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Activity and Accumulation of Cell Division-Promoting Phenolics in Tobacco Tissue Cultures[1]

Rita A. Teutonico[2], Matthew W. Dudley, John D. Orr[3], David G. Lynn, and Andrew N. Binns*

Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104 (R.A.T., A.N.B.; and Department of Chemistry, Searle Chemistry Laboratory, University of Chicago, Chicago, Illinois 60637 (M.W.D., J.D.O., D.G.L.)

ABSTRACT

Dehydrodiconiferyl alcohol glucosides (DCGs) are derivatives of the phenylpropanoid pathway that have been isolated from *Catharansus roseus* L. (*Vinca rosea*) crown gall tumors. Fractions containing purified DCGs have been shown previously to promote the growth of cytokinin-requiring tissues of tobacco in the absence of exogenous cytokinins. In this study, we utilized synthetic DCG isomers to confirm the cell division-promoting activity of DCG isomers A and B and show that they neither promote shoot meristem initiation on *Nicotiana tabacum* L., cv Havana 425, leaf explants nor induce betacyanin synthesis in amaranth seedlings. Analysis of cultured tobacco pith tissue demonstrated that DCG accumulation was stimulated by cytokinin treatment and correlated with cytokinin-induced cell division. Thus, the accumulation of metabolites that could replace cytokinin in cell division bioassays is stimulated by cytokinins. These data support the model that DCGs are a component of a cytokinin-mediated regulatory circuit controlling cell division.

Naturally occurring cytokinins are N^6-adenine derivatives that are involved in the control of a wide range of biological activities. Many plant tissues require cytokinin, as well as an auxin, to grow and divide in culture (1, 24). In addition to their role in promoting cell division, cytokinins have been shown to be crucial in shoot initiation, delay of leaf senescence, promotion of betacyanin synthesis, release of lateral buds from apical dominance (reviewed in ref. 11), regulation of β-1,3-glucanase and chitinase expressions (23), stimulation of cell wall lignification, and induction of specific phenylpropanoid pathway enzymes, including phenylalanine ammonia lyase, caffeic acid-*O*-methyl transferase, and peroxidase (12). These multiple effects make molecular analyses very complex, and, as of now, the biochemical bases for stimulation of these diverse processes are not known.

Elucidation of the mechanisms involved in cytokinin regulation of one process, such as cell division, would be facilitated by the separation of this action from the other activities of the hormones. One strategy is to determine whether a cellular metabolite that accumulates in the presence of cytokinin can replace it to stimulate a particular process. Such a metabolite could be a downstream component of a regulatory pathway specific to the process in question. More specifically, do cytokinin-stimulated tissues, or cytokinin-autonomous tissues, contain compounds that could substitute for cytokinins in cell division assays? This question is particularly relevant in light of the fact that certain cytokinin-habituated (nonrequiring) cell lines of tobacco and soybean have been reported that do not contain increased levels of either the isopentenyl adenosine or ZR-type cytokinins (9, 10, 30).

Candidates for such a non-adenyl metabolite are the cell division-promoting substances found in fractions isolated from hormone-autonomous *Catharanthus roseus* crown gall tumors by Wood *et al.* (27–29). These tumors, now also known to synthesize zeatin and ZR (18), were recently reinvestigated (16). The fractions isolated by Wood *et al.*(29) were shown to contain DCG[4] isomers capable of replacing cytokinin in the promotion of tobacco pith growth in culture. The individual DCG isomers differed in their ability to stimulate proliferation with isomers A and B (differing only in the enantiomeric nature of the phenylpropanoid dimer) being the most active (5). The relatively inactive isomers D and E differed from A and B only with respect to the location of the glucose moiety, at the C-13 hydroxyl and C′-4 hydroxyl, respectively (5). Moreover, the active DCGs were found in quiescent tobacco pith tissue at low levels (approximately 0.05 μM) but at 20- to 50-fold higher levels in actively growing cultured pith tissue (5). Such results indicated that these naturally occurring, phenolic derivatives could stimulate cell division. To demonstrate that the purified DCGs were, in fact, responsible for the biological activity observed, DCG isomers A and B (hereafter referred to as DCG A + B) have been synthesized chemically and their activities reexamined. The results presented here indicate that synthetic DCG A + B possess cell division-promoting activity but do not share several other biological activities with the adenine-derived cytokinins. In addition, we show that the accumulation of DCG A and DCG B in cultured tobacco pith explants is

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[2] Present address: Department of Agronomy, University of Wisconsin, Madison, WI 53706.

[3] Present address: Samuel Roberts Noble Foundation, Ardmore, OK 73402.

[4] Abbreviations: DCG, dehydrodiconiferyl alcohol glucoside; NAA, α-naphthalene acetic acid; ZR, zeatin riboside; THF, tetrahydrofuran; LS medium, Linsmaier-Skoog medium; t_R, retention time.

regulated by cytokinin in a fashion correlated with the induction of cell division.

MATERIALS AND METHODS

Syntheses

Methyl Ferulate

Ferulic acid was esterified according to the procedure of Brenner and Huber (6). The acid (1.9 g, 10 mmol) was dissolved in 25 mL of methanol. The solution was cooled in an ice bath for 15 min, before thionyl chloride (1.2 g, 10 mmol) was added. The ice bath was replaced with a heating mantle, and the solution was heated to reflux for 1 h. The solvent was removed by rotary evaporation, and the remaining material was chromatographed on silica gel, eluting with ether/pentane (2:1, v/v), to afford methyl ferulate (2.1g, 100%), as a pale yellow oil. ^{1}H-NMR (270 MHz, $CDCl_3$) δ 7.62 (d, 1H, J = 16 Hz, H-7), 7.04 (dd, 1H, J = 8.2, 2.0 Hz, H-5), 7.02 (d, 1H, J = 2.0 Hz, H-2), 6.91 (d, 1H, J = 8.2 Hz, H-6), 6.29 (d, 1H, J = 16 Hz, H-8), 3.93, (s, 3H), 3.80 (s, 3H).

Dehydrodiferulate Dimethyl Ester

Two procedures were used following the general protocol of Wasserman *et al.* (26). In one, methyl ferulate (500 mg, 2.4 mmol) was dissolved in 5 mL of methylene chloride, and the solution was diluted with 45 mL of chloroform. Potassium ferricyanide (1.5 g, 4.6 mmol), dissolved in 50 mL of saturated aqueous sodium bicarbonate, was added, and the biphasic mixture was stirred slowly (the two phases remaining distinct) for 6 h. The phases were separated, and the organic phase was washed with brine, dried with sodium sulfate, and concentrated *in vacuo*. The remaining material was chromatographed on silica gel, eluting with ether/pentane (2:1), to afford a white crystalline solid (180 mg, 36%).

In the alternative procedure, methyl ferulate (1.8 g, 8.6 mmol) was dissolved in 50 mL of chloroform. Saturated sodium carbonate solution (20 mL) and saturated sodium bicarbonate solution (30 mL) were combined, and potassium ferricyanide (8.3 g, 25 mmol) was dissolved in the combined solution. The chloroform solution and the aqueous solution were combined, and the biphasic mixture was stirred slowly (the two phases remaining distinct) for 5 h. The phases were separated, and the solvent was removed by rotary evaporation. The remaining material was chromatographed on silica gel, eluting with ether/pentane (3:2), to afford methyl ferulate (1.05 g) and dehydrodiferulate dimethyl ester (480 mg, 26% conversion, 64% yield), melting point 148 to 150°C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.64 (d, 1H, J = 16 Hz, H-10), 7.18 (bs, 1H), 7.02 (bs, 1H), 6.90 (s, 3H), 6.31 (d, 1H, J = 16 Hz, H-11), 6.11 (d, 1H, J = 8 Hz, H-2), 5.66 (s, 1H, -OH), 4.34 (d, 1H, J = 8 Hz, H-3), 3.92 (s, 3H), 3.87 (s, 3H), 3.83 (s, 3H), 3.80 (s, 3H).

Dimethyl Dehydrodiferulate Tetra-O-Acetyl-β-D-Glucopyranoside

Dehydrodiferulate dimethyl ester proved to be unstable to conditions for a coupling reaction with bromo-tetraacetyl-glucose ($NaOH/H_2O$/acetone, Ag_2O/CH_2Cl_2; 7, 14). Glucosylation was effected instead through a dehydrative coupling with 2,3,4,6-tetra-*O*-acetylglucose, under Mitsunobu conditions (19). Dehydrodimethyl ferulate (750 mg, 1.8 mmol) and 2,3,4,6-tetraacetylglucose (1.25 g, 3.6 mmol) were each dissolved in 4 mL of methylene chloride, and the two solutions were combined. Diethylazodicarboxylate (500 mg, 2.9 mmol) was added to the reaction mixture, which was then stirred for 10 min. Triphenyl phosphine (1.0 g, 3.8 mmol) was dissolved in 5 mL of methylene chloride and then added dropwise to the reaction mixture, pausing when the reaction mixture began to boil until solvent evaporation ceased. The reaction mixture was stirred for 2 h and then added directly to a silica gel column that was eluted with ether/pentane (2:1). The glucoside band was chromatographed on silica gel, eluting with benzene/ethyl acetate (6:1, v/v), to afford a mixture of aryl glycosides, of which dimethyl dehydrodiferulate tetraacetyl-β-glucoside was the major component (490 mg). ^{1}H-NMR (500 MHz, acetone-d_6) δ 7.55 (d, 1H, J = 16 Hz, H-10), 7.26 (bs, 1H, H-5 or H-7), 7.23 (bs, 1H, H-5 or H-7), 7.14 (d, 1H, J = 8.3 Hz, H-5′), 7.09 (d, 1H, J = 1.7 Hz, H-2′), 6.93 (dd, J = 8.3, 1.7 Hz, H-6′), 6.37 (d, 1H, J = 16 Hz, H-11), 6.03 (d, 1H, J = 7.6 Hz, H-2), 5.30 (t, 1H, J = 9.4 Hz, H-3′′), 5.20 (d, 1H, J = 8.0 Hz, H-1′′), 5.14 (dd, 1H, J = 9.4, 8.0 Hz, H-2′′), 5.06 (t, 1H, J = 9.6 Hz, H-4′′), 4.41 (d, 1H, J = 7.6 Hz, H-3), 4.24 (dd, 1H, J = 12.2, 5.2 Hz, H-6′′), 4.10 (dd, 1H, J = 12.2, 2.2 Hz, H-6′′), 3.78 (s, 3H), 3.76 (bs, 6H), 3.69 (s, 3H), 1.95 to 2.05 (four acetate singlets).

Dehydrodiconiferyl Alcohol Glucopyranoside

Dimethyldehydrodiferulate tetraacetyl-glucopyranoside (130 mg, 0.175 mmol) was dissolved in 5 mL of THF. The solution was cooled in an ice bath for 15 min, before diisopropyl aluminum hydride (1.5 mmol, as a 1 M solution in cyclohexane) was added, in four portions at 15-min intervals. The reaction mixture was terminated by the sequential addition of 30 mL of water, 90 mL of 15% aqueous sodium hydroxide solution, and another 30 mL of water. The solid formed was separated from the solution by vacuum filtration, washed with THF, suspended in 20 mL of methanol/water (1:1), and sonicated for 15 min. The insoluble material was removed by filtration through a filter sterilizer, and the mixture was purified by C-8 reverse-phase HPLC, eluting with methanol/water (3:7), to afford dehydrodiconiferyl alcohol glucopyranoside (12.0 mg, 23 mmol, 13%). ^{1}H-NMR (peracetylated, benzene-d_6) δ 7.15 (d, 1H, J = 8.9 Hz, H-5′), 6.94 (d, 1H, J = 1.8 Hz, H-2′), 6.90 (dd, 1H, J = 8.3, 1.8 Hz, H-6′), 6.77 (s, 1H, H-7), 6.76 (s, 1H, H-5), 6.48 (d, 1H, J = 15.9 Hz, H-10), 6.09 (dt, 1H, J = 15.9, 6.5 Hz, H-11), 5.57 (t, 1H, J = 7.8 Hz, H-2′′), 5.45 (t, 1H, J = 7.8 Hz, H-3′′), 5.43 (d, 1H, J = 6.9 Hz, H-2), 5.29 (dd, 1H, J = 7.9, 7.8 Hz, H-4′′), 4.90 (d, 1H, J = 7.7 Hz, H-1′′), 4.66 (d, 2H, J = 6.5 Hz, H-12), 4.32 (dd, 1H, J = 11.2, 5.2 Hz, H-13), 4.20 (dd, 1H, J = 12.3, 4.8 Hz, H-6′′), 4.12 (dd, 1H, J = 11.2, 7.2 Hz, H-13), 3.99 (dd, 1H, 12.3, 2.3 Hz, H-6′′), 3.59 (m, 1H, H-3), 3.47 (s, 3H, H-14), 3.31 (s, 3H, H-7′), 3.25 (m, 1H, H-5′′), and 1.6 to 1.9 (six acetate singlets).

Dihydroconiferyl Alcohol

Coniferyl alcohol (45 mg, 250 mmol) was dissolved in 10 mL of ethanol, in a three-necked flask equipped with a magnetic stirbar, a nitrogen line, a vaccuum line, and a hydrogen-filled balloon. After 15 mg of 5% palladium on carbon was added to the solution, the flask was evacuated and flushed with nitrogen three times, evacuated a fourth time, and filled with hydrogen. This solution was stirred for 1 h. The reaction vessel was flushed with nitrogen three times as above, the reaction mixture was filtered through celite to remove the catalyst, the celite was washed with more ethanol, and the solvent was removed by rotary evaporation to afford dihydroconiferyl alcohol as a yellow oil in quantitative yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.79 (d, 1H, J = 7.9 Hz, H-5′), 6.67 (d, 1H, J = 1.7 Hz, H-2′), 6.65 (dd, 1H, J = 1.7, 7.9 Hz, H-6′), 3.85 (s, 3H, H-7′), 3.65 (t, 2H, J = 6.4 Hz, H-1), 2.63 (t, 2H, J = 7.7 Hz, H-3), 1.85 (m, 2H, H-2). UV (MeOH) λ_{max} = 281 nm, e = 2800.

Enzymatic Production of Dehydrodiconiferyl Alcohol

Coniferyl alcohol (50 mg) was dissolved in 17.5 mL of ethanol. To this solution was added 17.5 mL of water and approximately 250 μg horseradish peroxidase (reinheitszahl, A_{403}/A_{275} (RZ) = 1.2, 95 purpurogallin units/mg solid; Sigma Chemical Co.; 22). Hydrogen peroxide (20 mL of a 0.02% solution) was added dropwise during 30 min. The ethanol was removed by evaporation *in vacuo*. The precipitate formed during the evaporation was taken up in ethyl acetate, and the solution was extracted twice with ethyl acetate. The combined solutions were washed with saturated NaCl, dried with sodium sulfate, and concentrated *in vacuo*, to afford an oil that solidified during prolonged standing (45 mg). ^{1}H-NMR (500 MHz, acetone-d_6) δ 7.07 (bs, 1H), 7.01 (s, 1H), 6.98 (s, 1H), 6.91 (dd, 1H, J = 8.0, 1.5 Hz, H-6′), 6.83 (d, 1H, J = 8.0 Hz, H-5′), 6.56 (d, 1H, J = 16 Hz, H-11), 6.28 (dt, 1H, J = 16, 5.2 Hz, H-12), 5.60 (d, 1H, J = 6.5 Hz, H-2), 4.23 (d, 2H, J = 5.2 Hz, H-13), 3.91 (s, 3H), 3.86 (s, 3H), 3.57 (m, 1H, H-3).

Synthesis of ^{3}H-DCG and Aglycone

[12-^{3}H]-Dehydrodiconiferyl alcohol (2110 Ci/mol) and [12-^{3}H]DCGs A and B (A/B, 1:1; 4400 Ci/mol) were synthesized as described before (21).

Plant Material

Nicotiana tabacum L., cv Havana 425 (H425), plants were maintained by selfing and grown in the greenhouse in 8-inch pots containing a mixture of potting soil/sand/vermiculite (4:2:1). Temperature was maintained between 22 and 30°C, and supplemental lighting (General Electric High Intensity Discharge metal halide lamps, 1000 W) was used to maintain 14-h days. Plants were fertilized with Peters (Fogelsville, PA) 9–45–16 (N-P-K) weekly for the first 6 weeks and Peters 20–20–20 weekly thereafter. Vegetative plants 35 to 45 cm tall, with 19 to 20 leaves >25 mm long, 85 to 100 days old, were used for bioassays. To ensure that plants used for the bioassays were at a similar developmental stage, the number of leaves initiated that were >25 mm long was used as an indicator of age rather than chronological age alone.

N. tabacum L., cv H425, leaf callus cultures were initiated January 26, 1988, and maintained on LS (15) medium containing 1 μM NAA and 0.5 μM kinetin for monthly subcultures. All tissue cultures were incubated at 25 ± 1°C with a light/dark cycle of 16:8 h using fluorescent lights (Cool White, Sylvania, Fall River, MA) at approximately 7 to 9 W/cm^2 (PAR).

Glycine max. L., cv "Mandarin Ottowa," cotyledon-derived callus line C1 was kindly provided by Dr. Richard Amasino, University of Wisconsin, Madison, WI, and cultured on a modified LS medium containing 2× the normal phosphate, 1 mg/L each of nicotinic acid and pyridoxine, 10 mg/L of thiamine, 10 μM NAA, and 1 μM ZR, under the conditions described above.

Assays

Leaf Explants Bioassays

Leaves of *N. tabacum* L., cv H425, plants were surface sterilized as described previously (17). Leaf explant squares, 3 mm per edge, weighing approximately 5 mg, were cut from 12- to 14-cm long leaves and placed two per plate in plastic Petri dishes (35 mm diameter × 10 mm high) containing 2.6 mL of agar-solidified test medium. Test media consisted of LS medium supplemented with NAA plus either ZR or a 1:1 mixture of synthetic DCGs A + B as cell division factors. The ZR and DCGs were filter sterilized and added aseptically to cooling, autoclaved medium. After a 28-d incubation, callus proliferating on each leaf explant was removed, cut into approximately 2-mm^3 (approximately 8 to 10 mg) pieces, with samples from each treatment subcultured onto medium of the exact composition as in the first culture or onto medium lacking the cell division factor, *i.e.*, containing NAA only. After a 21-day incubation, the subcultured callus was harvested, and the fresh weight was measured. Growth data are presented as ln W/W_0, where W is the final fresh weight and W_0 is the fresh weight of the explant at day 0. All bioassays were conducted at least twice with a sample size of six or greater per treatment.

Callus Proliferation Assays

N. tabacum L., cv H425, leaf callus was grown on LS maintenance medium (described above) until the late stationary phase of growth (35 to 40 d postsubculture). Tissues were cut into 3-mm^3 pieces and cultured on experimental media for two transfers as described above for leaf explants.

Soybean callus assays were performed essentially as the tobacco tissue culture assays but on the modified LS medium described above containing 10 μM NAA, and either ZR or DCG A + B was added aseptically to cooling autoclaved medium.

Pith Bioassays

The stems of *N. tabacum* L., cv H425, plants were surface sterilized as described above for leaves. Pith explant cubes, 2 mm per edge, weighing approximately 8 mg, were cut from

the stem segments and placed three per plate in plastic Petri dishes (60 × 15 mm) containing 10 mL of agar-solidified test medium and incubated in the dark for the growth period. Test media were prepared as described for leaf explant bioassays.

For the DCG accumulation experiments, pith tissue was excised from the stems and cut into 20- to 25-mg explants. These were placed, 16 per plate, in 20- × 100-mm plastic Petri dishes containing 25 mL of agar-solidified LS (15) medium supplemented with either 1 μM NAA or NAA + 1 μM ZR. Fresh weights, mitotic indices, DCG A + B, and DCG aglycone levels were measured at the start of the experiment (day 0) and during the culture period on both media.

Mitotic Index

N. tabacum L., cv. H425, leaf callus tissues or pith issues, grown as described above, were fixed in a 1:3 solution of acetic acid and ethanol for 2 to 3 h at room temperature. Cultured leaf tissues were stained using the 4′-6-diamidino-2-phenyl indole protocol (8, 25), and the cultured pith tissues were stained with aceto-orcein (3). For each sample, approximately 1000 nuclei were examined and the number of mitotic figures counted. At least three different callus pieces were counted for each time point. All counts were carried out on samples that were coded in a double-blind fashion.

Shoot Organogenesis Assays

Surface-sterilized *N. tabacum* L., cv H425, leaf explants were cultured on 5 mL of LS medium lacking NAA, but supplemented with either ZR or DCG A + B, incubated for 35 d, and then scored for shoot formation.

Betacyanin Pigment Induction

Surface-sterilized *Amaranthus cruentus* L. seeds (R158, Johnny's Select Seed, Albion, ME) were germinated on moist filter paper for 3 d in the darkness. Seedlings were treated with various doses of either ZR or DCG A + B, in the presence of tyrosine at 1 mg/mL in sodium phosphate buffer, and incubated in darkness for 24 h. Betacyanin pigment was then water extracted from the seedlings and the extract's absorbance read at 542 and 620 nm to correct for particulates (2).

DCG Accumulation

Tissues were removed from culture, weighed, frozen in liquid nitrogen, and stored at −80°C until all samples were collected. The purification and quantitation of the DCGs were carried out using modifications of the method of Lynn *et al.* (16) In this modified procedure, 2 to 8 g of material (approximately 80 to 300 pith cubes) were homogenized in 2 volumes of 1% aqueous β-mercaptoethanol, and an internal standard of approximately 6 nCi [^{3}H]DCG B (specific activity 4400 Ci/mol) was added. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C and the supernatant reduced in volume. This sample was then loaded onto a C_{18} octadecylsilane Sep-Pak column (Waters, Inc.), washed with water and eluted with 50% methanol. The eluent was reduced to dryness *in vacuo*, redissolved in 30% methanol, and analyzed by C_8 and C_{18} HPLC as described (16). The last step was reverse-phase C_{18} HPLC with UV detection to quantify the DCG A + B levels. At this step, the peaks were collected and counted to estimate recovery of the internal standard. The identity of the peaks believed to be DCG A + B was verified by coinjection of a known amount of synthetic DCG A + B with the sample extract.

Dehydrodiconiferyl Alcohol Quantitation

H425 pith tissues were excised and cultured as described above. Tissues were ground in 0.5 to 1.5 mL of 1% aqueous β-mercaptoethanol in a glass Tenbroeck tissue grinder and rinsed with 9.5 to 8.5 mL of the same extraction medium, respectively, into 15-mL centrifuge tubes. Each sample received [12-^{3}H]DCGs A and B (A/B, 1:1, 4400 Ci/mol, 3.78 nCi, 0.66 pmol) and (+)-[12-^{3}H]dehydrodiconiferyl alcohol (2110 Ci/mol, 5.67 nCi, 2.67 pmol). Insoluble material was removed by centrifugation in an IEC clinical centrifuge, and the soluble material was loaded into an octadecylsilane cartridge (Rainin, 1 × 1 cm). The column was flushed with 6 mL each of 15%, and then 60% (v/v), methanol. The latter fraction was dried *in vacuo* and chromatographed on HPLC (DuPont, C8; MeOH:$H_2$0, 32:68, v/v, 1.5 mL/min, t_R DCG A/B 6.6 min and t_R aglycone 14.5 min). Final purification was accomplished with HPLC (Whatman, SiO_2; cyclohexane:$CHCl_3$:MeOH:AcOH, 32:63:4.5:0.5 [v/v/v/v], flow rate 2 mL/min, t_R aglycone 8.2 min).

RESULTS

Dehydrodiconiferyl Alchohol Glucoside A and B and Aglycone Syntheses

The DCGs were synthesized by the overall route shown in Figure 1. The oxidative dimerization (26) followed by Mitsunobu glycosylation (19) gave reasonable yields of the DCGs A and B. The material and its peracetate were spectroscopically and chromatographically identical with the natural product (16). Dehydrodiconiferyl alcohol was produced either by hydrolysis of the phenolic glucoside or by the oxidation of coniferyl alcohol with horseradish peroxidase and dilute hydrogen peroxide (22). The enzymatic reaction was immediate, produced the coupling product in good yields, and was conveniently scaled up. In contrast, attempts to oxidize coniferyl alcohol with potassium ferricyanide resulted in the immediate formation of polymer.

Growth and Cell Division

A mixture of synthetic DCG isomers A and B (1:1) was tested for its ability to stimulate callus proliferation on *N. tabacum* L., cv H425, leaf explants. Because the ratio of cytokinins to auxin is known to be important for growth of cells *in vitro*, a range of NAA doses was incorporated into media containing the DCGs to determine whether the biological activity of the synthetic DCGs was affected by the level of this auxin. The results (Fig. 2) showed that 1 μM NAA was the optimal auxin concentration for DCG activity and was used in all subsequent assays. Additional bioassays were performed to determine whether DCG A + B affected callus

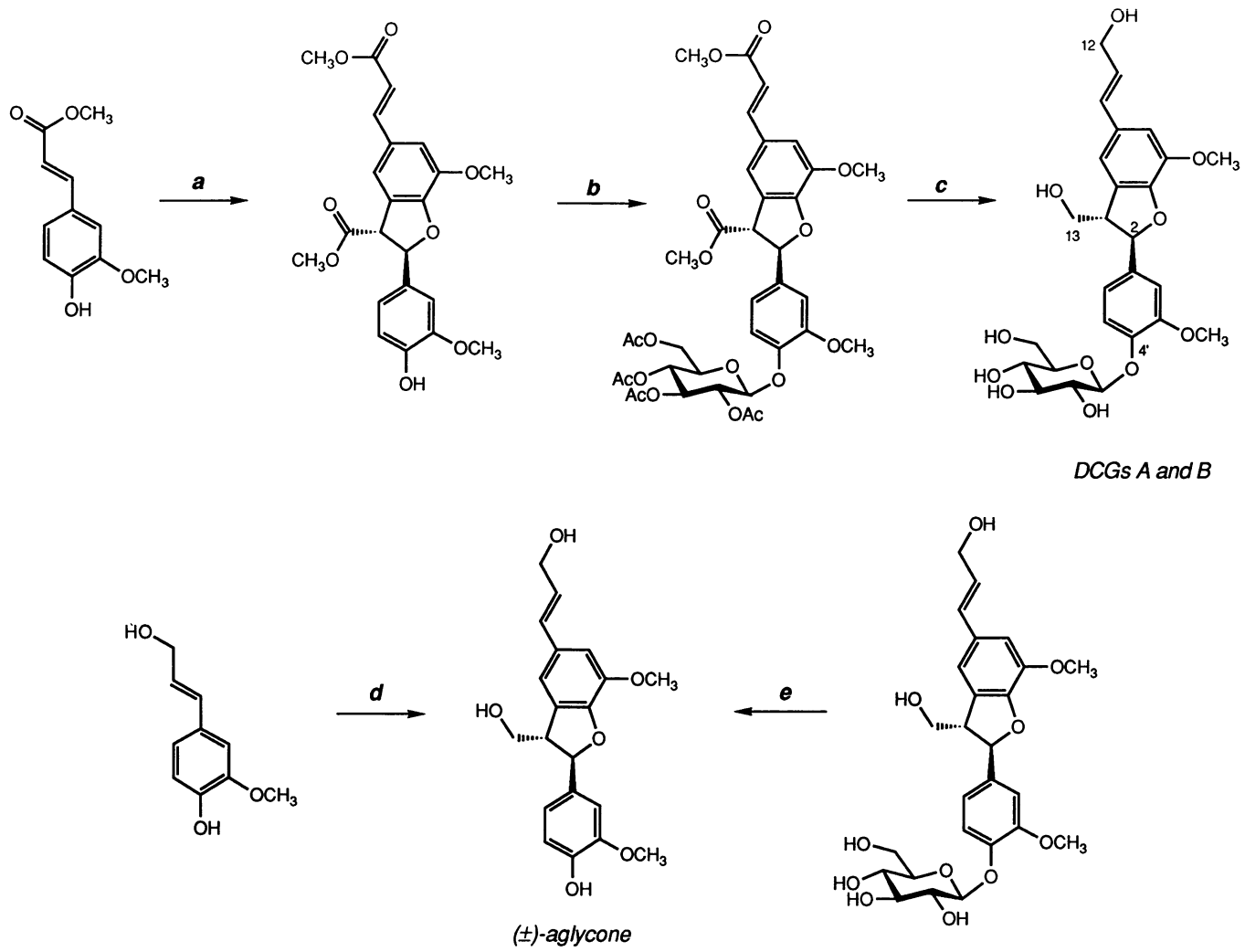

Figure 1. Synthesis of DCGs A + B and the aglycone. a, $K_3Fe(CN)_6$, $CHCl_3$, aqueous CO_3^{-2} 5 h; b, DEAD, Ph_3P, $(Ac)_4$-glucose, CH_2Cl_2, 2 h; c, DIBAL, THF, 0°C, 1 h; d, horseradish peroxidase, H_2O_2, ethanol/H_2O, 30 min; e, β-D-glucosidase (Sigma), 37°C, 45 min.

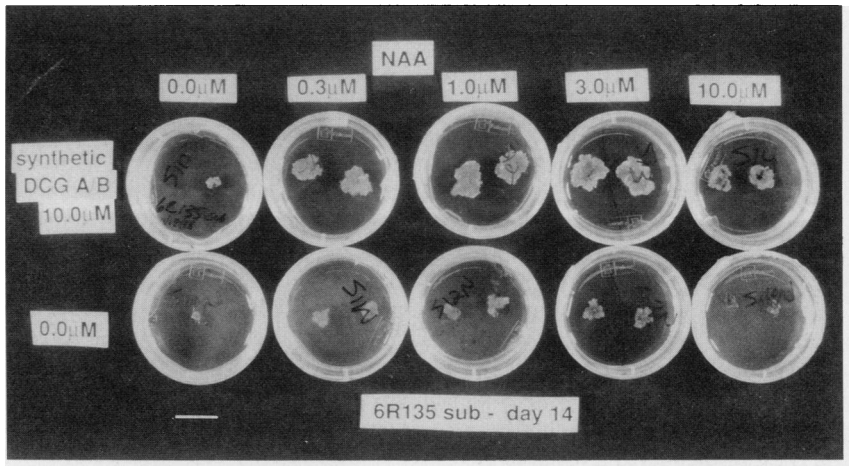

Figure 2. Callus growth after subculture from *N. tabacum* L., cv H425, leaf explants on various doses of NAA +/− 10 μM DCG A + B onto like medium, 21 d after subculture. Bar = 1 cm.

growth on H425 leaf explants in a dose-dependent manner. Typical results indicated that 10 to 30 μM DCG A + B (with 1 μM NAA) was required for callus proliferation and that the highest concentration of DCG A + B tested, 60 μM, was still not growth inhibiting (Fig. 3). The adenine cytokinin, ZR, was optimal at 1 μM and supraoptimal at ≥3 μM. DCG A + B, tested up to 100 μM, did not stimulate growth to the levels achieved at the optimal ZR concentration. Primary pith explants of H425 tobacco, also known to require cytokinin for continuous growth and cell division (4), were provided with either an artificial cytokinin (kinetin) or synthetic DCGs A + B, and their growth response was monitored. DCGs A + B stimulated proliferation of tobacco pith tissue but again, were required at higher concentrations than the adenylate cytokinin (data not shown). Growth of the tissues cultured on the highest levels of DCG tested mimicked the growth observed on suboptimal cytokinin concentrations in terms of both morphology—friable, translucent callus—and fresh weight accumulation.

To test the possibility that the response to the DCGs was due to substances found only in primary explants, we examined the response of a cytokinin-requiring tobacco leaf callus. The results showed that this cell line is stimulated by synthetic DCGs A + B in a dose-dependent fashion (data not shown). To confirm that the active DCGs promoted growth through the stimulation of cell division, this leaf callus line was cultured on 80 μM synthetic DCG A + B or 0.3 μM ZR, with 1 μM NAA, for 21 d and then subcultured onto fresh medium of the same composition. The mitotic index and growth of the cells were then monitored during the subculture. These experiments showed that DCG treatment resulted in exponential growth (Fig. 4A) and stimulation of mitotic activity in the leaf callus (Fig. 4B). DCGs A + B induced a peak of cell division at day 10 of culture, whereas the mitotic index of the auxin-treated tissues remained at a constant low level at the times measured. The ZR-treated tissues exhibited a faster growth rate and earlier peak of mitotic activity.

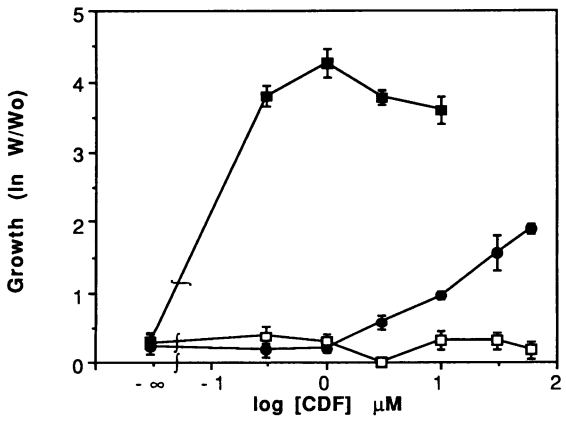

Figure 3. Callus growth after subculture from DCG A + B-stimulated *N. tabacum* L., cv H425, leaf explants onto either the same dose of DCG A + B (+NAA) (●) or NAA alone (□) and from ZR onto the same dose of ZR (■). Growth is measured 22 d after subculture and is expressed as ln W/W_0, where W = final fresh weight (mg) and W_0 = initial fresh weight (10 mg) (±SE, n = 6).

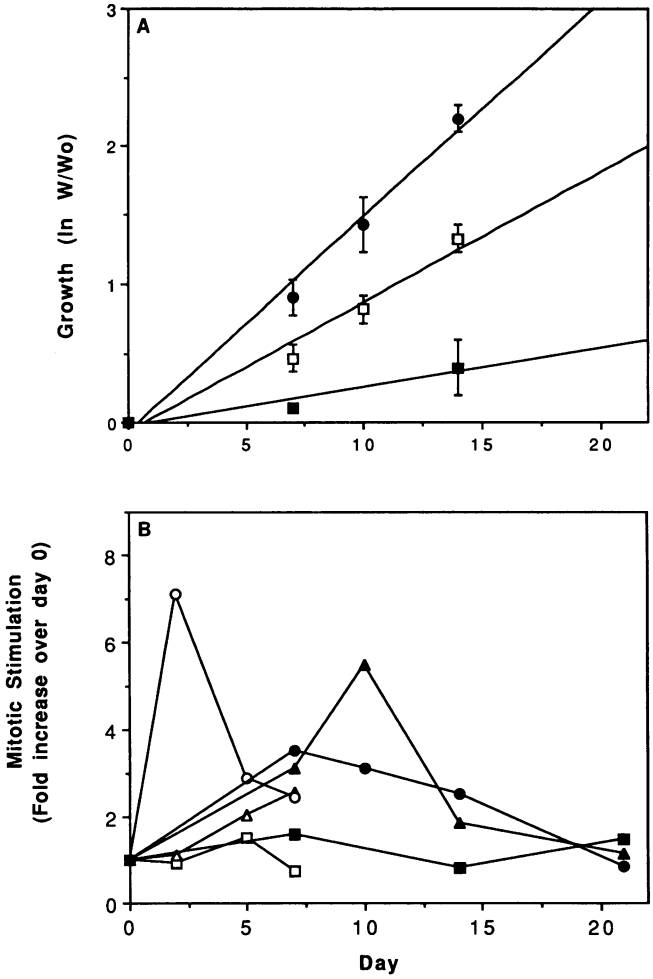

Figure 4. A, *N. tabacum* L., cv H425, leaf callus growth after subculture on medium containing 1 μM NAA alone (■), 1 μM NAA and 80 μM DCG A + B (□), or 0.3 μM ZR (●) for 1 to 21 d (±SE, n = 6). B, Mitotic stimulation of H425 leaf callus subcultured on either 1 μM NAA (□, ■), NAA plus 80 μM DCG A + B (△, ▲), or NAA + 0.3 μM ZR (○, ●). The data from two different experiments, experiment 1 (open symbols) and experiment 2 (closed symbols), are presented as -fold increase over the mitotic index of the starting tissue. The mitotic index of the starting tissue for experiment 1 was 1.13 and for experiment 2, 0.27.

The ability of DCG A + B to substitute for cytokinin and stimulate growth of a cytokinin-requiring soybean cell line was also tested. Line C1, derived from *G. max* L., cv Mandarin Ottowa, was tested over a wide range of ZR and DCG A + B concentrations. Growth stimulation by ZR was observed at approximately 0.001 μM and optimal at 1 μM, whereas the DCGs were not effective at stimulating growth at concentrations up to 100 μM (data not shown). In addition, this cell line did not respond to dihydroconiferyl alcohol, a phenolic known to replace cytokinin in growth promotion of a cytokinin-requiring soybean callus line (13). These results suggest that there can be cell line-specific variation in the response of plant tissues to cell division-promoting phenolics.

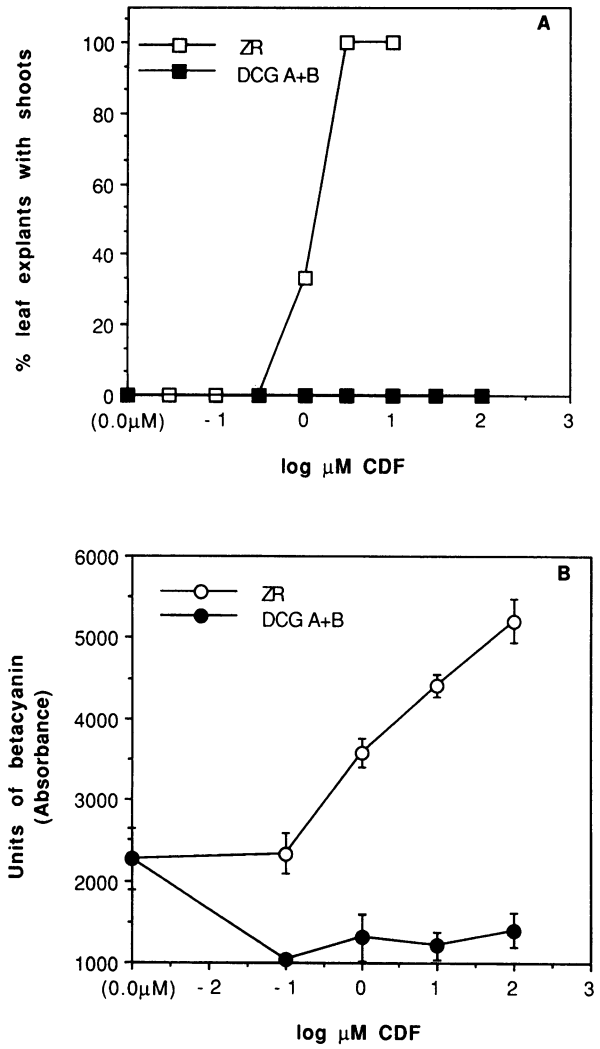

Figure 5. Effect of DCGs in other cytokinin bioassays. A, Shoot initiation in H425 tobacco leaf explants cultured on ZR (□) or DCG A + B (■). B, Betacyanin accumulation in *Amaranthus* seedlings treated with ZR (○) or DCG A + B (●).

Other Cytokinin Bioassays

Cytokinins stimulate shoot organogenesis from cultured leaf explants (24). When H 425 leaf explants were cultured on synthetic DCGs A + B, but without auxin, no shoots were initiated on the explants at concentrations up to 100 μM (Fig. 5A), whereas ZR was required at a minimum concentration of 1 μM. These experiments confirm that the DCGs do not possess the organogenic properties of the cytokinins (5). Cytokinins are also known to induce betacyanin pigment synthesis from tyrosine in *Amaranthus* seedlings (2). At concentrations up to 100 μM, neither synthetic DCGs A + B nor their aglycone stimulated the accumulation of betacyanin in *A. cruentus* L. seedlings (Fig. 5B).

Growth, Mitosis, and DCG A + B Accumulation in Cultured Tobacco Pith

Pith tissue from vegetative tobacco plants is composed of a population of quiescent cells that are primarily in the G_1 or G_0 phase of the cell cycle (1). To examine the possible role of DCGs in cytokinin stimulation of cell division, H425 pith tissues were grown in the presence of either auxin (1 μM NAA) or auxin plus ZR (1 μM), and the levels of DCGs A + B were measured after 0, 1, 3, 5, and 7 d of treatment. In addition, growth and mitotic index were measured every day for the 7 d of incubation. The results of four replicate experiments were combined to determine the average pattern of growth, mitotic index, and DCG accumulation. On a fresh weight basis, the tissues cultured on either NAA or NAA + ZR exhibited similar growth characteristics during the 7-d growth period (Fig. 6A). To determine whether the observed growth of these pith tissues was due to cell division or cell expansion, their mitotic indices were measured daily. The NAA-treated tissues exhibited a low level of mitotic activity during the 7-d culture

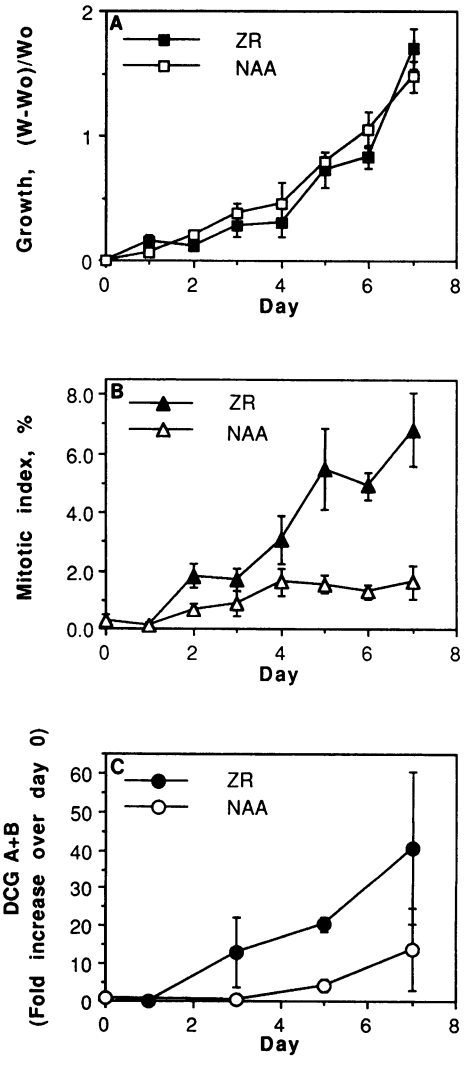

Figure 6. Growth, mitosis, and DCG accumulation of H425 pith tissues cultured on 1 μM NAA (NAA) or 1 μM NAA and 1 μM ZR (ZR) for 7 d. A, Growth; presented as $(W - W_0)/W_0 \pm$ SE ($n = 4$), where W = final fresh weight and W_0 = initial fresh weight (20 mg). B, Mitotic index; the percentage of nuclei in mitosis (±SE, $n = 4$). C, DCG A + B accumulation; -fold increases in DCG A + B levels (over day 0 DCG A + B level) (±SE, $n = 4$).

period (Fig. 6B), indicating that the growth observed in Figure 6A was primarily cell elongation. In contrast, the ZR-treated pith showed substantial mitotic activity, with the first peak of mitotic activity observed at day 5 of culture (Fig. 6B) followed by a subsequent increase at day 7. These results clearly document the cytokinin-mediated control of mitosis in these tissues.

Quantitation of the DCGs in these tissues demonstrated that their absolute amounts varied between experiments, ranging from 0.04 to 1.3 μM on day 0 and from 0.8 to 24 μM at the peak of DCG accumulation in ZR-treated pith. Therefore, the data were plotted as the -fold increase in concentration of DCG A + B over the day 0 level, to compare relative changes in DCG levels with growth and cell division. In tissues grown on NAA and ZR, the DCG A + B levels began to accumulate at day 3 and then increased further through day 7 to approximately 40 times the day 0 levels (Fig. 6C). This increase was temporally correlated with mitotic activity observed in these tissues (Fig. 6B). In the NAA-treated tissue the absolute DCG levels were much lower (0.2–2 μM at peak), and there was a much smaller increase in the DCG A + B level (Fig. 6C). The presence of some mitotic activity did correlate with low levels of DCG A + B observed in these samples.

Control of DCG Accumulation

The results described above indicated that cytokinin stimulates the accumulation of DCG A + B in a fashion that is correlated in time with the stimulation of mitosis by this hormone. To investigate the level at which this accumulation was controlled, we monitored the levels of the aglycone (dehydrodiconiferyl alcohol) in pith tissues grown in the presence or absence of cytokinin. The pattern of DCG A + B accumulation was also monitored in the same tissues so that a direct comparison could be made between the aglycone and DCG levels. The results of these tests showed that the aglycone accumulated to approximately the same extent in tobacco pith tissue grown on either NAA or NAA and ZR, increasing from approximately 0.5 to 1.0 μM to 8 to 12 μM within the first 7 d of culture in both cases. As expected from the results described above, the DCGs accumulated to a much greater extent in the ZR-treated tissues. Particularly striking was the observation that the ratio of the aglycone to DCG A + B was relatively low in both auxin- and auxin plus cytokinin-treated tissues in the first 3 d of the culture period but then increased to quite high levels in the auxin-treated tissues (Fig. 7).

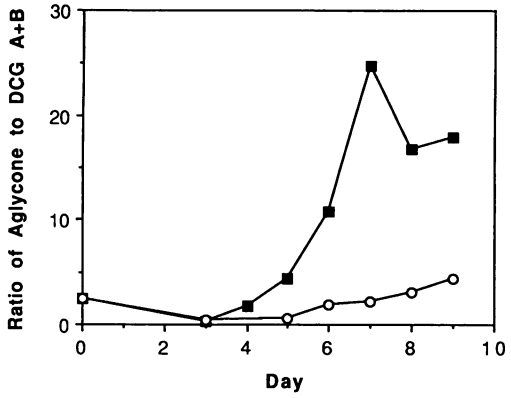

Figure 7. Ratio of aglycone (dehydrodiconiferyl alcohol) to DCG A + B in pith tissue during 9 d of culture on either NAA (1 μM; ■) or ZR + NAA (1 μm each; ○).

DISCUSSION

The bioassays performed with the synthetic DCGs confirm the results of Binns *et al.* (5) that DCGs A + B can sustain the proliferation of cytokinin-requiring tobacco tissues. The synthetic DCG A + B stimulates cell division of cultured leaf callus, as demonstrated by the logarithmic growth and mitotic activity of the tissues cultured in its presence (Fig. 4). In addition, the morphological phenotype and magnitude of the growth responses of the DCG-stimulated tissues differed from those treated with ZR or kinetin. Tissues grown even on the highest DCG concentrations tested closely resembled those stimulated by suboptimal cytokinin levels. Thus, the DCGs could not replace all of the growth-promoting activities of cytokinins but, rather, had only some subset of the growth-promoting activity of the adenine cytokinins. The DCGs also did not exhibit other activities of cytokinins. Of particular interest is the fact that tobacco leaf explants would respond to the DCGs in growth and cell division assays but would not respond to them in shoot initiation assays, even at concentrations of 100 μM. These results suggest that the activity of the DCGs is specific to promotion of growth and cell division.

DCGs A + B are present at low levels in quiescent or auxin-treated pith tissues but accumulate to high levels in auxin plus cytokinin-treated pith in a pattern that is temporally correlated with the cytokinin-induced mitotic activity of the cells. However, because the pith tissue is a heterogeneous mixture of dividing and quiescent cells, it is not known whether the DCGs are localized to tissue regions active in mitosis. This will require the development of techniques that will allow us to estimate the *in situ* distribution of these phenolics. The DCGs can be present at times when cells are not actively dividing. For example, we have found high levels of DCGs at day 21 of the culture period on auxin- and cytokinin-containing medium, whereas cell division is not active in such tissues (data not shown). Thus, although an increase in DCG levels may be necessary for cell division to occur, other factors, which could vary during the cell cycle, may also be required. These results can be compared to the situation in animal cells in which the level of Maturation Promoting Factor or $p34^{cdc2}$, the catalytic subunit of a protein kinase, is constant through the cell cycle but the levels of the factors, *e.g.* cyclins, it interacts with change with stage of the cell cycle (20).

Whereas cytokinin treatment caused extensive DCG accumulation in cultured tobacco pith in relation to its original concentration, the absolute amounts of DCG varied greatly. This suggests that it is not the concentration of DCG A + B that is critical but either the relative increase in its level or the ratio of DCG A + B to some other cellular component. Most striking in this regard is the fact that the ratio of aglycone to DCG was consistently high in the auxin-treated tissue and low in the auxin plus cytokinin-treated tissues. Preliminary data have revealed that feeding exogenous DCGs, along with

auxin to tobacco pith tissues, results in a lower aglycone/DCG A + B ratio. The relative lack of importance of the absolute level of growth-regulating substances is not unprecedented: the ratio of auxin/cytokinin, rather than their absolute values, controls a variety of developmental processes, including growth, organogenesis, and lignification.

The finding that dehydrodiconiferyl alchohol—the aglycone of DCGs A + B—accumulates in tobacco pith tissue cultured on either auxin or auxin plus cytokinin, whereas the DCGs accumulate to high levels only in the latter, suggests that cytokinin controls either the glycosylation of the aglycone and/or the stability of the DCGs. At this point we cannot distinguish between these possibilities. Orr (21) observed glucosyl transferase activity in tobacco pith that can glycosylate the aglycone to yield DCG A + B. It is not clear, however, whether cytokinin actually controls the specific activity of this enzyme(s). The alternative is that cytokinin blocks degradation of DCG A + B that is made continuously in the presence of auxin.

Despite years of intensive investigation, the mechanism whereby cytokinins control cell division in cultured plant cells has not been elucidated. The experiments reported here demonstrate that synthetic DCGs can stimulate growth and cell division in cytokinin-requiring tobacco cell lines, supporting the conclusion that DCGs A + B are specific, naturally occurring cell division factors in tobacco. Moreover, cytokinin induces DCG accumulation in tobacco pith tissue in a time frame that correlates with the cytokinin induction of mitosis. Thus, the accumulation of a metabolite known to influence cell division is regulated by cytokinin. Taken together, these observations are consistent with the hypothesis that the DCGs are downstream components of a cytokinin regulatory cascade controlling cell division. On the other hand, the inability of the DCGs to stimulate growth of a cytokinin-requiring soybean callus line indicates either that these molecules are not general cell division factors or that their activity is susceptible to other processes we were unable to control. For example, lack of uptake, metabolism into an inactive form, or degradation of the DCGs could lead to a negative result. An alternative possibility is that the DCGs are one component of a multifactor pathway involved in cytokinin-stimulated cell growth and division. A lack of, or more stringent requirement for, these other proposed factors could be responsible for the lack of activity by DCGs seen in the soybean callus culture assays.

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