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BARD

FINAL REPORT

PROJECT NO. US-711-83

**Immunochemical Methods to Elucidate
Hormonal Mechanisms in Plant Senescence and
to Monitor Responses to Stress**

L. Brandon, A. Maoz

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Bet Dagan, ISRAEL

BARD Project No. US-711-83

Title of Research Project:

Immunochemical methods to elucidate hormonal mechanisms in plant senescence and to monitor responses to stress

Principal Investigator: David L. Brandon

Cooperating Investigator: Arie Maoz

Name and Address of Affiliated Institutions:

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Cooperating Institution: Israel Agricultural Research Organization, Volcani Center, Bet Dagan

Project's Starting Date: 19 September 1984

Type of Report: Final

Signatures:

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Institution's Authorizing Official

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Director, WPRC

630.72 : 581.372 : 581.134.1 : 581.134.15:
BAR/BRA 574.192

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PROJECT ABSTRACT

Immunochemical methods to elucidate hormonal mechanisms in plant senescence and to monitor responses to stress

A series of 9-substituted derivatives of four common naturally occurring cytokinins was prepared and tested for growth response in the soybean callus assay. The nature of the dose-response curves indicate that the activities of the derivatives are a function of their affinities to a common receptor site and that their steric and polar properties determine that affinity.

The 9-2-carboxyethylcytokinins provided a practical source of haptens for raising antibodies to cytokinins, and an ideal method for preparation of antibodies directed against cytokinin-O-glucosides. Since glucosylation of zeatin may be a key regulatory step in hormonal control of senescence, E-9-2-carboxyethyl-O- β -D-glucopyranosylzeatin was synthesized and monoclonal antibodies specific for this cytokinin were prepared and characterized. The antibodies were used to determine zeatin-O-glucoside in several plant tissues.

A new procedure for coupling small molecules with carboxyl groups to proteins was developed. It can be used with molecules that are soluble or insoluble in water. The procedure produces highly immunogenic hapten-conjugates. The problems of insoluble and antigenic isourea compounds associated with carbodiimide reagents are avoided by this procedure. The method was applied to the production of hapten conjugates of cytokinins, auxins, and other small molecules for antibody production. The procedure is also effective in the production of enzyme-labeled ligands for ELISA. Cytokinin conjugates of horseradish peroxidase produced in this way were highly active and stable in solution. The immunochemical procedures should be readily adaptable to kit form.

Immunochemical methods were used to quantitate changes in cytokinin concentrations in a model system for soybean senescence and in preliminary experiments concerned with maturation and responses to nutritional and environmental stresses in several species. We are currently using the chemical and immunochemical methods developed during the project to define regulatory enzymes and receptors which mediate cytokinin actions.

The standardization and improved resolution of monoclonal antibody-based analytical techniques, including immunoassays, immunoaffinity methods, immunocytochemistry, and anti-idiotypic antibodies, should contribute to the elucidation of hormonal mechanisms in translocation of photosynthate, senescence of the photosynthetic apparatus, post-harvest senescence, and responses to pathogens and environmental stress.

OBJECTIVES

1. To develop permanent hybridoma cell lines for preparation of monoclonal antibodies directed against naturally occurring phytohormones (indoleacetic acid and cytokinins) and their presumptive storage forms (ester and glucosides) for use in high-resolution immunoassay.
2. To develop monoclonal antibodies directed against cytokinin receptors which mediate the effects of these phytohormones.
3. To use these antibodies for quantitation of endogenous hormone levels and for isolation and characterization of cytokinin receptors in plant tissues undergoing senescence and responses to stress.

Our working hypothesis was as follows. Endogenous hormone levels are determined by complex pathways and interactions, one feature of which is the presence of conjugated forms of phytohormones (such as cytokinin glucosides). Quantitation of all naturally occurring forms of the hormones is probably necessary to relate hormone concentration to physiological action. In addition, their sites of synthesis and action, signals which regulate these processes, and transport phenomena must be characterized.

The overall objectives were somewhat broadened from our original proposal because we believed that the antibodies produced under Objective 1 (the objective of the original proposal) would provide a route to the antibodies in Objective 2. The work focussed primarily on the cytokinins, compounds with which we had more experience in synthesis, purification, and analysis, but some work was done on indoleacetic acid derivatives and conjugates. Objective 3 is consistent with the original proposal, with the additional emphasis on cytokinin receptors.

In accordance with our original plans, we have applied immunochemical methods to analysis of cytokinins in several systems. Selection of systems and species (soybeans, corn, pepper, and pine) was motivated in large measure by the facility with which collaborations developed during the course of the project. Much work remains to be done to relate immunochemical, physicochemical, and bioassay data, and to improve the sensitivity and reliability of immunoassays as applied to diverse experimental systems.

established method of synthesizing cytokinin haptens is to oxidize the ribose ring of the corresponding nucleoside to a dialdehyde with periodate. The dialdehyde is then coupled to a carrier protein and the resulting aldimine is reduced with borohydride to complete the antigen (Erlanger and Beiser 1964; Hacker et al. 1972). It is obvious that other sugar moieties would not survive the oxidation steps. The method is thus inappropriate for attaching intact cytokinin glucosides to proteins. Cytokinin analysis is especially complicated since there are many diverse forms that must be estimated individually (Letham and Palni, 1983) and the immunoreagent specificities, or lack of them, must be taken into account. Of necessity, a number of reagents may be required for analysis, even if some determinations can be made by difference: e.g., analyses done before and after treating with a specific enzyme (Badenoch-Jones et al. 1984). Alternatively, a preliminary separation of cytokinins may be done (HPLC) and then the assay performed using appropriate conversion factors (Badenoch-Jones et al. 1987; Sayavedra-Soto et al. 1988). A direct and specific analysis of individual components is to be preferred, and precise structuring of haptens can lead to monoclonal immunoreagents that will make it possible.

Materials and Methods

Chemicals

Cytokinin standards were synthesized in this laboratory by methods described adequately in the literature. Commercial chemicals were obtained as indicated, where important. Analytical and melting point data are given in Table I. Proton NMR spectra were measured at room temperature on Varian EM 390 (90 MHz) and Nicolet NMC-200 (200 MHz) spectrometers. Since the modifications of the O-glucoside syntheses are of sufficient importance, these syntheses are described in detail. The syntheses of 9-substituted cytokinins were carried out in the usual manner by reaction of one of the substituted 6-chloropurines with the amine or amino alcohol of choice, to give the desired product, using 1-BuOH as solvent and N,N-diisopropylethylamine as a proton sink. The 9-(2-carboxyethyl)cytokinins were isolated by adjusting the cold, concentrated aqueous soln of the reaction mixture (after removal of the 1-BuOH and amine in vacuo) to a pH of 4.0-4.2. All of the cytokinin derivatives were purified by chromatography on silica using appropriate EtOAc-MeOH gradients.

6-Chloro-9-(2-carbomethoxyethyl)purine, 6-chloro-9-(2-carbo-t-butoxyethyl)purine and 6-methylthio-9-(2-carbomethoxyethyl) purine were synthesized by the Michael addition of methyl acrylate and t-butyl acrylate to the 6-substituted purine using conditions reported by Baker and Tanna (1965) for the addition of acrylonitrile to 6-chloropurine.

6-Chloro-9-(2-carboxyethyl)purine. A soln of 10.4 g (0.0432 mol) of 6-chloro-9-(2-carbomethoxyethyl)purine in 400 mL dioxane, 20 mL H₂O

Table 1. Melting points and elemental analysis^a

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	9C ₂ CO ₂ tBu	80-81	C ₁₇ H ₂₇ N ₅ O ₃	58.43	7.79	20.04	58.4	7.80	20.0
	9C ₂ CO ₂ H	172-173	C ₁₃ H ₁₉ N ₅ O ₃	53.23	6.53	23.88	53.4	6.58	23.8
21p	9C ₂ CN	148-150	C ₁₃ H ₁₆ N ₆	60.92	6.29	32.79	60.8	6.23	32.7
	9C ₂ CO ₂ tBu	90-91	C ₁₇ H ₂₅ N ₅ O ₂	61.61	7.60	21.13	61.6	7.61	21.2
	9C ₂ CO ₂ H	193-194	C ₁₃ H ₁₇ N ₅ O ₂	56.71	6.23	25.44	56.8	6.26	25.6
Z-Z	9C ₂ CN	170-172	C ₁₃ H ₁₆ N ₆ O	57.34	5.92	30.86	57.6	5.95	30.7
	9C ₂ CO ₂ tBu	111-112	C ₁₇ H ₂₅ N ₅ O ₃	58.77	7.25	20.16	58.5	7.08	20.1
	9C ₂ CO ₂ H	204-206	C ₁₃ H ₁₇ N ₅ O ₃	53.6	5.88	24.04	53.6	5.99	23.8
E-Z	9C ₂ CN	123	C ₁₃ H ₁₆ N ₆ O	57.34	5.92	30.86	57.3	5.85	30.5
	9C ₂ CO ₂ tBu	95-97	C ₁₇ H ₂₅ N ₅ O ₃	58.77	7.25	20.16	58.9	7.23	19.9
	9C ₂ CO ₂ H	182	C ₁₃ H ₁₇ N ₅ O ₃	53.6	5.88	24.04	53.6	5.86	23.7
6-ClP	9C ₂ CO ₂ CH ₃	93.5	C ₉ H ₉ ClN ₄ O ₂	44.92	3.77	23.28	45.1	3.93	22.9
	9C ₂ CO ₂ tBu	92	C ₁₇ H ₂₅ ClN ₄ O ₂	50.97	5.35	19.82	51.1	5.54	19.8
	9C ₂ CO ₂ H	163-165	C ₈ H ₇ ClN ₄ O ₂	42.4	3.11	24.72	42.7	3.40	24.7
6-MeSP	9C ₂ CO ₂ CH ₃	123-125	C ₁₆ H ₁₂ N ₄ O ₂ S	47.6	4.80	22.21	48.0	4.91	21.8

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and 50 mL N NaOH was stirred 2 h at room temp. Nearly all the solvent was removed by roto-evaporation and 50 mL of N HCl was added. The mixture was chilled and the precipitate collected; 9.00 g (92%); recryst MeOH-pet ether; mp 163-165°; ¹H NMR (200 MHz, DMSO-d₆) δ 8.80 (1H, s, 7 or 8-H), 4.52 (2H, t), 2.97 (2H, t, CH₂CO).

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E-2-Methyl-4-phthalimidobut-2-enyl 2,3,4,6-tetra O-acetyl-β-D-glucopyranoside. The general procedure of glucuronide formation of Conrow and Bernstein (1971) was followed. A mixture of 11 g (0.048 mol) of E-2-methyl-4-phthalimidobut-2-enol (mp 104-105°, Corse and Kuhnle, 1972) and 17.2 g of CdCO₃ (Johnson and Matthey, Seabrook, N. H.) in 1200 mL of toluene was dried by distilling about 200 mL of toluene from the flask (stirring). A soln of 39.7 g (0.1 mol) of 2,3,4,6-tetra-O-acetyl-α-glucopyranosyl bromide (acetobromoglucose) in 200 mL of dry toluene was added to the stirred, refluxing mixture over 1 h. The hot mixture was filtered through a Celite pad and the toluene was removed in vacuo from the filtrate. The resulting tan gum (46 g) contained the desired product, starting alcohol and its acetate. Purification was simplified by acetylating the entire mixture with 50 mL of acetic anhydride, 135 mL of pyridine and 1 g of 4-dimethylaminopyridine (steam bath, 1/2 h). The volatiles were removed in vacuo and the residue was dissolved in EtOAc and the soln washed with H₂O, dil HCl, KHCO₃ soln and H₂O. After drying and removal of the solvent, 29.8 g remained, which was divided into 4 portions and chromatographed on silica (J. T. Baker, flash chromatography grade, 40 μm, 50 cm x 4 cm column, hexane-EtOAc (65-35); yield 14.36 g (53.3%); mp 118-120°; ¹H NMR identical to that reported by Duke et al. (1978).

E-2-Methyl-4-phthalimidobut-2-enyl-β-D-glucopyranoside. A soln of 4 g of E-2-methyl-4-phthalimidobut-2-enyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside in 500 mL MeOH was stirred 16 h with 5 g Amberlyst A-26 (OH) prepared in MeOH (Reed et al. 1981). The catalyst was removed by filtration, the volatiles removed in vacuo and the residue recrystallized from MeOH-Et₂O; yield 2.27 g (74%); mp 143-145°; m/z (chemical ionization using NH₃) 411.1745 (calcd for C₁₉H₂₇N₂O₈; 411.1767); ¹H NMR (200 MHz, DMSO-d₆) δ 7.86 (4 H, m, Ar), 5.50 (1H, t, C=CH), 5.03 (1 H, d, glucose OH), 4.96 (2 H, m, glucose OH), 4.46 (2 H, t, CH₂N), 4.22 (2 H, dd, CH₂OH, J=28), 4.12 (1 H, anomeric H, J=8), 3.63 (1H, q, glucose OH), 2.8-3.4 (4 H, m, glucose H), 1.78 (3H, s, CH₃).

E-9-2-Carboxyethyl-O-β-D-glucopyranosylzeatin.

E-2-Methyl-4-phthalimidobut-2-enyl-β-D-glucopyranoside (1.17 g; (3mmol) dissolved in a mixt of 26.9 mL 2-PrOH and 4.54 mL H₂O was

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PROJECT STATEMENT

A. Synthesis and biological activity of 9-substituted cytokinins

J. Corse, R. S. Pacovsky, M. L. Lyman, and D. L. Brandon

Introduction

Structure-activity studies for small organic molecules have been very rewarding in a number of fields--medicine, pesticides research and the study of a wide range of plant growth regulators. Similarly, much work has been done with the cytokinins. After the discovery of kinetin (Miller et al. 1955), there was a flurry of syntheses that led to the finding of N⁶-benzyladenine (BA)¹, an active, easily obtainable cytokinin. A tremendous amount of research in plant physiology was done with BA, much of which may not be closely related to actual plant biochemistry. The isolation and identification of the natural cytokinin E-zeatin (Letham 1964) led to another surge of syntheses as well as the search for other natural cytokinins. The search for high-activity synthetic cytokinins with potential use in agriculture was dampened by the reports that BA was found in the tRNA of callus treated exogenously with BA (Armstrong et al 1976). However, the usefulness of structure-activity relationships still remains, both to elucidate the mechanism of cytokinin action and to provide information about the receptor sites involved in physiological responses.

Curiously, very little research has been done on non-glycosidic substitution at the 9-position in natural cytokinins (Matsubara 1980). Two members of this class do occur naturally, lupinic acid, the 9-alanyl conjugate of E-zeatin, and dihydrolupinic acid (Duke et al. 1978).

The present research had two goals. The first sought to explore a neglected region of the natural cytokinins--the vicinity of the 9-position--to determine what influence substituents might have on dose-activity relationships. A short series of related substituents was selected for observation. The 9-2-carboxyethyl-, 9-2-carbo-t-butoxyethyl- and 9-2-nitriloethyl- derivatives of EZ, ZZ, DHZ and 2ip were synthesized and assayed (soybean callus) for cytokinin activities.

The second goal, closely related to the first from a chemical standpoint, was to develop a general method of synthesizing haptens useful in preparing immunochemical reagents for the analysis and localization of cytokinins, particularly the glycosides. The

¹Abbreviations used: EZ, E-zeatin; ZZ, Z-zeatin; DHZ, (R,S)-dihydrozeatin; 2ip, N-(3-methyl-2-butenyl)-1H-purin-6-amine; -C₂CO₂CH₃, 2-carbomethoxyethyl; -C₂CO₂tBu, 2-carbo-t-butoxyethyl; -C₂CO₂H, 2-carboxyethyl; -C₂CN, 2-nitriloethyl; BA, N⁶-benzyladenine; LA, lupinic acid; EZOG, E-O-β-D-glucopyranosylzeatin; EZOG9C₂CO₂H, E-9-2-carboxyethyl-O-β-D-glucopyranosylzeatin; DMSO, methyl sulfoxide.

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Attempts to hydrolyse the t-butyl group from 6-chloro-9-(2-carbo-t-butoxyethyl)purine by aqueous acid catalysis resulted in the simultaneous displacement of the 6-chloro group to yield 9H-hypoxanthin-9-ylpropionic acid (Baker and Tanna 1965). Treatment of an anhydrous ethyl ether soln of the t-butyl ester with anhydrous HCl in ether gave a precipitate that, when isolated, also gave 9-H-hypoxanthin-9-ylpropionic acid upon treatment with ice-H₂O.

E-2-Methyl-4-phthalimidobut-2-enyl 2,3,4,6-tetra O-acetyl-β-D-glucopyranoside. The general procedure of glucuronide formation of Conrow and Bernstein (1971) was followed. A mixture of 11 g (0.048 mol) of E-2-methyl-4-phthalimidobut-2-enol (mp 104-105°, Corse and Kuhnle, 1972) and 17.2 g of CdCO₃ (Johnson and Matthey, Seabrook, N. H.) in 1200 mL of toluene was dried by distilling about 200 mL of toluene from the flask (stirring). A soln of 39.7 g (0.1 mol) of 2,3,4,6-tetra-O-acetyl-α-glucopyranosyl bromide (acetobromoglucose) in 200 mL of dry toluene was added to the stirred, refluxing mixture over 1 h. The hot mixture was filtered through a Celite pad and the toluene was removed in vacuo from the filtrate. The resulting tan gum (46 g) contained the desired product, starting alcohol and its acetate. Purification was simplified by acetylating the entire mixture with 50 mL of acetic anhydride, 135 mL of pyridine and 1 g of 4-dimethylaminopyridine (steam bath, 1/2 h). The volatiles were removed in vacuo and the residue was dissolved in EtOAc and the soln washed with H₂O, dil HCl, KHCO₃ soln and H₂O. After drying and removal of the solvent, 29.8 g remained, which was divided into 4 portions and chromatographed on silica (J. T. Baker, flash chromatography grade, 40 μm, 50 cm x 4 cm column, hexane-EtOAc (65-35); yield 14.36 g (53.3%); mp 118-120°; ¹H NMR identical to that reported by Duke et al. (1978).

E-2-Methyl-4-phthalimidobut-2-enyl-β-D-glucopyranoside. A soln of 4 g of E-2-methyl-4-phthalimidobut-2-enyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside in 500 mL MeOH was stirred 16 h with 5 g Amberlyst A-26 (OH) prepared in MeOH (Reed et al. 1981). The catalyst was removed by filtration, the volatiles removed in vacuo and the residue recrystallized from MeOH-Et₂O; yield 2.27 g (74%); mp 143-145°; m/z (chemical ionization using NH₃) 411.1745 (calcd for C₁₉H₂₇N₂O₈; 411.1767); ¹H NMR (200 MHz, DMSO-d₆) δ 7.86 (4 H, m, Ar), 5.50 (1H, t, C=CH), 5.03 (1 H, d, glucose OH), 4.96 (2 H, m, glucose OH), 4.46 (2 H, t, CH₂N), 4.22 (2 H, dd, CH₂OH, J=28), 4.12 (1 H, anomeric H, J=8), 3.63 (1H, q, glucose OH), 2.8-3.4 (4 H, m, glucose H), 1.78 (3H, s, CH₃).

E-9-2-Carboxyethyl-O-β-D-glucopyranosylzeatin.

E-2-Methyl-4-phthalimidobut-2-enyl-β-D-glucopyranoside (1.17 g; (3mmol) dissolved in a mixt of 26.9 mL 2-PrOH and 4.54 mL H₂O was

stirred overnight with 0.57 g of sodium borohydride (Osby et al 1984). Glacial AcOH (3.14 mL) was added and the soln heated 2 h at 80° and then evaporated to dryness in vacuo. The dry residue was triturated several times with 5 mL portions of ether to remove phthalide. The remainder was heated 10 h at 100-105° with 0.6 g of 6-chloro-9-2-carboxyethylpurine, 20 mL of 1-BuOH and 2 mL of N,N-diisopropylethylamine. The volatiles were removed in vacuo and the residue dissolved in 4.5 mL of DMSO and the filtered soln applied to a 21.4 mm x 50 cm HPLC column (C-18, 8 micron). Elution was carried out with CH₃CN-H₂O (6%-94%); yield 0.199 g (14.6%); mp 195-198° (dec) with sintering at 165°; m/e 453.1839 (calcd for C₁₉H₂₇N₅O₈: 453.1758; ¹H NMR (200 MHz, DMSO-d₆ + D₂O) δ 8.20 (1H, s, 7 or 8-H), 8.03 (1H, s, 7 or 8-H), 7.38 (1H t, NH), 5.62 (1H, t, =CH), 4.36 (2H, t, NCH₂C), 4.21 (2H, d, NCH₂C=), 4.14 (H, d, anomeric H), 4.02 (2H, dd, J=25, CH₂O), 3.54 (2H, q, glucose H), 2.9-3.3 (3 H, cmplx, glucose H), 2.85 (2H, t, CH₂CO₂), 1.73 (3H, s, CH₃).

Bioassays.

The cytokinins were dissolved in DMSO (Aldrich Chem. Co., Milwaukee, WI 53210) and these solns were incorporated into the basal medium (Miller, 1968) to achieve final concentrations of 10, 1.0, 0.1, and 0.01 μM. The concentrations of all test solutions were checked by UV spectrophotometry using published or calculated extinction coefficients. A basal medium control, in triplicate, was included with each assay replication.

The soybean (Glycine max (L.) Merr cv. Acme) callus, derived from the cotyledons of germinating seedlings was a gift from Dr. S. S. Hua (WRRRC, Albany, CA) and has been maintained for over 8 years in our laboratory. This callus was cytokinin-dependent and has been maintained on basal medium solidified with agar (1.0%, w/v) and supplemented with α-naphthaleneacetic acid (2 mg L⁻¹) and kinetin (0.6 mg L⁻¹). All cultures were grown at 28°C in the dark. Callus tissue for the bioassay was used 28 days after sub-culture.

Cytokinin bioassays were performed according to Miller (1968). Three callus explants, approximately 30 ± 4 mg each, were placed in each 125 ml Erlenmeyer flask containing 30 ml of medium. Test solutions of cytokinins were added to autoclaved medium (120°C for 20 min) in individual flasks just prior to gelation. The concentration of DMSO in the final medium was kept below 0.35% (v/v) and DMSO was also added to the control flasks. Callus fresh weights were determined after growth for 28 days at 28°C in the dark. The results presented are the average of eight flasks for each cytokinin concentration tested.

Means and variances were determined for each compound x derivative x concentration combination (60 total treatments). Transformation of the means and variances for each treatment to log₁₀ values was used in an analysis of variance (ANOVA) to determine main effects, independent of concentrations, using the procedures of the Statistical Analysis Systems (Freund and Littell 1981). To measure these interactions, ANOVA was performed separately on untransformed data at 1.0 and 10 μM incorporated cytokinin.

Results and Discussion

All of the 9- β -substituted ethyl derivatives of the four natural cytokinins tested showed considerable activity (Figs. 1-4). The pattern of activities was fairly uniform: $H > -C_2CO_2H > -C_2CO_2tBu > -C_2CN$, except for E-zeatin, wherein the t -butyl ester was more active than the free acid. The underlying general activity series of $EZ > ZZ \approx DHZ > 2ip$ for the parent cytokinins was followed in the derivatives. Thus, the activities of the 9-2-carboxyethylcytokinins were $EZ9C_2CO_2H > ZZ9C_2CO_2H > \approx DHZ9C_2CO_2H > 2ip9C_2CO_2H$.

The very low activity (Fig. 1) of E-9-carboxyethyl-O- β -D-glucosylzeatin is surprising in view of the reported response in assays of the parent O-glucoside (Van Staden and Papaphilippou 1977; Palni et al. 1984). A difference in the callus tissue strain we used toward glycosidic hydrolysis could account for the results. Mok et al. (1982, 1978) and Palni et al. (1984) have shown quite divergent chemical reactivities in strains of related calluses. For this reason we have used a callus strain sensitive to 9-substitution and kept variations in the 6-position limited to those occurring naturally. Further, the 9-substituent used here cannot be hydrolysed by glucosidases that might unduly complicate the results.

The results clearly show the importance of 9-position steric and polarity effects in implementing cytokinin response. It appears that steric influence (size), $-C_2CO_2tBu > -C_2CO_2H > -C_2CN$ (Fig. 5A and 5B) was of less importance than polarity, $-C_2CO_2H > -C_2CO_2tBu > -C_2CN$. The 9-alanyl conjugates of E-zeatin (lupinic acid, LA) and dihydrozeatin occur naturally as metabolic products of exogenously supplied E-zeatin (MacLeod et al. 1975). The cytokinin activity of LA is dependent on the type of bioassay, being inactive in the tobacco-callus and radish-cotyledon assays but having appreciable activity in the soybean-callus (Palni et al. 1984) and Amaranthus assays (Letham et al. 1983). The alanyl group is very slightly larger than the $-C_2CO_2H$ group (Fig. 5A), but much more polar because of the zwitterion contribution to structure. A direct correlation of the activity of LA with our results is hardly possible because of test strain differences.

A number of 9-substituted BA derivatives have been reported, but no correlations of structure-activity relationships can be made with the present work due to the diversity of bioassay methods used in testing. For example, 9- β -carboxyethyl BA and its ethyl ester were found to be inactive in the radish-cotyledon assay; the ethyl ester showed slight activity in the oat-leaf senescence assay (Letham et al. 1983). Neither compound was tested on soybean callus.

In the wide variety of cytokinin assays commonly used, callus tissue culture, beta cyanidin formation in Amaranthus, cotyledon expansion, seed germination, retention of chlorophyll or ethylene production, response plotted against log [cytokinin] gives similarly shaped curves. For a given assay the slopes of the curves in the maximum response sections are parallel, as in this research. These results may be interpreted as being due to structure-dependent affinities for receptor sites in the test tissues (see Romanov et al. 1988 for a discussion and references; Corse et al. 1983).

Firn (1986) described the changes in dose-response curves obtained using the same growth substance and measuring the same response, where there are changes in the numbers or affinities of receptors. The curve in Fig. 1B (Firn, 1986) illustrates a situation wherein the receptor has 10 times less affinity for the growth substance; the dose-response curve is thus displaced along the X-axis but the shape remains the same. This is formally analogous to decreasing the binding between growth substance and receptor by 10-fold due to changes in the structures of growth substances. The use of the word "affinity" in this paper refers to Firn's "growth substance-receptor interaction" and not to the changes of that interaction due to modifications of the receptors themselves.

If it is necessary for biochemical reactions to occur (oxidation, hydrolysis, etc.) in order to convert a derivative or precursor to a form of the hormone with high affinity to the receptor site, the slopes of the activity curves would vary if those reactions were slow in comparison with the biological response of the test tissue (see Fig. 7, Letham et al. 1983). If those reactions were very fast, as with the oxidation of the 9-methyl group to carbon dioxide in the 9-methyl cytokinins, the derivatives would exhibit the properties of the parent (Shaw et al. 1968; Fox et al. 1971; Pietraface and Blaydes 1981). It follows then, if one cytokinin is not as active as another and they have the same activity-concentration slopes, they are probably binding to the same receptor and the differences in activity reflect the differences in affinity. With this guideline we can design probes to explore receptor site topology and estimate parameters for molecular modeling.

The intensive research on adenylate cyclase receptors, summarized by Ukena et al. (1987) provides an excellent example of the information about receptors that can be gained from structure-activity studies. A sound basis has already been laid by the research team led by Letham since his first characterization of E-zeatin, by the groups of Leonard and Skoog, and the cooperation among Matsubara, Fujita, and Koshimizu at Kyoto.

The localization of cytokinins in maize root cells (Zavala and Brandon, 1983) with labelled antibodies suggests that a cytokinin bound to a receptor site can still be recognized by an antibody. The answer may well be indicated by the involvement of different regions of even small molecules like cytokinins in antibody and receptor site specificities (see Discussion, part B). The literature is replete with dose-response data showing the effect of structure on activity (and hence on receptor site recognition) relevant to 6-substitution in cytokinins. The present work shows 9-position receptor site binding is similarly sensitive to structural variations. A polyclonal antibody (as used by Zavala and Brandon) would probably contain antibodies responsive to either the 6- or 9-regions of adenines. Thus, a cytokinin bound to a receptor site by either region would still have the other region open for an antibody responsive to the open region. Having monoclonal antibodies of known specificities (e.g., Brandon et al. 1986; Trione et al. 1985, 1987) should permit regional specifications of the receptors to be determined.

We thank G. E. Secor, M. Benson, and S. Witt for excellent technical assistance and Dr. W. Haddon for the mass spectrometry.

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Figure 1. Weight of Glycine max Merr cv Acme callus 28 days after subculture in the presence of EZ and 9-substituted EZ derivatives at the concentrations noted (see text for details). Bars indicate standard deviations.

Figure 2. Weight of Glycine max Merr cv Acme callus 28 days after subculture in the presence of ZZ and 9-substituted ZZ derivatives.

Figure 3. Weight of *Glycine max* Merr cv Acme callus 28 days after subculture in the presence of DHZ and 9-substituted DHZ derivatives at the concentrations noted (see text for details). Bars indicate standard deviations.

Figure 4. Weight of *Glycine max* Merr cv Acme callus 28 days after subculture in the presence of 2ip and 9-substituted 2ip derivatives.

Figure 5A. CPK molecular models of the 9-substituents
 $-\text{C}_2\text{CO}_2\text{H}$, $-\text{C}_2\text{CO}_2\text{tBu}$, $-\text{C}_2\text{CN}$, alanyl

Figure 5B. CPK molecular models of EZ, EZOG, EZOG9C₂CO₂H.

(These figures are on pp. 18-19.)

EZ 9C₂CO₂H

EZ

EZOG

—C₂CO₂tBu

—C₂CN

—C₂CO₂H

Alanyl

B. MONOCLONAL ANTIBODIES FOR CYTOKININ ANALYSIS

D. L. Brandon and J. Corse

INTRODUCTION

Attaching haptens to protein renders them immunogenic, an old observation (reviewed by Landsteiner, 1947), yet the basis of antigen recognition and processing by the immune system is still undergoing investigation. Hapten conjugates are also required for immunoassay of low molecular weight compounds. Hapten conjugates which elicited antibodies directed against purines and pyrimidines were first described by Erlanger and Beiser (1964). Cytokinin-specific antibodies were reported by Hacker et al. (1972) and an RIA for cytokinins reported by Khan et al. (1977). In these studies, oxidation of the ribose ring was followed by Schiff's base formation with protein and reduction with borohydride to form a stable hapten-protein conjugate. Monoclonal antibodies which bind cytokinins were first described in the literature by Woodsworth, et al. (1983) and in a summary of a workshop (Brandon, 1984), and subsequently by other groups (see Discussion).

As discussed in Part A, alternative methods of coupling cytokinins to protein are required when an oxidizable sugar ring is lacking or must be preserved. In order to prepare antibodies which would recognize the O-glucoside of zeatin, we investigated the suitability of 2-carboxyethyl substituents in the 9-position as functional groups for coupling cytokinins to protein.

In addition, we investigated an alternative to the conventional procedures using carbodiimide reagents (reviewed by Williams and Ibrahim, 1981). Carbodiimides induce the formation of amide bonds, and thus can be used to couple haptens containing carboxyl or amino groups to proteins. One of the most extensively utilized reagents is the water-soluble 3,3'-ethyl-dimethylaminopropyl carbodiimide. The use of water-soluble carbodiimides is limited by the sparing solubility of some haptens in water and by the formation of the intermediate O-acylisourea, which can be less soluble than the hapten and which can also act as an unwanted hapten. To overcome these problems, we have utilized 2-morpholinoethyl isocyanide (MEI) and a suitable catalyst to effect amide bond formation, based on procedures for peptide synthesis (Aigner et al., 1982). The reaction conditions permit coupling nonwater soluble compounds to proteins in a simple, homogeneous system. We have demonstrated that this procedure is widely applicable for preparing enzyme-labeled and immunogenic conjugates of cytokinins and other biologically relevant compounds with an available carboxyl group.

In addition to the utility of MEI as a coupling reagent for immunoassay development, the technique should have applications in many immunochemical procedures such as production of synthetic vaccines. We hypothesize that conjugates prepared in this manner will not induce allergic responses and unwanted crossreactivities associated with the use of carbodiimides.

METHODS

Chemicals

N⁶-2-isopentenyladenine (IPA) and its riboside (IPAR) were synthesized by the method of Hall and Robbins (1968). E-zeatin (EZ) and its riboside (EZR) were synthesized as described by Kuhnle et al. (1977). (RS)-dihydrozeatin (DHZ) and its riboside (DHZR) were synthesized essentially as described by Corse et al. (1983). These compounds were all purified by HPLC and structures verified by NMR spectroscopy. The O-glucoside of zeatin (ZOG), and 9-substituted cytokinins were synthesized as described above (Part A) and summarized in Figures 1-3. Cytokinin ribotides and the O-glucosides of (RS)-DHZ and (RS)-DHZR were obtained from Apex Organics (Oxford, U. K.). Other purines and ribosides were obtained from Sigma Chemical Co. (St. Louis, MO). [³H]-isopentenyladenosine (20 Ci/mmol) was from Moravsek Biochemicals (Los Angeles, CA). [³H]-dihydrozeatin was from Amersham Corp. (Arlington Heights, IL). Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem (La Jolla, CA).

Preparation of conjugates using oxidized ribosides

Conjugation of cytokinin ribosides to bovine serum albumin (BSA), equine serum albumin (ESA), and KLH was performed as summarized in Figure 4, essentially according to Erlanger and Beiser (1964). These conjugates contained 10 to 20 mol cytokinin per 100,000 g protein. Conditions were modified to produce highly active enzyme conjugates, chiefly horseradish peroxidase (HRP) (Sigma or Scripps Laboratories, La Jolla, CA), with lower degrees of conjugation (1-3 mol cytokinin/mol enzyme). In brief, conditions for these enzyme conjugations included increased buffer concentration, with periodate and reducing agent added in just slight excess.

Preparation of conjugates using MEI and 9-(2-carboxyethyl)cytokinins

Conjugates of 9-(2-carboxyethyl)-E-zeatin (CEZ) and its O-β-D-glucoside (CZG) to BSA and to HRP were performed in a similar manner. Details of one reaction are as follows. CZG (55 μmol) was dissolved in 0.4 mL of dimethylformamide (DMF)-dimethylsulfoxide (3:1), and 55 μmol of N-hydroxysuccinimide in DMF and 66 μmol of MEI were added. The mixture was stirred in an amber vial at room temperature (22° C) for 30 min. Dimethylaminopyridine (55 μmol) in DMF was then added to the mixture. The reaction mixture was next added dropwise, with stirring, to a solution of BSA in water (2 mg/mL). A control reaction was conducted similarly, but without the addition of CZG. The mixtures were stirred for 16 h, then dialyzed against PBS.

The conjugates and control samples were analyzed by UV spectrometry, using E₂₆₈, 1 mM = 13 for CZG. The degree of conjugation was estimated as 32 mol CZG/mol ESA. The HRP conjugate had an apparent composition of 29 mol of glucoside per mol HRP. It was stored at 4°, and was stable for at least 9 months. The degree of conjugation is stated as apparent because

reaction conditions appeared to affect the protein spectrum. Other experiments, producing conjugates which could be analyzed without reference to the UV spectrum, indicated that conjugation ratios of 20-30 mol hapten per mol BSA were readily obtainable.

Measurement of enzyme activities

A unit of HRP activity is defined as oxidation of 1 μ mole of 2,2'-azino-bis(3-ethylbenzothiazolinesulfonic acid) (ABTS) per min under standard assay conditions. A solution of unconjugated HRP was stored refrigerated in PBS and used as control. Its initial specific activity was 4.5 u/mg and at the conclusion of these studies it was 2.9 u/mg.

Alkaline phosphatase (Scripps Institute, La Jolla, CA) was assayed at pH 10.4 and 25°, using 5 mM p-nitrophenylphosphate as substrate. 1 unit hydrolyzed 1 μ mole of substrate per min.

Storage conditions for conjugates

To test storage conditions, conjugates of EZR and IPAR were dialyzed against PBS, and merthiolate was added. Conjugates were then diluted with equal volumes of PBS, or PBS containing 10 mg/mL BSA (PBS + BSA) or 10% dimethylsulfoxide (PBS + DMSO). Aliquots were prepared and stored either at 4° or -20° C. At intervals of one month, aliquots were analyzed for HRP activity and activity in ELISA.

Preparation of antibodies in mice

Mouse antibodies were elicited in BALB/c mice by two intraperitoneal inoculations at 3-week intervals with the BSA- or KLH-cytokinin conjugates emulsified in complete Freund's adjuvant. The dosage was 100 μ g of conjugate in 0.2 mL per injection. The mice were bled 1 week after the second injection, and sera were obtained, and stored frozen.

Preparation of hybridomas

Hybridomas were produced by modification of techniques described by Oi and Herzenberg (1980). In brief, an antibody-producing mouse was inoculated intravenously with 50 μ g of cytokinin conjugate in PBS 3 or 4 days prior to the cell fusion. Equal numbers of immune spleen cells and myeloma cells (P3-NS1-1-Ag4-1 or the light chain nonproducing variant, P3-X63-Ag8.653; Kearney et al., 1979) were fused by treatment with 50% poly(ethyleneglycol) 1500, and plated at a density of 1.5×10^6 cell/mL in 96-well tissue culture plates containing 0.2 mL/well.

Antibody-producing cultures were detected by solid-phase enzyme immunoassay. These cultures were expanded, and then cloned by limiting dilution. Antibodies are denoted by their culture of origin (e.g., 240 for the products of cloned cell line 240). Monoclonal antibodies were characterized with regard to isotype and light-chain composition using β -galactoside conjugates of specific antibodies (Southern Biotechnology,

Birmingham, AL), using myeloma proteins (Litton Bionetics, Kensington, MD) as standards.

Immunoassays.

Antibodies were screened and further analyzed by solid-phase enzyme immunoassays as follows. Polyvinylchloride assay plates (Costar, Cambridge, MA), were coated with appropriate protein conjugate (10 $\mu\text{g/mL}$ in PBS, 50 $\mu\text{L/well}$) for 4 h at room temperature or overnight at 4°. Plates were then washed with PBS containing 0.05% Tween-20 (PBS-Tween). Remaining sites were blocked by incubation for 1 h with 100 $\mu\text{L/well}$ of 1% BSA in PBS-Tween solution (BSA-PBS-Tween). Plates were then rewashed and stored for up to 3 weeks at 4° in PBS containing sodium azide (PBS-N₃).

Direct binding (titration) and inhibition assays were performed with antibodies dissolved in BSA-PBS-Tween, a diluent selected to minimize nonspecific interactions with the solid phase, and to suppress binding by BSA-specific antibodies to the solid phase.

Inhibition assay format was used to assess antibody specificity. Antibody at an appropriate dilution determined by titration was mixed with hapten solution and preincubated for 1 h at room temperature prior to application to the coated wells. In both formats, samples (50 $\mu\text{L/well}$) were generally applied to plates in triplate and were incubated 1 h, with shaking. Plates were emptied, washed, rinsed with distilled water, and drained. Horseradish peroxidase-labeled rabbit anti-mouse Ig (Zymed Laboratories, South San Francisco, CA) was added at an appropriate dilution (50 $\mu\text{L/well}$) and incubated for 1 h with shaking. The plates were then washed as before, and bound peroxidase detected by addition of 50 $\mu\text{L/well}$ of substrate (1 mM ABTS and 6.7 mM H₂O₂ in 60 mM sodium citrate buffer, pH 4.2). The absorbance was determined at 415 nm with an EIA reader (Model 308, Bio-Tek, Winooski, VT), generally after 15 min. In some cases, reactions were terminated by addition of 50 $\mu\text{L/well}$ of 10% sodium dodecylsulfate.

Competition ELISA using cytokinin conjugates of HRP was performed using polystyrene assay plates coated with mouse monoclonal antibodies specific for cytokinins. Generally, plates were first coated with 100 $\mu\text{L/well}$ of 5 $\mu\text{g/mL}$ rabbit anti-mouse IgG (Zymed Laboratories) and blocked with 200 $\mu\text{L/well}$ of BSA-PBS-Tween, as described above. Immediately before use, assay plates were coated with 1 $\mu\text{g/mL}$ of monoclonal IgG in BSA-PBS-Tween. Samples were mixed with an appropriate concentration of cytokinin-HRP conjugate and applied to assay wells in replicate (100 $\mu\text{L/well}$). After 1 h, wells were drained, washed, and rinsed. Bound conjugate was detected with ABTS substrate solution and quantified by determining absorbance at 415 nm.

Calculations were performed as follows. Assay data was fit to a logistic model (Finney, 1964). Asymptotic values were determined by inspection of the assay curves, and other coefficients were determined by linear regression. Apparent binding affinity was estimated by determining the concentration of cytokinin which resulted in 50% inhibition (I₅₀) of binding in either inhibition or competition ELISA formats. Relative affinities are expressed using E-zeatin riboside or E-zeatin-O-glucoside as standards.

Preparation of corn root tip extracts

Seeds of Zea mays were surface sterilized by brief treatment with sodium hypochlorite, then sprouted under aseptic conditions for 4-5 d at approximately 25° C in the dark. Roots were decapped and the terminal 2 mm section excised and frozen until extracted. Samples of approximately 100 mg were homogenized in 1 mL of ice-cold extraction medium (80% methanol, 20% water, containing 1 mg/L butylated hydroxytoluene as anti-oxidant) with a Tissuemizer (Tekmar, Cincinnati, OH). Extraction slurries were then spiked with [³H]-dihydrozeatin, about 5 x 10⁵ cpm per g. Samples were stirred overnight in the dark at 4°. Residue was removed by centrifugation and the extract was dried in a centrifugal evaporator (Savant Instruments, Hicksville, NY).

Fractionation of extracts

Dried extracts were resuspended in about 0.2 mL of 40 mM ammonium acetate, pH 6.5, treated with alkaline phosphatase (0.03 units/mL for 30 min), and applied to a column (approx. 1 x 3 cm) of DEAE-cellulose (Whatman, Maidstone, UK). The column was eluted with ammonium acetate buffer, and the fractions containing tritium were dried in a centrifugal evaporator and redissolved in phosphate buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, pH 7.0) for assay. Aliquots were assayed for tritium, to determine recovery of [³H]-DHZ.

RESULTS

Conjugate stability

The stability of EZR-HRP and IPAR-HRP conjugates was studied to determine conditions for good preservation of peroxidase and antigenic activities. Frozen and refrigerated samples, stored in any of the three buffers, exhibited gradually declining HRP activity during the seven-month study. The buffer and temperature (4 vs. -20° C) of storage did not have a major effect on preservation of activity. After three months, EZR-HRP and IPAR-HRP conjugates retained $59 \pm 5\%$ of initial peroxidase activity, with storage conditions having little effect.

The stability of the conjugates was also assessed by determining the titer of the conjugate by ELISA using antibody 94 or antibody 40 as solid phase. The results of titrations after three months storage is shown in Table 1. Storage of conjugate in PBS + BSA resulted in excellent preservation of activity at -20° for three months. Loss of ELISA

Table 1. Preservation of activity of enzyme conjugates

Buffer	Temperature (°C)	Remaining activity (%) ^a
PBS	4	38
	-20	31
PBS + BSA	4	48
	-20	79
PBS + DMSO	4	38
	-20	62

^aActivity is expressed as a % of control value, and is based on the titer (reciprocal of dilution giving 0.8 absorbance units under standard assay conditions).

activity did not appear to be caused by simple reversal of the conjugation of cytokinin and enzyme. Dialysis of conjugates after several months storage at 4° did not affect binding to antibody-coated wells, as determined by titer and concentration of free cytokinin needed for 50% inhibition of conjugate binding (I₅₀).

The HRP conjugates of CEZ and CZG were stable on storage at 4°

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for at least 9 months, with merthiolate as a preservative. No time course was performed with these conjugates, but these conjugates appear to be more stable than ones produced using oxidized ribosides.

Characterization of monoclonal antibodies

Tables 2-6 summarize quantitative measurements of typical monoclonal antibodies: 93, 94, 240, and 45. Antibody 93 is IgG_{2a}. The others are IgG₁, and all have the kappa light chain. These antibodies are thus useful for quantitating cytokinins with the zeatin side chain (antibodies 93 and 94), the zeatin-O-glucoside side chain (antibody 240), and the 2-isopentenyl side chain (antibody 45).

The ratio of I₅₀'s for antibodies 93 and 94 (Tables 2-4) was not equal to 1 for several cytokinins. Thus, there appear to be subtle differences in the binding sites of these antibodies, with respect to the substituents in the 2- and 9-positions and the stereochemistry of the zeatin side chain. Antibody 240 showed great specificity for zeatin-O-glucoside. Significant binding of the intermediate (PBG) used in synthesizing ZOG indicates that the binding site has contact with the purine skeleton as well as with the glucosylated side chain.

Table 2. Side chain specificity - Antibodies 93 AND 94

Compound	Relative I ₅₀ ^a		Ratio ^b
	Ab 93	Ab 94	
E-ZEATIN	.992	1.000	.992
Z-ZEATIN	14.2	92.5	.154
CIS-NORZEATIN	834	737	1.13
ISOPENTENYLADENINE	884	676	1.31
N6-(3-ME-3-BUTENYL)AMINOPURINE	83.8	237	.353
N6-(3-OH-3-MEBUTYL)AMINOPURINE	64.2	210	.306
E-ISOZEATIN	82.8	74.5	1.11
(S)-DIHYDROZEATIN	22.5	61.3	.368
(R)-DIHYDROZEATIN	1.99	2.90	.685
N6-((+)2,3-DIOH-3MEBU)AMINOPUR	1490	1235	1.20
O-T-BUTYL-E-ZEATIN	3090	1430	2.16
N6-BENZYLADENINE	384	2310	.166
ISOPENTENYLADENOSINE	363	531	.684
N6-FURFURYLADENINE	749	5250	.143
ADENINE	* ^c	*	

^aI₅₀ relative to EZR

^bRatio of relative I₅₀ (Ab 93:Ab 94)

^c* indicates no measurable inhibition

Table 3. Purine ring specificity - Antibodies 93 AND 94

Compound	Relative I ₅₀ ^a		
	Ab 93	Ab 94	Ratio ^b
E-ZEATIN		.992	1.000
2-METHYLTHIO-E-ZEATIN		36.5	15.1
2-HYDROXY-E-ZEATIN	1460.	4610.	0.32
ADENINE	* ^c	*	
HYPOXANTHINE	*	*	
INOSINE	*	*	
GUANOSINE	*	*	

^aI₅₀ relative to EZR^bratio of relative I₅₀'s (Ab 93:Ab 94)^c* indicates no measurable inhibition

Table 4. Specificity for the 9-position - Antibodies 93 and 94

Compound	Relative I ₅₀ ^a		
	Ab 93	Ab 94	Ratio ^b
E-ZEATIN	.992	1.00	.992
O-β-D-GLUCOPYRANOSYL-E-ZEATIN	11600.	1860.	6.22
9-β-D-GLUCOPYRANOSYL-E-ZEATIN	8.07	1.32	6.13
E-ZEATIN RIBOSIDE	1.000	1.000	1.000
9-β-CE-E-ZEATIN	1.55	.404	3.84
9-β-CYANOETHYL-E-ZEATIN	.552	.187	2.95
E-ZEATIN RIBOSIDE-5'-MONOPHOSP	1.89	5.91	0.74

^aI₅₀ relative to EZR^bratio of relative I₅₀'s (Ab 93:Ab 94)

Table 5. Specificity of antibody 240

Compound	I ₅₀	Relative I ₅₀ ^a
E-ZEATIN	3.76E-04	14600.
ISOPENTENYLADENINE	6.60E-03	256000.
O-β-D-GLUCOPYRANOSYL-E-ZEATIN	2.58E-08	1.000
9-β-D-GLUCOPYRANOSYL-E-ZEATIN	1.10E-03	42600.
N6-BENZYLADENINE	1.10E-03	42600.
E-ZEATIN RIBOSIDE-O-GLUCOSIDE	2.98E-07	11.6
N6-FURFURYLADENINE	5.90E-04	22900.
E-2-ME-4-PHTH'IM'BUT-2-ENE-BDG ^b	1.20E-06	46.5

^aI₅₀ relative to Z06^bintermediate in Z06 and C26 synthesis, containing the zeatin-O-β-D-glucoside side chain (abbreviation: PBG)

Table 6. Specificity of antibody 45

Compound	I ₅₀	Relative I ₅₀ ^a
Z-ZEATIN	>1E-04	>1000
CIS-NORZEATIN	>1E-04	>1000
ISOPENTENYLADENINE	4.3E-08	1
S-(-)-DIHYDROZEATIN	>1E-04	>1000
R-(+)-DIHYDROZEATIN	>1E-04	>1000
E-ZEATIN RIBOSIDE	9.6E-06	224
ISOPENTENYLADENOSINE	4.3E-08	1

^aI₅₀ relative to IPA

Figures 5-7 illustrate typical standard curves obtained using these antibodies. Curves are illustrated for the competition ELISA format using HRP conjugates of EZR (for antibodies 93 and 94) and CZG (for antibody 240). An assay curve in the inhibition ELISA format is illustrated for antibody 45. When compared, both formats gave comparable results, though the competition ELISA format is faster since it combines the sample incubation and enzyme label steps. The inhibition format is more generally applicable, and it avoids problems caused by sample components which interact directly with the enzyme label.

Optimal reproducibility of results depended on scrupulous attention to conditions of the assay. When this was done, I₅₀ values could be reproduced with the same reagents over periods of many months. The highest quality polystyrene assay plates, with use of only the inner 6 x 10 array of wells contributed to this reproducibility. Relative values for different cytokinins were more easily reproduced, requiring only routine vigilance, with considerably more choice and economy possible in selection of assay plates. An example of this reproducibility is illustrated in Table 7. The data are calculated from competition ELISA's using antibody 94 and HRP-EZR conjugate over a period of two weeks.

Table 7. Reproducibility of I₅₀ values for E-zeatin riboside

Experiment	I ₅₀ (M x 10 ⁸)
1	3.0 ± 0.7 ^a
2	2.9 ± 0.5
3	4.5 ± 0.9
4	2.5 ± 0.5
5	3.3 ± 0.6
6	2.7 ± 0.6
mean	3.2 ± 0.7 ^b

^aestimated error in calculation of I₅₀^bstandard deviation

Analysis of zeatin and zeatin-O-glucoside in corn root tips

Figure 8 illustrates standard curves following logit transformations for zeatin and zeatin-O-glucoside ELISA's. Midpoints for these curves (I_{50} 's) are 3.1×10^{-9} M for zeatin and 2.6×10^{-8} M for zeatin-O-glucoside. Samples were assayed with and without addition of various concentrations of zeatin and the glucoside. Representative logit-transformed curves for unspiked and spiked samples are illustrated in Figure 9. The nearly parallel curves suggest that the same antigenic substance is reacting in each assay. This criterion is more critical than parallelism of curves representing different dilutions of the same unspiked sample.

The zeatin and zeatin-O-glucoside concentrations were estimated from curves fit following logistic transformation of the data. The results are summarized in Table 8.

Table 8. ELISA analysis of E-zeatin and E-zeatin-O- β -D-glucoside in corn root tips

cytokinin	content (nmol/g)
zeatin	5.5 ± 1.5
zeatin-O-glucoside	263 ± 191

Data are means and standard deviations from 3 independent samples of corn root tips.

The ratio of glucoside to zeatin in these samples was 45 ± 27 . This compares with typical ratios of about 1-2 in leaf samples (see section C of report). This ratio is unexpectedly high, and it remains to be confirmed by other techniques. It should be pointed out that the antibodies are measuring primarily on the basis of the isopentenyl side chain. Derivatization of the 9-position of ZOG reduces its binding to antibody 240 (Table 5). The large variance in ZOG concentration appears to represent differences among the samples, rather than within-assay variance.

Additional studies using these antibodies must include the following:

1. Comparison of results with physicochemical measurements.
2. Assessment of other possible antigenic substances which bind to antibody 240.
3. Analysis of immunoassay results on samples previously treated with glucosidase.

4. Determination of effects of growth and harvest conditions on cytokinin levels.

Nevertheless, it appears significant that root and leaf tissues differ in their cytokinin contents as determined by ELISA. We plan to conduct follow-up studies using modified assay conditions with smaller sample size, which will permit better sample clean-up and replication.

Relationship of antibody binding sites to phytohormone receptors

In order to relate the three-dimensional geometry of antibody combining sites to putative hormone receptor sites, relative activities of cytokinins in immunoassays and bioassays must be compared. There are clearly several reasons why activities in the two types of assay could differ, even if the combining sites are similar:

1. Cytokinins must be translocated from site of application to site of action.
2. Cytokinins are metabolized in plant tissues, involving activation of inactive forms and degradation or derivatization of active forms.
3. Cytokinin actions may be indirect, involving combinations of growth regulators.
4. Only rate-limiting steps can be detected with bioassays.
5. Hormone response is not necessarily proportional to receptor occupancy.
6. Immunoassays--especially ELISA's--do not necessarily reveal intrinsic affinity constants.

This list is a precautionary note. It indicates that hormone receptor sites are difficult to identify and relate to antibody sites. However, it is worth noting the topographic similarity of animal hormone receptors and antibody binding sites for ligands such as acetylcholine and dopamine.

An example of this approach is illustrated in Figure 10. Plotted are the relative concentrations which produce the same response as E-zeatin (defined as 1 for both assays). Thus, the higher numbers correspond to less active compounds. The data indicate the general parallelism of activities of 4 naturally occurring cytokinins in the soybean callus assay and competition ELISA using antibody 93 or 94 (E-zeatin > (RS)-dihydrozeatin > isopentenyladenine > E-zeatin-O-glucoside). The parallelism breaks down, however, when a more complete panel of cytokinins is compared (e.g., Z-zeatin is about 50 times more active in the bioassay than in the immunoassays with these antibodies).

We are currently analyzing data for additional antibodies and bioassays, and the results are not yet complete. One example of profoundly different binding sites, however, is illustrated in Figure 11. Antibody 94 is very sensitive to the stereochemistry of the DHZ side chain, whereas antibody 101 binds equivalently to the two enantiomers. Other data concerning the combining site of antibody 101 suggest that its combining site, discriminating poorly between E- and Z-zeatins, resembles the putative "receptor" of soybean callus. (Again, the term "receptor" is used loosely, and refers to the rate-limiting component. See the discussion in Part A.)

DISCUSSION

Now that there are a number of monoclonal antibodies specific for cytokinins as described herein and in the literature, one can reach some general conclusions and suggest directions for using these reagents and on improving methodology. In general, similar monoclonal antibodies have been reported independently. A note of caution is that different groups have used different methods to estimate antibody affinity. Each method is subject to different biases, and true affinities have not been reported. In view of the use of BALB/c mice in these studies and similar immunization protocols, the similarity of results is not surprising.

Differences among monoclonal antibodies have been reported. Knox et al. (1987) and our group (Brandon et al., 1986) have pointed out that even small molecules like GA's and cytokinins can have multiple epitopes. Observations along these lines have been made by many workers, with Landsteiner (1947), a classic reference on the subject. While the antibody specificity is greatly influenced by the particular hapten derivative, different specificities can be obtained from a single cell fusion. These results are consistent with current concepts of antigen processing and maturation of the immune response.

Trione et al. (1985) reported both specific and relatively nonspecific monoclonal anti-zeatin riboside. Eberle et al. (1986) reported 3 specific anti-zeatin riboside monoclonals, and one with specificity for DHZR. The most specific of the monoclonals reported by the above groups appear similar to antibodies 93 and 94 described in this report. Even so, antibody 94 may well be a new specificity, with its lowered binding to the phosphate ester of zeatin riboside and its dramatic sensitivity to side chain modification.

Antibodies for IPA reported by Wang et al. (1987), Trione et al. (1987), and Eberle et al. (1987) are generally similar to each other, and to our antibodies, of which antibody 45 is an example. The binding of BAP to anti-isopentenyladenosine (Trione et al., 1987) illustrates again that a variety of monoclonal antibodies can be elicited with even a well-defined haptenic antigen, provided a suitable screening method is used to detect the antibody of interest.

Antibody 240 represents a new specificity, and will hopefully lead to further understanding of the role of conjugation and derivatization

in phytohormone action. Experimental priorities include further definition of the components which bind to this antibody and the preparation of antibodies which bind to other endogenous cytokinin conjugates and metabolites.

As the quantity and quality of analytical data improve (e.g., Hocart et al., 1988), cytokinin glycosides appear increasingly important. Understanding the interconversions among cytokinins and their relationship to the actions of other plant growth regulators will be essential to delineating cytokinin function. For example, O-glycosylated cytokinins are converted to free forms during later shoot initiation (Taylor et al., 1984). Inhibitors of enzymes which influence cytokinin derivatization (especially the 9-alanyl transferase) include cytokinin analogues and natural and synthetic auxins (Parker et al., 1986). Once the significance of the metabolic pathways are elucidated and enzymes identified, both chemical and genetic (e.g., Barry et al., 1985) manipulations can be performed.

Antibodies which bind cytokinins have recently been used for localizations of this group of hormones (Zavala and Brandon, 1983; Eberle et al., 1987; Sossountzov et al., 1988). It is clear that the methodology is still evolving, but the application of antibodies to problems of hormone reception will undoubtedly be fruitful. One of the first definitive results obtainable with anti-receptor probes will be distinguishing between receptor number and receptor activity. (For example, see Pengelly et al., 1986, for a discussion of auxin sensitivity.)

Anti-idiotypic antibodies may be the most promising, since they represent stable, readily labeled cytokinin-like probes. Since anti-idiotypic, receptor-specific antibodies can be elicited simultaneously with anti-ligand antibodies (see for example Shechter et al., 1982), we may have already inadvertently applied them in the localizations using polyclonal antibodies.

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Figure 1. Synthesis of 9-(2-carboxyethyl) cytokinins.

Figure 2. Synthesis of zeatin-O-β-D-glucoside side chain.

Figure 3. Synthesis of 9-(2-carboxyethyl) derivative of zeatin-O-β-D-glucoside.

Figure 4. Protein conjugates based on oxidized cytokinin ribosides.

Figure 5. Some typical standard curves for cytokinin ELISA using EZR-HRP conjugate and antibodies 93 and 94. The cytokinins assayed are (a) 9-(2-cyanoethyl) zeatin; (b) E-zeatin riboside; (c) E-zeatin riboside-O-glucoside; (d) isopentenyladenosine; (e) hydroxybenzyladenine; and (f) kinetin.

Figure 6. Standard curve for inhibition ELISA of isopentenyladenine with antibody 45.

Figure 7. Standard curves for competition ELISA using CZG-HRP conjugate and antibody 240.

Figure 8. Standard curves for ELISA's of zeatin and zeatin-O-glucoside, following logit transformation.

Figure 9. Logit-transformed assay curves for analysis of zeatin and zeatin-O-glucoside in extracts of corn root tips, with (large symbols) or without (small symbols) addition of authentic analyte.

Figure 10. Relationship of antibody combining sites and "receptor" sites. The figures show the correlation between ELISA and soybean callus activities of 4 cytokinins ($r = .99$ for both linear regressions). In order of decreasing activity, the points represent E-zeatin, (RS)-dihydrozeatin, isopentenyladenine, and E-zeatin-O-glucoside.

Figure 11. Example of an additional specificity of anti-cytokinin monoclonal antibody. Antibody 94 is highly selective for (R)-DHZ.

C. Senescence in soybeans--investigation of isolines and a model system

Wittenbach (1974) described a model system which duplicated some of the physiological changes associated with senescence in wheat plants. Dark stress induced chlorophyll and protein degradation, which was reversible up to about 4 days of dark treatment. Beyond that time, irreversible changes occurred, and the plants became unable to re-green and thrive. Wagner and coworkers in Nebraska have used a similar experimental system to study senescence of soybean root nodules (Pfeiffer et al., 1983). We wished to adapt this system to elucidation of the role of cytokinin glucosylation in whole plant senescence. For initial studies, we selected plants which were available as isolines (Phillips et al., 1984; Hymowitz, 1986), so that the effects of single gene changes might be observable. Ultimately, manipulation of plant senescence will depend on definition of regulation at the genetic level, and experiments using isolines could help identify these genetic factors.

We were interested in studying varieties with known differences in the stability of the photosynthetic apparatus, those with delayed leaf senescence, and those which differ in protease inhibitory activity. The paramount importance of degradative processes in monocarpic senescence is widely accepted, but it is unclear whether nucleolytic and proteolytic activities initiate senescence responses. The involvement of proteases, including serine proteases in leaf senescence (Miller and Huffaker, 1982; Shurtz-Swirski and Gepstein, 1985; Mae and Hageman, 1986) led us to include protease inhibitors as factor which should be evaluated for roles in senescence. The availability of new, sensitive immunoassays (Brandon et al., 1988) for soybean protease inhibitors, along with isolines differing in Kunitz trypsin inhibitor (KTI) content (Hymowitz, 1986), impelled us into a preliminary evaluation of the influence of KTI on leaf senescence.

In this report we describe measurements of cytokinins during lifetime studies of soybeans, a model system which may prove useful in designing more rapid experiments to study soybean senescence, and preliminary data regarding the influence of endogenous protease inhibitors on senescence.

Plants

Table 1 shows the varieties used. Six isolines were of the Clark-L1 subline. Two isolines were of the Harosoy-L2 subline. Two isolines were of the Williams-82 subline and differ at the Ti locus which codes for the Kunitz soybean trypsin inhibitor. Seeds were kindly provided by the USDA Soybean Germplasm Collection (Urbana, IL).

Growth of plants

Plants grown to maturity were grown in Sunshine Mix (Fison's Western Corp., Vancouver, BC, Canada) in a greenhouse under natural lighting conditions, and were fertilized regularly with Plantex 20-20-20 (Plantex, Inc., Bremerton, WA).

Table 1. Isolines used in soybean senescence study

<u>Strain</u>	<u>Genotype</u>	<u>Phenotype</u>
Clark isolines:		
CL1	Dt ₁ dt ₂ e ₁ E ₂ E ₃ Y ₃	Normal
L67-1474	Dt ₁ dt ₂ E ₁ E ₂ E ₃ Y ₃	Late maturity
L63-2346	Dt ₁ dt ₂ e ₁ E ₂ E ₃ Y ₃	Yellow-green
L65-778	dt ₁ dt ₂ e ₁ e ₂ E ₃ Y ₃	Delayed leaf senescence
L63-3016	dt ₁ dt ₂ e ₁ E ₂ E ₃ Y ₃	Intermediate senescence
L66-531	dt ₁ dt ₂ E ₁ e ₂ E ₃ Y ₃	Intermediate senescence
Harosoy isolines:		
H	Dt ₁ dt ₂ e ₁ e ₂ E ₃ Y ₃	Normal
L63-1016	Dt ₁ dt ₂ e ₁ e ₂ E ₃ Y ₃	Yellow-green
Williams isolines:		
Williams 82	Ti ^a	Normal
L81-4590	ti	Lacks Kunitz TI

mid-May) were made, with leaf samples obtained at various times. Leaves were cut, frozen on dry ice, and maintained at -80° C until extracted.

For the dark-induced senescence model studies, young plants in 10 cm pots were grown in a protected courtyard area, with 14-16 hours of natural daylight. For the dark period, plants were transferred near the end of a natural day to a dark incubator maintained at 28° C. At the end of the dark period, plants were returned to outdoor ambient environmental conditions. Plants were occasionally exposed to a green safelight during the dark period. All plants in these studies were fertilized with modified Johnson's solution at four-day intervals.

Chlorophyll analysis

Leaves were harvested and photocopied for determination of area. A portion of the leaf sample was extracted with 95% ethanol for measurement of total chlorophyll a and b according to extinction coefficients reported by Lichtenthaler (1987).

Cytokinin analysis

Extraction of leaves. Extraction medium consisted of ice-cold 80% methanol in water, containing 1 mg/ml butylated hydroxytoluene. Samples were processed and stored in polypropylene or silanized glass test tubes. Leaves were homogenized with a Tissuemizer (Tekmar, Cincinnati, OH), using

2.5 ml of extraction medium per gram fresh weight. Extraction slurries were then spiked with [^3H]-dihydrozeatin (DHZ, Amersham Corp., Arlington Heights, IL), about 50,000 cpm per g. Samples were stirred overnight in the dark at 4°. Residue was removed by centrifugation and the extract was dried in a centrifugal evaporator (Savant Instruments, Hicksville, NY).

Fractionation of extracts. Dried extracts were resuspended in 40 mM ammonium acetate, pH 6.5, treated with alkaline phosphatase as described above, and applied to two columns (approx. 1 x 3 cm) which were eluted in tandem. The first column was DEAE-cellulose (Whatman, Maidstone, UK) and the second was crosslinked vinylbenzene (XAD-2, Mallinckrodt, Inc., Paris KY). The columns were eluted with ammonium acetate buffer, and the material retained on XAD-2 was eluted with methanol. The methanol eluates were dried and redissolved in phosphate buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, pH 7.0) for assay. Aliquots were assayed for tritium, to determine recovery of [^3H]-DHZ.

Immunoassays

Samples were assayed by preparing a dilution series in PBS containing 0.05% Tween-20 and 10 mg/ml bovine serum albumin (BSA-PBS-Tween). The diluted samples were mixed with an appropriate concentration of cytokinin-horseradish peroxidase (HRP) conjugate. For analysis of E-zeatin-like cytokinins, EZR-HRP conjugate was used; for analysis of E-zeatin-O- β -D-glucoside (ZOG) crossreactive cytokinins, ZOG-HRP was used. Assays were conducted on 96-well polystyrene microtitration plates (Nunc, Roskilde, Denmark or Immulon II, Dynatech, Chantilly, VA) which had been coated with rabbit-anti mouse IgG (Zymed Laboratories, South San Francisco, CA) at 5 $\mu\text{g}/\text{ml}$. For zeatin analysis, a secondary coating of antibody 94 was used (1 $\mu\text{g}/\text{ml}$). For zeatin-O-glucoside analysis, antibody 240 was used (1 $\mu\text{g}/\text{ml}$). Sample-conjugate mixtures were incubated for 1 h with shaking at room temperature. Assay wells were then washed with PBS-Tween and rinsed with water. Bound conjugate was detected by addition of 100 $\mu\text{l}/\text{well}$ of ABTS substrate solution. Absorbance at 415 nm was generally determined using a microplate reader (Model 308, Bio-Tek, Winooski, VT) after 30 min. Standard solutions of ZR and ZOG were included with assay samples. Cytokinin concentrations in unknowns was determined by reference to the standard curve. Recovery and linearity of response was checked in some samples by analysis of samples to which known quantities of authentic ZR and ZOG had been added.

Measurement of protease inhibitors

Kunitz trypsin inhibitor (KTI) was determined by competition ELISA (Brandon et al., 1988), using antibody 129 (Brandon et al., 1987). This combination of technique and antibody was selected for maximal sensitivity. Bowman-Birk inhibitor (BBI) was determined by competition and inhibition ELISA methods described in detail elsewhere (Brandon et al., manuscript submitted for publication). Leaf and cotyledon extracts were prepared by homogenization of tissues in a Tissuemizer in 50 M

Tris-Cl buffer containing 0.02 g polyvinylpolypyrrolidone, essentially according to Tan-Wilson et al. (1985). The extracts were clarified by centrifugation prior to immunoassay.

RESULTS AND DISCUSSION

Measurement of zeatin and zeatin-O-glucoside

The Clark isolines were examined in most detail, and these results are summarized in Table 2. There appeared to be no dramatic difference among the three isolines in either glucosylated or nonglucosylated zeatin or in their ratio. Table 3 shows some of the results obtained on the yellow-green variant (L63-2346). There was a progressive increase in the

Table 2. Zeatin and zeatin-O-glucoside in leaves of Clark isolines

Isoline	Zeatin	ZOG	Z:ZOG ratio
	(pmol/g)		
C-L1	431	687	.627
L63-2346	291	538	.540
L67-1474	342	520	.658

Table 3. Age dependence of zeatin:zeatin-O-glucoside ratio in leaves of L63-2346

Age (weeks)	Z:ZOG ratio
16	.370
17	.463
19	.810
21	1.16

zeatin:zeatin-O-glucoside ratio as the plants senesced. Results with the other isolines are incomplete at this writing. While the results are preliminary, they do suggest the practicality of differentiating between zeatin and its glucosides by immunoassay.

In the case of the senescing soybean L63-2346, the change in ratio during transition from mid-podfill to late senescence was unexpected. There are several possible explanations for these results.

- (1) Recovery of individual cytokinins (especially zeatin vs. ZOG) may

differ from the overall recovery of [^3H]-DHZ used to compute the results.

- (2) Whole senescing leaves may not be appropriate for detection of regulatory levels of cytokinins. Finer analysis of the leaf, determination of subcellular localization, and measurement of flow into (or out of) the leaves must be made.
- (3) The presence of related and unrelated metabolites may be causing spurious results in the immunoassays. These metabolites may change during leaf senescence.

Aside from the above considerations, results must be replicated and validated by other techniques. We are proceeding in this direction.

The dark-induced senescence model

Dark-induced chlorophyll loss was used as an indicator of the senescence response in this system. The time course of the response was studied using the Williams 82 isoline. After dark period of various lengths, plants were allowed to recover under control conditions. The results are depicted in Figure 1. The dark period of 3.7 d (89 h) was used in subsequent experiments. This was a period which resulted in about 80% loss of chlorophyll, but plants were still viable, and the chlorophyll loss was reversible.

Table 4 illustrates the response of various isolines to the 89 h dark period. Where indication of experimental error is given, results are

Table 4. Dark-induced chlorophyll loss of soybeans

Isoline	Chlorophyll loss (percent)	
	per cm^2	per g fresh wt
Clark isolines:		
C - L1	70 $\pm$ 9	69
L67-1474	33	37
L63-2346	96 $\pm$ 2	96
L65-778	69 $\pm$ 20	76
L63-3016	57 $\pm$ 13	58
L66-531	73 $\pm$ 9	73
Harosoy isolines:		
H - L2	60	60
L63-1016	97 $\pm$ 1	97
Williams isolines:		
Williams 82	68	69
L81-4590	85	87

are averages and standard deviations of determinations on 3 or 4 individual plants. Other values of chlorophyll loss per unit area are average values for groups of 3 plants. Chlorophyll loss per unit fresh weight is given for comparison. The close agreement between the two sets of numbers indicates that leaf density (probably influenced most by water content) did not vary greatly among samples.

The results are noteworthy because they do not appear to differ among the plants representing different genetic backgrounds (Clark, Harosoy, and Williams), but do differ within the groups depending on known genetic differences. Thus, the isolines L63-2346 and L63-1016 which are phenotypically yellow-green and which undergo qualitatively early leaf senescence--beginning at 16 weeks, during podfill--also exhibit dramatically increased chlorophyll loss (96-97%) in this model system. Secondly, isolate L67-1474, a late maturing variety, sustains only a relatively small loss of chlorophyll (33%) under these conditions.

The results obtained with other Clark isolines were less dramatic. These isolines (L65-778, L63-3016, and L66-531) exhibit phenotypic late or intermediate senescence. In the model system, there was no difference between this group and the normally senescing isolines. However, it may be that the experimental time period or particular endpoint (chlorophyll loss per cm^2) was not appropriate to correlate with lifetime senescence.

The results with the Williams isolines, though preliminary, suggest that the absence of Kunitz trypsin inhibitor permitted more rapid degradation of chlorophyll in L81-4590. Replication of these results is needed to establish the statistical significance of these observations. To test the possibility that specific protease inhibitors could influence chlorophyll stability in soybean leaves, we determined the protease inhibitors content of leaves and cotyledons of soybean plants (26 d old). The results are shown in Table 5, and indicate that measurable amounts of the inhibitors

Table 5. Protease inhibitors in leaves and cotyledons of Williams 82 isolines on day 26 of growth

<u>Isoline</u>	<u>Tissue</u>	<u>Inhibitor (μg per g)</u>	
		<u>KTI</u>	<u>BBI</u>
Williams 82	leaf	0.38	0.36
	cotyledon	0.9	0.2
L81-4590	leaf	n.d. ¹	0.08
	cotyledon	n.d.	1.8

¹not determined, but expected to be $< .001$.

KTI and BBI are found in leaves and cotyledons. Our preliminary experiments also indicate that levels of KTI and BBI decline below the limit of detection by the time Williams 82 plants reach 8 weeks of age. The observations are consistent with reports (e.g., Tan-Wilson et al.,

(1985) that seed protease inhibitors decline rapidly in senescing cotyledons of soybeans and are undetectable in leaves by immunodiffusion. Our 100-fold more sensitive assays suggest that two specific protease inhibitors are present in plants nearing maturity.

Even very low levels of specific protease inhibitors could influence the stability of selected organelles or macromolecular complexes including cellular membranes. The involvement of specific proteases in leaf senescence is suggested by much data (e.g., Miller and Huffaker, 1982 in barley leaves, Lamattina et al., 1985 in wheat leaves, Mae and Hageman, 1986 in corn leaves), but evidence concerning a regulatory role is scanty. Even within a single enzyme complex, ribulose biphosphate carboxylase, individual polypeptides undergo proteolysis differentially (Shurtz-Swirski and Gepstein, 1985). The influence of inhibitors of proteolysis in leaf senescence is thus a viable but largely unexplored hypothesis.

The next steps in this study are:

- (1) Analysis of zeatin and zeatin glucosides at various times during the dark period and recovery in the model system and during natural senescence. The correlation of changes in endogenous cytokinins and pod set (Wyllie et al., 1988) and the importance of flows of nutrients and growth regulators (Neumann and Stein, 1983) suggest that better quantitation of endogenous cytokinins involving smaller sample size would be productive.
- (2) Further study of the possible influence of endogenous protease inhibitors. Since a Ti-null isolate of the Clark variety is available, we will be able to test the hypothesis on plants of two different genetic backgrounds, Williams and Clark. Differences among cultivars of soybeans in responses to senescence-delaying strategies such as flower removal (Crafts-Brandner and Egli, 1987) indicate the importance of observations in multiple cultivars.

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Figure 1. Time course of chlorophyll loss during dark stress (solid line) and recovery in light (dashed line). Soybeans plants (Williams 82) were 28-days old on day 0.

2. The effect of root pruning on the maturation of loblolly pine

L. A. Wisniewski, D. L. Brandon, S. E. McKeand, and H. V. Amerson

INTRODUCTION

Cytokinins have been implicated in the maintenance of the juvenile state of trees (Franclet, 1979; Abo El-Nil, 1982). In addition, evidence has been presented relating the maturation state of plantlets to their relatively deficient root system (McKeand, 1985). Since the major source of cytokinins in plants is the roots, especially root apices (Van Staden and Davey, 1979), it seemed possible that a reduced cytokinin supply might account for the aberrant maturation pattern of plantlets.

In some plants, the number of root apices can be increased by root pruning (Forsyth and Van Staden, 1981), with concomitant changes in cytokinin levels. This study tested the hypothesis that the number of root apices and lateral roots influences the maturation state of loblolly plantlets, possibly by altering cytokinin levels. The experiments also tested whether exogenous cytokinins would produce similar effects.

METHODS AND MATERIALS

Plant samples

Loblolly pine (*Pinus taeda* L.) tissue culture plantlets, rooted hypocotyls, and seedlings were produced, established in a greenhouse, and transferred to hydroponic growth conditions. After the first two weeks of the 4-month growth period, half of the plants were maintained on nutrient solution with the addition of benzyladenine (BA) at 1 mg/L. Root pruning was performed monthly, with experimental groups consisting of plants with 100% of their roots pruned, plants with 50% of their roots pruned, and unpruned controls. At the end of the experimental period, shoot apical meristem tissue was analyzed for E-zeatin + E-zeatin riboside. Techniques for extraction and analysis of cytokinin were adapted from MacDonald and Morris (1985).

Immunochemical methods

Rabbit anti-cytokinin antibodies were prepared as described previously (Zavala and Brandon, 1983). Rabbit 312 anti-zeatin riboside was selected for immunoaffinity purification of cytokinins (see below). The IgG fraction was isolated by ammonium sulfate fractionation and chromatography on diethylaminoethylcellulose. It was coupled to microcrystalline cellulose as described by Kohn and Wilchek (1982). Extent of coupling was estimated by immunoassay (see below). Capacity of the columns prepared in this manner were determined by chromatography of cytokinin solutions containing zeatin or dihydrozeatin (DHZ) and [³H]-DHZ (Amersham). Quantitation of cytokinins was by UV spectrophotometry following fractionation by HPLC (MacDonald and Morris, 1985).

RESULTS

Antibody characterization

Table 1 illustrates the characterization of rabbit-anti-zeatin riboside antibodies. It is noteworthy that this antibody is specific for the zeatin side chain, with ribosylation or derivatization of the 9-position nearly irrelevant to binding.

Table 1. Characterization of rabbit antibody to E-zeatin riboside

Cytokinin	I ₅₀ (M)	relative I ₅₀
E-zeatin	2.2×10^{-9}	1
E-zeatin riboside	2.2×10^{-9}	1
9-carboxyethylzeatin	1.4×10^{-9}	0.64
isozzeatin	1.4×10^{-8}	6.4
dihydrozeatin riboside	7.1×10^{-8}	32
Z-zeatin	1.5×10^{-7}	68
dihydrozeatin	1.8×10^{-7}	82
<u>cis</u> -norzeatin	6.3×10^{-7}	290
<u>cis</u> -norzeatin riboside	6.3×10^{-7}	290
benzyladenine	7.9×10^{-7}	360
2-isopentenyladenine	1.1×10^{-6}	500
kinetin	1.1×10^{-6}	500

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Avidity was estimated as the concentration of cytokinin producing 50% inhibition of binding (I₅₀) in a liquid-phase enzyme immunoassay. The assay used a conjugate of 9-carboxyethyl-E-zeatin and horseradish peroxidase (CEZ-HRP). CEZ-HRP was incubated with appropriately diluted antibody in the presence of buffer or analyte. Antibody-bound CEZ-HRP was then precipitated with ammonium sulfate, redissolved in buffer, and assayed for peroxidase activity.

Analysis of derivatized cellulose by immunoassay

As part of this study, we developed a procedure for measuring the degree of conjugation of protein-microcrystalline cellulose matrices. This was done by inhibition ELISA assay, using the cellulose matrix as analyte. Experiments were first done with BSA to test the feasibility.

Conditions for ELISA of BSA conjugates were as follows. Polyvinyl chloride microtitration plates were coated with 50 μL /well of 10 $\mu\text{g/mL}$ BSA. Following the washing procedure, remaining sticky sites were blocked with 1% normal rabbit serum. In the inhibition ELISA, samples were diluted in buffered saline containing 2 mg/mL ovalbumin. (When analyte is not BSA, BSA can be used in the diluent and blocking steps.) Samples to be assayed in the inhibition ELISA were mixed with mouse anti-BSA antiserum at a final dilution of 1:500. After a 1 h preincubation, the mixtures were transferred to assay wells (50 μL /well) and incubated for one hour. Plates are then washed and rinsed, and bound mouse IgG detected with horseradish peroxidase-labeled rabbit anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) and ABTS substrate solution.

Figure 1 illustrates a standard ELISA curve for BSA in this assay system, and the effect of adding unconjugated microcrystalline cellulose. It is apparent that the cellulose (10% by volume) has no effect on the assay. Figure 2 illustrates the analysis of two conjugates, produced using 5 and 25 mg/mL BSA in the initial coupling step. The results are 11 and 100 μg BSA/mL cellulose by reference to the standard curve.

Figure 3 illustrates the results of applying the method to the analysis of rabbit anti-cytokinin affinity matrices. The results are shown for (A) standard conditions, (B) 2.5-fold increase concentration of CNBr in the coupling step, and (C) 5-fold reduced concentration of cellulose relative to protein. The shift of curves B and C to the left are indicative of a 10-fold higher degree of coupling (5 $\mu\text{g/mL}$) compared to the initial reaction conditions. (That is, there was equivalent inhibition in the ELISA at 1:100 for B and C as at 1:10 for A.)

Capacity of immunoaffinity column

The capacity of immunoaffinity columns to bind cytokinins was determined with mixtures of unlabeled cytokinins and [^3H]-DHZ. Results from the first batch of R312-cellulose (A, Figure 3) are shown in Table 2.

The results indicate that the column capacity is at least 20 pmol/mL of packing. The column appeared to be most efficient in the concentration range of 10^{-7} M. Higher concentrations exceeded the capacity of the column and substantially lower concentration resulted in reduced binding. The binding was apparently reduced as the zeatin concentration approached the apparent affinity of R312 (about 2×10^{-9} M).

Table 2. Binding capacity of R312-cellulose

Concentration of unlabeled ZR (M)	[³ H] recovered (cpm)
10 ⁻⁶	625
10 ⁻⁷	15,700
10 ⁻⁸	4,280

=====

1 mL samples in 40 mM ammonium acetate,
pH 6.5, were chromatographed on a 5 mL
immunoaffinity column. Cytokinins were
eluted with 50% methanol in acetate
buffer.

In a separate experiment, it was determined that more densely conjugated R312-cellulose (5 ug/mL) had a higher capacity at 10⁻⁸ M zeatin, retaining 16,100 compared to 12,200 cpm of [³H].

A representative data set indicating the recovery of [³H]DHZ added as an internal standard to the plant samples is indicated in Table 3.

Table 3. Recovery of cytokinins in plant samples

Sample No.	[³ H] recovery (%)
11	80.5
17	82.7
59	59.3
73	100
118	100
141	95.1

Figure 4 indicates a chromatogram showing the fractionation of immunoaffinity-purified cytokinins from a typical sample. Predominant cytokinins are E-zeatin (6.03 min), DHZ (7.21 min), and E-zeatin riboside (8.82 min). Earlier, more polar peaks were not characterized.

Figure 5 shows that the root pruning treatments had the expected effect--increasing the number of apices. Morphological measurements (data not shown) indicated that the root pruning did result in a more juvenile state of the plantlets. Table 4 summarizes the measurements of E-zeatin in this study.

Table 4. E-zeatin in shoot apical meristem

=====	
Experimental group	Zeatin (ng/g)

All treatment groups:	
Plantlets	7.45
Rooted hypocotyls	8.15
Seedlings	7.45

All plant types:	
Not pruned	7.28
50% root pruned	7.45
100% root pruned	7.89

Seedlings:	
Not pruned	6.96
50% root pruned	7.54
100% root pruned	8.02
=====	

Differences among the treatment groups were significant for the seedlings only. Within this plant type, zeatin concentration correlated significantly with the number of root apices. Exogenous benzyladenine did not affect the maturation state of any of the plants (data not shown).

The negative results obtained with rooted hypocotyls and plantlets--the lack of correlation between zeatin concentration and state of maturation--means that further work is needed to define the relationship between cytokinins and maturation. A possible explanation is that zeatin alone may not be crucial, but the ratio of zeatin to other phytohormones (ZOG, IAA, etc.) may be important. In addition, the number and activity of zeatin "receptors" may be more closely linked to maturational state than the concentration of zeatin. Since the results indicate that root pruning does influence maturation, further work should focus on processes occurring within the roots, including nutrient uptake and cytokinin synthesis.

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Figure 1. Inhibition ELISA analysis of BSA in the presence or absence of 10% by volume of packed microcrystalline cellulose.

Figure 2. Inhibition ELISA analysis of two BSA-cellulose matrices. Initial BSA concentrations were (A) 5 mg/ml and (B) 25 mg/ml.

Figure 3. Inhibition ELISA analysis of rabbit anti-zeatin antibody-cellulose matrix. (A) Standard conditions, (B) increased CNBr (2.5-fold), (C) increased protein:cellulose ratio (5-fold).

Figure 4. Chromatography of cytokinins isolated from loblolly shoot apical meristem on C-18 column.

Figure 5. Mean number of root apices and standard errors for loblolly seedlings, plantlets, and rooted hypocotyls in the three root pruning treatment groups.

E. OTHER STUDIES AND WORK IN PROGRESS

Description of these facets are presented in the form of brief summaries.

1. Effect of nutritional stress in pepper plants (with A. Bar-Tal).

Pepper plants were grown under different conditions of nutrient--control, low N or P, or grown with restricted root volume. Roots were lyophilized, pulverized, and extracted to prepare samples for immunoassay of cytokinins. Monoclonal antibody 93 was used in some of these analyses. Preliminary studies suggested that restricting the root volume lowers the cytokinin content, and that nutritional supplementation may counter this effect. The immunoassays did not result in statistically significant data, however, with considerable plant-to-plant variation. We have not received the complete write-up of Bar-Tal's experiments, including comparison with other techniques for quantitating cytokinins.

2. Immunoassay of indoleacetic acid and its conjugates.

Haptens were synthesized (J. Corse) for eliciting antibodies and preparing enzyme-labeled ligands which may permit improved specificity of immunoassays for IAA. In addition, this approach should permit direct immunoassay of IAA-2'-myo-inosityl ester. These haptens were synthesized by derivatization of IAA in the 5 and 6 positions:

Figure 1. IAA haptens based on derivatization of the benzene ring. (I) methyl 5-carboxymethoxyindole-3-acetate; (II) methyl 6-succinoylaminoindole-3-acetate; (III) 6-β-alanylaminoindole-3-acetic acid; and (IV) 2'-myo-inosityl 6-succinoylaminoindole-3-acetate.

During my visit to the Maoz laboratory in Israel, collaboration with E. Epstein led to the observation that the perfluorotoluy ester (PFT) of IAA crossreacts with antibodies which recognize IAA

methyl ester. Since the PFT ester can be readily measured by GC, antibodies with this specificity pattern could be used to correlate immunochemical and physicochemical measurements or for immunological quantitation of IAA derivatives fractionated by GC.

3. Probes for use in receptor studies.

Two idiotypes of zeatin-specific monoclonal antibodies have been defined. One of these idiotypes (the antigenic site of the antibody combining site itself) is similar in its binding specificity to the putative cytokinin receptor measured by a number of bioassays. Techniques have been developed to screen for monoclonal antibodies which recognize this idio type. Production of such antibodies would provide a tool for identification of cytokinin receptors, whether they are enzymes or other transducers of cytokinin action.

In addition to immunochemical probes, fluorescent and paramagnetic cytokinins have been synthesized (J. Corse). These can be used to detect changes in cytokinin binding sites as well as to quantitate cytokinins (with J. Windle).

4. Cold stress in Zea mays (with M. E. Zavala).

This project is aimed at determining the genetic basis of cold tolerance of roots. Information gained could contribute to producing plants with a longer growing season. A related practical objective is to produce varieties subject to earlier and more uniform establishment of seedlings. One focus of this project is the morphological and quantitative study of cytokinins and their receptors in meristematic root tissue. Quantitative measurements of cytokinins in roots of seedlings have been made. The techniques will also be applied to roots grown in vitro.

DESCRIPTION OF COOPERATION

Cooperation and contacts with Israeli investigators

Arieh Maoz (Institute of Plant Protection, ARO Volcani Center) was Cooperating Investigator. Collaboration included discussions and exchanges of letters and occasional phone conversations concerning planning and design of experiments. Exchanges of experimental reagents were made in person or through the mail. Antibodies from the U. S. were referred to other collaborators (principally A. Bar-Tal), with assistance in setting up ELISA's for cytokinins.

Visits were exchanged--I visited the Maoz laboratory in September-October 1985; Dr. Maoz visited my laboratory in July 1986, with occasional visits and conversations during his sabbatical in the U. S. (7/86 - 7/87). During each of the visits, the host facilitated contacts with other investigators, through informal discussions or by formal seminar (Volcani Center, 20 October 1985).

During my visit to Israel, Arieh Maoz enthusiastically facilitated contacts with Drs. E. Epstein, A. Vardi, A. Carmi, A. Bar-Tal (then a graduate student with B. Bar-Yosef), R. Ben-Arie, and S. Ben-Yehoshua (Volcani Center); O. Sagee, J. Riov and S. Mayak (Hebrew University); B. Epel (Tel Aviv University); S. Gepstein and P. Neumann (Technion Institute); S. Fuchs and F. Kohen (Weizmann Institute). Discussions with most of these investigators was concerned with plant senescence, stress, and phytohormones. Other areas of interest were post-harvest senescence, immunoassay procedures, and development of anti-receptor antibodies. Preliminary studies were conducted with Drs. Epstein and Bar-Tal, and are mentioned elsewhere in this report.

The BARD collaboration led to visits to Western Regional Research Center by Drs. G. Ben-Hayyim (1985), S. Mayak (1987) and O. Sagee (1988), including seminar presentations and discussions with a number of investigators. Plans for future collaboration have been made with Dr. Sagee, and can be realized now that he has returned to the Volcani Center after his U. S. sabbatical (9/88). Future collaboration with Dr. Maoz will be most influenced by interests and policies of his new, industrial employer in the area of immunodiagnosics.

It is my hope that some of the contacts which have not yet developed into collaborations will still do so. My recent access to BITNET should enhance the prospects for effective collaboration. Economical, yet speedy written communication is important in exchanging data and ideas and would complement contacts in person or by phone.

Project staff and collaborations in the U. S.

It was a pleasure to be associated with the following co-workers as part of this project. Their direct, indirect, and informal contributions are gratefully acknowledged.

Western Regional Research Center:

Joseph W. Corse, Research Chemist, provided unflagging enthusiasm and encouragement, perceptive ideas, and exhaustive work on cytokinins and indole derivatives for eliciting antibodies and making conjugates. Dr. Corse headed the in-house ARS project corresponding to the BARD project.

Raymond S. Pacovsky, Biologist. Conducted soybean callus assays.

Marion L. Lyman, Physical Science Technician. Assistance with organic chemistry and bioassays.

Judith P. Tweedie (1985-86) and James M. Oliphant (1987-88), Biological Laboratory Technicians. Assistance with tissue culture and immunoassay. (Funded by BARD)

Undergraduate student assistants: Susan Prohaska (1985), Mac Axelrod (1985), Anthony Hsieh (1986-87), and Darin Latimore (1988). (Funded in part by BARD)

USDA Research Apprentices (Graduating high school students, funded by ARS Equal Employment Opportunity Program to encourage minorities and females to enter agricultural sciences) : Elizabeth Chiao (1986) and Tina Chin (1988).

Anne H. Bates, Chemist, helped to train and supervise technicians and student aides and to respond to crises.

Glenn Fuller, Research Leader of the Plant Development-Quality Research Unit, and Thomas A. McKeon, current leader of the in-house plant senescence project, are acknowledged chiefly for their help with integrating the project into the research unit.

The administrative and clerical support of secretaries Rose DeLemos, Gisela Travis, Lillie Davis and Emily Grey, and the helpful people of our Budget, Procurement, Personnel, and Patents offices are acknowledged.

Other collaborators:

Lisa A. Wisniewski, graduate student (present address, Del Monte Agricultural Research, San Leandro, CA), and faculty members S. E. McKeand and H. V. Amerson, Department of Forestry, North Carolina State University, Raleigh, NC. Conducted studies on maturation of loblolly pine in relationship to root morphology and cytokinin production.

Maria Elena Zavala, Visiting Professor, Department of Biochemistry, Michigan State University, East Lansing, MI (present address, Department of Biology, California State University, Northridge, CA). Studying regulation of responses to cold stress in roots of Zea mays, including the role of cytokinins.

EVALUATION OF RESEARCH ACHIEVEMENTS

Significant technical achievements have been made as follows:

1. New derivatives of cytokinins and indoles were synthesized.
2. Spin-label probes for cytokinin binding sites were synthesized.
3. A widely applicable method of promoting amide bond formation between proteins and haptens containing carboxyl groups was developed.
4. Immunoassay procedures were developed for characterizing antibody-polysaccharide conjugates useful in affinity chromatography.
5. Monoclonal antibodies and immunoassay methods for cytokinins--especially cytokinin-O-glucosides--were developed.
6. A model system for studying the function of cytokinins and relating cytokinin metabolism to specific genes was developed.
7. The putative cytokinin receptor which stimulates cell division in soybean callus was shown to be sensitive to substitution in the 9-position.
8. Endogenous zeatin and zeatin riboside were found to undergo small changes in concentration during nutritional stress in pepper plants.
9. Root pruning of seedlings (Pinus taeda) was found to alter endogenous zeatin concentration in shoot apical meristem, but not alter maturation characteristics.

Relationship to objectives:

With respect to the original objective (#1) of the project, we have completed the proposed chemical and immunochemical work related to cytokinins. As stated earlier in this report, we decided to concentrate on the cytokinins rather than on indoleacetic acid. We have a monoclonal antibody for quantitating and purifying one of the major conjugated forms of zeatin. We have done preliminary studies applying the immunochemical methods to physiological systems. We have used two of the three model systems originally proposed for studying plant senescence and one of the two model systems proposed for studying responses to stress.

Work is in progress to extend these preliminary studies and to compare analytical results by different techniques (immunoassay, bioassay, physicochemical analysis). Further work will give more emphasis to post-harvest senescence.

With respect to additional objectives (#2 and #3), not part of our original proposal, we have completed chemical syntheses and developed chemical and immunochemical procedures (such as immunoaffinity chromatography, etc.) for detection, isolation, and characterization of putative cytokinin receptors. Collaboration with Dr. M. E. Zavala (California State University, Los Angeles) and J. Windle (WRRC) will help us in this direction.

Benefits to agriculture

The long-run benefits from work undertaken in this project can be extrapolated from our preliminary results.

1. Increase in product yield.

Increase in yields could be expected from the uncoupling of leaf senescence from seed development in monocarpic crop plants. The role of phytohormones in this process has begun to reveal mechanisms and suggest practical solutions, but further work is needed to develop agronomically superior crop species based on the research. Conjugation of phytohormones, regulation of biosynthesis and translocation of hormones, and changes in receptor number and affinity are the areas which relate the present work to the expected benefit.

It should be noted that delay or acceleration of senescence in seed crops prior to harvest could affect production. Delay of senescence would give longer times for seed fill; acceleration could promote double cropping and permit crops to succeed where shorter growing seasons prevail.

2. Improvement in product quality and increase in exports.

This benefit would accrue primarily as a result of regulating of the post-harvest senescence of leafy vegetables. The considerations discussed above (1) apply here, too. From an economic point of view and from a food safety and regulatory perspective, manipulation of endogenous plant growth regulators would probably be more acceptable than developing artificial sprays, for example. Thus, the long-range outcomes of this line of research could contribute to minimization of the use of agricultural chemicals which degrade the environment or jeopardize the safety of agricultural commodities.

3. Reduction in crop losses due to poor seedling establishment.

There is evidence that nutritional stress (such as N or P deficiency) and cold stress may cause poor seedling establishment in some crops. It is known that roots can be affected by such environmental stresses, and that resistance to some stresses is heritable. Meristematic regions involved with cytokinin biosynthesis are sensitive to environmental stresses.

Methods for measurement and localization of phytohormones, their conjugates and receptors may help define the genetic basis of resistance to the environmental stresses which lead to poor seedling establishment. Work begun during this project is proceeding with this objective.

PUBLICATIONS

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