Uptake and metabolism of N^6-benzyladenine and 1-naphthaleneacetic acid and dynamics of indole-3-acetic acid and cytokinins in two callus lines of *Actinidia deliciosa* differing in growth and shoot organogenesis

María L. Centeno[a], Ana Rodríguez[b], Isabel Feito[c], Ricardo Sánchez-Tamés[b] and Belén Fernández[b,*]

[a]*Departamento de Biología Vegetal, Universidad de León, Facultad de Ciencias Biológicas y Ambientales, Campus de Vegazana, 24071 León, Spain*
[b]*Laboratorio de Fisiología Vegetal, Departamento de Biología de Organismos y Sistemas, Facultad de Biología, Universidad de Oviedo, C/Catedrático Rodrigo Uría s/n, 33071 Oviedo, Spain*
[c]*Departamento de Hortofruticultura, Servicio Regional de Investigación y Desarrollo Agroalimentario, 33300 Villaviciosa, Asturias, Spain*
Corresponding author, e-mail: bmfernan@sci.cpd.uniovi.es

Uptake, metabolism and accumulation of N^6-benzyladenine (BA) and 1-naphthaleneacetic acid (NAA) as well as changes in endogenous indole-3-acetic acid (IAA) and several isoprenoid-type cytokinins (Cks) were characterized in two callus lines of *Actinidia deliciosa* cv. Hayward showing different growth and shoot organogenic responses to exogenously applied 2.7 µM NAA and 4.4 µM BA. Studies were carried out in callus 0, 1, 12, 24 and 48 h after the onset of their fifth subculture on a medium containing [³H]NAA or 8-[¹⁴C]BA. Kiwifruit callus of line A presented high caulogenic response and lower growth that was positively associated with faster BA uptake, with transient accumulation of BA and isoprenoid-type Cks, mainly zeatin, exceeding three- and four-fold that of the non-caulogenic callus, and with values of the BA/NAA ratio exceeding 1, in fact higher than the BA/NAA ratio in the culture medium. The accumulation of BA took place in both callus lines during the first 24 h of subculture and before the re-initiating of callus proliferation. The higher growth and the low or null caulogenic response shown by line B callus were correlated with faster NAA uptake, with endogenous NAA levels two-fold higher than in A calli, with higher IAA amounts and with values of the BA/NAA ratio below 1. Moreover, at 48 h free NAA in both kinds of callus reached levels close to those found after 35 days of subculture. Results suggest that temporal accumulation of BA and endogenous Cks is involved in the initiation of cell division leading to callus growth, whereas the maintenance of high NAA and IAA levels are related to the support of long-term callus development. It also appears that for callus cells to become committed to shoot organogenesis, they must have a concentration of active Cks higher than a threshold value during the first 2 days of culture on fresh medium, while at the same time the concentration of auxins must not exceed a certain maximum.

Introduction

Organogenesis *in vitro* depends on the application of phytohormones and also on the ability of the tissues to respond to these hormonal changes during culture (Sugiyama 1999). Specifically, the presence of auxins and cytokinins (Cks) is necessary for 'indirect organo-genesis' (Krikorian 1995): the process involves the development of callus from an initial explant and the induction of new organs from the newly formed callus. In *Actinidia deliciosa* (kiwifruit) tissues, each of these steps depends on the different concentrations of the

exogenously supplied N^6-benzyladenine (BA), and 1-naphthaleneacetic acid (NAA) (Centeno et al. 1996). The hormonal control of formation and development of initial callus from kiwifruit petioles has been previously studied (Centeno et al. 1998, 1999). The aim of the current study was to ascertain the control of growth and shoot organogenic response of established callus by auxins and Cks

Morphogenic responses of cultured tissues have been typically described in relation to the kind and the concentration of the plant growth regulator(s) (PGRs) in the medium. Thus, application of Cks has been associated with the cellular dedifferentiation and proliferation that lead to callus initiation, as well as with the adventitious production of buds, whereas auxins or auxin-like compounds such as NAA are usually correlated with callus growth and maintenance and with the formation of adventitious roots (Krikorian 1995). However, this approach is simplistic, since the developmental response of explants to exogenous PGRs is a result of a variety of biochemical processes, including hormone uptake, distribution and metabolism, that affect the free hormone concentration in the tissues, as already demonstrated in several systems (Auer et al. 1992, Ribnicky et al. 1996, Centeno et al. 1998, 1999). For instance, most of the NAA taken up by tissues is converted to amino and ester-conjugates (Venis 1972, Smulders et al. 1990, Ribnicky et al. 1996, Centeno et al. 1999) and, BA is transformed into BA glucosides, ribosides and ribotides (Auer et al. 1992, Feito et al. 1994, Centeno et al. 1998, Moncaleán et al. 1999), with the physiological activities of these compounds differing significantly. Moreover, in order to assess the physiological relevance of studies that use exogenously added PGRs, more knowledge is needed concerning the naturally occurring fluctuations of endogenous phytohormones, since the active molecules within the explants controlling the morphogenic response are probably derived from both exogenous and endogenous sources (Auer et al. 1999). Comparative analysis of the previously cited factors in organogenic and non-organogenic areas, explants or genotypes, still provide valuable, useful information about the control of in vitro organogenesis now that the molecular mechanisms underlying these processes are beginning to be known through analysis of hormone-independent mutants (see Sugiyama 1999).

We have selected two callus lines of Actinidia deliciosa differing in their morphogenic responses, line A presented high shoot regeneration, whereas line B presented scant caulogenic response, but a higher growth ability (Centeno et al. 1996). A relationship was established between endogenous BA content and caulogenic expression of callus, since BA levels were two-times greater in A calli than in B calli, although no other differences were found between them. However, these analysis were carried out at the end of the fifth subculture, when the endogenous hormonal content might be a consequence of callus response, rather than their cause.

To better ascertain the participation of exogenous and endogenous PGRs in the determination of the in vitro behaviour of calli, we established the following objectives: (1) to initiate the two lines of callus once more and to re-evaluate their caulogenic potential along several subcultures on callus proliferation and shoot induction medium (CPM); (2) to compare the uptake, metabolism and accumulation of NAA and BA during the first 2 days of the fifth subculture; and (3) to compare the fluctuations of indole-3-acetic acid (IAA) and isoprenoid-type Cks at the same time. Data showed that callus lines A and B present differences in the analysed factors. The results are subsequently discussed with the aim of clarifying the role of auxins and Cks in indirect shoot organogenesis and callus growth and maintenance.

Materials and methods

Plant material and culture conditions

Two callus lines, A and B, of *Actinidia deliciosa* (A. Chev) Liang and Ferguson cv. Hayward, differing in their growth and shoot organogenic abilities, were used in all experiments.

The tissue culture conditions and methods were those previously reported (Centeno et al. 1996, 1998, 1999). Calli were derived from the basal end of young petioles of four female kiwi plants (called 1, 2, 3 and 4). These plants originated from cuttings and had already been growing in a greenhouse for 6 years at the time of harvesting (August). Only the five youngest petioles were harvested and used for tissue culture (Centeno et al. 1996). After 30 days of culture, the induced calli were cultured on CPM, consisting of the Murashige and Skoog (MS) medium containing sucrose (2.5% w/v) and supplemented with 4.4 μM BA (Sigma, Madrid, Spain) and 2.7 μM NAA (Sigma). The medium was gelled with agar (0.7% w/v) and the culture vessels contained 30 ml of medium in all cases. Two callus portions per flask were transferred to fresh CPM every 5 weeks and cultures were maintained along nine subcultures. All cultures were grown in a chamber at $25 \pm 2°C$ with 16 h of light ($33 \mu mol\,m^{-2}\,s^{-1}$).

The number of shoot-forming calli and the buds formed per callus were separately recorded at the end of each subculture for the A and B callus lines. Callus lines were selected on the basis of differences in tissue culture regeneration. Thus, line A groups together calli with a high organogenic response proceeding from kiwi-plant no. 3, whereas line B groups together those calli with a small or null response which originated from plants 1, 2 and 4.

To determine whether the growth characteristics of both callus lines were similar to those tested in Centeno et al. (1996), calli from six flasks [two pieces, 1 ± 0.05 g fresh weight (FW) per flask] were collected and weighted at the end of the fifth subculture, frozen in liquid N_2, lyophilized and calculated their dry weight (DW).

Extraction and measurement of NAA and BA uptake

Analysis of NAA and BA uptake and metabolism in kiwifruit callus were carried out during the fifth subculture on CPM, when lines A and B were well defined and established. For NAA uptake studies, three pieces of callus (1.5 ± 0.07 g FW) were cultured on 30 ml CPM additionally containing unlabelled NAA, 16.5 kBq 1-[^{3}H]NAA (836 GBq mmol^{-1}, Amershan Ibérica, Barcelona, Spain). Likewise, for the BA studies, 16.5 kBq 8-[^{14}C]BA (2 GBq mmol^{-1}, Amersham Ibérica) were added together with unlabelled BA (final concentration 4.4 μM) to the CPM, and three calli were placed in each culture vessel. The calli were harvested after 0, 1, 12, 24 and 48 h of culture. The experiments were repeated three times.

Calli from each incubation period on 1-[^{3}H]NAA or 8-[^{14}C]BA were washed for a few seconds with distilled water to remove medium residues, dried with filter paper, weighed, deep-frozen in liquid N_2, powdered and lyophilized. One hundred milligrams of material were extracted twice with 25 and 15 ml of 80% (v/v) methanol containing 10 mg l^{-1} butylated hydroxytoluene, for 14 and 7 h by repeated inversion at 4°C in darkness. At this point, three aliquots (1 ml) were taken from the combined extracts, reduced to dryness and re-suspended in 0.5 ml of distilled water and 1.5 ml Pico-Aqua (Packard, Madrid, Spain) as scintillating liquid. The radioactivity was measured with a liquid scintillation counter (Packard 2500 TR). Data were corrected according to the extraction volume and the sample weight to determine total NAA or BA uptake per culture.

Subsequently 0.1 kBq tritiated *t*-zeatin riboside (^{3}H-[9R]Z) (128 GBq mmol^{-1}, synthesized in our laboratory according to MacDonald and Morris 1985) was added to the methanolic extracts containing labelled BA to correct for isoprenoid-type Cks losses. All methanolic extracts were evaporated under vacuum (30°C) and the resulting aqueous solutions were brought to a final volume of 5 ml with distilled water and then cleared by centrifugation (10 000 g for 15 min at 4°C), collecting the supernatants.

Analysis of NAA metabolites and IAA

Samples containing labelled NAA metabolites were reduced to dryness by Speed-Vac concentration (Savant (Biexbeck, Belgium) SC-200) and re-dissolved in 500 µl 80% (v/v) methanol. After filtration (0.2 µm Anatop-Plus filters; Whatman International Ltd., Maidstone, England), three aliquots (25 µl) were taken to measure the radioactivity once again.

The analysis of NAA derivatives in methanolic extracts of callus were carried out by silica gel thin-layer chromatography (TLC) as previously reported (Centeno et al. 1999). Volumes of extracts containing 0.2 kBq combined with NAA standard were spotted in triplicate on TLC plates (0.25 mm thickness and 20 cm × 20 cm, Merck 60F254 (Merck, Barcelona, Spain)) and developed with the solvent system called

S1: chloroform:methanol:acetic acid [75:20:5, v/v] (Caboche et al. 1984, Smulders et al. 1990). Migration profiles and Rf values of radioactive compounds were determined by autoradiography of the TLC plates (films Kodak XAR Omat; Radisolver developer; Valca Co, Burgos, Spain). Subsequently, the plates were divided according to the Rf values and radioactivity was eluted from each silica fraction with 3 ml of methanol. All collected silica extracts were reduced to dryness and radioactivity was counted as described above. Tentative identification of NAA derivatives was carried out using three different solvent systems and by their alkaline hydrolysis in 1 mM NaOH at room temperature (ester hydrolysis) and in 7 mM NaOH at 100°C (amide hydrolysis), as described previously (Centeno et al. 1999). Quantification was carried out by calculating the percentage of the radioactive labelling associated with each spot with respect to the total radioactivity measured in each sample.

After analysis of NAA metabolites, the remaining methanolic extracts were dried, dissolved in 5 ml distilled water, acidified to pH 3 with diluted HCl, and extracted four times with diethyl ether at 4°C in darkness. The ether was then removed under a N_2 stream and samples were methylated with diazomethane. Samples containing methylated IAA were dried, re-suspended in tris-hydroxymethyl-aminomethane saline buffer (TBS, 25 mM, pH 7.5) and quantified in triplicate by enzyme immunoassay (ELISA) with monoclonal antibodies (Chemical Co test kits; Sigma). The guidelines given by the manufacturers of the antibodies were followed. The data were corrected according to the volume of each methanolic extract and the IAA losses. To calculate IAA losses during the extraction process, six culture vessels were prepared with 30 ml of complete CPM but without 1-[^{3}H]NAA, and each one of the six corresponding callus samples was analysed separately after addition of 0.17 kBq [2–^{14}C]IAA (2 GBq mmol^{-1}; Amersham Ibérica) to the initial extracts. The mean of the recovery values obtained was applied for the individual samples.

Analysis of BA metabolites and Cks

Extracts containing labelled BA metabolites were adjusted to pH 3 with acetic acid and percolated through a 5-ml column of mono-ammonium ionic form cellulose phosphate (Whatman P11) equilibrated with acidified water (pH 3 with acetic acid). Once the sample had been applied, 25 ml of acidified water (pH 3) were passed to elute the cytokinin nucleotides (acidic fraction). Subsequently, the cytokinin bases and nucleosides retained in the column were eluted with 25 ml 2 M ammonium hydroxide (basic fraction). Acidic and basic fractions of each sample were further purified using Sep – Pak C$_{18}$ cartridges (Feito et al. 1994).

Separation of the different Cks was carried out by a Waters 600 liquid chromatograph equipped with a diode array detector (Waters 996; Madrid, Spain) and a radioactivity monitor (LB 507B; Berthold, E.G. and

G., Sandridge, UK) with a solid scintillation cell. Separation of the isoprenoid-type Cks, dihydrozeatin (DHZ), dihydrozeatin riboside ([9R]DHZ), zeatin (Z), and [9R]Z, and the BA metabolites, adenine, adenosine (all furnished by Sigma), BA, N^6-benzyladenosine ([9R]BA), N^6-benzyladenine-7-glucoside ([7G]BA), and N^6-benzyladenine-9-glucoside ([9G]BA) (all supplied by Apex Organics, Devon, UK) was accomplished by HPLC. The column used was a Spherisorb ODS-2 (150×4.6 mm; 5 µm) and the flow rate was $1.5 \, \text{ml min}^{-1}$. . The mobile phase was acetonitrile:water (pH 7 with triethylamonium bicarbonate) in a linear gradient from 5 to 13% (v/v) in 5 min, then 2 min at 13% acetonitrile, followed by a 18-min linear gradient to reach 20% acetonitrile, and finally 5 min at 20% acetonitrile. The eluted Cks were monitored at 265 and 275 nm and the radioactivity associated with BA metabolites was detected by the radioactivity monitor. Fractions corresponding to Z-type Cks were collected at 1-min intervals and reduced to dryness.

Separation of N^6-benzyladenosine-5'-monophosphate ([9R-MP]BA) (Apex Organics) was accomplished using the same column. The mobile phase was methanol: aqueous buffer (0.2 M acetic acid adjusted to pH 3.5 with triethylamine). A linear solvent gradient from 5 to 45% (v/v) methanol over 45 min and a flow rate of $1 \, \text{ml min}^{-1}$ were used. Each basic and acidic fraction from each cytokinin extract was injected on HPLC in duplicate.

Isoprenoid-type Cks, BA and its metabolites were identified by their retention times and by their UV absorption spectra. Quantification of BA and its metabolites was done by measuring the radioactive peak area (Centeno et al. 1998). Quantitative determination of isoprenoid-type Cks was accomplished by ELISA. The dried HPLC fractions were re-suspended in TBS buffer (25 mM, pH 7.5) and quantified using polyclonal rabbit antibodies raised in our laboratory (Fernández et al. 1995): (1) anti [9R]Z to measure [9R]Z and Z; (2) anti [9R]DHZ to measure [9R]DHZ and DHZ. Assays were carried out using Immunolon M129 Micro ELISA plates (Nunc Ruskilde, Denmark) and according to a modification of the ELISA method described by Eberle et al. (1986) and published by Centeno et al. (1996). Furthermore, the cytokinin-alkaline phosphatase tracer conjugates for the ELISA assays were prepared following Eberle et al. (1986), except that 3 mg of protein were used and therefore the volumes of other reagents were proportionally scaled up.

Results

Growth and organogenic response of calli

The *in vitro* behaviour of kiwifruit callus initiated in two consecutive years was similar, as can be seen in Fig. 1. The percentage of shoot-forming calli in line A along nine subcultures was 75–100% and 50–100% in the first and the second year, respectively. Calli produced 1–10 buds/callus visible after 8–10 days of culture in fresh medium. The proportion of calli with caulogenic
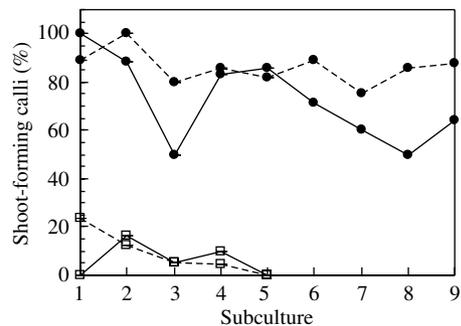

Fig. 1. Caulogenic response (percentage of shoot-forming calli) in *Actinidia deliciosa* callus from lines A (●) and B (□) during nine subcultures on callus proliferation and shoot induction medium (CPM). Line A calli originated from kiwiplant no.3 and those from line B from plants 1, 2 and 4. Calli were obtained in two consecutive years (– – – first year, — second year) from the basal end of petiolar explants cultured on callus induction medium, and were then transferred to CPM and further maintained by subculturing each 35 days on fresh CPM.

response in line B was lower than in line A, such that it was 4–23% in the first year and 10–16% in the second. This organogenic response of B callus disappeared after five subcultures.

The growth of organogenic calli along their fifth subculture on CPM was lower than that of the-non-organogenic ones, since the gain in fresh weight for line A calli was 1.68 ± 0.08 g, whereas it was 2.45 ± 0.12 g for those from line B. However, A calli accumulated more dry matter per gram of fresh weight than B calli, with the ratio dry weight (DW)/FW being 0.099 ± 0.01 and 0.075 ± 0.003, respectively, at the end of the fifth subculture. The appearance of the calli demonstrated this last fact, since callus A was less friable and more nodular than callus B, although both were green. Therefore, the higher caulogenic potential, as well as the lower growth and water content, was associated with line A, and the reverse characteristics with line B.

NAA uptake and metabolism

To evaluate differences in NAA absorption and metabolism by kiwifruit callus, calli from lines A and B were placed after four subcultures on fresh CPM containing 1-[^{3}H]NAA and analysed after 1, 12, 24 and 48 h. Figure 2A shows that initial NAA uptake by calli was very efficient, since the rate of NAA uptake was high and similar in both callus lines during the first hour ($38.9 \, \text{nmol h}^{-1} \, \text{g}^{-1}$ DW in A and $36.2 \, \text{nmol h}^{-1} \, \text{g}^{-1}$ DW in B). Afterwards, these rates slowed down and varied only slightly, by about $4.8 \, \text{nmol h}^{-1} \, \text{g}^{-1}$ DW in A callus and $6.9 \, \text{nmol h}^{-1} \, \text{g}^{-1}$ DW in B callus. After 48 h, organogenic callus (A) accumulated less NAA (32.5%) than-non-organogenic callus (B) (44.6%).

The 1-[^{3}H]NAA taken up was metabolized by the calli. Six NAA derivatives were separated and tentatively characterized (Centeno et al. 1999) as NAA, α-naphthylacetyl-β-D-glucose (NAGlu), α-naphthylacetylaspartic
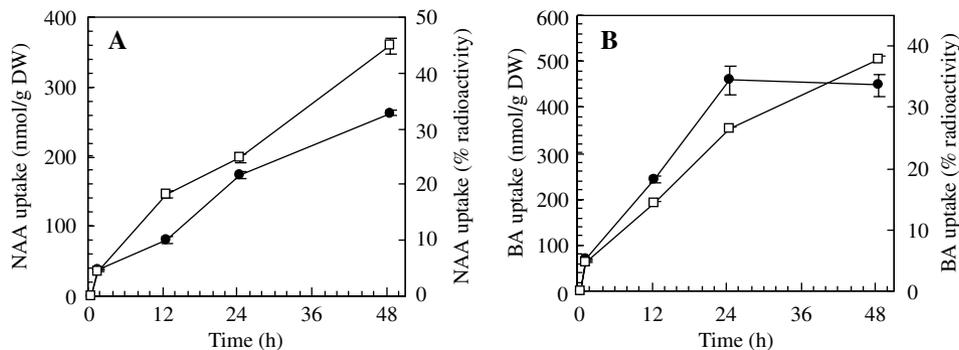
Fig. 2. Uptake of [³H]-NAA (A) and 8-[¹⁴C]BA (B) by organogenic (●) and-non-organogenic calli (□) of kiwifruit during the first 48 h of their fifth subculture on CPM. The results are expressed as nmol of total NAA and BA taken up per g of dry weight ± SE and as percentage of total radioactivity applied in each case. Values are the mean of the three independent experiments and vertical bars indicate standard errors when larger than the symbols.

acid (NAAsp), metabolite 1 of NAA (Met 1), metabolite 2 of NAA (Met 2) and metabolite 3 of NAA (Met 3). Metabolites 1, 2 and 3 were NAA-ester conjugates, as they released NAA after ester hydrolysis, as well as NAGlu (Centeno et al. 1999).

The kinetics of NAA metabolism is shown in Fig. 3A and B, where Met 1 and Met 2 are represented as the sum of both compounds, given that they evolved similarly. NAA was rapidly metabolized by calli, since after 1 h less than 25% of the NAA taken up remained as free NAA. At this point, Met 3 was the major metabolite, representing about 37% of the total radioactivity. Subsequently, the percentage of Met 3 decreased in proportion to the increase in NAAsp. The Met 1 + Met 2 percentage remained relatively low and constant, and that of NAGlu changed from 8–12% at 1 h to 2.5–3% at 48 h, while being undetectable at 24 h.

In spite of the similarities described, lines A and B of kiwifruit calli showed a number of differences in NAA metabolism. Thus, the percentage of free NAA in the-non-organogenic line (B) was maintained at greater than 20% during the first 24 h of culture (Fig. 3B), whereas in the organogenic line (A) it was already lower than 10% at this point in time (Fig. 3A). Another noteworthy

difference was that NAAsp became the major NAA derivative in line B calli after 24 h, reaching 58% of the radioactivity taken up at 48 h (Fig. 3B). The formation of NAAsp was slower in line A calli, in which this NAA conjugate only reached 30% of the metabolites at the end of the experiment (Fig. 3A). Thus, the NAA conversion to NAAsp was faster in-non-organogenic kiwifruit callus.

BA uptake and metabolism

The kinetics of BA uptake by kiwifruit callus cultured on fresh CPM containing 8-[¹⁴C]BA is shown in Fig. 2B. During the first hour, the rate of BA uptake in caulogenic calli was 68.89 nmol h⁻¹ g⁻¹ DW, whereas in-non-caulogenic calli it was 62.3 nmol h⁻¹ g⁻¹ DW. The rate subsequently slowed to 17 and 12 nmol h⁻¹ g⁻¹ DW in lines A and B, respectively. At 24 h line A calli reached maximal absorption of BA (34% of applied to the medium), whereas line B accumulated 26% of radioactivity but continued to absorb BA up to 48 h, reaching 37% of the radioactivity. Data not shown indicated that these calli did not absorb BA from 48 up to 96 h.
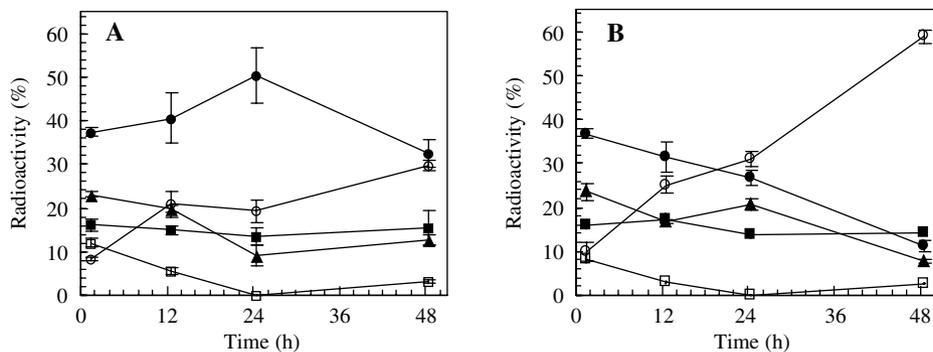

Fig. 3. Distribution of the radioactivity detected in samples between the NAA metabolites in organogenic (A) and-non-organogenic (B) kiwifruit calli. Results are expressed as percentage of the total radioactivity extracted in samples. Values are the mean of the three independent experiments and standard errors are indicated by vertical bars when larger than the symbols. σ, NAA; ○ NAAsp; ●, Met3; ■, Met1 + Met2; □, NAGlu.

The BA metabolites detected were the same in both lines. In the basic fraction of samples, four peaks were detected after HPLC separation and the compounds were identified by their retention times and UV-absorption spectra as BA, [9R]BA, [7G]BA and [9G]BA. In the acidic fraction [9R-MP]BA was identified as the main BA nucleotide, containing between 74 and 87% of the radioactivity associated with these fractions.

The kinetics of BA metabolites showed some similarities and some differences between kiwifruit callus lines A and B (Fig. 4A and B). In both, BA was transformed as fast as NAA, since less than 23% of the BA uptake occurred as free base after 1 h of culture. Subsequently, the proportion of BA decreased, although it was always higher in A than in B calli. The [9G]BA and BA nucleotides, mainly [9R-MP]BA, were the major metabolites present in the samples. [7G]BA was only found after 12 h in a very small proportion, and the percentage of radioactivity associated with [9R]BA was also lower than 7.5% at all times assayed.

In organogenic calli the percentage of [9G]BA increased steadily throughout the experiment from 15.5 to 68%, whereas that of BA-nucleotides decreased from 59% at 1 h to 22% at 24 h (Fig. 4A).-Non-organogenic calli showed a similar pattern for [9G]BA and BA-nucleotides, although the percentage of [9G]BA increased just until 12 h (from 8.1 to 46%) and subsequently remained almost constant (Fig. 4B). The proportion of nucleotide also declined in B calli during the first 24 h, as well as in A calli, but to a lesser extent (up to 31%). Therefore, organogenic calli showed faster BA uptake and a greater ability to transform BA into their 9-glucoside than-non-organogenic calli.

Concentrations of NAA and BA in kiwifruit callus. Relationships between NAA and BA

Uptake and metabolism of NAA and BA gave rise to the endogenous NAA and BA concentration in callus shown in Table 1. Initially there were no differences in NAA levels between callus lines, although they reached at least double in B calli than in A calli at 12 and 24 h. The endogenous NAA exceeded $30 \, nmol \, g^{-1}$ DW in both callus tissues but in organogenic calli this occurred with a delay of 24 h in relation to non-organogenic calli. BA content per gram of callus DW increased throughout the first 24 h and declined during the next day to values that were very close to those detected at 1 h (Table 1). However, the highest BA reached by A calli was $66.06 \, nmol \, g^{-1}$ DW at 24 h, whereas it was one-third in B calli at the same time ($22.37 \, nmol \, g^{-1}$ DW).

In the induction of organogenesis, the endogenous BA/NAA balance is more important than their absolute concentration. In organogenic calli, the BA/NAA ratio (Table 1) increased and was favourable for BA during the first 24 h of the fifth subculture. Moreover, it was higher than the BA/NAA ratio in the medium. This never occurred in-non-organogenic calli, which showed a ratio in favour of NAA, except at 1 h. At 48 h, the BA/NAA ratio was the lowest and similar in both callus lines. The most noticeable difference between the two callus lines was observed at 24 h, when the BA/NAA ratio was 7.5-fold higher in organogenic (A) than in-non-organogenic calli (B).

Levels of IAA and isoprenoid Cks

The content of endogenous IAA in callus lines A and B was measured after 0, 1, 12, 24 and 48 h of culture (Table 2). IAA found in both lines was around $30 \, nmol \, g^{-1}$ DW at the start of the fifth subculture. During the first 12 h of subculture, endogenous IAA declined 1000-fold in organogenic and 50-fold in-non-organogenic calli, but the decrease continued in the latter until 24 h. Subsequently, the IAA content increased again to reach values between 5 and $20 \, nmol \, g^{-1}$ DW. It was noticeable that IAA levels obtained at 1, 12 and 48 h in line B greatly exceeded those of line A. The contrary was observed at 24 h.

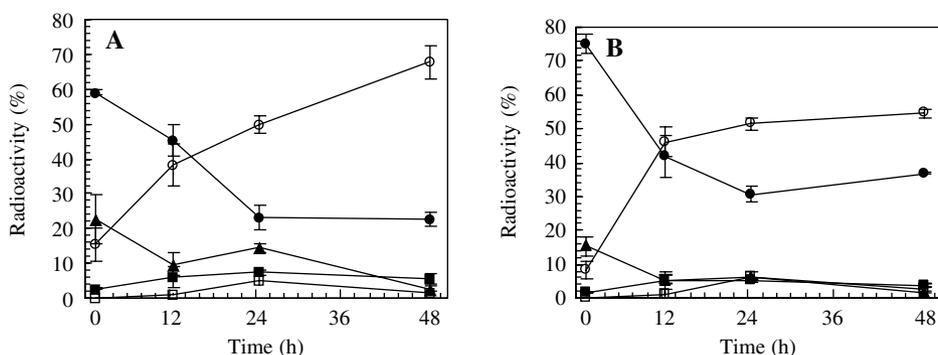Using ELISA analysis, four isoprenoid-type Cks were identified as DHZ, Z, [9R]DHZ and [9R]Z by comparing

Fig. 4. Distribution of the radioactivity detected in samples between the BA metabolites in organogenic (A) and no organogenic (B) kiwifruit calli. Results are expressed as percentage of the total radioactivity extracted in samples. Values are the mean of the three independent experiments and standard errors are indicated by vertical bars when larger than the symbols. σ, BA; ●, BA nucleotides; ○, [9G]BA; ■, [9R]BA; □, [7G]BA.

Table 1. Concentrations of exogenously applied BA and NAA in callus proliferation and shoot induction medium (CPM), endogenous concentrations (nmol g^{-1} DW) of free BA and NAA detected in organogenic (A) and-non-organogenic (B) calli of *Actinidia deliciosa* during the first 48 h of their fifth subculture on CPM, and the relationship between both plant growth regulators in the medium and in calli. Values are the mean of the three independent experiments $\pm$ SE.

| | Added to the medium | Callus line | BA and NAA concentration (nmol g^{-1} DW) and BA/NAA ratio inside the tissues | | | |
			1 h	12 h	24 h	48 h
BA	4.4 μM	A	15.51 ± 4.41	23.76 ± 5.84	66.06 ± 11.83	11.77 ± 4.48
		B	9.56 ± 4.51	10.24 ± 4.81	22.37 ± 7.16	8.91 ± 2.51
NAA	2.7 μM	A	9.01 ± 0.16	10.34 ± 0.67	15.99 ± 2.97	33.18 ± 1.02
		B	8.50 ± 0.72	24.37 ± 0.57	39.63 ± 3.45	28.43 ± 2.48
BA/NAA	1.63	A	1.72	2.29	4.13	0.35
		B	1.12	0.42	0.56	0.31

Table 2. IAA concentration in organogenic (A) and-non-organogenic (B) calli of *Actinidia deliciosa* during the first 48 h of their fifth subculture on callus proliferation and shoot induction medium. Values are the mean of the three independent experiments $\pm$ SE.

| Callus line | Concentration of IAA (nmol g^{-1} DW) | | | | |
	0 h	1 h	12 h	24 h	48 h
A	28.32 ± 4.21	0.27 ± 0.13	0.02 ± 0.01	4.77 ± 2.37	5.62 ± 1.37
B	33.08 ± 7.45	4.79 ± 2.61	0.65 ± 0.25	0.12 ± 0.06	19.08 ± 5.62

their retention times with the immunoreactive fractions found in the samples. Figure 5A and B show the amount of these natural isoprenoid-type Cks in A and B kiwifruit calli, respectively, during the first 48 h of their fifth subculture on CPM.

In organogenic calli (Fig. 5A) Z, DHZ, [9R]DHZ and [9R]Z contents evolved as was previously described for endogenous BA, showing a larger increase during the first 24 h and a subsequent decrease. Z was the predominant Ck at the time of maximum accumulation, representing nearly 50% of the Cks measured. The increase in isoprenoid-type Cks observed in A calli 24 h after the onset was 17-fold, whereas only a 4.3-fold increase was found in B calli (Fig. 5B). Moreover, the major Ck present at 24 h in B calli was not Z but [9R]DHZ. Another difference between the two callus lines was that Z and [9R]Z contents continued to increase from 24 to 48 h in B calli, although Z never reached the values

found in A calli at 24 h. As for free BA, the highest content of isoprenoid-type Cks, mainly that of Z, was found in organogenic calli at 24 h, the levels being a quarter and one-tenth, respectively, in-non-organogenic calli at the same point.

Discussion

Two callus lines obtained from kiwifruit petioles, defined according to their differences in growth and shoot organogenesis in response to NAA and BA, were previously established in our laboratory (Centeno et al. 1996), where it was shown that a high percentage of calli proceeding from kiwifruit no. 3 (line A) showed caulogenic response along nine subcultures in CPM, whereas this proportion was small or null with calli derived from plants 1, 2 and 4 (line B). Furthermore, callus growth
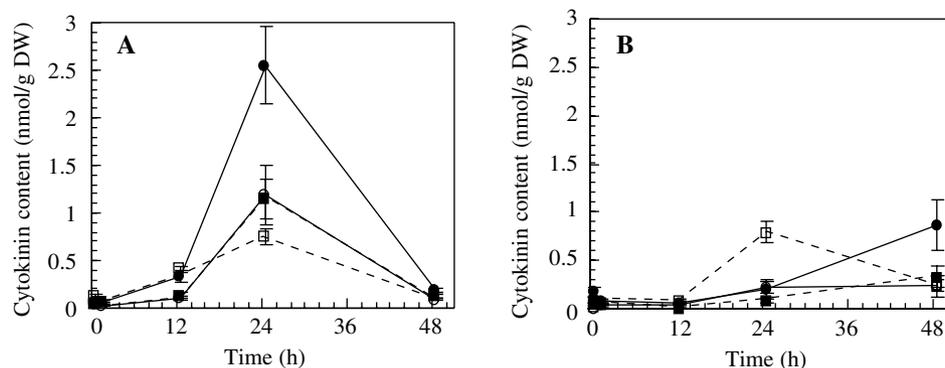

Fig. 5. Endogenous amounts of isoprenoid-type cytokinins in organogenic (A) and-non-organogenic (B) kiwifruit callus during the first 48 h of their fifth subculture on callus proliferation and shoot induction medium. Values are the mean of the three independent experiments ± SE. ●, Z; ■, [9R]Z; ○, DHZ; □, [9R]DHZ.

along a subculture was lower in line A than in line B, which in turn had more water content per gram fresh weight. In the present study, callus cultures were newly initiated, obtaining similar results (Fig. 1). On the basis of these findings and those reported by Barbieri and Morini (1988) and Leva and Bertocci (1988), competition between callus growth and shoot organogenesis in kiwifruit calli may be suggested. Since callus growth was enough to mask caulogenesis in B but not in A calli, lines A and B represented a good system for carrying out a comparative test to clarify the relationships of NAA and BA with each process.

Differences in the uptake of applied PGRs were observed between A and B calli, since organogenic calli accumulated more BA after 24 h but showed less uptake of NAA at the end of the experiment (Fig. 2). A positive correlation between exogenous BA uptake and caulogenic response was also found by Auer et al. (1992) in leaves of two *Petunia hybrida* lines. For NAA, Smulders et al. (1988) showed that tobacco explants developed friable calli and reduced the formation of flower buds by increasing the exogenous NAA concentration from 1 to $10 \mu M$, which was clearly associated with a higher NAA uptake. Therefore differences in PGR absorption may be decisive for the different responses of kiwifruit calli. Nevertheless, metabolism must be borne in mind as a significant factor affecting endogenous concentrations.

NAA was quickly metabolized by kiwifruit calli, since after only 1 h of culture on fresh CPM more than 75% of NAA uptake appeared as the conjugated form. Ester conjugates, mainly Met 3, were the first major metabolites after feeding NAA, although their relative amounts decreased in parallel to the increase in the proportion of NAAsp (Fig. 3). Comparable patterns of NAA conjugation have been published before in kiwifruit petioles (Centeno et al. 1999) and in carrot and tobacco tissue cultures (Smulders et al. 1990, Ribnicky et al. 1996). The NAA metabolism in line A calli showed a delay with respect to that of B calli, which may be explained by their lower rate of NAA uptake and by the characteristics of the enzyme responsible for NAAsp formation, as the acylaspartate synthethase is a substrate-induced enzyme (Venis 1972, Smulders et al. 1990).

Regardless of the callus line, NAA conjugation gave rise to very close free growth regulator levels after two (Table 1) and 35 days (Centeno et al. 1996), namely about $30 \, \text{nmol g}^{-1} \text{DW}$. In line with this result, Ribnicky et al. (1996) and Centeno et al. (1999), respectively, found that callus-forming carrot hypocotyls and kiwifruit petioles each have similar endogenous levels of free NAA after several short and long culture periods. All this data supports the thesis of Smulders et al. (1990), who proposed that the formation and hydrolysis of NAA conjugates play an important role in maintaining optimal levels of biologically active NAA, as has been pointed out for IAA conjugates (see Kleczkowski and Shell 1995, Normanly 1997, Normanly and Bartel 1999). The current results were also in agreement with the need to maintain a particular level of free NAA, which was in this case optimal to sustain the proliferation and growth of calli.

The metabolism of BA has been studied in a variety of *Actinidia deliciosa* explants in relation to several morphogenic processes (Feito et al. 1994, Centeno et al. 1998, Moncaleán et al. 1999, Cañal et al. 2000). In line with results obtained for calli (Fig. 4), BA was generally metabolized to [9R]BA and [9R-MP]BA, which can be converted back to the putative free base form, and to its 7- and mainly 9-glucosides, which are biologically inactive BA conjugates and detoxification or inactivation products of BA (McGaw and Burch 1995, Kaminek et al. 1997, Zazimalová et al. 1999, Mok and Mok 2001). In contrast with petioles and microshoots, kiwifruit calli did not have labelled adenine and adenosine as a result of BA degradation.

Initially, the proportion of free BA in kiwifruit calli was relatively high, but the conjugation with glucose to form [9G]BA gave rise to an abrupt decrease in its content from 24 to 48 h (Table 1). Moreover, free BA levels in calli decreased even more from the second day to the end of the subculture (Centeno et al. 1996). Thus, the initial accumulation of BA seemed to be transient, as opposed to that of NAA. Moreover, this occurred before the onset of callus proliferation, which took place between the third and fourth day of subculture (histological data not shown). This data supports our previous proposal explaining the role of BA and NAA in the formation and development of callus (Centeno et al. 1999): temporal accumulation of BA triggers cellular division leading to callus formation or re-initiating callus growth, whereas maintaining a high level of NAA is required to support long-term callus development. The current results also agree with the model proposed by Kaminek et al. (1997) describing the relationship between the regulation of Cks levels and the induction of physiological responses by Cks in plant cells.

Although caulogenic response cannot be separated from callus growth, some relationships may be established from organogenesis and Cks and auxins supplied to the calli. Kiwifruit calli from line A had a higher commitment to shoot organogenesis, which was correlated with faster BA uptake; transient accumulation of free BA exceeding by three-fold that of the-non-caulogenic calli and, high values of the BA/NAA ratio exceeding two-fold that applied to the medium at 24 h of subculture (Table 1). It thus appears that BA accumulation in line A calli was sufficient not only to trigger cell division but also to render cells competent to undergo shoot organogenesis, whereas it was insufficient to promote the latter effect in line B calli. There is a great deal of evidence indicating the ability of exogenously applied Cks to induce shoot formation. Furthermore, the 'shooty' phenotype shown by callus from tobacco plants, expressing the Ck biosynthesis *ipt* gene, has been associated with a highly endogenous Cks/IAA ratio (Smigocky and Owens 1989). Furthermore, calli from several mutant lines of *Arabidopsis* forming shoots in the absence of exogenous Cks have increased Ck

content or enhanced Ck signalling (Kakimoto 1996, Sugiyama 1999, Frank et al. 2000).

In contrast to organogenic calli, the higher growth capacity and relative lack of caulogenic response shown by kiwifruit B calli were associated with higher NAA absorption, with endogenous content of free NAA double that of A calli at 12 and 24 h, and with BA/NAA ratios lower than 1 (Table 1). It appears that there was a initial concentration of NAA above which callus cells cannot acquire caulogenic competence and thus only calli with a NAA concentration below this maximum could subsequently develop shoot organogenesis. In agreement with the negative effect of auxins or auxin-like compounds upon caulogenesis, IAA-overproducing calli originating from plants double transformed with *iaaM* and *iaaH* genes produced shoots only when they were cultured in a medium that lacked auxin (Sitbon et al. 1992). In addition, Ribnicky et al. (1996) showed that for the formation and long-term maintenance of carrot calli, a highly endogenous concentration of each auxin assayed was needed, including NAA, although this concentration must decrease below a certain threshold level for organogenesis or embryogenesis to occur. Thus, it seems that kiwifruit calli must simultaneously fulfil the two requisites referring to the initial concentration of endogenous BA and NAA to become committed to the development of shoot, a fact taking place only in line A calli.

Changes in endogenous IAA and isoprenoid Cks were also measured, as it is likely that the active PGRs within calli controlling their *in vitro* response were derived from both exogenous and endogenous sources (Auer et al. 1999). In fact, it may be inferred that they could play similar roles to those previously attributed to BA and NAA, respectively, on the basis of the following coincidences: (1) the patterns of endogenous Cks and free BA matched in caulogenic calli, including the accumulation observed at 24 h (Fig. 5); (2) IAA amounts were usually lower in these than in-non-organogenic calli during the first 2 days of culture (Table 2), as occurred with NAA; and (3) IAA levels were equal in the two callus lines and as high as those of NAA after 35 days (Centeno et al. 1996). However, this does not imply that the PGRs supplied act indirectly by affecting endogenous hormones. On the contrary, BA has been recognized as an active Ck for several years now and it has been shown that NAA can induce morphogenic responses directly like an auxin analogue (Ribnicky et al. 1996).

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