryogenic oil palm callus 25 h after a 2-h incubation with [8-^{14}C]benzyladenine. The sample was separated by reverse-phase HPLC on a 150- × 4.6-mm C_{18} ODS 5-μm microsphere column using a gradient of 15% methanol increasing to 36.25% in 15 min then to 40% in the next 25 min. The solvents contained 0.02 M triethylammonium acetate, pH 7.0, flow rate 1.5 mL·min^{-1}. Fractions were collected each minute, 4 mL of Optiphase Hi-Safe scintillant was added, and the radioactivity was counted in a Packard TriCarb 3000 scintillation counter.

There was an increase in the label appearing in the insoluble fraction to about 15% of total radioactivity.

In the second experiment, the time course of metabolism was explored in more detail using tissues from both the normal and abnormal flowering clones. The objective was to apply the labeled cytokinin at 0.1 mg·liter^{-1} (0.44 nmol·mL^{-1}) as frequently used in tissue culture media. Because of the large number of samples required and the time-critical nature of the experimental protocol, the experiment was done in separate runs on the two clones. The radiolabeled medium was prepared by injection of 50 μL of prediluted radiolabel into sterile medium immediately before dispensing the aliquots of 2 mL of medium into a sufficient number of separate Sterilin pots. The actual concentration of cytokinin obtained in the two experiments was determined by counting control samples of medium and by measuring the UV absorbance at 268 nm. The cytokinin concentration was calculated from the molar extinction coefficient of BA. These measurements showed that the cultures were supplied with 0.78 nmol of BA (Clone 4) and 1.03 nmol of BA (Clone 128) in 2 mL of incubation medium at approximately 1.85 MBq/culture. A series of samples was set up, each containing approximately 0.5 g of tissue and incubated for 2 h. After washing and transfer back to the original medium duplicate samples were taken at 5-h intervals over a 25-h period. "Time 0" samples were taken after 1 min in the radioactive medium and immediately after washing.

The results were subjected to analysis of variance and revealed a significant clone × time interaction in the internal concentration of BA but not in [9G]BA, [9R-MP]BA, or the ethanol-insoluble fraction. In this experiment [9R]BA showed as a small shoulder on the BA peak and could not be effectively resolved.

The clonal difference was the result of a slight difference in the rate of BA entry into the tissues, the uptake rate being slower and the later decline in label in BA greater in the abnormal than in the normal clone.

The results are summarized in Fig. 2. The differences between clones were generally insignificant. In order to show the metabolic pattern more clearly the clonal results were pooled, and only the main effect means for sampling time are shown.

As expected, the label in BA was highest immediately after the feed and then fell steadily. Ribotide appeared very rapidly (significant levels after 1 min). After the 2-h feed it remained relatively constant, although subject to large sample variation. The amounts of label in [9G]BA and in the insoluble fraction increased rapidly at first and then more slowly. The total radiolabel in cells declined only slowly (not significant) during the period on "cold" medium, suggesting that there was little leakage into the agar medium.

At the end of the uptake period 44% of the radioactivity was in the cells in Clone 4 (abnormal flowering) and 50% in Clone 128 (normal flowering). The ratios of the concentrations of BA in the tissues to that in the external medium were 2.3:1 in clone 128 and 1.95:1 in Clone 4.

From this experiment there was no evidence of a difference in intracellular BA metabolism between normal

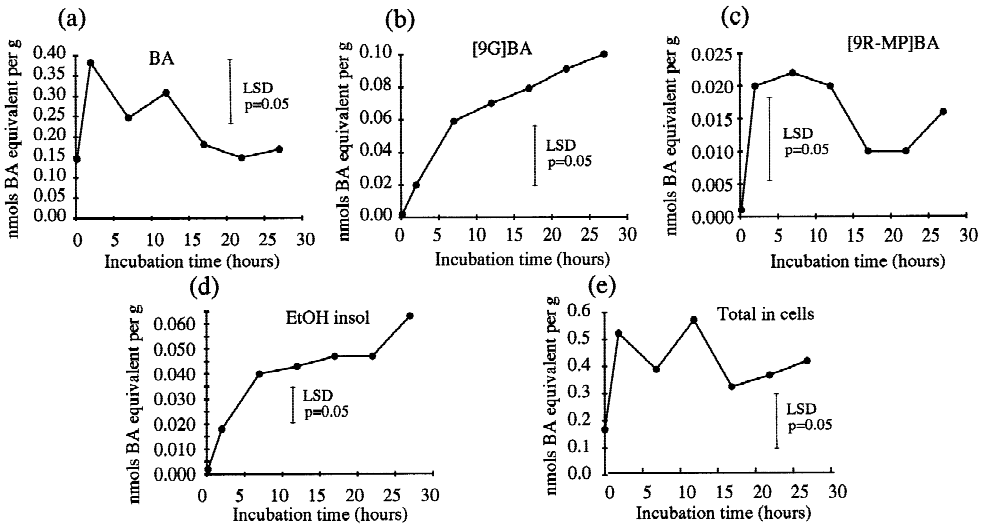

Fig. 2. Time course of 6-benzyladenine metabolism in embryogenic oil palm callus cultures. Cultures were fed with [8-^{14}C]BA (200 GBq·mmol^{-1}) for 2 h, rinsed in sterile water, and transferred to fresh MS medium with 3% sucrose. Each culture (approximately 0.5 g fresh weight) was fed 1.85 MBq in 2 mL of medium, representing a concentration of 0.5 μM BA. The concentrations in cells were calculated as nmol of BA equivalent/g fresh weight. Mean values were averaged over two clones × 2 replicates. The least significant differences ($p < 0.05$) were obtained from analysis of variance. There were no significant differences between clones. *a*, 6-benzyladenine; *b*, 6-benzyladenine 9-glucoside; *c*, 6-benzyladenine ribotide; *d*, ethanol-insoluble fraction; *e*, total radioactivity in cells.

and abnormal clones although there may be a difference in BA transport.

The third experiment was designed to follow the capacity of the cells of the two clones to metabolize BA to its 9-glucoside over a wide range of cytokinin concentrations in a system unable to use cytokinin oxidase as an inactivation mechanism. By dilution of the isotope with unlabeled BA it was possible to supply BA over the range 0.5×10^{-3} M to 0.73×10^{-7} M. The highest concentration attainable was limited by the solubility of BA. The experimental protocol was as before. Samples were taken at time 0 (within 1 min of transfer to isotope), immediately after the uptake period (2 h), and 15 h later. As in the previous experiment, there was a significant difference between clones in BA uptake over the period of the 2-h feed, resulting in different internal concentrations of BA in response to dose.

Fig. 3 shows the ratio of internal to external BA after (*a*) 2 h and (*b*) 15 h later plotted against the initial external BA concentration. Initial uptake was faster in Clone 128 (normal flowering) than in Clone 4 (abnormal flowering). The difference was greatest at the lowest concentration, suggesting that it might become significant at physiological concentrations.

The initial analysis of variance of log.-transformed data (log. transformations were required to equalize the treatment variances over the wide range of BA concentrations used) showed significant dose × clone and time × clone interactions for [9G]BA, but when the rate of [9G]BA synthesis was related to the internal BA concentration the clonal differences disappeared.

Fig. 4 shows the mean rates of [9G]BA synthesis over (*a*) the first 2 h and (*b*) the subsequent 15 h plotted against the mean internal BA concentrations over these periods. In calculating mean rates it was assumed that the changes in BA and [9G]BA were linear with time. Logarithmic axes are used in order to accommodate the range of values. There were no significant differences between clones in their ability to 9-glucosylate the benzyladenine.

Using the initial rate data (0–2 h, Fig. 4*a*) an asymptotic dose-response relationship was found analogous to a first-order reaction with Michaelis-Menten kinetics. Data from the period 2–17 h showed some evidence of product inhibition at the highest BA concentrations.

Attempts to linearize the response using Lineweaver-Burk plots gave negative intercepts; but fitting an asymptotic curve to the equation $y = ax/(b + x)$, where y is the mean rate of synthesis of [9G]BA (time 0 to 2 h) and x is the mean internal BA concentration, produced values for an in vivo equivalent of V_{max} of 3.54 nM·g fresh weight^{-1}·h^{-1} and a value analogous to a K_m of 0.12 mM for both clones (Fig. 5). These values give some indication of the capacity of oil palm cells to inactivate exogenous cytokinins. Entsch et al. (1979) found a K_m value of 1.5×10^{-4} M for the enzyme-glucosylating zeatin.

Following the experiments with BA, in which there was no possibility of degradation by cytokinin oxidase, an experiment was conducted using high specific activity [9R]iP at low dose in order to explore the natural metabolism of cytokinins at physiological concentration.

The protocol used was similar to the previous time course experiment, using embryogenic tissue culture ma-

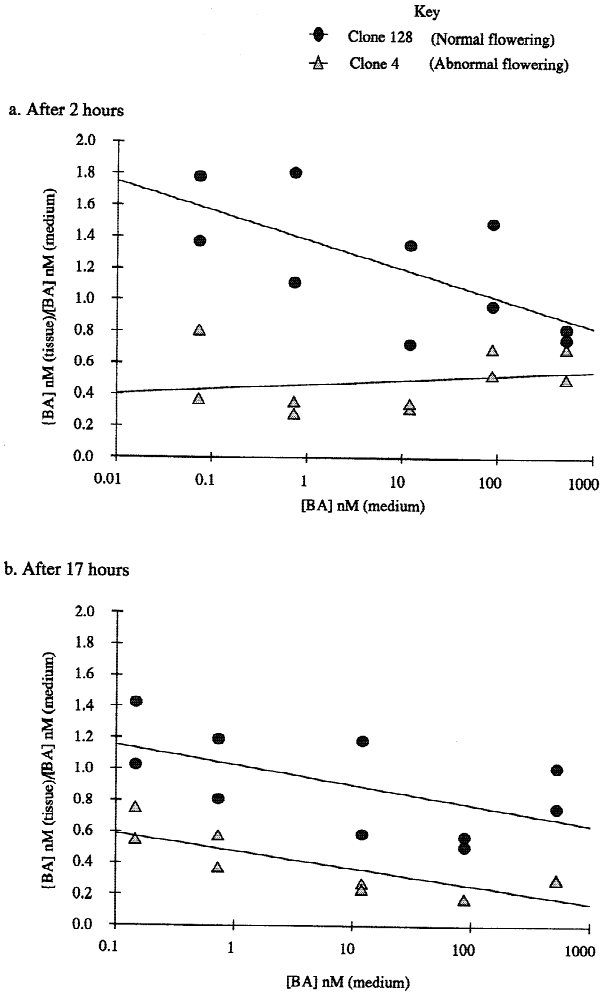

Fig. 3. Ratio of BA in tissues to initial external BA in the medium after (*a*) 2 h and (*b*) 17 h at external concentrations from 10^{-3} to 10^{-7} M, plotted against the original medium concentrations. Clone 128 (normal flowering) is shown by *solid circles;* clone 4 (abnormal flowering) is shown by *triangles.*

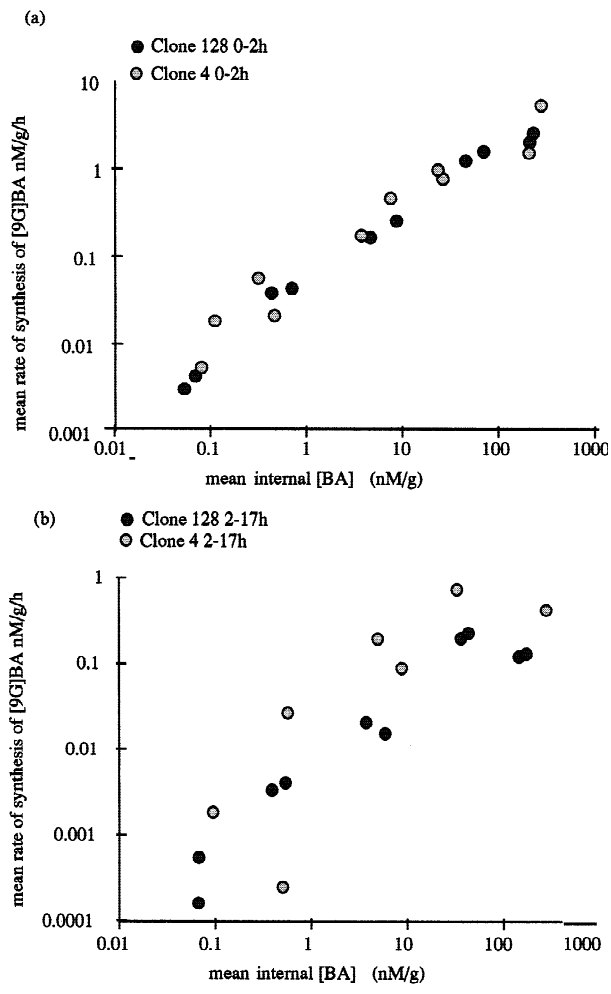

Fig. 4. Relation between mean rate of synthesis of [9G]BA and mean internal BA concentration (log-log plots) (*a*) over the first 2 h and (*b*) the following 15 h. Clone 128 (normal flowering) is shown by *solid circles;* clone 4 (abnormal flowering) is shown by *shaded circles.*

terial of Clones 4 and 128. Approximately 4 g of tissue, representing five culture tubes, was incubated for 2 h in 15 mL of MS liquid medium containing 3% sucrose in 50-mL Sterilin pots. A time 0 sample was taken 1 min after immersion in the radioisotope. At the end of the 2-h period the tissues were rinsed in sterile water and returned to the same volume of fresh MS liquid medium. The pots were sampled at 5-h intervals over the next 25 h. Duplicate samples of approximately 0.25 g fresh weight were taken at each time. Cytokinin (^{3}H-[9R]iP, 1.1 TBq·mmol^{-1}) was applied at 0.26×10^{-9} M.

After the uptake period the radioactivity in the tissues declined rapidly with time, and the HPLC data were very noisy, with a large variability between replicates. After 25 h there was too little radioactivity remaining in the tissues to resolve the individual peaks. Much of the radioactivity appeared in the suspending medium. For this reason the data in Figs. 6 and 7 are shown as the percentage distribution of radioactivity in the ethanol-soluble fraction for the period up to 20 h after the primary incubation. Data were analyzed by ANOVAR, and where significant clone or clone × time interactions occurred this is indicated on the diagrams. Where no significant effects were found the clonal data were pooled and are represented by a *single line.* The *error bars* indicate the least significant difference between means ($p < 0.05$).

The distribution of the radioactivity in the suspending medium after 25 h showed differences between clones. The medium from Clone 4 (abnormal) contained primarily adenine and iP, whereas Clone 128 (normal) medium contained both iP and some residual [9R]iP and approximately equal proportions of adenine and adenosine.

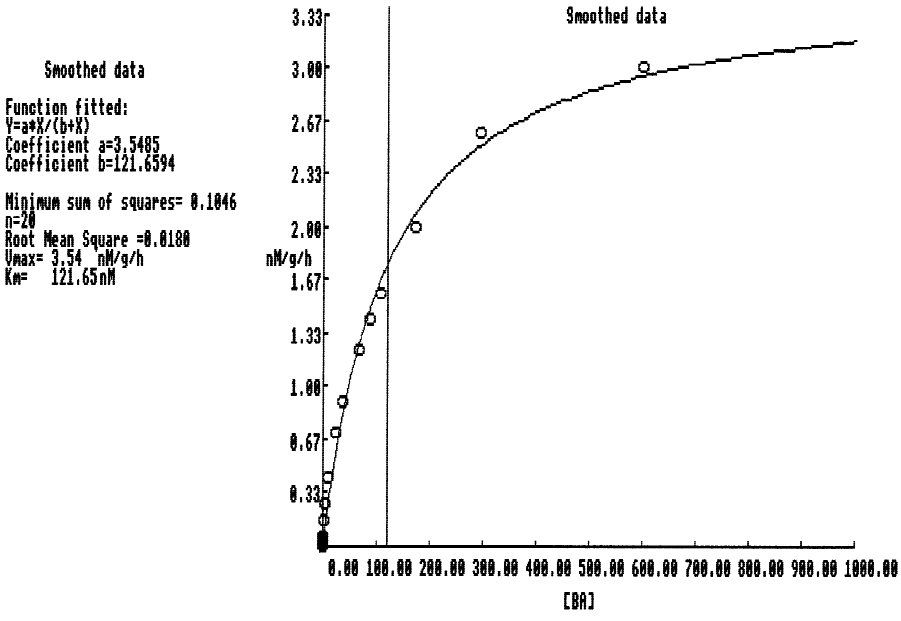

Fig. 5. Asymptotic curve $y = ax/(b + x)$ fitted to smoothed data from log-log transforms in Fig. 4*a*. Coefficient *a* is the asymptote equivalent to V_{max}, and *b* is the concentration of BA at half-maximum rate, equivalent to K_m.

The time 0 samples, which were immersed in the isotope for only 1 min, picked up about 3.3Bq·g^{-1} fresh weight. In Clone 4 about half of the label was still in [9R]iP, but in Clone 128 it was almost entirely in iP.

The time course is illustrated in Figs. 6 and 7. Fig. 6 shows the proportions of label in [9R]iP, iP, adenine, and adenosine. These represent the primary reactions of side chain removal and deribosylation.

The proportion of label in [9R]iP declined rapidly with time. The disappearance was faster in Clone 4 than 128. This difference could be accounted for by the appearance of the transient peak eluting at 22 min. Adenosine and iP were formed rapidly. There were no significant clonal differences, nor did they change significantly with time after the 2-h incubation period, although there was great sample variation.

Glucosylation, putative *trans*-hydroxylation, and other reactions are shown in Fig. 7, which shows the proportions of radioactivity in [9G]iP and in peaks eluting at the retention times of Z and [9R]Z, together with an unknown transient peak eluting at 22 min. [9G]iP appeared during the first 2 h, and although subsequently showing an upward trend with time this was not statistically significant. The other data in Fig. 7 show significant clonal differences and clone × time interactions. The putative zeatin peak (12 min) rose rapidly in the normal clone (Clone 128) to a peak of 15% after 5 h. In Clone 4 the rise was slower and only reached 4% after 15 h. Meanwhile, the putative [9R]Z (retention time 14 min) showed a steady increase in Clone 4, but in Clone 128, after an early rise, it fell to zero by 20 h. The most dramatic difference was in the transient peak eluting from the HPLC at 22 min, which in Clone 4 accounted for more than 40% of the radioactivity after 5 h and then declined rapidly. In Clone 128 there was a slower rise to a 15% peak after 15 h, also followed by a decline. Unfortunately, time did not permit further experiments before termination of the project.

Discussion

The data presented here were accumulated over a period of several years. During this period there has been a widespread assumption that the abnormal flowering induced by some component of the tissue culture process is in some way associated with disturbed cytokinin metabolism, and several attempts were made to establish a connection (see the Introduction). The evidence for this assumption is still tenuous at best.

Two lines of argument were popular. One was that the abnormality was caused by the addition of growth regulators to the culture medium, in particular kinetin and 2,4-D. There is now at least good circumstantial evidence that the addition of kinetin to the medium does increase the probability of some clones showing the abnormality. The interesting evidence for 2,4-D as an inductive agent (Duval et al. 1995) and its interaction with cytokinin concentrations is not considered in this paper.

The second argument was that the abnormality is associated with disturbed cytokinin metabolism. This could be the result of a totally different inductive mechanism not related to exogenous cytokinin application. Measur-

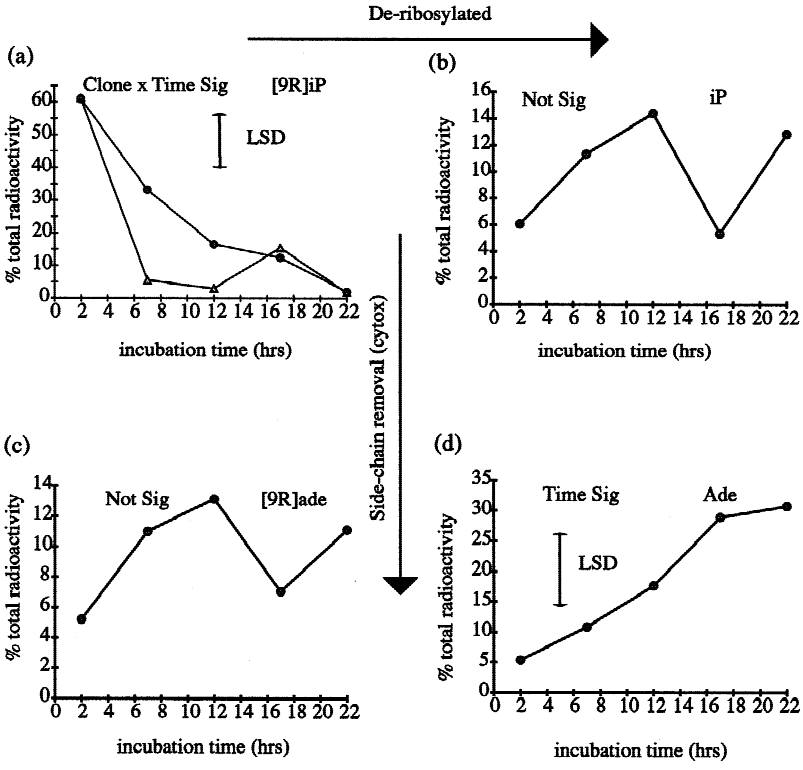

Fig. 6. Time course of metabolism of ^{3}H-[9R]iP by embryoids of two oil palm clones. Cultures were fed with ^{3}H-[9R]iP (1.1 TBq·mM^{-1}) equivalent to 150 fmol of [9R]iP in 40 mL of liquid MS medium with 3% sucrose and incubated on an orbital shaker. After 2 h, cultures were rinsed in sterile medium and transferred to fresh medium. Samples were taken at 5-h intervals up to 20 h after the initial incubation. Data are shown as percentage distribution of the total enthanol-soluble radioactivity at each time point. The data were analyzed by ANOVAR for clone, time, and clone × time interactions ($p < 0.05$). Where no significant clone differences were found data were pooled and shown in a *single line. a,* side chain oxidation and deribosylation of isopentenyl-adenosine; *b,* isopentenyladenine; *c,* adenosine; *d,* adenine.

able changes in cytokinin pool sizes or metabolism may simply be a secondary consequence of whatever primary process is affected.

There has also been a tacit, but totally unwarranted, assumption that if cytokinin (e.g., kinetin) in the culture medium was the causal agent then this would automatically be reflected in disturbed cytokinin metabolism some years and hundreds of cell division cycles after the regenerant plantlets were removed from the medium.

Although results from different laboratories proved inconclusive, and in some instances contradictory, there were some indications of differences in pool sizes of some cytokinins, particularly zeatin riboside, in tissues from normal and abnormal palms and also in tissue cultures giving rise to normal and abnormal palms (Besse et al. 1992, Jones et al. 1995). The consensus was that tissues from normal palms contain higher concentrations of [9R]Z than abnormal tissues and, possibly, lower amounts of [9G]iP.

In a previous paper (Jones et al. 1995) we argued that the observed differences in cytokinin concentrations in tissues from normal and abnormal oil palm clones were smaller than between-clone differences within normal clones and therefore were not directly responsible for the abnormality.

The work described in the present paper was prompted by both lines of approach. Initially it was important to discover how exogenous cytokinin supplied to oil palm tissue cultures was metabolized. It quickly became apparent that added kinetin was quickly converted to the 9-glucoside with few other metabolic products (Jones and Hanke 1992). Analysis of the cytokinin content of callus and embryoids from tissue culture demonstrated that the most abundant natural cytokinins in tissues were the 9-glucosides of iP and Z (Jones 1990). It was only later that the family of naturally occurring aromatic cytokinins was found to be an important component of oil palm tissues (Jones et al. 1996).

The commercial availability of radiolabeled benzyladenine allowed an investigation of the short term metabolism of exogenous cytokinin at a concentration similar to that used in many tissue culture protocols. Although the evidence of identification from retention times in a single solvent system cannot be regarded as secure, the putative compounds identified are the expected short term metabolites of BA and confirm the findings of other workers. Auer et al. (1992), working at a longer time scale (days rather than hours) with *Petunia* leaf explants, found a very similar pattern of metabolism of radiolabeled BA (cf. Fig. 3 in their paper with Fig. 2 here).

These experiments showed that although [9G]BA was formed quickly from the free base there was rapid initial phosphoribosylation. It was also found that in oil palm there was virtually no formation of the 7- or 3-gluco-

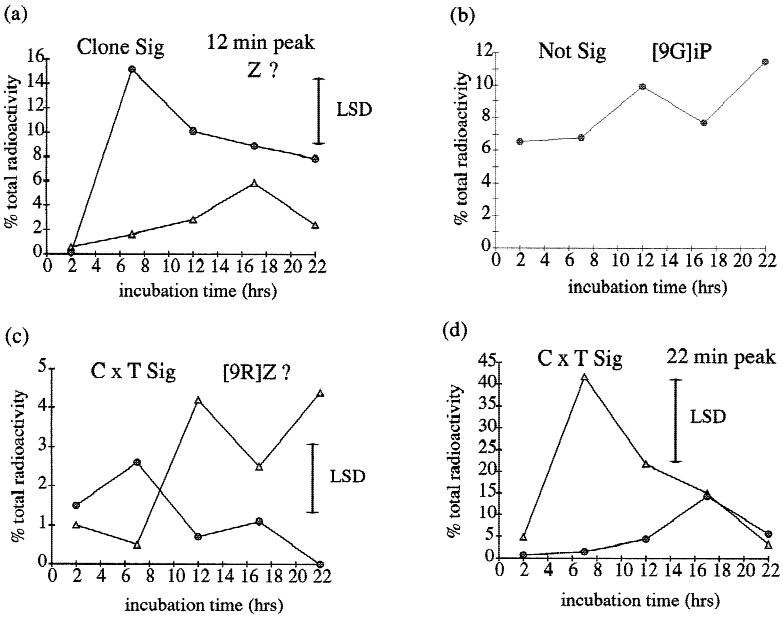

Fig. 7. The procedures are as described for Fig. 6. Shown are the glucosylation; *trans*-hydroxylation, and other reactions of *a*, putative zeatin; *b*, isopentenyladenine 9-glucoside; *c*, putative zeatin riboside; *d*, peak eluting after 22 min. Clone 128 (normal flowering) is shown by *solid circles;* clone 4 (abnormal flowering) is shown by *triangles.*

sides. Because the immunoassays used previously were not able to detect 7- or 3-glucosides this was an important finding. Whereas the enzyme from radish described by Entsch and Letham (1979) and Entsch et al. (1979) produces both 9- and 7-glucosides, this does not occur in all species, and in particular *Rhododendron* and *Musa* produce only 9-glucosides (Blakesley 1991, Blakesley and Constantine 1992). It seems that oil palm is similar to the latter species in this respect.

The two experimental clones showed consistient differences in uptake rates of the cytokinin. Although there was a small concentration difference in the treatment applied to the two clones in the first experiment, the same effect was seen over a very wide range of concentrations (several orders of magnitude) in the dose-rate experiment. The normal tissues had a consistently faster rate of uptake of BA than the abnormal tissues, and this was seen again in the experiment with [9R]iP. In the experiment set up to examine the kinetics of 9-glucosylation, using a wide concentration range (from 10^{-7} to 10^{-3} M), it was found that the different uptake rates were more marked at the lowest concentrations, which approached physiological concentrations. Thus, if the difference is truly associated with the normal/abnormal flowering response rather than a random difference between clones, there may be a difference in cell permeability to, or transport of, cytokinins. Because the ratios of internal to external BA concentrations approached 2:1 in a relatively short time, an active uptake process can be assumed. On the other hand, when the rate of 9-glucosylation was related to the internal BA concentration there was clearly no difference between the clones.

The experiments and observations made so far indicate that 9-glucosylation is an important cytokinin inactivation mechanism in oil palm tissues. Although this can occur rapidly in the presence of free base, the cytokinin ribosides and ribotides must be deribosylated before the glucosylation reaction can occur. One of the main advantages of kinetin and benzyladenine, which are both commonly used in tissue culture, is that they are not subject to degradation by cytokinin oxidase, which is regarded as the major inactivation route in most plants.

A gift of high specific activity isopentenyladenosine from Dr. M. Strnad permitted a more realistic investigation of the role of the 9-glucosylation mechanism in tissues at physiological concentrations of the natural isoprenoid cytokinins. Unfortunately, only one preliminary experiment could be completed before the program ended, but the findings suggest several important avenues for future exploration.

The use of the riboside of iP permitted comparison of the relative importance of different competing pathways. These can include side chain removal by cytokinin oxidase, deribosylation allowing other reactions at N-9, and *trans*-hydroxylation to zeatin or zeatin riboside.

Comparison of the normal and abnormal clonal tissues in this experiment gave evidence of both the overall balance of metabolism of [9R]iP in oil palm tissues and an unexpected clonal difference.

Both side chain removal and deribosylation occurred rapidly, with the appearance of radiolabeled peaks at the retention times of adenine and adenosine. There was some evidence of *trans*-hydroxylation, because labeled compounds were formed with retention times similar to

those of Z and [9R]Z. As in the BA experiments, the uptake rate was faster in the normal than the abnormal clone. A relatively small proportion of the [9R]iP was converted to [9G]iP. The prevalence of this compound in oil palm tissues possibly results from a long term low rate of accumulation of excess cytokinin.

In summary, in both the BA and iP feeding experiments there appeared to be differences between clones in uptake rates, suggesting a difference in cytokinin transport. The steady increase in a compound at the retention time of adenine in the cultures fed with ^{3}H-[9R]iP demonstrates that both deribosylation and side chain removal were major metabolic routes. The clonal differences were focused on a possible indication of slower deribosylation in the abnormal clone, which was particularly noticeable in the differences in the kinetics of putative Z and [9R]Z formation, and in the initial appearance of iP in the earliest sampling (time 0 sample). The accumulation of label in what is assumed to be [9R]Z in the abnormal cell line is at variance with previous analytical estimates of the size of the [9R]Z pool and may indicate that the assumption of the identity of this peak as [9R]Z is insecure. Although the accumulation of cytokinin 9-glucosides is a feature of all oil palm tissues, it appears to have no connection with the development of the flowering abnormality. The most obvious difference was in the rapid appearance of an unidentified transient compound eluting from the HPLC at 22 min in the abnormal line. In any further study of cytokinin metabolism in normal and abnormal oil palm cell lines it would be important to confirm this finding, to identify the compound concerned, and to discover its fate.

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