Plant Physiol. (1992) 98, 1035–1041
0032-0889/92/98/1035/07/$01.00/0

Received for publication May 2, 1991
Accepted October 21, 1991

Comparison of Benzyl Adenine Metabolism in Two *Petunia hybrida* Lines Differing in Shoot Organogenesis[1]

Carol A. Auer*, Jerry D. Cohen, Michel Laloue, and Todd J. Cooke

Department of Botany, University of Maryland, College Park, Maryland 20742 (C.A.A., T.J.C.); Plant Hormone Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705 (J.D.C.); and Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, Route de Saint-Cyr, F 78026 Versailles Cedex France (M.L.)

ABSTRACT

The uptake and metabolism of the cytokinin benzyl adenine (BA) was compared in two lines of *Petunia hybrida* Vilm. differing in their shoot organogenic response. Leaf transfer experiments using shoot induction medium containing 4.4 micromolar BA showed that leaf explants from petunia line St40 required a shoot induction period of 6 to 10 days for commitment to shoot organogenesis; whereas leaf explants from petunia TLV1 required 12 to 28 days. The short induction period of petunia St40 and the higher organogenic response was positively associated with a threefold higher absorption of BA from the medium, an increased BA ribotide metabolite pool, the presence of BA within the explant during the shoot induction period, and the production of an unidentified metabolite C. However, the study of petunia TLV1 leaf explants showed that neither BA nor metabolite C are required during the shoot induction period for eventual shoot development. The longer shoot induction period of TLV1 was associated with low BA uptake during 24 days, a decreasing ribotide metabolite pool, the absence of benzyl adenosine triphosphate and metabolite C throughout the study, and the absence of BA within the explant during the shoot induction period. Differences in the shoot organogenic response of these related plant lines have been shown to be associated with differences in exogenous cytokinin uptake and the subsequent metabolism of that hormone.

Many tissue culture experiments have attempted to examine the relationship between exogenous plant hormones in the medium and subsequent plant growth and development (9, 20, 21). The developmental response of explants to exogenous hormones is the result of a variety of biochemical processes including hormone uptake, transport, and metabolism (10). Thus, although explant growth is typically described in relation to the hormone concentration in the medium, it is important to realize that the hormone concentration does not necessarily reflect the level of active hormone within the explant (3, 24). Unfortunately, these biochemical processes are rarely investigated with respect to their role in controlling plant development.

For petunia leaf explants, the exogenous cytokinin BA[2] can control the commitment of leaf explants to produce shoots in tissue culture. Understanding the action of cytokinin requires that the actual concentration of active molecules within the tissue be characterized throughout the developmental process (9, 24). The major conjugates of BA and other cytokinins have been characterized in a few plant tissues, and their activities differ significantly (12–15). Whereas the BA glucosides are generally considered to be inactive, the ribosides (BAR), ribotides, and free base may be involved in the regulation of cell division or other developmental processes (12, 13, 16). One approach to obtaining information about the effects of exogenous hormones on organogenesis is to compare plants with altered organogenic responses in tissue culture. Genetic mutants have already been used to elucidate hormone biosynthetic pathways and to study other aspects of plant hormone biochemistry (11). However, the literature contains few studies of hormonal control of shoot organogenesis using selected mutants or variant lines. Petunias provide a convenient system for studying shoot organogenesis because they can be rapidly and reliably induced to produce shoots with exogenous cytokinins, a genetic map with markers has been constructed, and the plant is amenable to molecular analysis (19, 21).

In this study, we used two *Petunia hybrida* lines, St40 and TLV1, which differed in their shoot regeneration responses, to evaluate BA uptake and metabolism in relation to shoot organogenesis. The objectives were to: (a) determine the timing of shoot induction in each line, (b) compare the uptake of [^{3}H]BA during shoot induction and development, and (c) identify and compare the BA metabolites throughout the process of shoot organogenesis. The results showed that petunia lines St40 and TLV1 had markedly different shoot induction periods and that BA uptake differed appreciably between the lines. Distinct differences in the presence of BA and its metabolites during commitment to organogenesis were also observed between the two lines. These results are discussed with respect to the active form of BA and the Christianson and Warnick model (8) of shoot organogenesis.

[1] This research was supported in part by DCB-8917378 to J.D.C. and U.S. Department of Agriculture-Competitive Research Grants Office grant No. 89-37261-4791 to T.J.C. Scientific article No. A6174, Contribution No. 8343, of the Maryland Agricultural Experiment Station, College Park, MD.

[2] Abbreviations: BA, benzyl adenine; BAR, benzyl adenosine; BA-7G, benzyl adenine-7-β-D-glucopyranoside; BA-9G, benzyl adenine-9-β-D-glucopyranoside; BAMP, benzyl adenosine-5′-monophosphate; BADP, benzyl adenosine-5′-diphosphate; BATP, benzyl adenosine-5′-triphosphate.

MATERIALS AND METHODS

Shoot Induction Experiments

Leaves of *Petunia hybrida* Vilm. lines St40 and TLV1 were used in all experiments. These lines were obtained from a collection located in Dijon, France, and were selected based on the presence of complementary genetic markers on all seven chromosomes and on differences in tissue culture regeneration (H. Dulieu, personal communication). The plants were grown in soilless medium under cool-white fluorescent lights at 150 $\mu mol/m^2 \cdot s^{-1}$ with a 16 h light/8 h dark photoperiod. Leaf explants in tissue culture were grown under the same light conditions. To obtain leaf explants, fully mature leaves were surface sterilized with 0.53% NaOCl for 20 min and then rinsed three times in sterile distilled water. Explants (1 cm^2) containing the midrib were cut from the leaves. Preliminary experiments indicated that explants of this size represented the minimum size capable of shoot organogenesis. Each explant was placed on 10 mL of medium in a 60 × 15 mm Petri dish. The basal culture medium consisted of Murashige-Skoog salts (17) supplemented with 1 mg/L nicotinic acid, 1 mg/L pyridoxine, 10 mg/L thiamine, and 30 g/L sucrose and solidified with 7.5 g/L Difco bacto-agar[3] at pH 5.8 (2). All chemicals were purchased from Sigma unless otherwise noted.

To determine the timing of shoot induction, leaf explants from both lines were placed directly on basal medium without hormones or transferred from the inductive medium containing 4.4 μM BA (1 mg/L) to the basal medium. Following preliminary experiments to determine the induction periods for both lines, leaf explants of St40 were transferred from inductive to basal medium on days 4, 6, 8, 10, and 28; TLV1 leaf explants were transferred on days 8, 10, 12, 20, 24, and 28. In an additional experiment, TLV1 explants were exposed to 44 μM BA (10 mg/L) for 6, 10, 12, or 28 d. The number of shoots and their spatial distribution were recorded after 28 d. Each experimental treatment consisted of 15 replicate Petri dishes with one explant per dish.

BA Uptake and Metabolism Experiments

BA uptake and metabolism were determined by exposing leaf explants of St40 and TLV1 to [^{3}H]BA for 6, 10, 12, or 24 d. The [^{3}H]BA (labeled on the benzyl ring, specific activity 740 MBq μmol^{-1}; CEA, Gif-sur-Yvette, France) had a final specific activity of 21.4 MBq μmol^{-1} and when combined with cold BA provided a final concentration of 4.4 μM BA in the medium. Each explant was placed on 10 mL medium in a 60 × 15 mm Petri dish. Each treatment consisted of two explants which were weighed after BA exposure. Measurements of BA uptake and explant fresh weight were performed in three separate experiments.

BA uptake by the explants was measured during the initial extraction procedure (see details below) by counting the radioactivity in small aliquots of the supernatant and the pellet, solubilized in 5 mL Ready-Solv HP liquid scintillation fluid.

Observed differences in BA uptake led to an examination of the abaxial leaf explant surface. Using a light microscope attached to a camera viewer, we recorded the number of glandular hairs/mm^2 of leaf surface, the number of hairs/mm of midrib length, and the length of 10 glandular hairs for 10 leaf explants from each line. Using free-hand cross-sections, we recorded the thickness of leaf explants for 10 explants from each line.

BA Metabolite Extraction and Analysis

Two leaf explants of St40 or TLV1 were removed from the medium containing [^{3}H]BA after 6, 10, 12, or 24 d and rinsed in 10 mL distilled water, blotted to remove surface water, ground in liquid N_2, and extracted overnight in 20% perchloric acid. The BA metabolites were separated using a Waters HPLC system in conjunction with a Waters model 440 UV absorbance detector (254 nm), Beckman 171 radioactivity detector, Gilson model 201 fraction collector, and Beckman Chromatographics software. The extract was first fractionated on a 25-cm Partisil 10 SAX column (Whatman) to separate ribotide metabolites, using a 38-min linear gradient of 20% methanol to 0.55 M [K_2HPO_4 + KH_2PO_4] in 20% methanol at pH 5.4 with a flow rate of 1.2 mL/min. Unretained metabolites were injected onto a 15-cm LiChrospher 60 RP-Select B column (E. Merck) to separate base, riboside, and glucoside forms. A solvent gradient of 10 mM triethylamine in 10% methanol at pH 5.4 to 100% methanol with a flow rate of 0.8 mL/min during 27 min was used. Cold standards of BA, BAR, BAMP, ADP, ATP, BA-7G, BA-9G, BA-3-β-D-glucopyranoside, and adenosine were used to establish retention times (BA-7G, BA-9G, and BA-3-β-D-glucopyranoside were purchased from Apex Organics, Leicester, UK). Metabolites were quantified by multiplying the percentage of counts per metabolite peak by the total counts (dpm) in the extract and then using the specific activity to convert from dpm to picomoles. Radioactive metabolite fractions coeluting with standards were collected for further analysis. The limit of detection was approximately 0.7 pmol.

To provide additional evidence for the identity of the radioactive metabolite fractions, acid or enzymatic hydrolysis treatments were applied. Acid hydrolysis was performed on radioactive metabolite fractions which coeluted with cold standards of BAR, BA-7G, and BA-9G. The unidentified metabolite C also received acid hydrolysis treatment. Fractions containing approximately 5000 dpm of these radioactive metabolites were combined with 1 mL 1 N HCl. Hydrolysis occurred for 3 h at 100°C. After hydrolysis, the samples were evaporated *in vacuo* and resuspended in 1 mL H_2O. Evaporation and resuspension were repeated until the pH was 5.5. Samples were resuspended in 10% methanol, cold standards of 1 μg BA, BAR, BA-7G, and BA-9G were added, and the sample was analyzed by reverse phase HPLC as described above.

An enzymatic alkaline phosphatase treatment was performed on radioactive metabolite fractions coeluting with cold standards of BAMP, ADP, and ATP. Metabolite fractions containing about 5000 dpm were desalted by evapora-

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that might be suitable.

ting the sample, resuspending the sample in 1 mL 50% ethanol, freezing the sample for 15 min, followed by centrifuging and collecting of the supernatant. Fractions were then combined with 200 μL 0.5 M Tris HCl + 5 mM $MgCl_2$, 1800 μL H_2O, and 2 units alkaline phosphatase. The samples were held at 37°C for 3 h, after which 4 mL ethanol was added to stop the reaction. Enzymes were precipitated by freezing the samples for 30 min and then centrifuging. Supernatants were evaporated *in vacuo*, resuspended in 10% methanol, and injected onto a LiChrospher 60 RP-Select B HPLC column. Radioactive and UV absorbance peaks were recorded as described above. An enzymatic 5′-nucleotidase treatment was performed on the radioactive metabolite fraction coeluting with the standard BAMP. The procedure was the same as described for the alkaline phosphatase treatment except that 2 units of 5′-nucleotidase was used. The analysis of metabolites was repeated in two separate experiments with virtually identical results. Data presented in the figures represent an average of the two experiments.

RESULTS

Shoot Induction and BA Uptake

Leaf explants from petunia St40 and TLV1 were transferred from inductive medium containing 4.4 μM BA to basal medium to define the critical period of shoot induction (Fig. 1). St40 leaf explants exposed to basal medium for 28 d or inductive medium with 4.4 μM BA for 4 d did not produce shoots; whereas, the exposure to inductive medium for 6, 8, or 10 d increased the percentage of explants producing shoots from 27 to 60 to 100%, respectively. In addition, increased duration of exposure to BA produced an increased number of shoots per explant from 0.67 shoots per explant for the 6-d exposure to 18.7 shoots per explant for the 28-d exposure. In contrast, leaf explants of TLV1 required a much longer exposure of 12 d on BA for shoot induction (Fig. 1). Exposure to basal medium only or inductive medium with 4.4 μM BA for 8 or 10 d produced no shoots. BA exposure for 12, 16, 20, 24, or 28 d increased the percentage of TLV1 explants producing shoots from 7 to 27, 40, 73, and 75%, respectively. The number of shoots per TLV1 explant also increased from 0.07 shoots per explant to 18.5 shoots per explant as the duration of exposure increased from 12 to 24 d. After 28 d exposure, only 75% of the TLV1 explants produced shoots; whereas 100% response was possible on St40 explants with only 10 d exposure to BA.

Table I. *Percentage of Total BA Taken Up by Leaf Explants of Petunia St40 and TLV1 during Shoot Organogenesis*

Leaf explants were placed individually on 10 mL Murashige-Skoog medium containing [H^3]BA and cold BA for a final concentration of 4.4 μM BA. The explants were removed after 6, 10, 12, or 24 d exposure, and the percentage of BA in the explant from the total BA available in the medium was recorded. Values shown are the means of three replicates ± SE.

Petunia Line	% Uptake after Exposure to BA			
	6 d	10 d	12 d	24 d
St40	4.0 ± 1.6	11.7 ± 1.6	21.5 ± 1.0	53.5 ± 1.5
TLV1	2.6 ± 0.8	7.8 ± 0.7	7.7 ± 0.7	14.0 ± 1.0

To try to obtain a greater response from TLV1 explants, TLV1 explants were exposed to 44 μM BA for 6, 10, 12, or 28 d. With this BA concentration, none of the explants produced shoots.

To evaluate differences in BA absorption by the leaf explants during shoot induction, St40 and TLV1 leaf explants were placed on medium containing [^{3}H]BA, and the uptake of BA was measured after 6, 10, 12, or 24 d (Table I). For St40, the percentage of available BA absorbed increased from 4 to 53.5% from days 6 to 24. For TLV1, the increase during the same period was 2.6 to 14%. Because the cytokinin-free base and riboside are thought to diffuse freely across cell membranes (13, 14), the differences in diffusion rate could be caused by increased diffusive distance, altered concentration gradient, or altered surface properties. Physical characteristics of the leaf explant surfaces were examined to determine whether surface characteristics might explain the difference

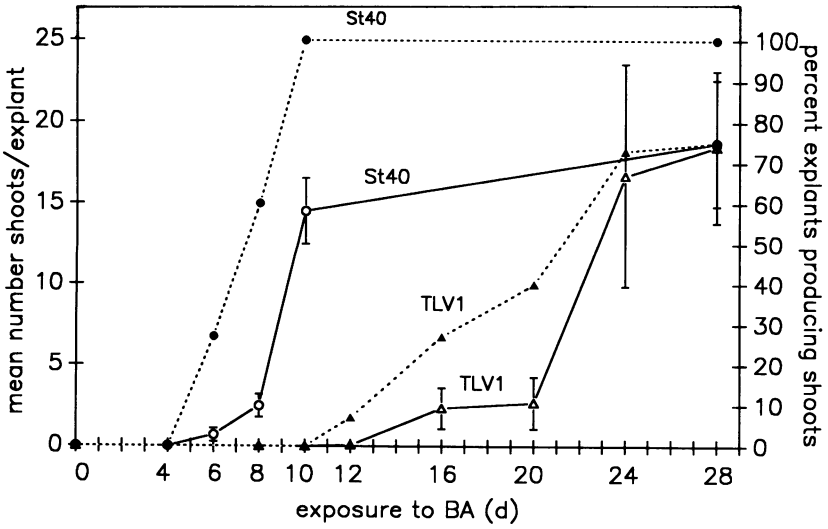

Figure 1. Petunia leaf explants from lines St40 and TLV1 were transferred from inductive medium containing 4.4 μM BA to basal medium. St40 leaf explants were transferred after 4, 6, 8, or 10 d exposure; whereas TLV1 explants were transferred after 8, 10, 12, 20, or 24 d exposure to BA. Explants from both lines were also exposed to inductive medium for 28 d without transfer to basal medium. The number of shoots per explant and the percentage of explants producing shoots were recorded after 28 d. ——, Mean number of shoots per explant; · · · · ·, percentage of explants producing shoots. Points, Means of 15 replicates (bars, ± SE).

Table II. *Structural Differences in Leaf Explants from Petunia St40 and TLV1*

Structural aspects of leaf explants from petunia St40 and TLV1 were observed using a light microscope. Measurements were made on 10 leaf explants from each line and the values represent the means ± SE. Letters represent significant differences at P = 1%.

	Petunia Lines	
	St40	TLV1
Hair density (No./mm^2)	14.4 ± 1.3a	4.0 ± 0.3b
Midrib hairs (No./mm)	36.0 ± 2.4a	11.5 ± 0.7b
Hair length (μm)	297.4 ± 9.2a	304.4 ± 5.0a
Leaf thickness (μm)	323.8 ± 13.0a	385.6 ± 14.6b

in BA uptake (Table II). The abaxial surface of St40 explants had 14.4 hairs/mm^2 of surface area, whereas TLV1 had only 4.0 hairs/mm^2. Likewise, St40 had more hairs per millimeter of midrib length. Glandular hair length was not significantly different in the two lines. The leaf explants of TLV1 were slightly thicker than those of St40. Thus, the increased number of hairs on the abaxial surface of St40 may provide an increased surface area and thereby increase BA uptake.

The fresh weight of leaf explants showed no significant differences between the two lines up to day 12 (Table III). However, between days 12 and 24, St40 leaf explants showed a much larger increase in fresh weight compared with TLV1, resulting in a final fresh weight of 0.6 g per explant in St40 as opposed to 0.14 g per explant in TLV1. This stage was accompanied by greater uptake of BA into St40 leaf explants than TLV1 explants.

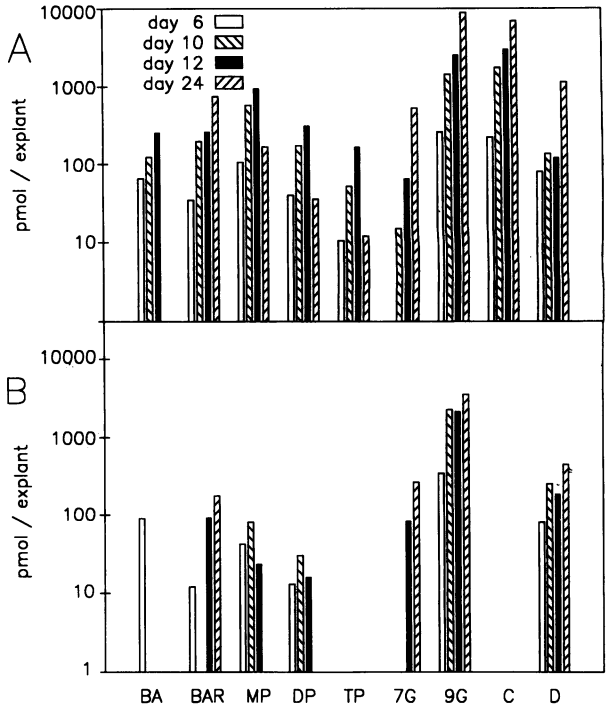

Figure 2. Changes in BA metabolites in petunia St40 and TLV1 leaf explants expressed as picomoles per explant. BA metabolites were extracted after 6, 10, 12, or 24 d exposure to [^{3}H]BA. A, BA metabolites in petunia line St40; B, BA metabolites in petunia line TLV1. Abbreviations: MP, BAMP; DP, BADP; TP, BATP; 7G, BA-7G; 9G, BA-9G; C, unidentified BA conjugate; D, unidentified metabolite containing tritiated BA side chain.

Identification of BA Metabolites

St40 and TLV1 petunia leaf explants exposed to [H^3]BA produced the metabolites BA, BAR, BA-7G, BA-9G, BAMP, BADP, and an unidentified metabolite D (1). A second unidentified metabolite C and BATP accumulated in St40 but were completely absent from TLV1. Additional evidence for the identification of these BA metabolites was obtained through acid hydrolysis of radioactive HPLC fractions containing BAR, BA-7G, BA-9G, and C and alkaline phosphatase treatment of fractions containing BAMP, BADP, and BATP that had coeluted with cold standards. In each of the treatments described, the results were consistent with the initial identification of the metabolite based on HPLC retention times and coelution with standards.

The identity of metabolites C and D was investigated. Compound D, which was detected in both lines and had a retention time of 11.4 min on the LiChrospher reverse phase column, was tentatively identified as a catabolic product containing the [^{3}H]benzyl group after side chain cleavage of BA (M. Laloue, unpublished observation). Metabolite C was identified in petunia line St40 only and had a retention time of 18.9 min on the reverse phase column. A fraction containing C was subjected to acid hydrolysis and the resulting product coeluted on reverse phase HPLC with BA standard, thereby demonstrating that the metabolite was a conjugate of BA.

Table III. *Fresh Weight of Leaf Explants of Petunia St40 and TLV1 during Shoot Organogenesis*

Leaf explants were placed individually on 10 mL Murashige-Skoog medium containing 4.4 μM BA. The explants were removed after 6, 10, 12, or 24 d exposure and fresh weight was recorded. Values shown are the means of three replicates ± SE.

Petunia Line	Fresh Wt after Exposure to BA			
	6 d	10 d	12 d	24 d
	g			
St40	0.07 ± 0.01	0.1 ± 0.01	0.13 ± 0.01	0.6 ± 0.01
TLV1	0.08 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.14 ± 0.01

BA Metabolism during Shoot Organogenesis

A comparative study of BA metabolism in St40 and TLV1 was conducted during the respective shoot induction periods (Fig. 2). BA increased in St40 from day 0 to day 12 but was not detected on day 24. In contrast, BA was detected only on day 6 in TLV1. BAR was present in increasing concentrations in St40 from day 0 to day 24. In TLV1, the concentration increased during the same time except for day 10 when no BAR was detectable. The pool of ribotide metabolites showed conspicuous differences in the two lines. BAMP, BADP, and

BATP increased in St40 from day 0 through day 12 and decreased from day 12 to day 24. In contrast, the ribotide pool was much reduced in TLV1, with BAMP and BADP increasing until day 10, decreasing on day 12, and becoming undetectable on day 24. In TLV1, no BATP was detectable throughout the study. Another example of metabolic differences between the two lines was shown in the unidentified metabolite C which accumulated significantly throughout the experiment in St40 but was never detected in TLV1. The glucoside forms, BA-7G and BA-9G, increased throughout the experiment in both lines, with the concentration of BA-9G greatly exceeding that of BA-7G at each time point. These results represent the average of two experiments with the percentage of deviation from the mean value <12%.

To make comparisons in BA metabolism without the effect of uptake differences, it is necessary to compare metabolites based on their percentage of total concentration of metabolites (Fig. 3). In St40, the percentage of BA increased until day 6, decreased from day 6 to day 12, and was not detectable at day 24; whereas BAR remained relatively constant. BA disappeared from TLV1 explants after day 6, whereas BAR remained at a relatively constant small percentage of the total metabolites. The ribotide pool remained relatively constant in St40 until day 12, making up about 18% of the metabolites. TLV1 was dissimilar with the ribotide pool decreasing from approximately 8% on day 6 to 1.5% on day 12. In addition, no BADP was detectable at day 12, and no BATP was evident at any time. In TLV1, BA-9G is the predominant metabolite, increasing from 59.3% at day 6 to 79.8% at day 24. In St40, BA-9G increased from 31.8 to 47.9%, and C made up 27.2 to 37.7% from days 6 to 24. It is possible that the metabolic pathway that produces C competes with the pathway for BA-9G in St40. BA-7G was not detectable on day 6 in either line but increased to 2.9% in St40 and 6% in TLV1 by day 24.

DISCUSSION

Shoot Induction and BA Uptake

Explant transfer experiments are often used to determine the critical time in which exogenous hormones mediate the commitment of explants to produce organs (7, 22, 24). The model of Christianson and Warnick (6, 8) defined the induction phase as the time during which the hormone concentration in the medium controls the developmental commitment to organ initiation. Our leaf explant transfer experiments demonstrated a marked difference between St40 and TLV1 in the induction period or duration of BA exposure required for commitment to shoot organogenesis (Fig. 1). St40 became

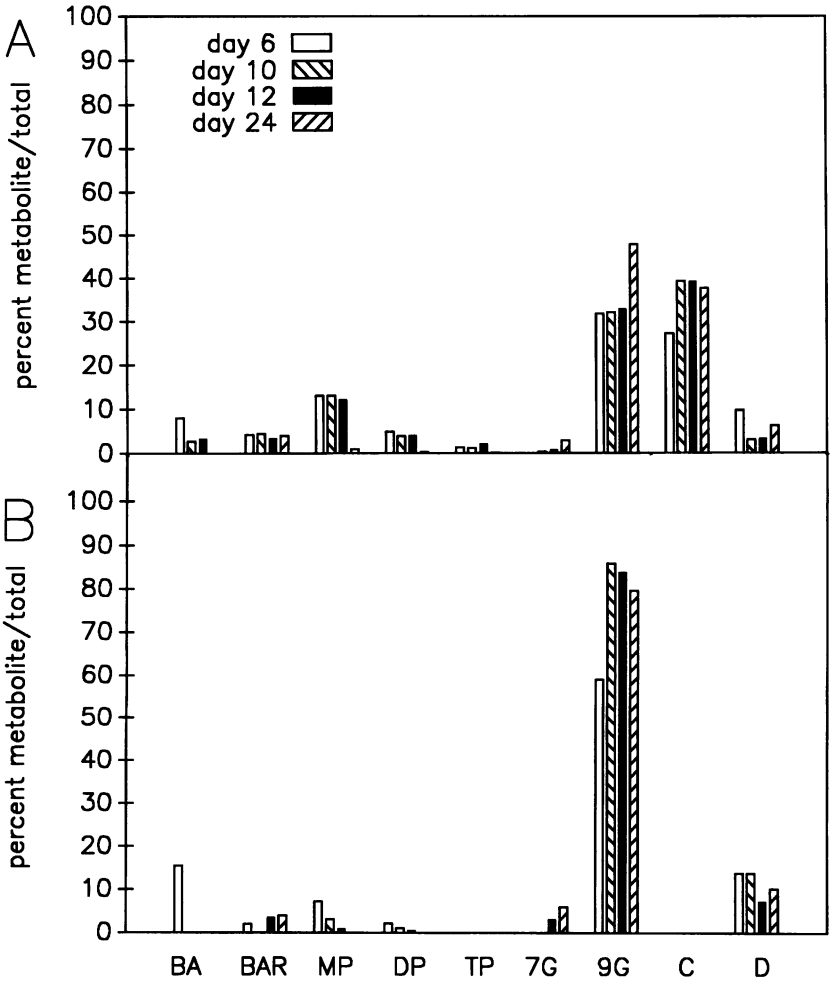

Figure 3. Changes in BA metabolites in petunia St40 and TLV1 leaf explants expressed as a percentage of the total metabolites extracted. BA metabolites were extracted after 6, 10, 12, or 24 d exposure to [^{3}H]BA. A, Petunia line St40; B, petunia line TLV1. For abbreviations, see Figure 2.

committed to shoot development between 6 and 10 d of exposure to 4.4 μM BA with 100% of the explants responding after 10 d exposure. TLV1 explants showed a low level of shoot induction after 12 d exposure (7%), and even 28 d exposure induced commitment to produce shoots in only 75% of the explants.

The study of BA absorption from the medium revealed very significant differences between the two lines (Table I). In St40, BA taken up by the explant increased from 4% of the total BA in the medium to 53.3% between days 6 and 24. This was in obvious contrast to TLV1 in which the BA taken up increased from 2.6 to 14% during the same period. The increase in fresh weight in the two lines was comparable until day 12 when St40 explants began a rapid increase in weight until day 24; TLV1 explants did not increase in fresh weight from days 12 to 24 (Table III). Skvirsky *et al.* (21) also reported equal weight increases until day 13 with petunia explants differing in organogenic response.

It was initially expected that uptake of BA due to diffusion would be the same in both lines; therefore, the differences were surprising. BA and BAR have been shown to easily diffuse through tobacco suspension culture cells, whereas metabolites such as ribotides and glucosides do not move out of the cells (13). In theory, passive diffusion could be affected by differences in BA metabolism or structural features of the abaxial leaf surface. For instance, it follows from Fick's first law of diffusion that high internal concentrations of BA would slow net uptake of exogenous BA into leaf explants by reducing the gradient from the medium to the explant. However, because the concentration of internal BA is greater in St40 than in TLV1 at days 6, 10, and 12, the greater uptake rate in St40 cannot be attributed to an enhanced rate of conversion to nondiffusible metabolites.

Physical characteristics of the leaf explant surfaces were examined to determine structural differences that might affect uptake. St40 leaf explants had approximately three times more abaxial surface and midrib glandular hairs than TLV1 (Table II). Increased hair density in St40 is equivalent to increased surface area, and it is possible that this allows increased diffusion of BA into the explants.

Despite the obvious differences in uptake between the two lines, it is important to note that the delayed induction of TLV1 cannot be attributed to its slow rate of BA uptake *per se*. For example, St40 explants had accumulated only 4% of the available BA in the medium by day 6 at the start of its induction period, whereas TLV1 had accumulated 7.7% of the available BA on day 12 at the start of its inductive period (Table I). Although these experiments do not provide insight into the reason for the delayed induction period of TLV1, they do highlight the possibility that differences in organogenesis in related plants may be affected by differences in cytokinin uptake and that total cytokinin absorption from the medium does not necessarily correlate with the commitment to produce shoots as defined by leaf transfer experiments. It is also possible that differences in endogenous cytokinins contribute to the observed patterns of shoot organogenesis.

Inherent limitations are associated with the use of a leaf explant system in the study of shoot organogenesis. For example, although the shoot organogenic response requires that the petunia leaf explant be of a certain critical minimum size so that one must examine the whole explant to fully understand the organogenic process, not all of the leaf tissue ultimately expresses its organogenic potential (1). Second, it is not possible to predict which cells within the explant will eventually produce shoots. Nevertheless, our results show that characteristics of the entire explant such as glandular hair density and metabolic activity may play roles in the organogenic response. Despite these limitations, leaf explants provide a reliable and easily manipulated model system for studying shoot organogenesis.

BA Metabolism

The *P. hybrida* lines St40 and TLV1 showed marked differences in the duration and timing of shoot induction and organogenic response. Therefore, these lines presented a useful system for investigating the importance of BA and its metabolites in the commitment to shoot development. Free BA was detectable in St40 leaf explants during the time of shoot induction (days 6–10) and could be the active cytokinin. However, the metabolites BAR and the ribotides were also present and cannot be eliminated as active cytokinins.

TLV1 presented an interesting contrast because BA was not detectable within the explant during the shoot induction period (days 12–24). The metabolites present at days 12 and 24 included BAR, BA-7G, and BA-9G. BAMP and BADP were detectable at day 12 but not day 24. Based on previous research, it is unlikely that the glucoside forms are active cytokinins in most systems (12–15, 23). Therefore, BAR or the ribotides are more likely candidates to be active cytokinins in shoot organogenesis of TLV1 and St40. Alternatively, these compounds may provide a pool from which BA can be derived in very low amounts through interconversion (4, 5, 14, 16). Certainly, determining the active form of BA continues to be one of the most significant unsolved problems in cytokinin metabolism (15).

The accumulation pattern of glucosylated BA metabolites in St40 and TLV1 differed from that observed in previous work with petunia (1). In the petunia line MD1, large concentrations of BA-7G accumulated between days 1 and 10 (1). In contrast, BA-7G was detected in small concentrations on days 10 to 24 in St40 and days 12 to 24 in TLV1, with the concentration of BA-9G greatly exceeding that of BA-7G. It would appear that the accumulation of glucosylated BA metabolites differs in the petunia lines studied.

Interestingly, compound C has not been previously described as a major metabolite in other plant tissues. Although this compound was detected in previous shoot organogenesis studies with *Petunia* MD1, it cannot be considered as required for organogenesis because TLV1 is capable of shoot development without production of this metabolite (1). However, it is associated with a shorter time period for shoot induction and a higher percentage of explant response in two petunia lines studied.

Shoot Organogenesis Models

Even though the competence and induction model as described by Christianson and Warnick (7, 8) is a useful working hypothesis for describing the timing of hormone activity in

organogenesis, the model itself has not been subjected to rigorous testing. Determination of key developmental periods is dependent upon the correlation of an exogenous hormone concentration to the active hormone concentration within the explant. The results of this research into the process of BA uptake and metabolism during shoot organogenesis present information that is difficult to interpret within the framework of the Christianson and Warnick model. First, the model assumes that the explants absorb the exogenous hormone in a well-defined manner. Our results clearly showed that petunia lines can differ significantly in total absorption of BA by leaf explants. Second, the model correlates the active cytokinin in the medium to the level of active hormone present in the explant during the induction period. However, this does not take into account the rapid and dramatic metabolic conversion of cytokinins into forms with different activity levels. For example, the petunia line TLV1 had no detectable free BA within the explant during induction, although large concentrations existed in the medium. Third, the model does not account for the delayed activity of cytokinins that have accumulated within the explant. For example, cytokinin effects can be observed in tobacco pith cell division when exposure has occurred for 1 d followed by transfer to medium with auxin alone for 20 d (18). The results of our experiments using petunia leaf explants suggest that the Christianson and Warnick model is distinctly limited in its ability to describe exogenous hormone control over shoot organogenesis because it is clear that cytokinin uptake and metabolism can be significantly different even between related plant lines.

In conclusion, the short induction period of petunia St40 and high organogenic response is positively associated with higher levels of BA uptake, an increased ribotide pool, the presence of BA during induction, and the production of metabolite C. However, the study of TLV1 showed that neither BA nor metabolite C are required during the induction phase for eventual shoot development. The relatively long induction period of TLV1 is associated with low BA uptake during 24 d, a decreasing ribotide pool, the absence of BATP and metabolite C, and the absence of BA during induction. Therefore, differences in the organogenic response of these plants have been shown to be associated with differences in exogenous cytokinin uptake and the subsequent metabolism of that hormone.

ACKNOWLEDGEMENT

We would like to thank Dr. Hubert Dulieu for generously providing the petunia lines used in this work.

LITERATURE CITED

1. **Auer CA** (1990) The role of benzyl adenine in shoot organogenesis of *Petunia* leaf explants. Ph.D. Thesis. University of Maryland, College Park, MD
2. **Ausubel F, Bahnsen K, Hanson M, Mitchell A, Smith H** (1980) Cell and tissue culture of haploid and diploid *Petunia* 'Mitchell.' Plant Mol Biol Newslett **1:** 26–32
3. **Bialek K, Meudt WJ, Cohen JD** (1983) Indole-3-acetic acid (IAA) and IAA conjugates applied to bean stem sections: IAA content and the growth response. Plant Physiol **73:** 130–134
4. **Burch LR, Stuchbury T** (1987) Activity and distribution of enzymes that interconvert purine bases, ribosides and ribotides in the tomato plant and possible implications for cytokinin metabolism. Physiol Plant **69:** 283–288
5. **Chen C** (1981) Biosynthesis and enzymic regulation of the interconversion of cytokinin. *In* J Guern, C Peaud-Lenoel, eds, Metabolism and Molecular Activities of Cytokinins, Springer-Verlag, New York, pp 34–43
6. **Christianson ML** (1987) Causal events in morphogenesis. *In* CE Green, DA Somers, WP Hackett, DD Biesboer, eds, Plant Tissue and Cell Culture. Alan R. Liss, New York, pp 45–55
7. **Christianson ML, Warnick DA** (1983) Competence and determination in the process of *in vitro* shoot organogenesis. Dev Biol **95:** 288–293
8. **Christianson ML, Warnick DA** (1985) Temporal requirement for phytohormone balance in the control of organogenesis *in vitro*. Dev Biol **112:** 494–497
9. **Fosket DE** (1980) Hormonal control of morphogenesis in cultured tissues. *In* F Skoog, ed, Plant Growth Substances 1979. Springer-Verlag, New York, pp 362–369
10. **Horgan R** (1987) Plant growth regulators and the control of growth and differentiation in plant tissue cultures. *In* CE Green, DA Somers, WP Hackett, DD Biesboer, eds, Plant Tissue and Cell Culture. Alan R. Liss, New York, pp 135–149
11. **King PJ** (1988) Plant hormone mutants. Trends Genet **4:** 157–162
12. **Laloue M** (1977) Cytokinins: 7-glucosylation is not a prerequisite of the expression of their biological activity. Planta **134:** 273–275
13. **Laloue M, Pethe C** (1982) Dynamics of cytokinin metabolism in tobacco cells. *In* PF Wareing, ed, Plant Growth Substances 1982. Academic Press, New York, pp 185–195
14. **Laloue M, Pethe-Terrine C, Guern J** (1981) Uptake and metabolism of cytokinins in tobacco cells: studies in relation to the expression of their biological activities. *In* J Guern, C Peaud-Lenoel, eds, Metabolism and Molecular Activities of Cytokinins. Springer-Verlag, New York, pp 80–96
15. **Letham DS, Palni LMS** (1983) The biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol **34:** 163–197
16. **Letham DS, Tao GQ, Parker CW** (1982) An overview of cytokinin metabolism. *In* PF Wareing, ed, Plant Growth Substances 1982. Academic Press, New York, pp 143–153
17. **Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497
18. **Nitsch JP** (1968) Studies on the mode of action of auxins, cytokinins and gibberelins at the subcellular level. *In* F Wightman, G Setterfield, eds, Biochemistry and Physiology of Plant Growth Substances. The Runge Press, Ottawa, Ontario, Canada, pp 563–580
19. **Sink KC** (1984) Petunia. Springer-Verlag, New York
20. **Skoog, F, Miller C** (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp Soc Exp Biol **11:** 118–131
21. **Skvirsky RC, Hanson MR, Ausubel FM** (1982) A genetic approach for studying regeneration: analysis of cytokinin-controlled shoot morphogenesis from tissue explants of *Petunia hybrida*. *In* ED Earle, Y Demarly, eds, Variability in Plants Regenerated from Tissue Culture. Praeger Publishers, New York, pp 101–120
22. **Smulders MJ, Croes AF, Wullems GJ** (1988) Polar transport of 1-naphthaleneacetic acid determines the distribution of flower buds on explants of tobacco. Plant Physiol **88:** 752–756
23. **Tao GQ, Letham DS, Palni LMS, Summons RE** (1983) Cytokinin biochemistry in relation to leaf senescence. I. The metabolism of 6-benzylaminopurine and zeatin in oat leaf segments. J Plant Growth Regul **2:** 89–102
24. **Van der Krieken WM, Croes AF, Smulders MJ, Wullems GJ** (1990) Cytokinins and flower bud formation *in vitro* in tobacco: role of the metabolites. Plant Physiol **92:** 565–569