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BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF EARLY STAGES OF GIBBERELLIN-INDUCED AND NATURAL FLOWERING IN WESTERN RED CEDAR (*Thuja plicata* Donn.)

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Biochemical and physiological aspects of early stages of Gibberellin-induced and natural flowering in Western red cedar (Thuja plicata Donn.)" submitted by Nora F. McGregor in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Shoot tip proteins were examined at different stages of gibberellin A_3 (GA_3)-induced conebud production in western red cedar (Thuja plicata Donn.) to determine whether there are protein differences that coincide with (i) the arrival of GA_3 at the shoot tips, (ii) stages of GA_3 metabolism, (iii) endogenous GA levels, and/or (iv) early stages of conebud development.

At weekly intervals after $[^3H]GA_3/GA_3$ injection, samples were harvested for $[^{35}S]$ in vitro protein labelling, GA extraction and paraffin embedding.

Both in vitro $[^{35}S]$-labelled proteins and total proteins (Coomassie blue stained) were separated by SDS one-dimensional polyacrylamide gel electrophoresis.

Gibberellin-like substances were purified using preparatory C18 and silica gel partition columns and separated using analytical C18 reversed phase HPLC then quantified using radiospectrometry (for $[^3H]GA_3$ and its metabolites) and Tan-ginbozu dwarf rice bioassay.

A $[^{35}S]$-labelled 36 kDa protein occurred in in vitro-labelled shoot tips three to seven weeks after GA_3 injection. This coincided not with the arrival of GA_3 at the apices (which occurred more than two weeks earlier) but rather during microsporophyll initiation.

Increased levels of less-polar GA-like substances, on the other hand, were at maximum levels within the first week after injection of GA_3. It is not known whether they were important in conebud initiation, or whether initiation was caused by the GA_3.

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I would like to thank my committee members, especially Dr. Dick Pharis, for their advice and inspiration. Thanks also goes to all my colleagues in the Botany division and other friends for being both patient and helpful during particularily the stressful times. You will all be glad to know that next time I write a thesis, I will at least double my allotted time for putting it together.

Special thanks goes to Francis Dunlop for preparation of most of the Figures (thanks Tigger!!!), and to Ms. Lee Siew Hwee for her typing.

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ABBREVIATIONS

DMSO = dimethylsulfoxide

dpm = disintegrations per minute

f.w. = fresh weight

GA = gibberellin

GC-MS = gas chromatography-mass spectrometry

HPLC = high performance liquid chromatography

kDa = kiloDalton

LD = long day

mRNA = messenger ribonucleic acid

PAGE = polyacrylamide gel electrophoresis

SD = short day

SDP = short day plant

SDS = sodium dodecylsulfate

SIM = selected ion monitoring

TBA = tertiary butyl alcohol

TCA = trichloroacetic acid

INTRODUCTION

With the present rapid depletion of Canada's natural forest resources, intensive and successful reforestation programs are a necessity. Some reforestation programs currently center around the use of seed orchards. Trees with desirable growth characteristics (but not necessarily with high reproductive capacities) are grown under natural conditions in order to collect large quantities of viable seed for seedling production and outplanting.

Western red cedar (Thuja plicata Donn.) produces cones sporadically after the age of 10 to 20 years (Pharis and Owens, 1966). Cone induction and development studies have been performed with the aims to improve seed yield and to induce sexual reproduction at an earlier chronological age.

T. plicata, a member of the Cupressaceae, is monoecious, producing separate strobilate male and female "flowers" on the same tree. Both organs arise from apices of small lateral branches due to a physiological transition of a previously vegetative apex to a reproductive apex. It is far from clear what causes this switch in development; indeed its control and specific events that take place during the switch are concerns central to physiology of reproduction in

any seed plant system.

It has been found that treatmemt of western red cedar (and other Cupressaceae species) with exogenous Gibberellin A_3 (GA_3) will increase cone production in mature trees and also can cause precocious cone induction in seedlings or saplings (Pharis et al., 1969; Owens and Pharis, 1971). There is some evidence to suggest that high gibberellin levels in conifer buds are associated with the transition to the reproductive state (see later). It is of interest to know whether this transition involves an alteration of gene expression, induced either directly or indirectly by gibberellin(s). In a system where the characteristic proteins associated with the vegetative and reproductive states have previously not been described, a comprehensive examination of these macromolecules is essential. If there are noticeable differences in proteins between vegetative and reproductive conebuds at an early stage of initiation and/or differentiation, then the significance of these differences, and how they are controlled, can eventually be determined.

The purpose of this thesis was to compare proteins in shoot tips of western red cedar at the vegetative stage and at early stages of GA-induced reproductive development (prior to conebud maturation). Differences seen in shoot tip proteins were related to some

physiological aspects of the samples including water potential and gibberellin levels. Also, the stage of conebud development (determined by anatomical analysis) was considered.

Cone Production in Western Red Cedar

Mature trees are naturally induced to initiate reproductive organs under LD ---> SD during early summer ---> fall (Owens and Pharis, 1971). On southern Vancouver Island, British Columbia, initiation takes place primarily in June for male conebuds and in July and August for female conebuds although the phenology of the reproductive cycle differs between climatic regions (Owens and Molder, 1984). Female conebuds appear at top and distal portions of the tree on short lateral branches whereas male conebuds appear on lower or more proximal positions on relatively long lateral branches (usually having approximately 30 leaf pairs) (Owens and Pharis, 1971). Ten months elapse between conebud initiation and pollination; mature seed cones have finished developing by the following autumn. For this thesis, only conebud initiation and early development (before SD and cold) were considered.

Early work by Pharis et al. (1965) and Kato et al. (1958) showed that conebud production in conifer

seedlings that had not reached sexual maturity (e.g. ripeness-to-flower), was dramatically promoted by treatment with GA_3. Mature trees also responded to similar treatments with increased cone production (Owens and Molder, 1984). Treatment was optimal for initiation and further development when it began at the natural time (and therefore day-length sequence) for initiation: in this case early June and early July for male and female cones respectively. To ensure complete development of female cones, extended treatment with GA_3 using a spraying method of application, was required (Pharis et al., 1969; Ross, 1983).

Events During Transition of Buds from Vegetative to Early Reproductive Modes

Early initiation events can only be seen with the aid of microscopic observations; transition of vegetative apices to pollen- and seed-cone reproductive apices occurred after 14 and 27 days, respectively, from inital GA_3 treatment in seedlings, as determined by increased rates of cell division and delay in sublateral primordial initiation (Owens and Pharis, 1971). After the sublateral primordia initiation resumed, phenolic substances accumulated in abaxial cells of microsporophyll and bract primordia. These

cells stopped dividing and subsequently microsporophylls and bracts began to grow upwards. After all microsporophylls, microsporangia, bracts and ovules had been initiated, phenolic substances accumulated in the cells of the small residual apex, which then in turn becomes inactive (Owens and Pharis, 1971). It is evident from anatomical studies that many structural and biochemical changes must take place during early stages of the transition. It is of interest then to see if specific changes in proteins accompany initiation of reproductive parts vs vegetative leaves and the permanent switch of the apex to the reproductive mode.

Pollen and seed cones can only be distinguished from vegetative buds with the naked eye 6 and 8 weeks after GA_3 treatment begins, although this depends on environmental conditions (Ross, pers. comm.) It is not clear which anatomical stage of development corresponds to this externally visible stage; initiation of reproductive organ parts may still be occurring.

Because frequency of initiation after GA_3 treatment varies between trees and even branches on the same tree (Ross, per. comm.), harvesting only truly initiated buds at very early stages of initiation would require dissection of each bud. Each shoot tip at early initiation weighs between 2 and 4 mg f.w. and

each apex only a small fraction of that (McGregor, unpublished data). Dissection to obtain homogeneous tissue samples seemed impractical when one also considers that wounding of the tissue will activate proteases (Davies, 1982). For these reasons, I chose to look at proteins in whole shoot tips, as opposed to apices, to get an overall view of biochemical changes during the transition of shoot tips to the reproductive mode.

Sex Expression

Although there is a positional factor involved in determining sex expression in T. plicata and in most gymnosperms (Owens and Molder, 1984; Marquard and Hanover, 1984; Lee, 1979) only one characteristic vegetative meristem has been described anatomically for Thuja (Owens and Pharis, 1971). From an evolutionary viewpoint, gynosporangia and androsporangia of seed plants were at one time homologous (Heslop-Harrison, 1957). Since 'ontogeny recapitulates phylogeny', during early stages of floral initiation, few biochemical differences between buds destined to become pollen or seed cones would be expected.

Mature male and female conebuds in T. plicata undoubtedly contain different proteins and mRNAs;

many structural features differ between the two sexes (Owens and Pharis, 1971; Owens and Molder, 1980, 1984). The question of relevance to this thesis is at what stage in development can sexuality be recognized biochemically? Anatomical distinction between bracts and microsporophylls can be made 20 days after initial apical transition (Owens and Pharis, 1971). The distinction was made on the basis of time of initiation and their position on the apex and on the whole plant.

Proteins and mRNA Associated with the Reproductive Transition

In the past most of the research related to the study of gene expression associated with the reproductive transition of the apex has been focussed on those species that flower after one inductive short photoperiod (SPDs) such as Lolium, Xanthium, Pharbitis and Sinapis. Many attempts to demonstrate specific qualitative differences in protein and mRNA patterns between non-induced and induced apices have not been successful (Vince-Prue, 1975; Cherry and van Huystee, 1965a, 1965b; Rijven and Evans, 1967; Stiles and Davies, 1976). Most of these studies have shown, however, that increased overall RNA and protein synthesis accompanies induction (Vince-Prue, 1975).

Studies using cycloheximide (an inhibitor of protein synthesis) and 5-flurouridine (an inhibitor of RNA synthesis) have shown that induction of flowering can be blocked without affecting vegetative growth (Cherry and van Huystee, 1965b; Vince-Prue, 1975). This suggests that pre-existing proteins and messages appear to sustain vegetative growth (for at least a limited period) but that new proteins or messages appear to be required for a switch to reproductive growth.

Recent work with Sinapis alba has shown that changes in concentrations of 3 specific proteins in the shoot apical meristem are evident immediately after photoperiod induction and until 10 days after induction (Pierard et al., 1977, 1980). The role of these proteins in flower initiation/differentiation (if any) has not yet been determined.

No work with inhibitors of protein or mRNA synthesis, examination of in vitro incorporation of radioactive precursors to protein and mRNA, or even characterization of proteins and mRNA associated with cone induction in western red cedar or any other Cupressaceae species has been reported. There have been a number of papers published that isolated proteins from conifer tissue, however, the particular tissues used were foliage, vegetative buds, or seeds

(McMullen and Ebell, 1970; Perry, 1963; King et al. 1984). Many possibilities exist for research on this aspect of reproduction in conifers.

Endogenous Gibberellin Levels in Relation to Cone Production

Successful cone induction in Cupressaceae by treatment with GA_3 has led to the suggestion that when trees are actively growing, GAs are used preferentially for vegetative growth, and are available for reproductive use only when produced in amounts in excess of that required for vegetative growth (Pharis and Owens, 1966; Pharis and Kuo, 1977). This would imply that mature flowering trees should contain a higher concentration of GAs than seedlings, which have active vegetative growth only. On the other hand, it is exogenous application of $GA_{4/7}$ and GA_9 that induces dramatic flowering responses in the Pinaceae family, not GA_3, implying that there is some specificity in the GA applied required to induce flowering (Ross and Pharis, 1985; Pharis and Ross, 1986; Pharis, 1979).

Application of GAs also causes shoot elongation in conifers (Ross, 1983; Kuo and Pharis, 1975; Dunberg and Eliasson, 1972). Pharis (personal comm.) has

hypothesized that in conifers, like in maize and pea (Phinney, 1985), the more polar GAs (e.g. GA_1, GA_3) are causal in inducing shoot elongation. Those GAs causal for producing flowering in Pinaceae conifers would appear to be less-polar in nature (e.g. similar in polarity to $GA_{4/7/9}$; see Pharis and Ross, 1986). However, in the Cupressaceae, where all bioactive GAs (and especially GA_3) can promote both vegetative shoot elongation and flowering, it is less clear as to which category of GA may be causal for flowering. Data of Kuo (1973) and Kamienska et al. (1974) showed that flowering induced in Cupressus arizonica by Nitrogen starvation was associated with an increase in less-polar GAs.

Endogenous gibberellins have not yet been characterized in T. plicata so it is not known whether the processes during GA_3 induced flowering in this species are similar to those occurring during naturally-induced cone production. Indeed, GA_3 may not be native to T. plicata and may exert its effects through conversion to other GAs or by causing higher levels of less polar endogenous GAs to increase, possibly due to a feedback inhibition mechanism. (Generally, less polar GAs are precursors to more polar GAs, Glenn, 1975; Pharis, 1979).

Specific aims of the Thesis

The main purpose of this thesis was to compare proteins present in shoot tips of western red cedar associated with different stages of the transition from vegetative apices to reproductive apices. The majority of the comparisons were made on shoot tips induced to flower by injection of los specific activity [^{3}H]GA_3 into branches of seedlings. This required finding a dosage of GA_3 which by injection would induce close to 100% flowering (conebud production in all actively-growing shoot tips).

The comparison of proteins associated with early conebud development was focussed mainly on proteins being actively synthesized in shoot tips at certain stages of development. This required determination of an efficient and effective procedure for <u>in vitro</u> labelling of proteins with [^{35}S] methionine.

The changes in [^{35}S]-labelled proteins with time after [^{3}H]GA_3/GA_3 injection were then related to changes in [^{3}H]GA_3 metabolism and to levels of putative less polar GAs in the same shoot tip samples. When greater amounts of putative less polar GAs were found in reproductive shoot tips (whether or not as a direct result of [^{3}H]GA_3/GA_3 metabolism) than in control (vegetative) shoot tips (by bioassay), then iden-

tification of these GA-like substances was attempted by GC-MS.

The changes in shoot tip proteins throughout reproductive development were also related to the anatomically determined stage of development of shoot tips in the samples of interest.

In addition, GA_3 and GA_7 were compared in their ability to cause cone production and shoot elongation in T. plicata seedlings.

MATERIALS AND METHODS

Plant Material

Three different stocks of western redcedar (Thuja plicata Donn.) were obtained by air shipment from various seed orchards near Victoria, British Columbia. Clonal propagules, rooted from branches of mature trees were donated by Lost Lake Seed Orchard. Harmac Seed Orchard and Cowichan Lake Seed Orchard donated three-year-old seedlings and one-year-old seedlings respectively. Plants were repotted in a 50:50 mixture of peat moss and sand using one or two gallon pots. Plants were then grown in the University of Calgary greenhouse under natural photoperiod or in an 8' X 4' growth chamber with an 18 hour photoperiod using fluorescent light. The day and night temperatures in the greenhouse were 22 °C and 20 °C whereas in the growth chamber they were 23 °C and 15 °C respectively. Light intensity at terminal apex level was 92 $\mu E/m^2/s$. Greenhouse and growth chamber plants were watered daily with Peter's fertilizer (20:20:20, 100 ppm Nitrogen with trace elements). Plants were moved in the growth chamber at least two weeks prior to

the beginning of any experiment.

Gibberellin Preparation and Application

Gibberellin A_3 (GA_3, from Imperial Chemical Industries) was purified by SiO_2 gradient-eluted partition chromatography (Durley et al. 1972). The resulting product was analyzed for purity by GC-MS. Radioactive GA_3 (6-^{3}H) obtained from Isocommerz, DDR, was purified by reverse-phase C18 HPLC. The specific activity of the main radioactive peak was determined to be 4.5 Ci/mmol by GC-MS. Gibberellin A_7 (GA_7, Abbott Chemical Company) was estimated to be 97.2% pure.

Primary branches of GA_3-treated three-year-old seedlings were injected with 5 µL of 2% DMSO in 95% ethanol containing the appropriate amount of GA_3 (and 10^6 dpm [^{3}H]GA_3 where applicable). Control trees received analogous injections of DMSO/ethanol and high specific activity [^{3}H]GA_3. The site of injection on a branch was chosen to be close to the most proximal secondary branch that showed active vegetative growth (Figure 1). One-year-old seedlings received 5 µL DMSO/ethanol injections of GA_3, GA_7 or solvent only. Because these seedlings were considerably smaller than the three year seedlings, there was only one injection

per tree and the site of injection was approximately mid-way up the stem; below that point was little active vegetative growth. All injections were performed by making a one cm incision through the bark, and extending the incision on both sides under the bark for a few mms. The syringe needle was then inserted under the bark and one half the injection was applied to each side of the incision (Figure 1).

In vitro and in vivo labelling of shoot tip proteins with [^{35}S]

L-[^{35}S] methionine (Amersham, 1.5 KCi/mmol, 15 µCi/µL in aqueous 0.1% 2-mercaptoethanol) was used for all labelling experiments. In vitro labelling was achieved by placing a known fresh weight of excised shoot tips in a 15 mL glass vial containing 0.5 ml distilled and deionized water (ddH_2O) and the appropriate amount of [^{35}S] methionine. Incubations were for three hours unless otherwise stated and were done on a 60 rpm shaker under diffuse light at room temperature. An attempt was made to keep fresh weights constant between samples for each experiment but this resulted in using variable numbers of shoot tips for different samples.

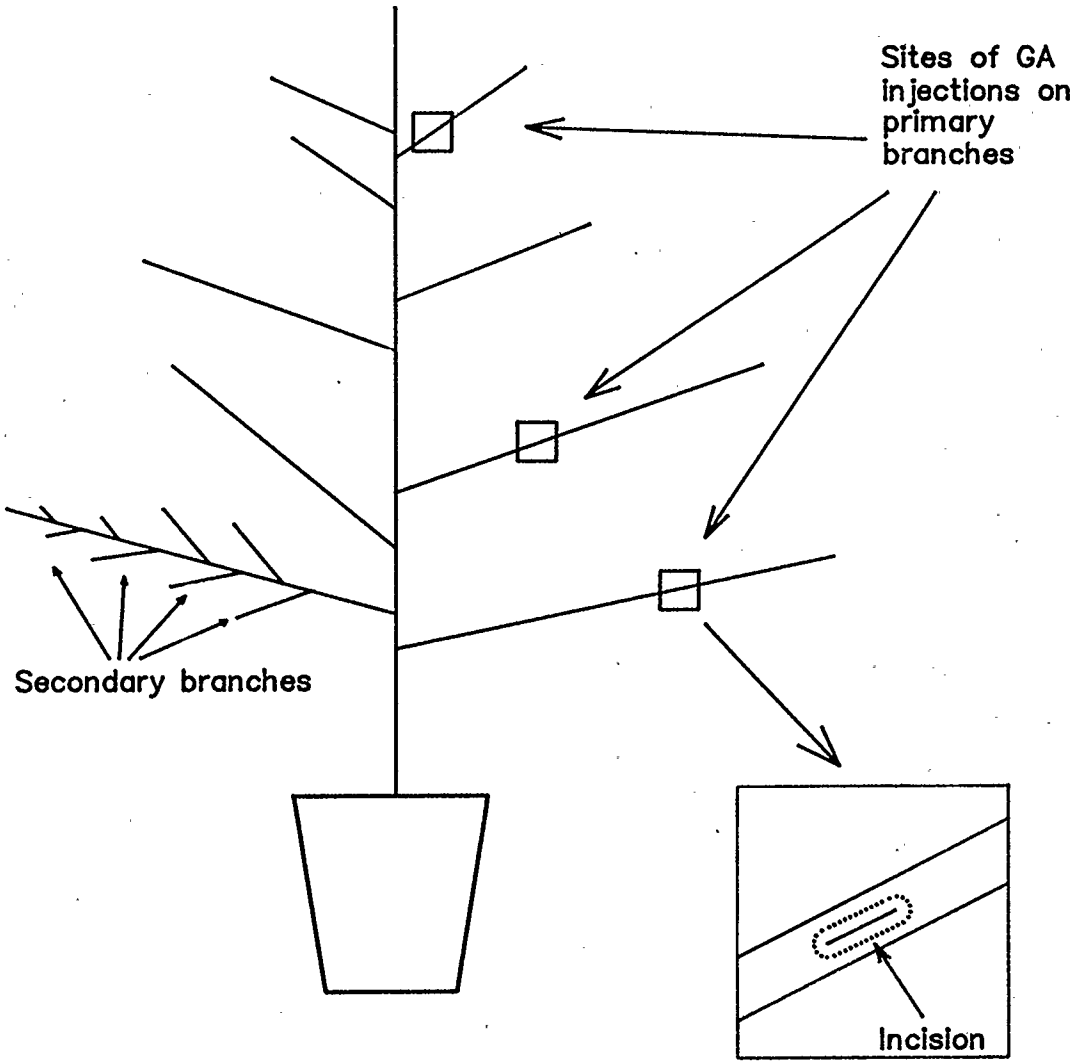

Figure 1. Sites of GA injection on three-year-old seedlings.

Shoot tip samples consisted of the excised distal 2 mm of potentially reproductive tissue (see the introduction) or of the excised distal 5 mm of terminal shoot apex tissue with two to four outer leaves removed. Potentially reproductive shoot tips were always classified as either GA_3-treated, GA_7-treated or control, and sometimes classified as either male or female region shoot tips depending on the experiment and the area of the tree from which the tips were excised. Samples were rinsed after incubation using vacuum filtration with a few mLs of ddH_2O then a few mLs of 95% ethanol. Samples were frozen in liquid Nitrogen and kept frozen at -20 °C until extracted for proteins less than four days later.

In vivo labelling of proteins was done on terminal apex tissue of control one-year-old seedlings. One-half µL or one µL of [^{35}S] methionine (15 µCi/µL) was injected one cm below the most distal portion of the shoot. These trees were then left under direct high light intensity (92 µE/m^2/s) for six hours. Samples were then excised and frozen as for the in vitro experiments. Each sample consisted of enough shoot tips to give a total of 1 µL of [^{35}S] applied.

Protein Extraction, Determination of Uptake and Incorporation of [^{35}S] label

To extract soluble proteins, the frozen shoot tip samples were ground with mortar and pestle, then SDS extraction buffer (composition appears in Appendix 1) was added, 100 µL for each 10 mg of sample, and ground again. Extracts were transferred to 1.5 mL Eppendorf centrifuge tubes. Mortars and pestles were rinsed with another equal volume of extraction buffer, the rinses and 0.05 g insoluble Polyvinylpolypyrrolidone (PVPP, Sigma, 100 mesh) were added to the original extracts before the contents were mixed well by inverting the tubes. Tubes were then placed in a boiling water bath for 5 minutes then centrifuged at 15000 X g for 10 minutes.

Aliquots were taken from supernatants to determine uptake of [^{35}S] label into shoot tips, for incorporation of [^{35}S] label into soluble protein, and for estimates of amounts loaded directly onto gels. Thus, 2 µL of sample (in duplicate) were added to 500 µL water for uptake determinations by liquid scintillation spectrometry. Ten µL of sample (in duplicate) were absorbed and dried onto Whatman No. 1 filter paper squares for determination of protein incorporation by TCA precipitation. The TCA precipitations were

accomplished with 2 consecutive 5 minute rinses of the filter papers in 10% ice-cold TCA, then immersing the papers for two minutes in boiling 10% TCA. Residual free [^{35}S] methionine was removed and the papers dried by rinsing twice with 95% ethanol and once with anhydrous ether. Filter papers and the 2 µL uptake samples were assayed on the Tracor Analytic Mark III Liquid Scintillation Counter after one hour of dark adapting with 10 mL of scintillation cocktail (either Scint-A, Packard, or Scinti-Verse E, Fisher). Samples were counted for one minute using a channel window of 0-167 keV.

Gel Electrophoresis and Autoradiography

Denatured proteins were electrophoresed using 7 to 15% polyacrylamide gradient SDS-PAGE 1.5 mm slab gels, using LKB 2001 Vertical Gel apparati. Composition of solutions used in preparing, running and fixing gels appear in Appendix 1. Proteins were separated using constant current of 20 mA per gel, fixed and stained with Coomassie Blue (R250) solution overnight then de-stained at least five hours. Before drying, gels were placed in 3% glycerol for five minutes. Gels were then dried for 1.5 hours on an LKB gel dryer.

Autoradiograms were made by exposing Kodak XAR

film to the dried gels for the appropriate amount of time. Generally, gels contained 150,000 dpm of TCA precipitable protein per lane and films were thus exposed for one week. Films were developed in a Pako X-ray film developer or by hand in Picker Developer and Fixer using the manufacturer's instructions.

Densitometry scans were prepared on a Joyce-Loebl Chromoscan 3 Laser Densitometer using a scan aperture of 0.5 mm.

Extraction and Purification of Gibberellin-like Substances

Shoot tip samples harvested for analysis of GA-like substances were analogous to that tissue excised for protein analysis although these samples consisted of many more tips than the amount needed for protein analysis. Samples were freeze-dried after harvesting into liquid Nitrogen for at least five days before their dry weights were determined and extractions performed.

The method used for some of the GA extractions and purifications was based on that of Koshioka et al. (1983). This method used two preparative columns of C18 reverse-phase material and one SiO_2 partition column to separate each crude extract into four

fractions: pigments, putative GA precursors, putative GA conjugates and free GAs (Figure 2). The free GAs were then separated by analytical C18 reversed-phase HPLC using a gradient program of 10-73% methanol (Appendix 2). A revised method that did not include a second C18 column to separate free GAs and conjugates from GA precursors, was used for the remaining samples (Figure 3). A mainly isocratic program of 64 % aqueous methanol on C18 reverse-phase HPLC then was used to separate polar free GAs from non-polar free GAs. and in turn from putative GA precursors (Appendix 2). This revised method improved losses of less polar and non-polar GAs which had occurred earlier due to difficulty in solubilization in the 10% aqueous methanol used for HPLC injection on the 10 to 73 % Methanol HPLC gradient.

Regardless of which method was used, separate glassware was used for GA-treated samples and control samples. Samples not containing [^{3}H]GA_3 and [^{3}H]GA_3 metabolites generally had 50,000 or 100,000 dpm of high specific activity [^{3}H]GA_1, [^{3}H]GA_4 (both from Amersham) or [^{3}H]GA_9 (Yokota) added to them as internal standards to follow losses when necessary. The C18 purification columns used 2.5 g of C18 material, and the pH of extracts was adjusted to neutrality before applying extracts to the columns and eluting with 80 % methanol.

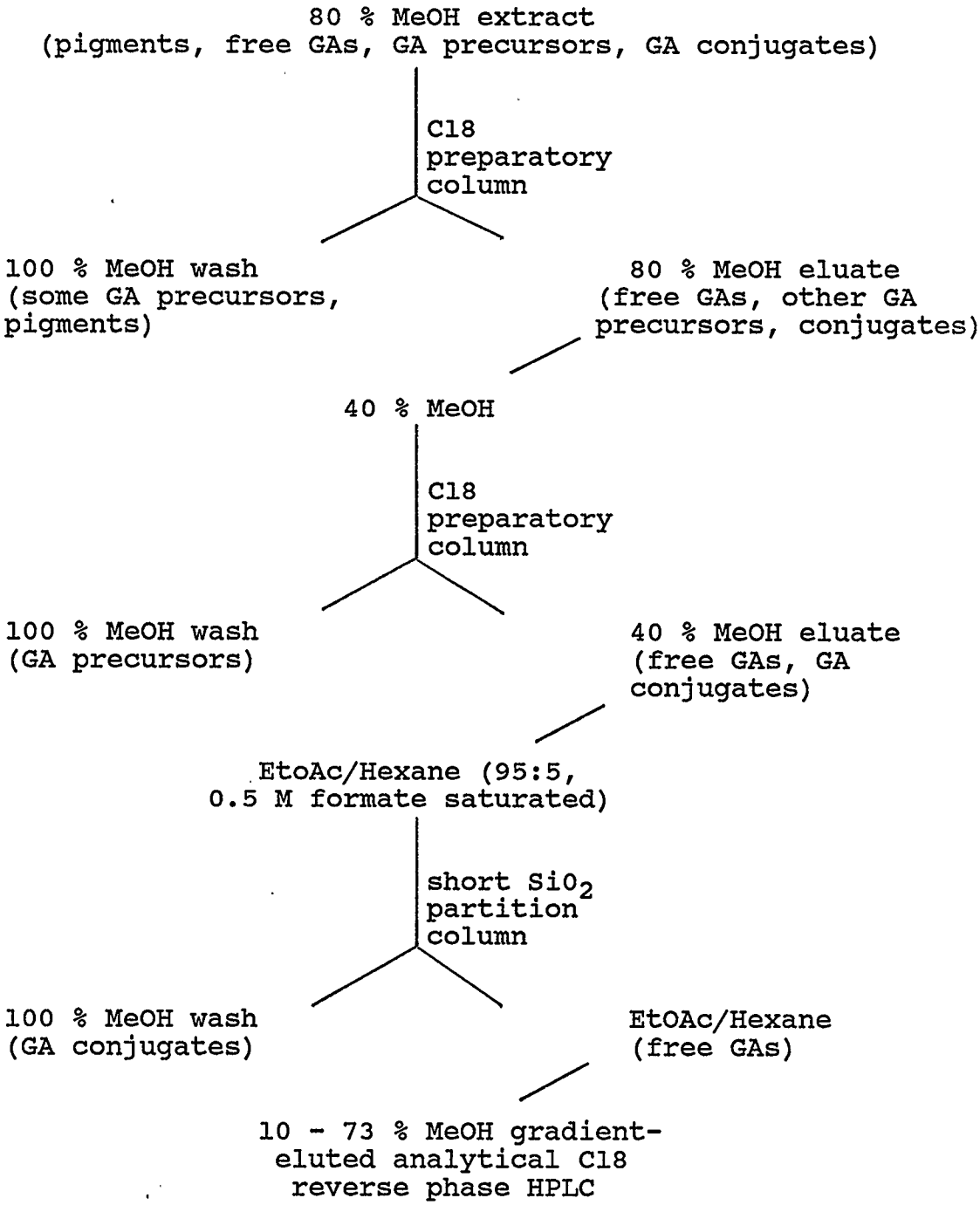

Figure 2. Purification and separation procedure used for isolation of GA-like substances and $[^3H]GA_3$ and its metabolites in Thuja. Most GA methyl esters would be included in the free GA fraction.

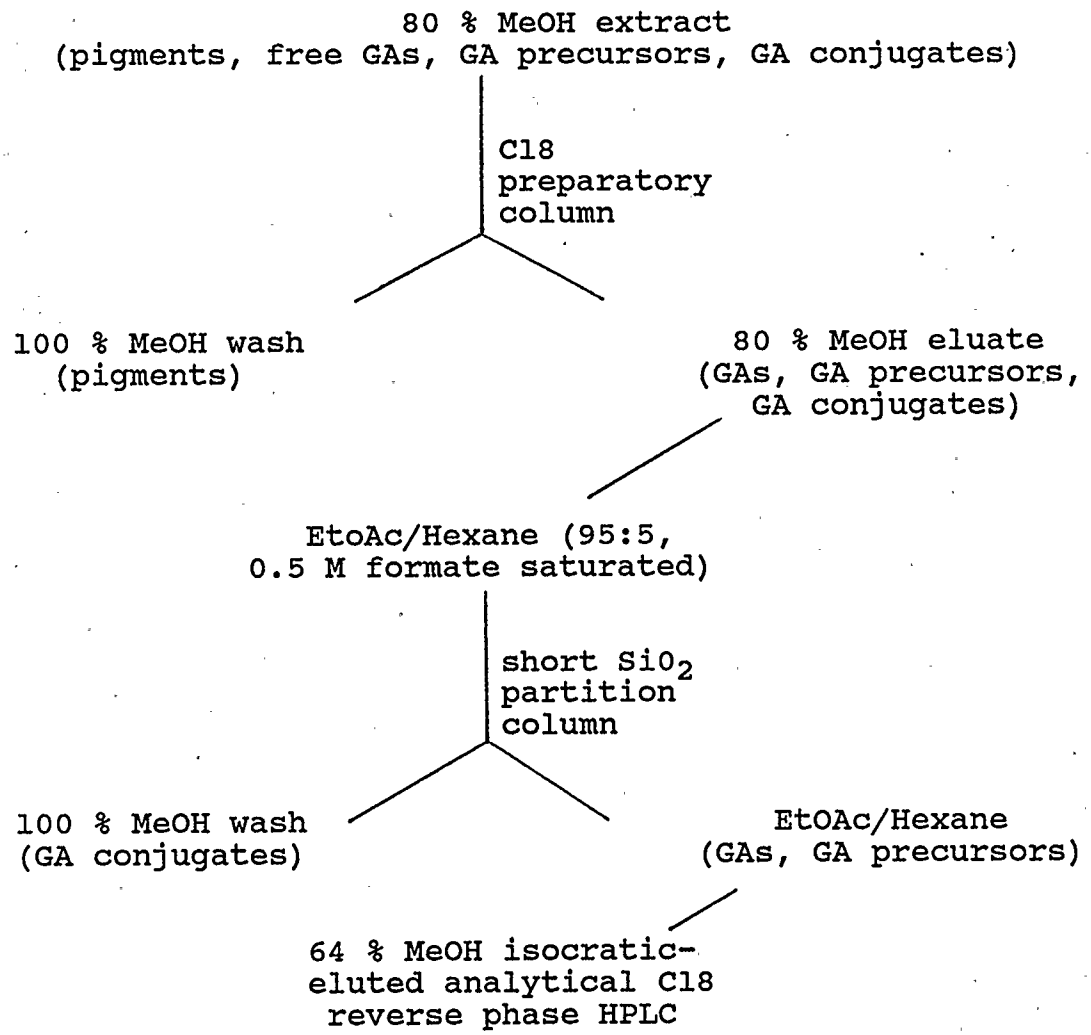

Figure 3. Revised purification procedure used in later experiments on some GA_3 treated samples from three-year-old seedlings and non-GA_3 treated material.

If a second C18 column was used, the extract was adjusted to 40 % methanol before the column was eluted with 40 % methanol.

Samples to be analyzed by GC-MS that were still too dirty after initial HPLC purification, were purified by preparative adsorption SiO_2 chromatography. The sample was dissolved in a minimal amount of dry isopropanol and loaded onto the top of a column of 4 g dry activated silica gel equilibrated with 30 mL dry isopropanol. The sample was eluted in 60 mL of 100% dry isopropanol. Occasionally, a second SiO_2 adsorption column was required.

GA Quantification and Identification

Determination of the amount of radioactive GA (internal standard or $[^3H]GA_3$ or its metabolites) present in fractions at various stages of purification was done by taking a suitably sized aliquot from the sample, adding scintillation cocktail and dark adapting as for measuring $[^{35}S]$. Samples were counted for one minute on a channel for tritium (0-18 keV). When counts of $[^3H]$ were found to be low and hence unreliable, larger aliquots were taken and the samples then counted for two minutes each.

To detect biologically active GA-like substances,

the Tan-Ginbozu dwarf rice bioassay was used (Murakami, 1968). Usually, 10 rice seedlings per aliquot dilution were used. To estimate the amount of GA-like biological activity present, the growth of the sheath of seedlings was measured for each fraction and compared to the growth obtained by applying standard amounts (10 pg to 100 ng) of GA_3 applied to rice seedlings the same day.

Identification of GAs was attempted by combined GC-MS. Generally, 50 or 100 ng of $[^2H]GA_1$ or $[^2H]GA_4$ was added to these samples prior to derivitization to monitor the efficiency of the derivitizing processes in the presence of contaminating substances. Samples were then methylated by adding 100 µL of ethereal diazomethane and incubating at room temperature for 0.5 hour. Samples were then silylated by dissolving the methyl ester in dry pyridine, adding 50 µL N,O-bis-trimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane (Pierce) and incubating at 60 °C in Nitrogen for 0.5 hour. Using a Selected Ion Monitoring program on the Hewlett-Packard GC-MS, attempts were made to identify specific GAs that might be expected (on the basis of HPLC retention times) to be in the extracts.

Anatomical Analysis

Representative shoot tip samples were excised and fixed in glutaraldehyde/formaldehyde fixative (see Appendix 1) for 24 hours. The first hour was under vacuum (20 inches Hg). The embedding series that followed included daily changes of the solutions to methyl cellulose, absolute ethanol, 25% ethanol/75% TBA/saffranin, absolute TBA, and finally paraffin (Tissue Prep-2). A second vacuum infiltration was used at the paraffin stage before the tips were poured into paper boats. Seven micron sections were cut using a Spencer microtome. A general histochemical stain was used. Slides were stained in 0.5 % safranin, 0.2 % basic fuschin and 0.2 % crystal violet in 50 % ethanol (Yeung and Peterson, 1972) followed by staining in Fast green (0.5 % in absolute ethanol; Yeung, 1985).

Water Potential Determinations

Shoot tip water potential for the three different types of plant material was estimated by the dew point method, using Wescor C-52 psychrometers attached to a Wescor HR-33T dewpoint microvolt meter (Wescor, Inc). Readings were taken every 15 minutes for the first hour and every 30 minutes thereafter until the equilibration

was reached (three consecutive readings with no change). Equilibration time ranged from 3 to 5 hours.

RESULTS

Effects of exogenous GA application on cone induction and shoot elongation

To find an optimal concentration for promotion of flowering by GA_3 injection, three dosages of GA_3 (10^{-4}, 10^{-3}, 10^{-2} mmol per tree) plus control injections of solvent only, were given to 16 seedlings such that there were 4 replicate seedlings per dosage. A further 4 seedlings served as uninjected controls. Injections were applied to 5 branches on each seedling to give 7, 70, or 700 µg to each branch. Cone production was scored 10 weeks after treatment when the conebuds were expected to have completed initiation of all reproductive organs that are possible (e.g. from actively growing shoot tips; Owens and Pharis, 1971). Seedlings remained in the growth chamber throughout the incubation period.

The cone induction response to these variable dosages of GA_3 are summarized in Figure 4. Only actively growing shoot tips were scored. Neither group of control seedlings had cones at this time. Cone production increased with increasing GA_3 concentration to a maximum of 100 % of the shoot

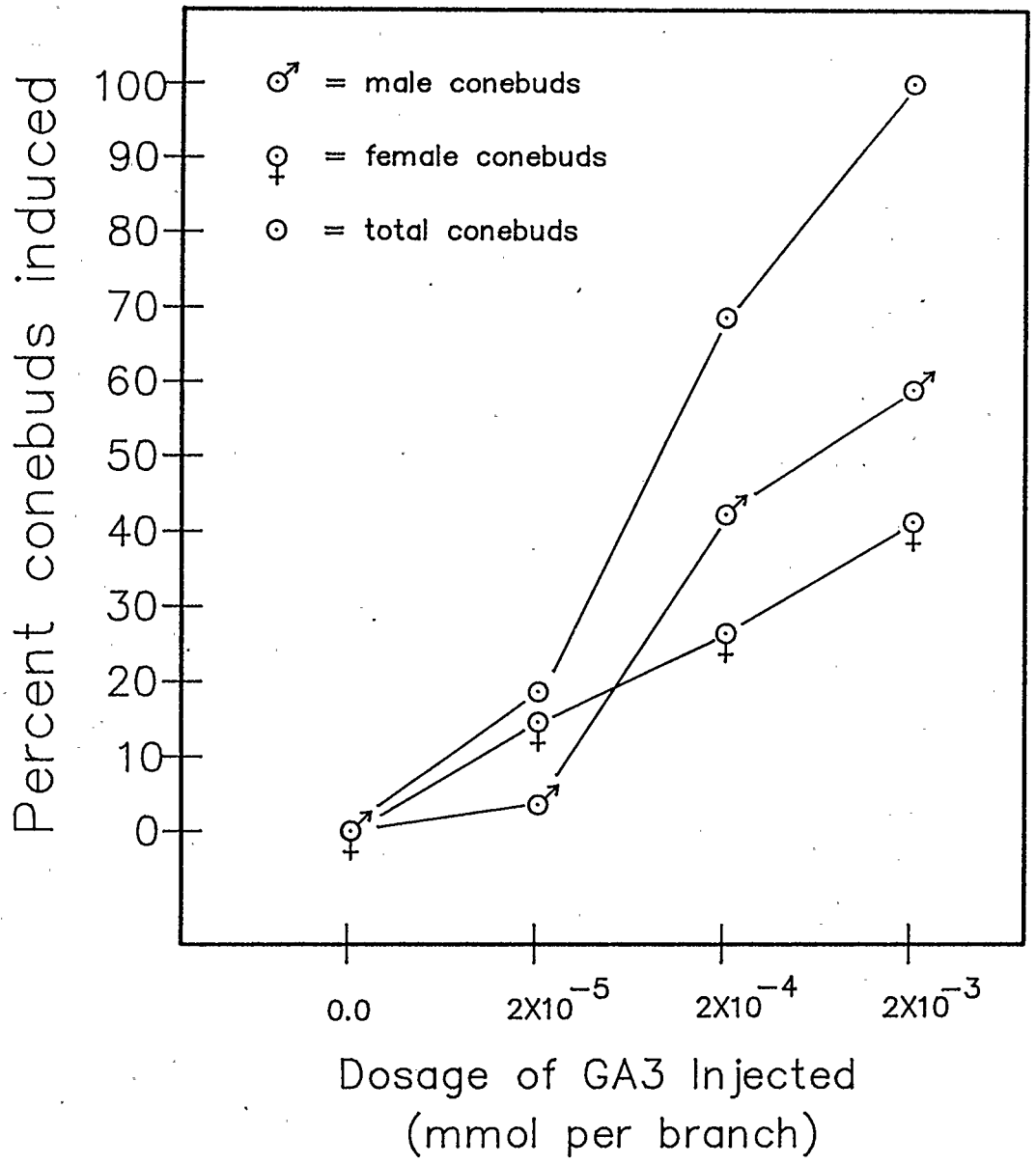

Figure 4. Flowering response of Thuja plicata shoot tips to variable GA_3 dosage. Data points shown represent the percentage of actively growing shoot tips scored as reproductive 10 weeks after GA_3 injections. Alternate secondary branches on treated branches were collected and grouped from four trees at each dosage.

tips scored induced by 10^{-2} mmol per tree, or 2 X 10^{-3} mmol per branch. No toxic concentration was seen; however only a limited number of concentrations were applied, and application of greater amounts of the hormone would require either more solvent or multiple injections to overcome difficulties in solubility.

In Figure 5, individual branch respones to the optimal GA_3 injection dosage (2 X 10^{-3} mmol or 646 µg per branch) were plotted. All injected branches (basal, intermediate and terminal) showed a 100 % flowering response on actively growing shoot tips. On all branches with the exception of the most apical branch, pollen conebuds outnumbered seed conebuds. At least 25 % of the reproductive shoot tips on all branches were seed conebuds. On the most apical branch, 58 % of total conebuds were female.

A second set of GA_3 branch injections were made on three-year-old seedlings, in the growth chamber. Gibberellin A_3 (2 X 10^{-3} mmol) plus 450 nCi of high specific activity [^{3}H]GA_3 per branch were injected into 3 branches on each of 16 seedlings. Four control seedlings received similar injections but with high specific activity [^{3}H]GA_3 only. A further 4 seedlings served as uninjected controls. Randomly selected pairs of GA_3/[^{3}H]GA_3-treated trees were chosen 2, 8, 15, 22, 29, 36, and 50 days after injection for tissue

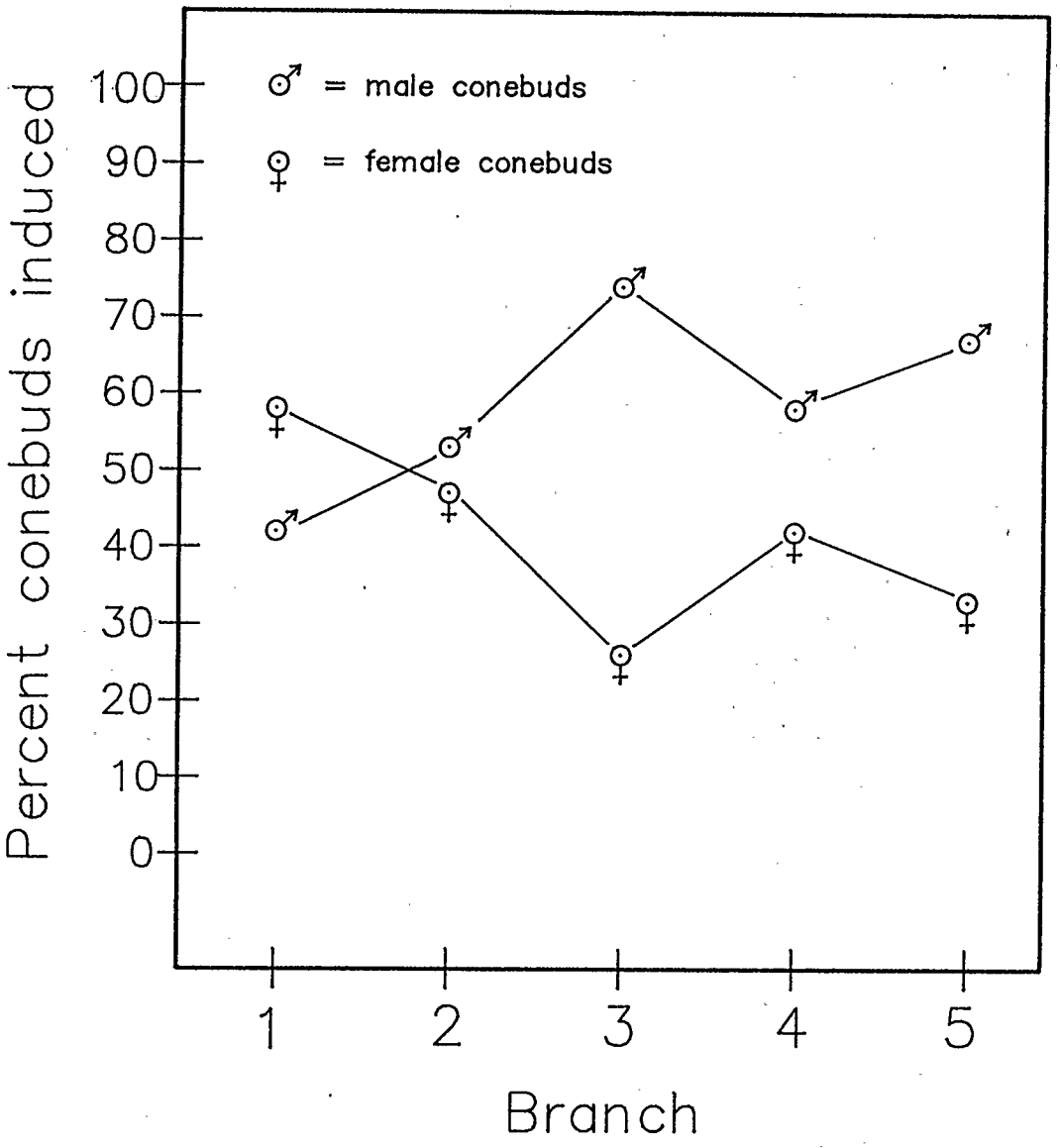

Figure 5. Positional variation in sex expression in GA_3-induced cones. Data points shown represent the total percentage of conebuds on branches one to five, scored as either male or female, 10 weeks after the "optimal" dosage ($2X10^{-3}$ mmol/branch) was injected into four seedlings.

harvests. Shoot tips were excised from female and male regions on each tree at these times and were prepared for anatomical analysis of the shoot apices. Shoot tips from the same trees were used for in vitro [^{35}S]methionine labelling experiments and for determining GA_3/[^{3}H]GA_3 uptake and metabolism (see later section).

In Figure 6 photomicrographs of representative apices are shown. Apices were scored as either vegetative (6a and 6b) or as reproductive (6c and 6d). Only when one pair of reproductive structures (bracts or microsporophylls) had been formed was an apex scored as reproductive. Distinct changes in the apices due to initiation alone, prior to bract or microsporophyll formation, were not detected. Young seed conebuds were not observed in sections of apices obtained from regions on treated trees where they would be expected (on short, distal branches); instead, young pollen conebuds were observed (see Figure 6d).

The percent of obviously reproductive apices (as determined by anatomical analysis) over time appears in Figure 7. In general, anatomically reproductive apices became abundant four weeks after injection. After 5 weeks, some shoot tips on the seedlings could be visually classed as reproductive without the aid of anatomical analysis. By six weeks after injection the

Figure 6. Photomicrographs of median longitudinal sections of T. plicata shoot tips. 6a: Vegetative shoot tip from control seedling showing vegetative apex (a), leaf initials (li) and vegetative leaves (l). X 80. 6b: Vegetative shoot tip from GA_3-treated seedling, female region, two weeks after injection showing vegetative apex (a), leaf initials (li), and vegetative leaves (l). X 60. 6c: Reproductive shoot tip from female region of GA_3 treated seedling, three weeks after injection showing enlarged and flattened reproductive apex (a), bract initials (bi), and surrounding vegetative leaves (l). X 60. 6d: Young pollen conebud four weeks after GA_3 injection showing reproductive apex (a), microsporophyll initials (mi), and microsporophylls (m). X 60.

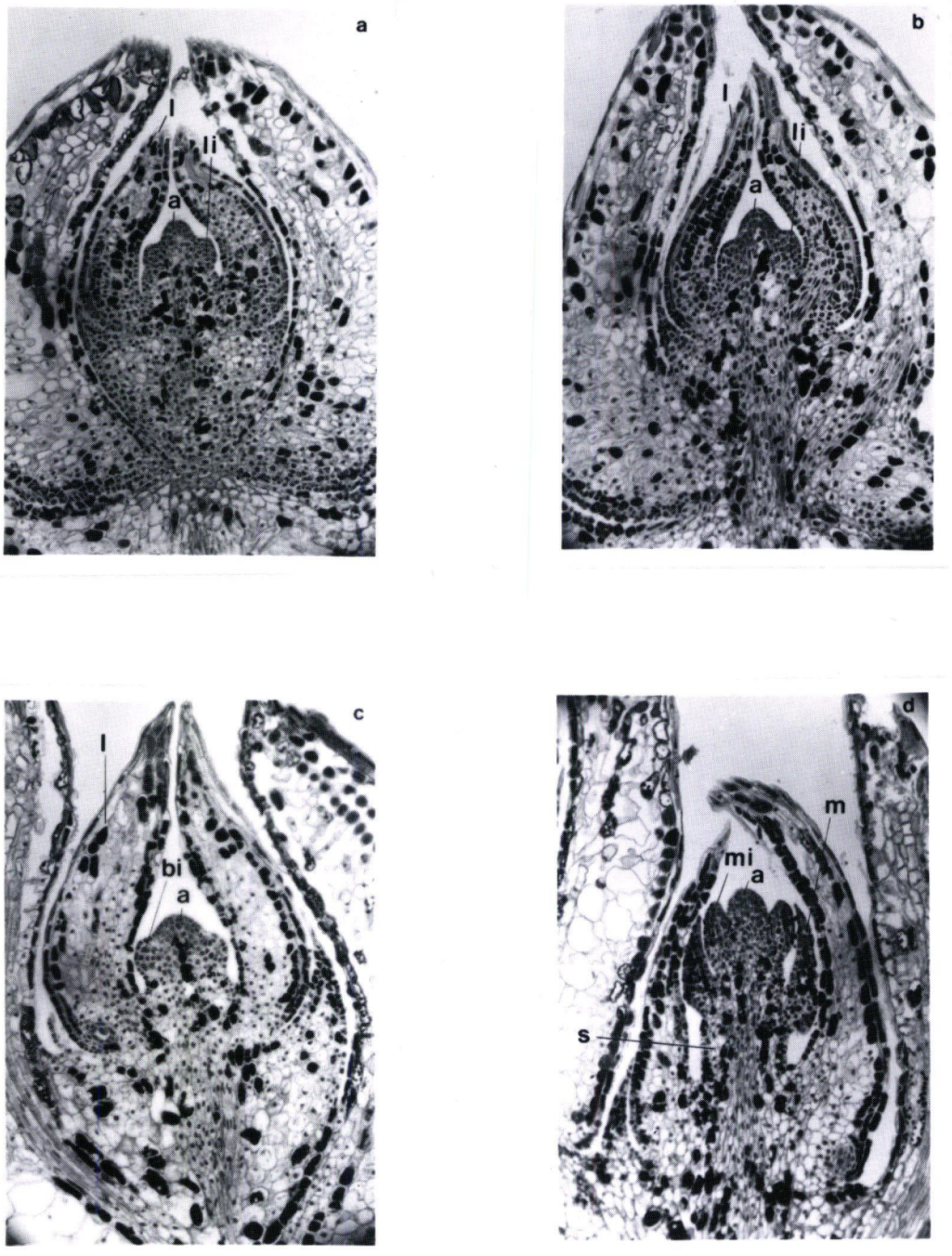

Figure 6

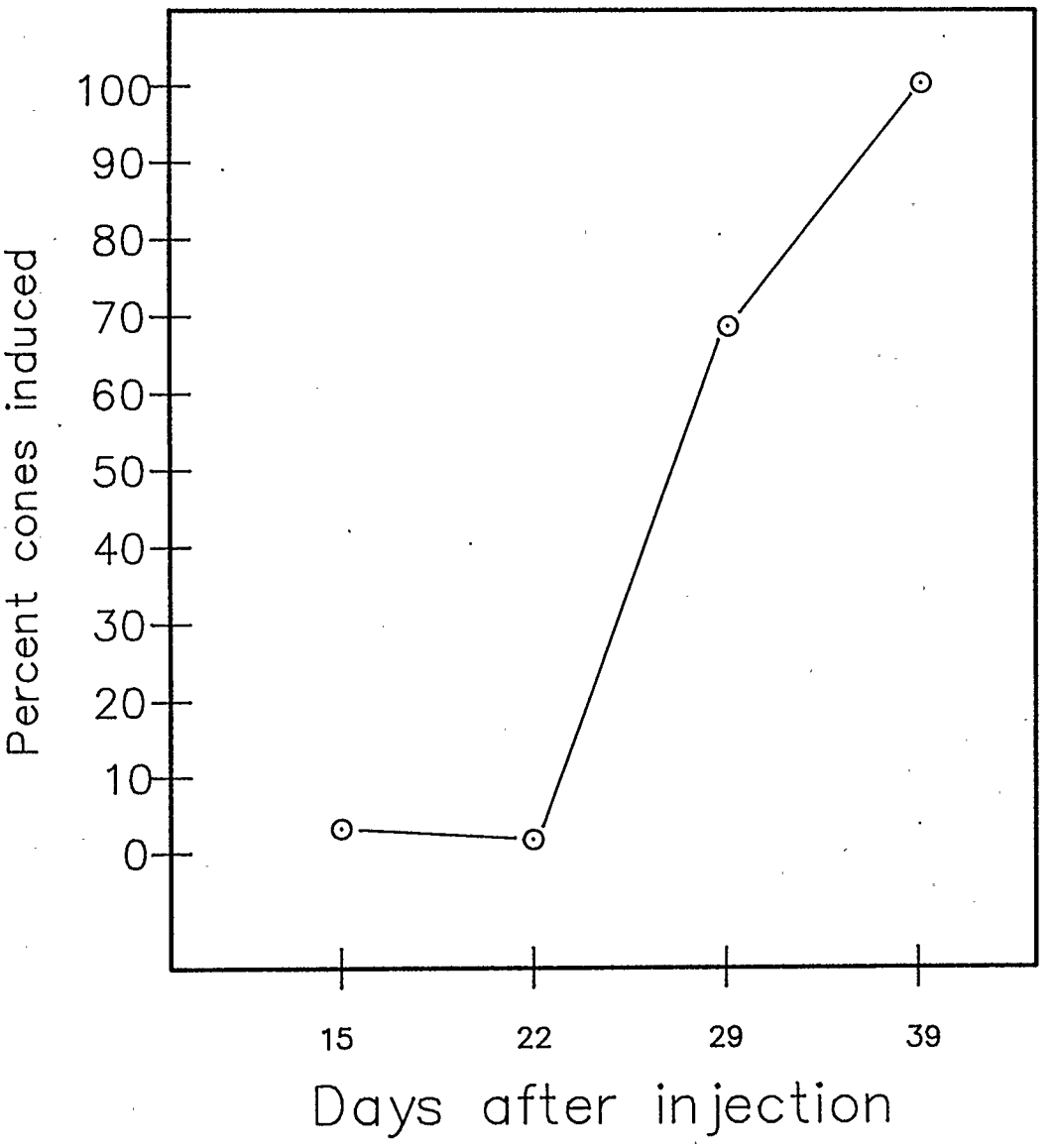

Figure 7. Flowering response of Thuja plicata shoot tips to GA_3 injection (determined by anatomical analysis) as percentage of total apices examined.

reproductive response to GA_3 had reached its maximum level.

Three-year-old seedlings that had not been completely destroyed for sampling purposes during the first 7 weeks after injection, were examined for overall flowering response 4 months after injection. There were no actively growing vegetative apices remaining on all treated branches. These showed 100 % cone induction. Non-treated branches on treated trees showed a high degree of cone induction if they were positioned above at least one treated branch. This probably indicates some back-translocation of GA_3 (or of a florigenic stimulus) to the main stem. If the treated branch had been removed shortly after an early sampling date, the translocation effect was considerably reduced. There were a few isolated female conebuds near the distal portion of control seedlings that had not had $[^3H]GA_3$ injections but none on the $[^3H]GA_3$ treated control seedlings.

The flowering in controls may have been caused by water stress, which in turn was caused by the large size of the plants in relatively small pots. Thus, water potential measurements were made on three-year-old seedlings, using mature rooted propagules, and a one-year-old seedling for comparison. Table 1 shows the results of these measurements. Water potential

Table 1. Water potential of Thuja plicata shoot tips in the greenhouse under natural photoperiod (approx. 16 hour daylight). Measurements were made in late afternoon in September.

Tree stock	Replicate no.	Water potential (bars)
Mature rooted propagule	1	-8.5
	2	-7.4
Three-year-old seedling	1	-7.6
	2	-8.6
	3	-9.2
One-year-old seedling	1	-5.4

(determined by the dewpoint psychrometer method) was similar and low in mature rooted propagules and three-year-old seedlings, but higher for the well-watered recently repotted one-year-old seedling.

The effectiveness of two gibberellins, GA_3 and GA_7, in promoting flowering and vegetative shoot elongation were compared using stem injections on one-year-old seedlings. A concentration of 2×10^{-3} mmol per tree was injected into the stems of six seedlings for each GA. Two sets of control trees, consisting of four solvent-injected and seven uninjected seedlings were used. Stem lengths (soil level to terminal shoot tip) were measured weekly for four weeks while the seedlings were under high light intensity and long days in the greenhouse. The seedlings were then moved into the growth chamber for another four weeks after which time final shoot length was measured and percent flowering was scored. The results of these measurements appear in Figure 8 as average weekly rates of shoot elongation, and in Table 2 as percent flowering. Elongation data for both injected and uninjected control seedlings were grouped together to simplify the graphic representation of the data points with 95 % confidence intervals. Percentage flowering observations were transformed by taking the arcsin of the square root of the proportion of flowering shoot tips. This

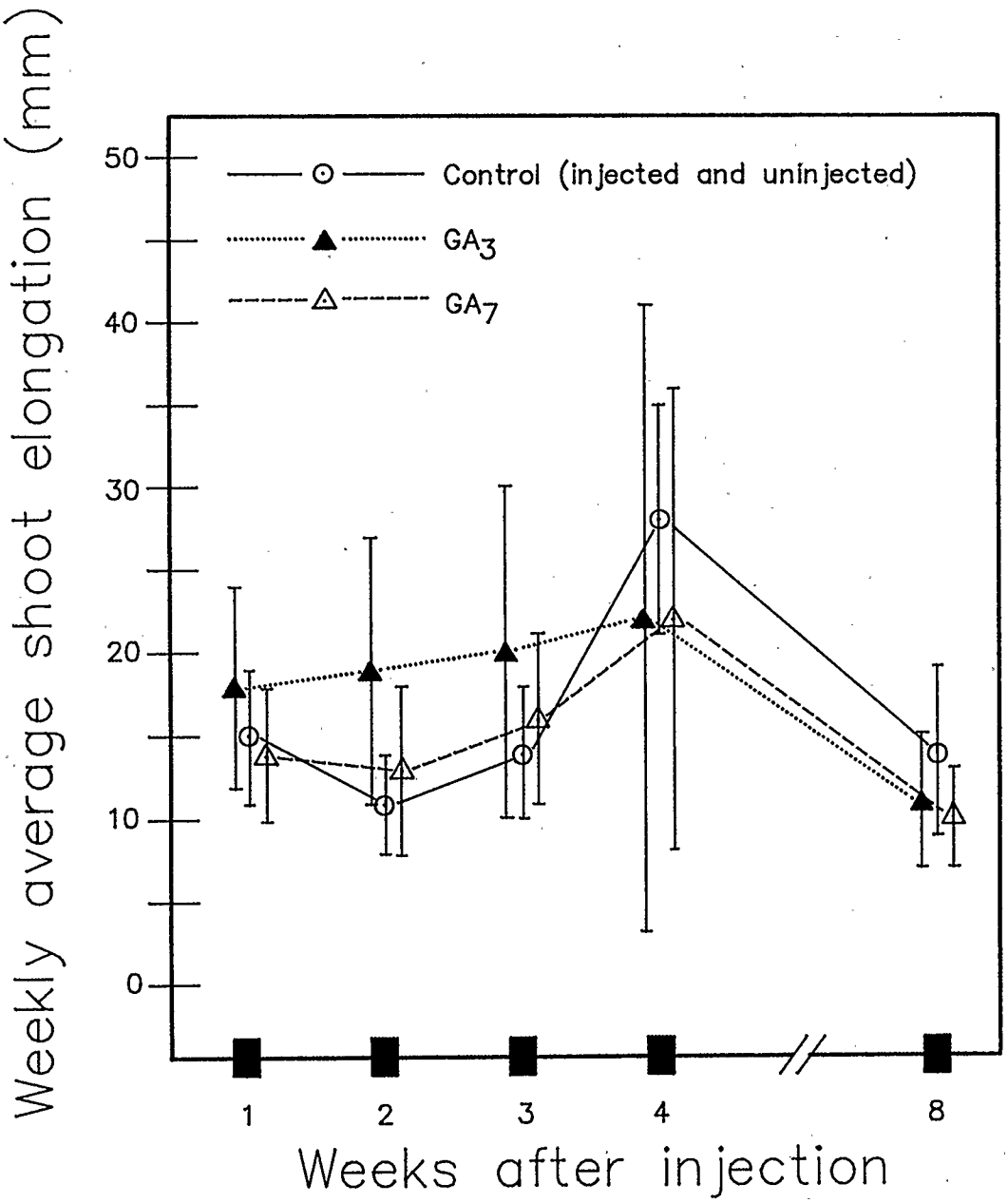

Figure 8. Weekly shoot elongation in response to GA_3 or GA_7 injection with 95% confidence intervals shown. Week 8 values were obtained by dividing individual values by four and proceeding with standard deviation calculations as usual.

Table 2. Percentage flowering by GA_3 or GA_7, measured 8 weeks after injection. Injections were made into stems of one-year-old seedlings maintained in the growth chamber.

Treatment	Replicate no.	% flowering	$\sin^{-1}\sqrt{\text{mean \% flowering}}$ ± 95 % confidence interval
GA_3 2×10^{-3} mmol/tree	1	75	82.8 ± 11.8
	2	100	
	3	95	
	4	100	
	5	100	
	6	100	
GA_7 2×10^{-3} mmol/tree	1	8	18.8 ± 12.4
	2	5	
	3	50	
	4	5	
	5	5	
	6	5	
Control DMSO/ethanol	1-4	0	0.0
Control no injection	1-7	0	0.0

transformation is applied in order to render the data into a normal distribution thus allowing the application of standard statistical analyses (e.g. standard error calculations; Zar, 1974).

Gibberellin A_3-treated seedlings showed greater shoot elongation during the first 3 weeks after injection than did GA_7-treated or control seedlings. By week four, variation in shoot elongation had become so large that trends in the data were obscured. The large variations in elongation of GA_3- and GA_7-treated seedlings at week 4 were due to one seedling's very great elongation for each group (60 and 50 mm respectively). If these two seedlings were removed, a much lower average elongation and standard deviation would be obtained for both GA_3- and GA_7-treated seedlings. Control seedlings would thus have significantly greater elongation than either GA-treated group. Even without removing the 'problem' seedlings, by week 8, controls elongated slightly more than either treated group of seedlings (Figure 8).

Conebud production was not obtained in all actively growing shoot tips in response to GA_3 treatment of one-year-old seedlings, although in general, a high proportion of the shoot tips were induced to flower (Table 2). Only nominal flowering was obtained in these seedlings from GA_7 injection with the exception

of one seedling in which 50 % of the actively growing shoot tips produced cones. There were no cones seen in either control group of seedlings.

[^{35}S] labelling of T. plicata shoot tip proteins

To determine the optimal incubation time for in vitro labelling of shoot tip proteins with [^{35}S] methionine, shoot tips were excised from mature clonal propagules and placed in water containing 50 µCi [^{35}S] methionine for varying lengths of time. Uptake was expressed as concentration of label in crude protein extracts (dpm/µL) where the ratio of fresh weight to extraction volume was constant (see Materials and methods). Uptake of label into shoot tips increased but the rate of uptake remained fairly constant with increased incubation time. Uptake was faster for male than for female conebuds and fastest in vegetative shoot tips (Figure 9). Incorporation of [^{35}S] methionine into TCA-precipitable proteins by protein synthesis was occurring in the in vitro system. Due to unknown rates of protein degradation, however, only a net incorporation rate could be calculated, not a rate of protein synthesis per se. Net incorporation into TCA-precipitable proteins was a nearly linear function of uptake and so all incorporation data were standard-

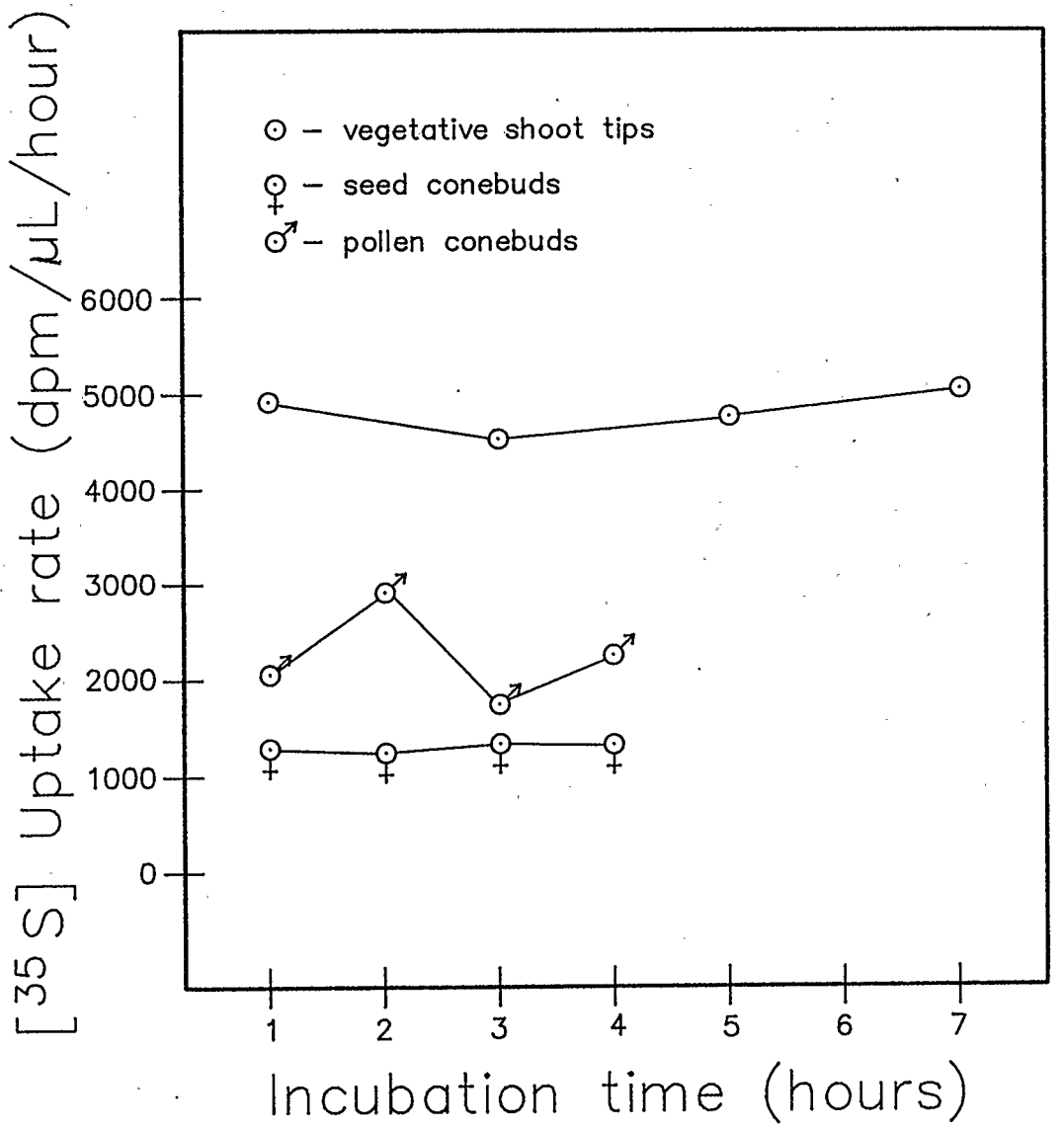

Figure 9. Effect of increased incubation time on uptake rate of [^{35}S] methionine into excised shoot tips of mature rooted propagules.

ized by dividing net incorporation (dpm/μL) for each individual sample by its individual uptake of label (dpm/μL) unless otherwise stated.

Figure 10 shows that the majority of net incorporation of [^{35}S] into newly-made proteins took place during the first hour of incubation. The overall net amount of [^{35}S] incorporated per hour of incubation, was lower when the incubation period exceeded one hour. Seed conebuds had the lowest rates of incorporation, male conebuds had the highest (Figure 10). For three hours of incubation, however, the three types of shoot tips had fairly similar rates of incorporation.

Since there was a decrease in net incorporation of [^{35}S] methionine with increased incubation time, it was of interest to determine the extent to which this was caused by an increased degradation of newly-made protein. Protein extracts of shoot tips, labelled for different times, were electrophoresed and the amount of degradation was found to be fairly consistent between samples incubated for 1 to 3 hours. Slightly more degradation, however, was visible in samples incubated for 5 or more hours, as evidenced by increased streaking and poorer resolution in the low molecular weight region.

Based on the above information, a 3 hour incubation period was chosen as a standard time for <u>in</u>

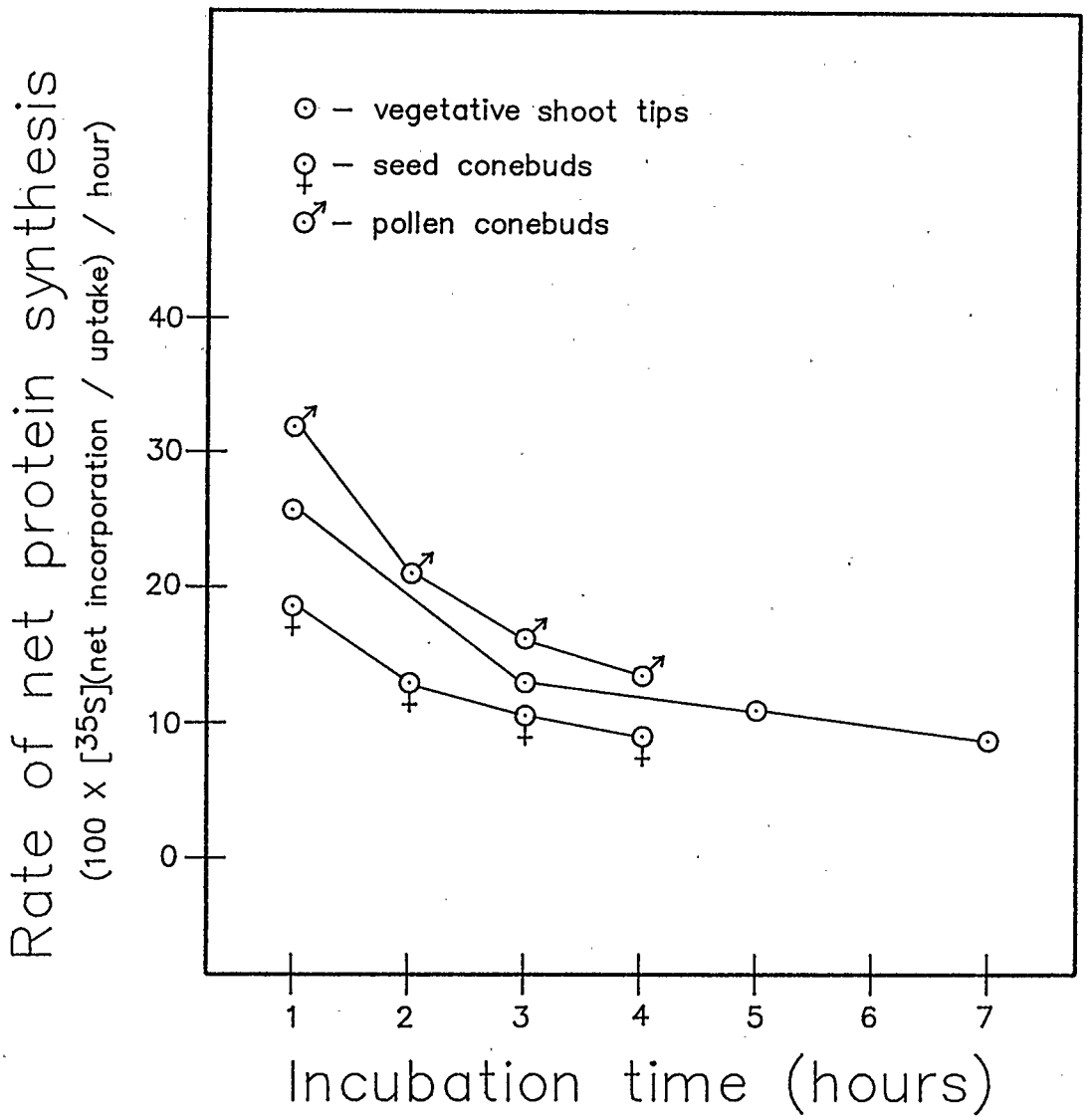

Figure 10. Net incorporation of [^{35}S] methionine into TCA precipitable protein in excised shoot tips from mature rooted propagules expressed as a percent of uptake occurring per hour of incubation.

vitro incorporation of [^{35}S] methionine into shoot tips.

In vitro uptake of [^{35}S] methionine in shoot tips of 3-year-old seedlings appeared to be influenced by GA_3 treatment (branch injection). For incubated samples that were excised from plants one week or more after GA_3 injection there was significantly greater overall [^{35}S] uptake than for control shoot tips or shoot tips of plants treated with GA_3 for only two days prior to harvest (Figure 11). Possible differences between male and female shoot tips were obscured by the very large 95 % confidence intervals although the average trends suggest that male-region shoot tips had higher uptake than female-region shoot tips. Uptake of [^{35}S] into terminal leading shoot tips was lower than uptake into potential conebud shoot tips. The average concentration of [^{35}S] for terminal shoot tips incubated for 3 hours was 11200 dpm/μL for GA_3-treated apices and 6700 dpm/μL for control apices.

Net incorporation into newly-synthesized protein was not affected in any predictable way (Figure 12). Again, significant differences between male and female-region shoot tips were not seen due to large 95% confidence intervals surrounding their respective means.

Uptake seemed to be approximately proportional to

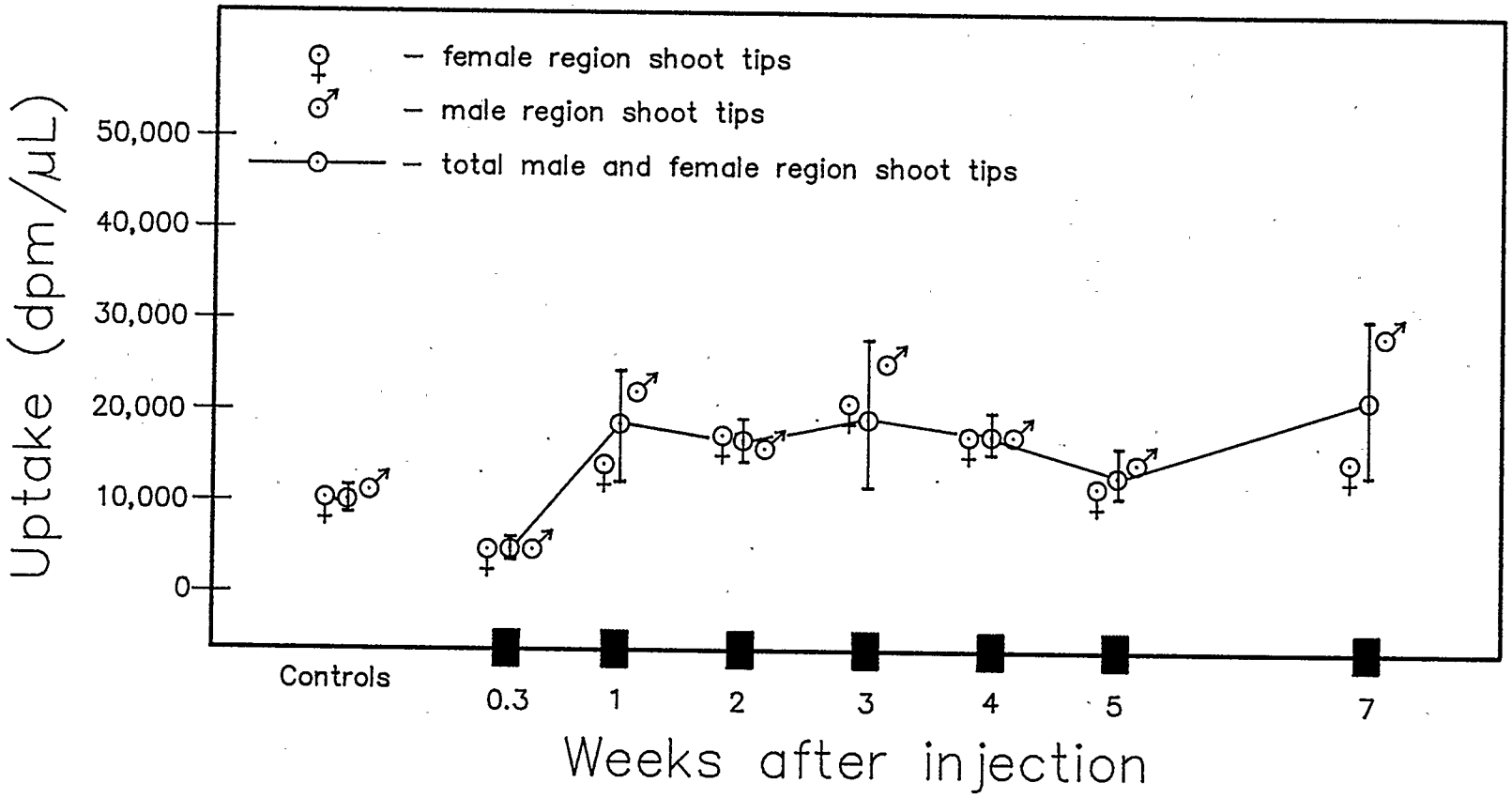

Figure 11. [^{35}S] uptake into excised shoot tips of three–year–old seedlings, GA_3– and non–GA_3–treated.

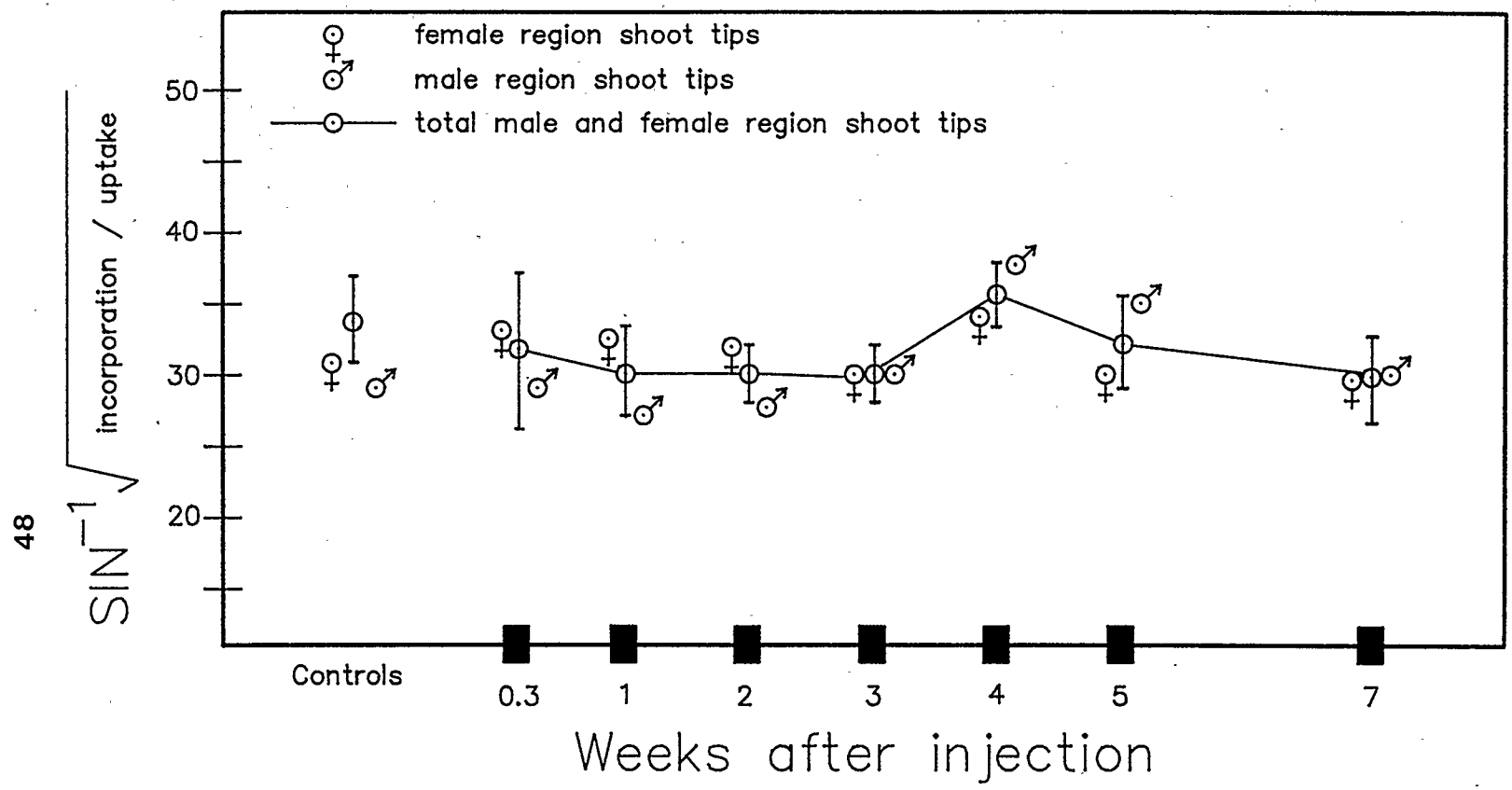

Figure 12. [^{35}S] incorporation as a percent of uptake in excised shoot tips of three-year-old seedlings, GA_3- and non-GA_3-treated. Numbers were transformed to the arcsin of the square root of the ratio of incorporation (dpm) to uptake (dpm).

the number of shoot tips in each sample. Due to the relative size differences of shoot tips (terminal shoot tips> female conebuds > male conebuds > vegetative shoot tips), samples of similar fresh weights consisted of only a few (6-12) terminal shoot tips or conebuds but many (20-40) vegetative shoot tips. This implied that surface area, and especially cut surface area, could be the major factor limiting uptake.

To determine whether uptake was occurring mainly through the cut surfaces of shoot tips or through the entire surface area, whole shoot tips were incubated under standard conditions for 3 hours. After removing residual [^{35}S] methionine by rinsing, each shoot tip sample was sectioned into three sub-sample regions by cutting shoot tips twice, parallel to the original cut surface such that either the distal or central section would contain the shoot apex. Three kinds of shoot tips were examined nine weeks after GA injection: vegetative controls, GA_7-induced female conebuds and GA_3-induced female conebuds. Proteins were then extracted from these sub-samples and uptake and incorporation of [^{35}S] were measured.

The majority of protein synthesis took place in the lower (proximal) portion of *in vitro* [^{35}S]-labelled shoot tips near the original cut surface (Table 3). If equal amounts of net incorporation had taken place in

Table 3. Analysis of incorporation of [^{35}S]methionine into protein of three different sub-sample of female region shoot tip samples from one-year-old seedlings nine weeks after GA injection.

Sample	Sub-sample	Sub-sample fresh weight and total fresh weight (mg)	% total f.w. (a)	Sub-sample incorporation per µL*	% total incorp. (b)	(b)/(a)
Control	distal	3.7	18.9	151.6	3.4	0.18
	central	7.3	37.2	673.2	15.2	0.41
	proximal	8.6	43.9	3604.8	81.3	1.85
		19.6		4429.6		
GA_7 treated	distal	8.3	35.8	60.6	1.6	0.04
	central	8.3	35.8	296.4	7.8	0.22
	proximal	6.6	28.4	3453.2	90.6	3.20
		23.2		3810.2		
GA_3 treated	distal	5.9	24.4	320.4	12.6	0.52
	central	8.9	36.8	851.6	33.6	0.91
	proximal	9.4	38.8	1363.6	53.8	1.39
		24.2		2535.6		

* Concentrations were normalized to fresh weight of tissue.

each sub-sample of shoot tips, the ratio of the sub-sample incorporation to the fresh weight would be approximately one (incorporation values were not standardized for variable uptake to allow for such a comparison). In GA_3-induced female conebuds, the ratios of incorporation to fresh weight in each sub-sample were the closest to one indicating almost equal amounts of [^{35}S] incorporation into protein occurred throughout the three regions of the shoot tips. Gibberellin A_7-induced conebuds showed sub-sample ratios of incorporation to fresh weight that were the furthest from one; 91 % of the net incorporation took place in the third of the shoot tips closest to the original cut surface (Table 3). In vegetative control shoot tips, 81% of the net incorporation took place in the half of shoot tips closest to the cut surface.

To determine if the early stages in cone production were accompanied by a specific change in shoot tip proteins, proteins were labelled with [^{35}S] methionine, <u>in vitro</u>, at different times after injection of three-year-old seedlings with GA_3. Proteins were electrophoresed by loading equal amounts of [^{35}S]-labelled protein on to each lane of gradient SDS-PAGE slab gels. However, this resulted in variable amounts of total protein being applied since the rates of [^{35}S]

uptake (and thus incorporation) differed so much between samples.

Autoradiograms from gels of female region shoot tips are shown in Figure 13. Only proteins of molecular weights of 15 to 140 kDa are shown; this range included all detectable differences associated with conebud production and GA_3 treatment. Labelled proteins with molecular weights of 25, 33, and 52 kDa were seen to be present in fairly consistent relative amounts throughout the time course examined, hence, these were used as internal molecular weight standards.

No detectable differences in [^{35}S]-labelled proteins were seen between control and GA_3-treated shoot tips of either male or female regions on the seedlings by week two after injection of GA_3. However, by five weeks after GA_3 injection, a labelled 36 kDa protein became abundant in most GA_3-treated samples, but was undetectable in control samples (Figure 13). Densitometry scans of the week five GA_3-treated and control samples shown in Figure 13, clearly show this difference (Figure 14). Seven weeks after GA_3 treatment, the 36 kDa bands were still seen on autoradiograms of GA_3-treated shoot tip extracts, but they appeared to be in smaller relative proportions than for the week five samples.

The relative abundance of this protein in the

Figure 13. Autoradiograms of SDS-PAGE gels using proteins from GA_3-treated shoot tips of female regions of 3-year-old seedlings. Molecular weights (kDa) of consistent internal marker proteins, and bands of interest are indicated. Elapsed time after GA_3 injection (weeks) are shown below each pair of lanes. Each pair of lanes represents replicate samples from one seedling except for the first pair of lanes in each row where samples from control non-GA_3-treated female region shoot tips are shown beside the analogous GA_3-treated samples. Control samples are not shown for weeks three to seven but the control samples at week four looked identical to week one and two control samples. Where there are two pairs of samples shown (weeks three to seven), both pairs represent samples from two replicate seedlings.

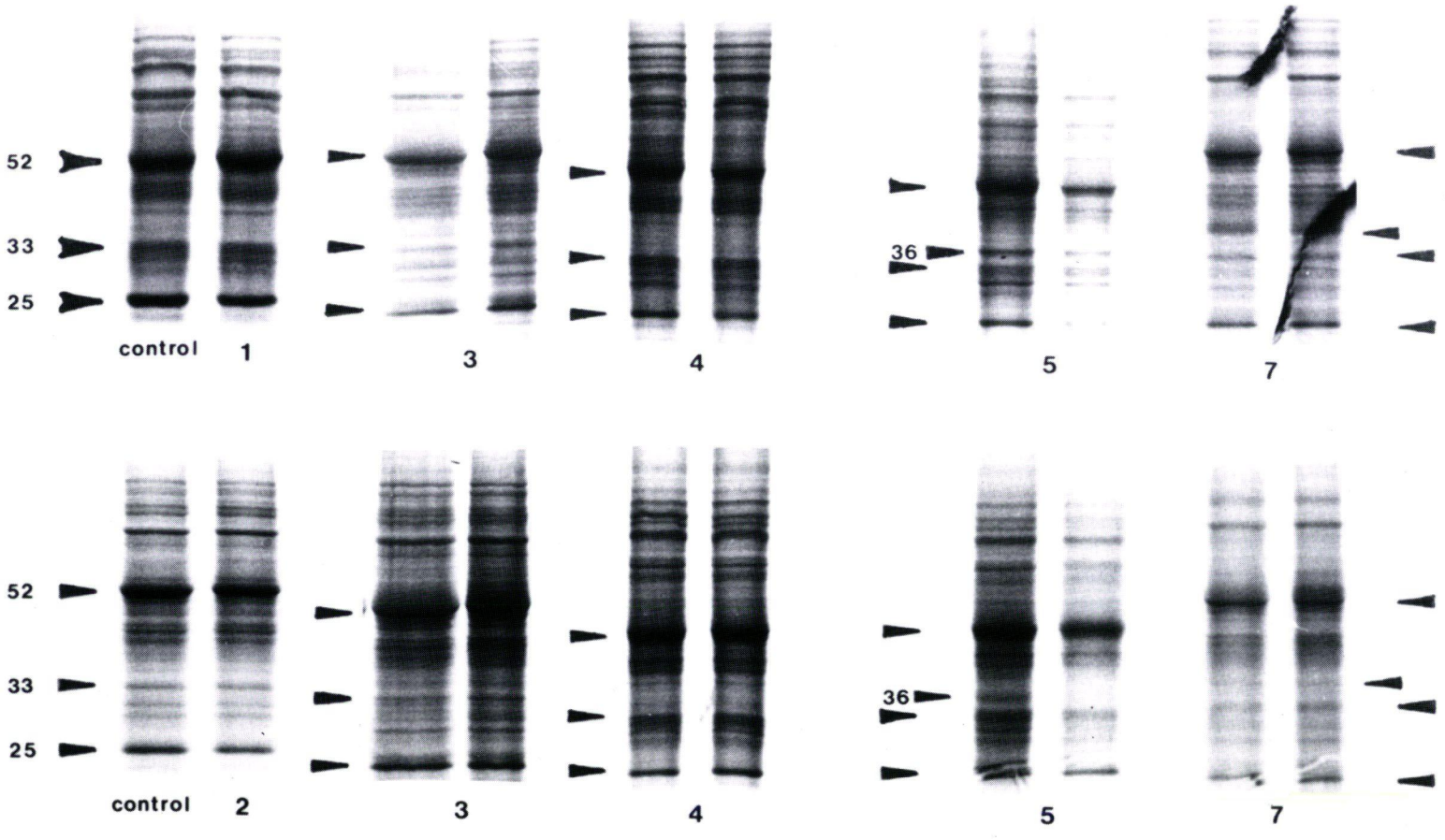

Figure 13

Figure 14. Densitometry scans of electrophoresed [^{35}S]-labelled proteins from Thuja. The dashed line represents GA_3-treated female region shoot tip proteins harvested five weeks after GA_3 injection. The solid line represents control non-GA_3-treated proteins harvested at the same time. Molecular weights (kDa) of bands of interest are shown.

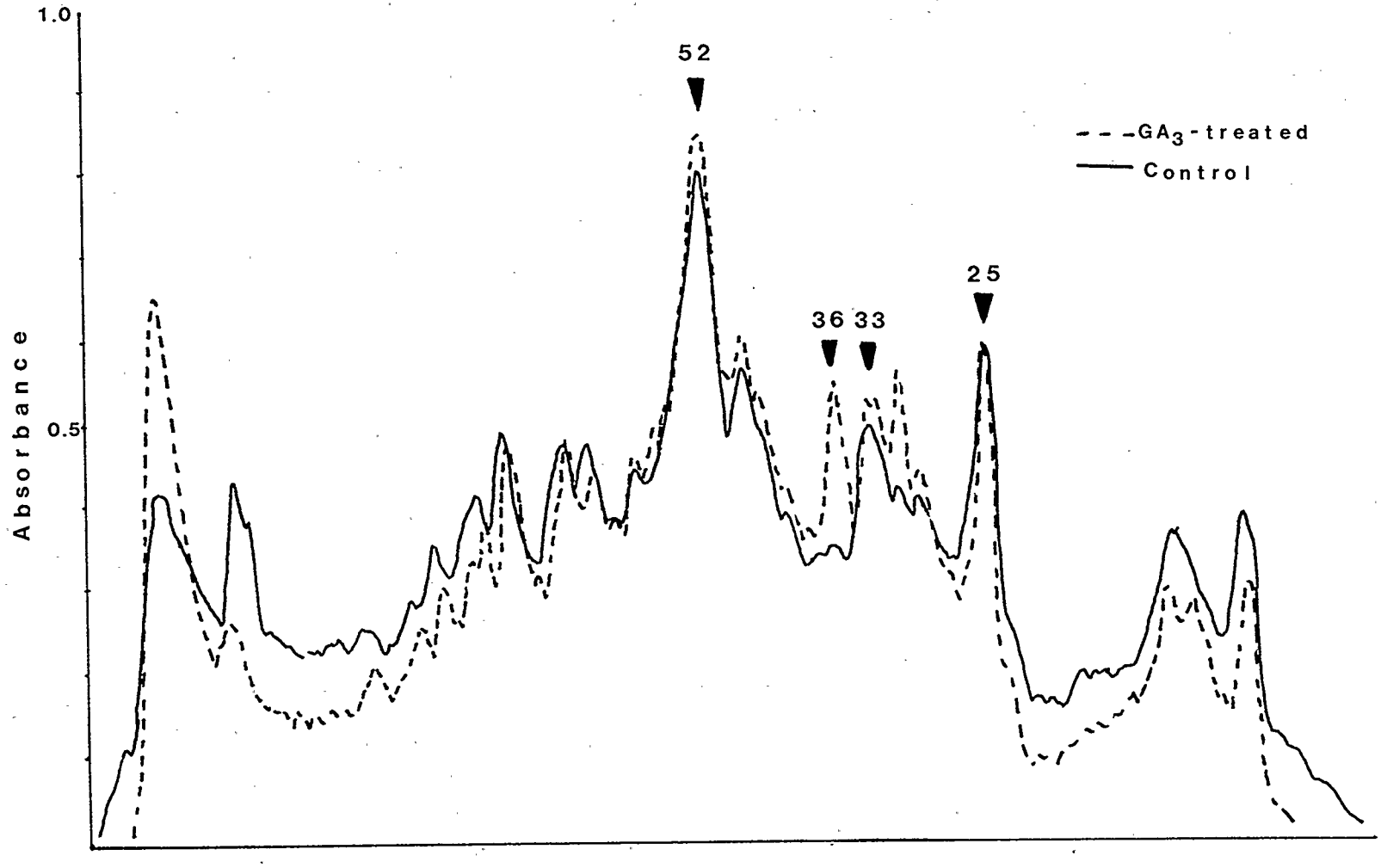

Figure 14

regions of the autoradiograms shown were quantified by laser densitometry. The Chromoscan 3 Laser Densitometer (Joyce-Loebl) automatically integrates the area under the curve produced by a full scan and then integrates peaks separately depending on user-specified peak-trough thresholds. Low thresholds were used to allow separate integration of small or closely-situated peaks. However, peaks representing less than 2 % of the total integral scanned were generally not resolved. A background level of 1 % relative abundance was assigned to all samples where a 36 kDa protein peak was not integrated.

The average relative abundance of 36 kDa labelled proteins increased gradually with time after GA_3 treatment to a maximum of 4.4 % for female region shoot tips harvested seven weeks after GA_3 injection (Figure 15). Amounts of 36 kDa labelled proteins seen in shoot tips collected from the female conebud region were variably different from amounts seen from the male conebud region, although insufficient data were available to perform a statistical analysis.

<u>In vitro</u> [^{35}S]-labelled proteins from naturally-induced female conebuds (flowering without the application of GA_3) of a single water-stressed 3-year-old seedling did not show a 36 kDa band.

To determine if the 36 kDa protein accumulated

Figure 15. Relative abundance of [^{35}S] *in vitro*-labelled 36 kDa protein in *T. plicata* shoot tips with increased time after GA_3 injection to 3-year-old seedlings. Points represent the average percentage relative abundance of a 36 kDa protein (2-6 samples). Relative abundance was determined from the total area integrated under the peaks derived from densitometry scans of autoradiograms.

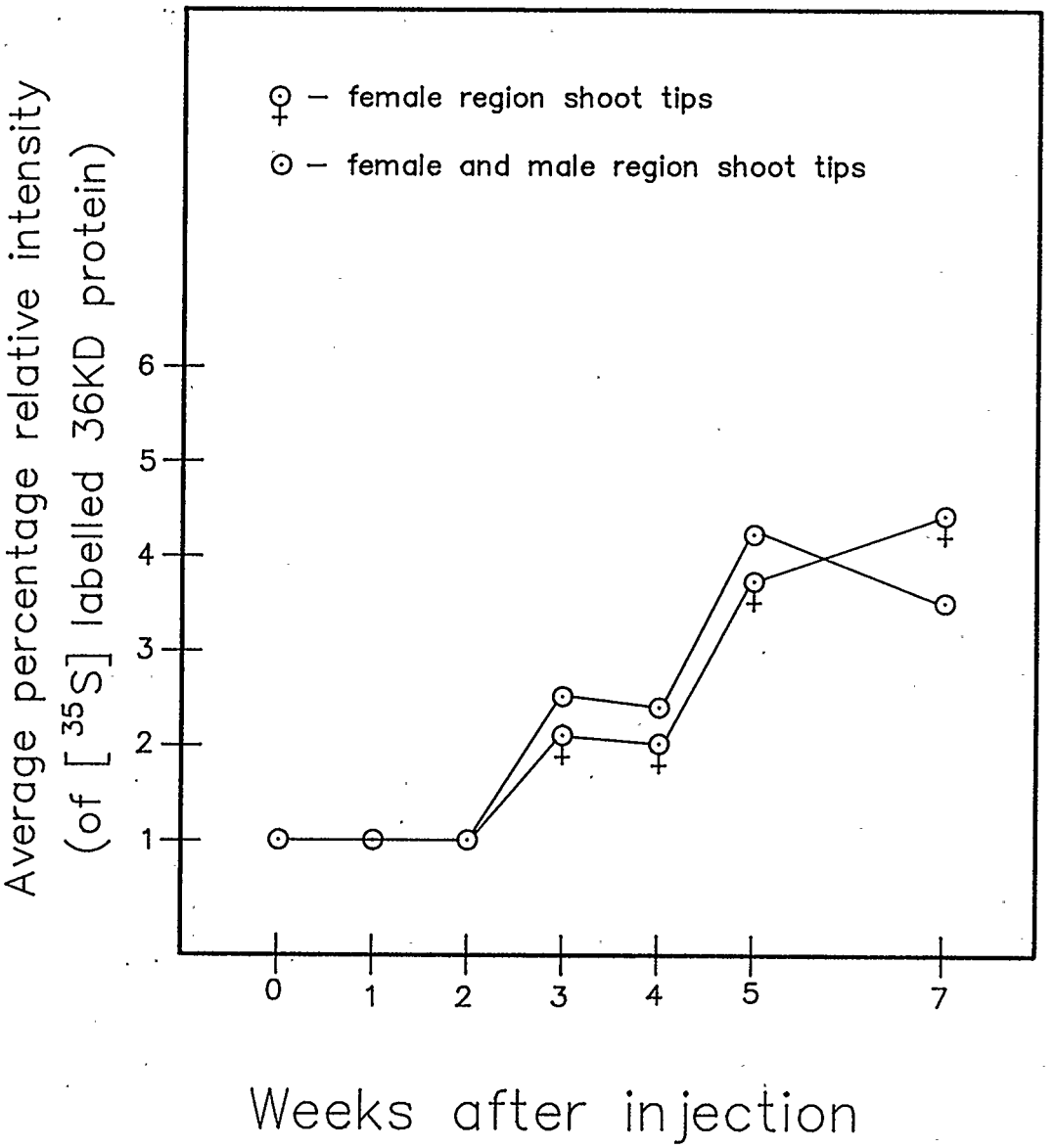
♀ – female region shoot tips
⊙ – female and male region shoot tips
Average percentage relative intensity
(of [35S] labelled 36KD protein)
1
2
3
4
5
6
0
1
2
3
4
5
7
Weeks after injection

Figure 15

with increased time after GA_3 injection (or if the 36 kDa protein had a fairly high turnover rate), Coomassie Blue stained gels of protein samples from GA_3-treated and control shoot tips were examined four, five and seven weeks after GA_3 treatment (Figure 16).

A 36 kDa protein staining for Coomassie Blue was seen in all lanes examined with approximately equal relative amounts being present in control and GA_3-treated shoot tips at any given week after GA_3 injection.

The presence of a 36 kDa protein in stained gels of control plant shoot tips may suggest that the presence of this protein per se is not be related to conebud production. Thus, autoradiograms of gels of additional samples were examined.

Figure 17 shows an autoradiogram made from a gel where individual protein bands were exceptionally well resolved. There were many radioactive protein bands present with molecular weights ranging from 33 to 38 kDa, and these differed in relative intensities between the different samples. Thus, a high degree of accuracy would be required to determine the molecular weights of any specific protein, and additionally, a high degree of precision would be required for any conclusions to be drawn about quantitative differences for any particular protein. Without additional

Figure 16. Photograph of Coomassie Blue stained protein gels from female region shoot tips samples four, five and seven weeks after GA_3 injection. Internal molecular weight standards (52, 33 and 25 kDa) are labelled as is a 36 kDa protein which is present in control and GA_3-treated shoot tips in relatively equal amounts. All lanes represent GA_3-treated samples unless marked directly below as control. No control was available for week five.

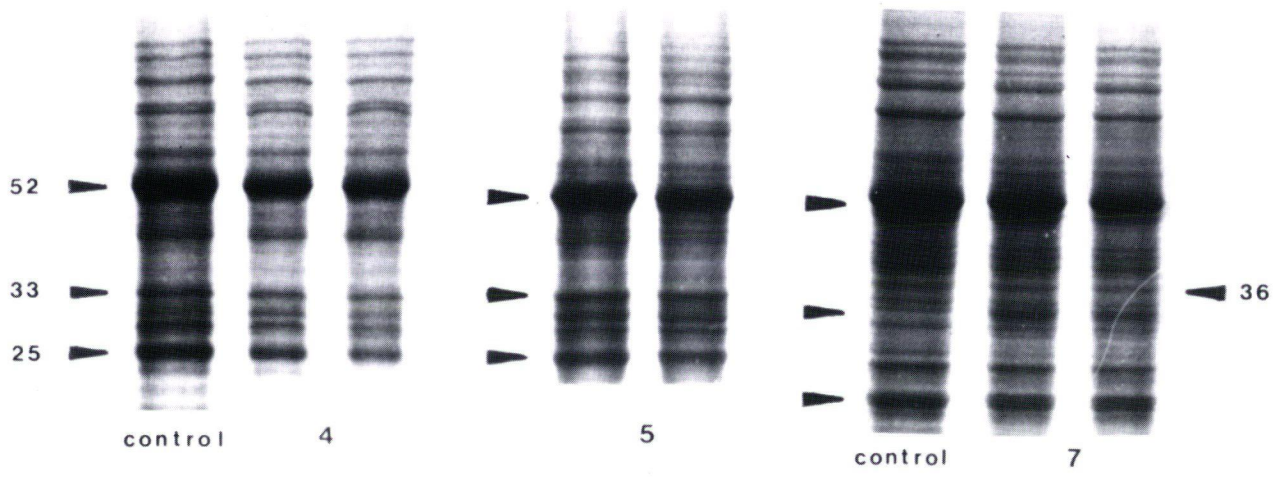

Figure 16

Figure 17. Autoradiogram of [^{35}S]-labelled shoot tip proteins extracted from tissue harvested nine weeks after GA injection. All samples were harvested from the female region of one-year-old seedlings except the lane marked Ct on the far right which was from terminal shoots. C = control, GA_3 = GA_3-injected (700 µg per seedling), GA_7 = GA_7-injected (700 µg per seedling). Internal molecular weight markers (52, 33, and 25 kDa) and protein bands that differ between samples (36, 35, and 34 kDa) are indicated.

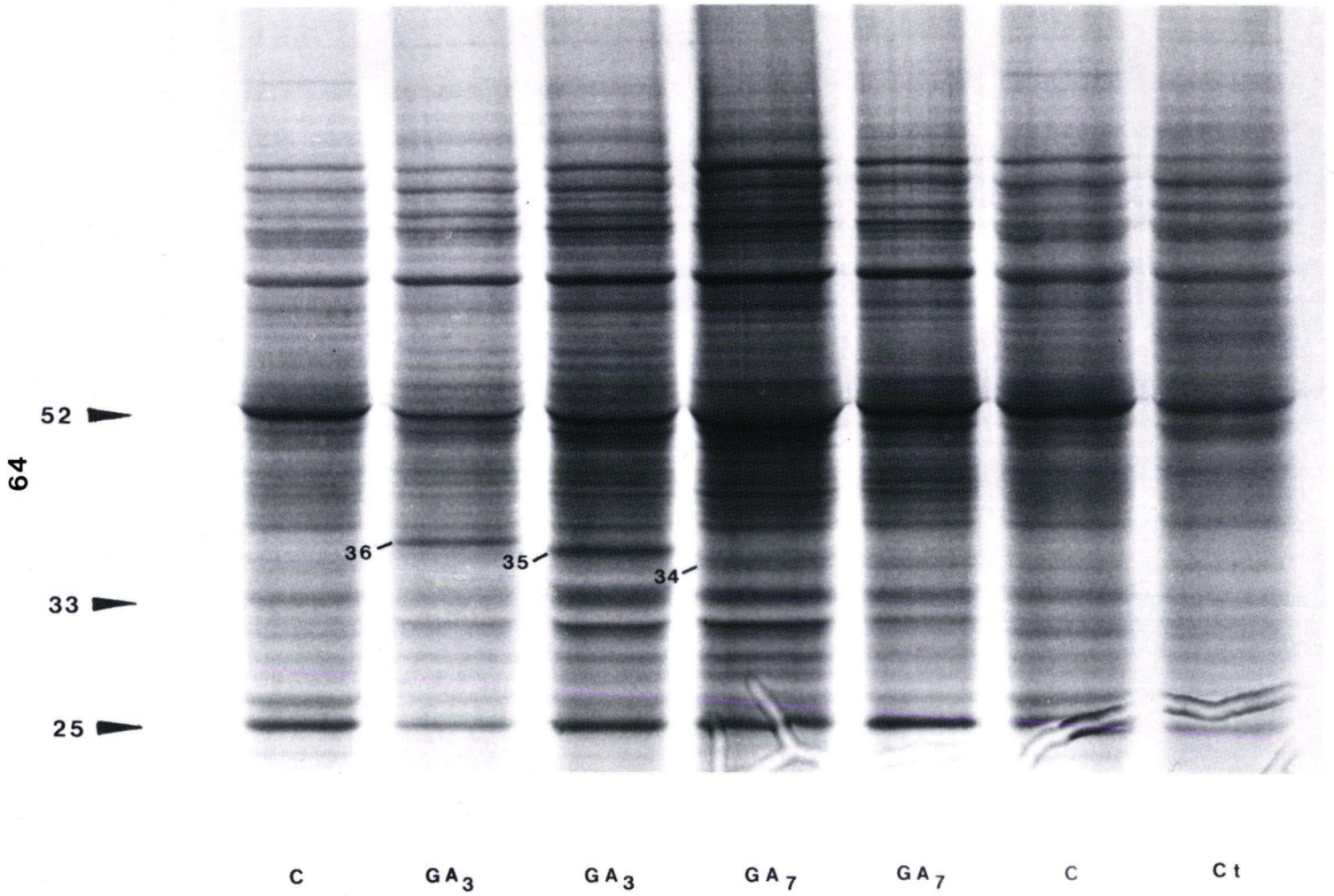

Figure 17

molecular weight markers between 43 and 25.7 (these were used to determine molecular weights of 52, 33, and 25 kDa proteins used as internal markers) and highly resolved protein bands, it was not possible to be certain that the protein band at the appropriate Rf for a 36 kDa molecular weight protein was in fact 36 kDa.

Table 4 summarizes the range of relative amounts of the 36 kDa-like [^{35}S]-labelled protein present in different shoot tip samples. In general, amounts greater than 1% were seen only in GA_3-treated shoot tips regardless of their position on the tree. Also, in male or female region GA_3-treated shoot tips, amounts of the [^{35}S]-labelled 36 kDa-like protein were greater than for terminal vegetative GA_3-treated shoot tips. Control seedling potentially reproductive or terminal shoot tips only had background levels of a [^{35}S]-labelled 36 kDa-like protein.

One or two [^{35}S]-labelled proteins with molecular weights slightly less than 36 kDa were often seen in control terminal shoot tips. It was difficult to determine whether the "36" kDa band seen in GA_3-treated terminal shoot tips (with no reproductive potential) was partly composed of either or both of these bands since many of these gels were poorly resolved.

Gibberellin A_7 injection of six one-year-old

Table 4. Occurrence of a [^{35}S] *in vitro*-labelled 36 KD-like protein in *T. plicata* shoot tips for all weeks after injection of GA_3.

Tree Stock	Treatment[a]	Region from which Sample Taken[b]	Range of Percentage Relative Abundance found for the 36 KD band[c]
3-year-old	GA_3	potentially reproductive	1-9
3-year-old	GA_3	terminal	1-5
3-year-old	control	potentially reproductive	0-1 (traces)
3-year-old	control	terminal	4 (one observation)
3-year-old	control	female conebuds	0
1-year-old	GA_3	potentially reproductive	1-5
1-year-old	GA_3	terminal	3 (one observation)
1-year-old	control	potentially reproductive	0-1
1-year-old	control	terminal	0

(a) Controls with or without solvent injection or GA_3-injected with 2×10^{-3} mmol/branch (3 year-old) or 2×10^{-3} mmol/seedling (1-year-old).

(b) Potentially reproductive = male and female region shoot tips.

(c) Abundance determined from the relative percentage of peak integral of whole integral of densitometry.

seedlings produced only one plant that flowered well. Proteins from the female region shoot tips of this plant, incubated with [^{35}S] nine weeks after GA_7 injection, did not have an obvious 36 kDa protein band. However, the radioactive protein profile from this tree looked almost identical to that of one analogous replicate sample of GA_3-induced female conebuds ([^{35}S]-labelled nine weeks after injection with GA_3, Figure 17). Hence, it is not possible to evaluate the importance (if any) of the absence of a 36 kDa labelled protein in the GA_7-treated shoot tips.

Proteins of vegetative shoot tips from mature propagules were labelled with [^{35}S] methionine and electrophoresed to allow an assessment of the protein differences due to genetic variability. Most of the shoot tip samples from at least three replicate clonal propagules of four genetically different mature trees showed nearly identical protein profiles between and among clones. Figure 18 shows overlayed densitometry scans from autoradiograms of three of these samples that showed the greatest differences. Both qualitative and quantitative differences seen in the 36 kDa region were present in clones of the same genetic origin. The 52 kDa protein was the most consistent band present in all samples, otherwise many slight differences in relative abundance of protein bands were

Figure 18. Variation in [^{35}S] labelled protein profiles of vegetative female region shoot tips taken from one genetically dissimilar propagule and two clonal (genetically similar) propagules of mature trees. Densitometry scans of the autoradiograms are shown and molecular weights (kDa) of bands of interest are labelled.

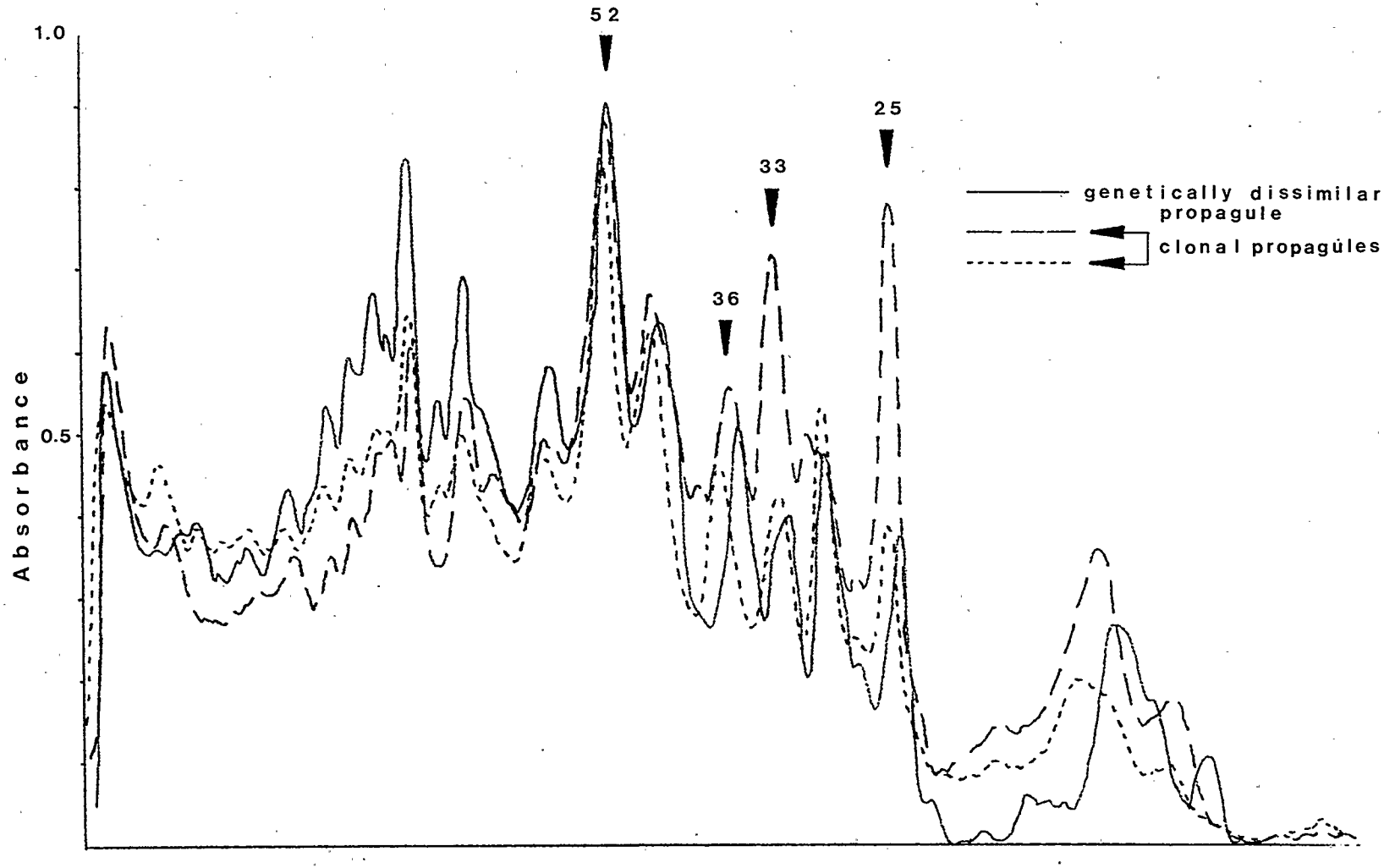
1.0
0.5
Absorbance
52
36
33
25
genetically dissimilar propagule
clonal propagules

Figure 18

seen in clonal samples particularly in the region of high and low molecular weights and in the 36 kDa region.

Gibberellin-like substances in GA_3-treated and control shoot tips of T. plicata.

To determine how much [^{3}H]GA-like substance had reached the by shoot tips by translocation after [^{3}H]GA_3/GA_3 injection, the amount of 80% MeOH-extractable tritium was compared for shoot tip samples harvested at different times after injection. The first harvest time was two days after injection and already shoot tips contained appreciable amounts of radioactivity (Figure 19). There was more [^{3}H] per gram dry weight in female than in male region shoot tips. The total amount of [^{3}H]GA_3-like substance found in male and female region shoot tips from one tree ranged from 1.6 to 6.4 percent of the original amount of [^{3}H]GA_3 injected into each plant. When the amounts of [^{3}H] were standardized by dividing by the sample dry weights, a slight overall decrease in relative amounts of [^{3}H] was seen for shoot tips harvested at increased times after [^{3}H]GA_3/GA_3 injection.

After the 80% MeOH extract was separated into fractions, no significant radioactivity (less than one

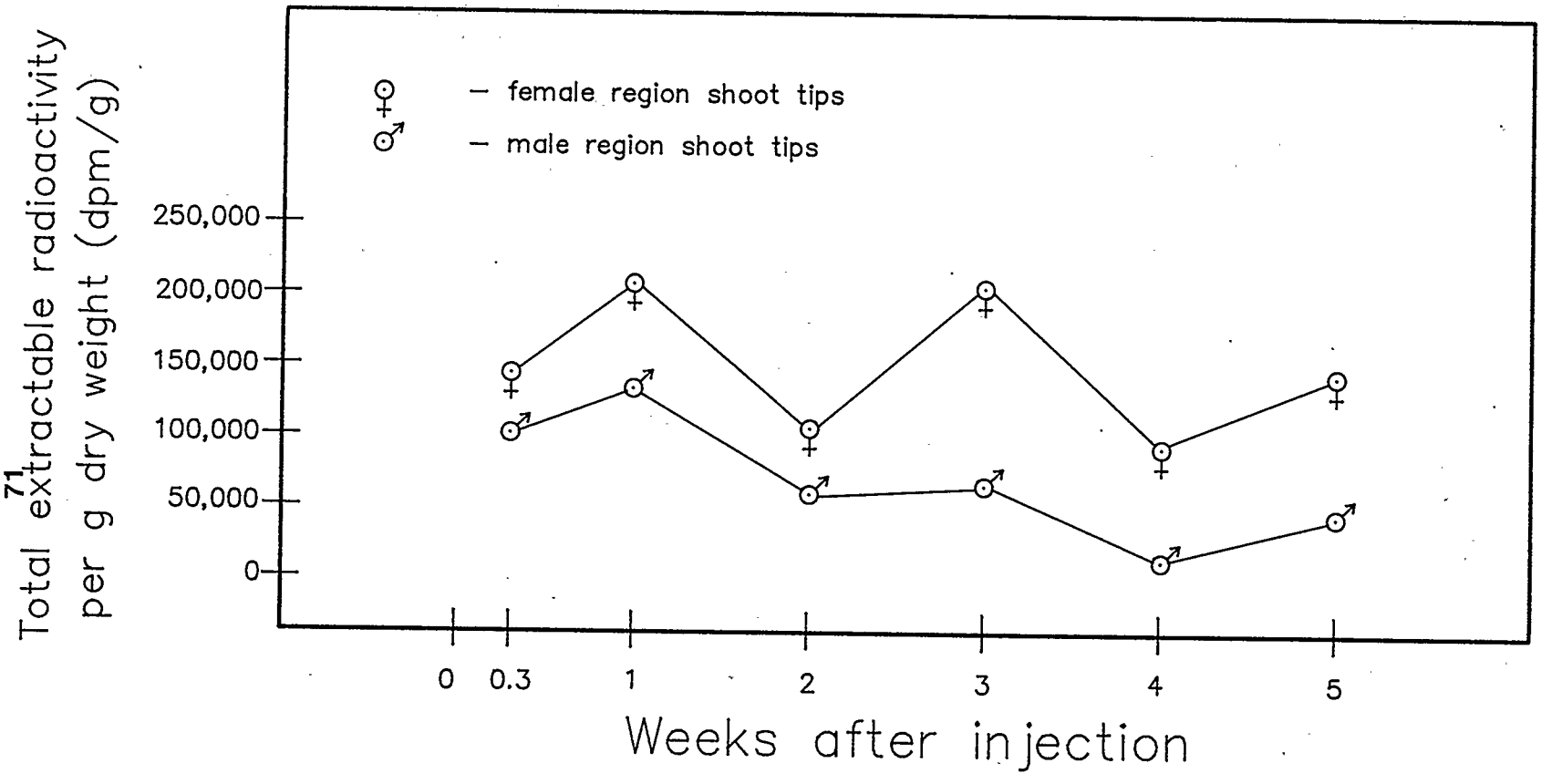

Figure 19. Amount of [^{3}H] found in 80% MeOH extracts of actively growing shoot tips of 3—year—old seedlings harvested at different times after injection of [^{3}H] GA_3/ GA_3. Each point is composed of one or the average of two measurements.

percent of the total amount extracted) was detected in those fractions containing pigments or putative GA precursors. The relative amount of tritium associated with putative GA conjugates, however, increased from 3.5 to 16 percent of the total sample radioactivity with harvests of increased time after [^{3}H]GA_3/GA_3 injection (Figure 20). Control samples (injected with high specific activity GA_3 only) had comparable proportions of [^{3}H] associated with their putative GA conjugate fractions.

Free GA-like substances from [^{3}H]GA_3/GA_3-treated shoot tips were separated by 10-73% MeOH gradient-eluted C18 HPLC. At least 52 percent (of the total free GA-like radioactivity) was [^{3}H]GA_3-like regardless of how many weeks after GA_3 injection the shoot tip samples were harvested. Radioactive profiles from HPLC fractions of [^{3}H]GA_3/GA_3-treated shoot tips harvested on each of two days or four weeks after GA_3 injection are shown in Figure 21.

The major [^{3}H]GA_3 metabolites were present in the more polar fractions eluting just before [^{3}H]GA_3. Only very small amounts of radioactivity were detected in other HPLC fractions. The only detectable difference in HPLC radioactivity profiles from shoot tip samples harvested with increased time after GA_3 injection, was a gradual increase in the amount of the more polar

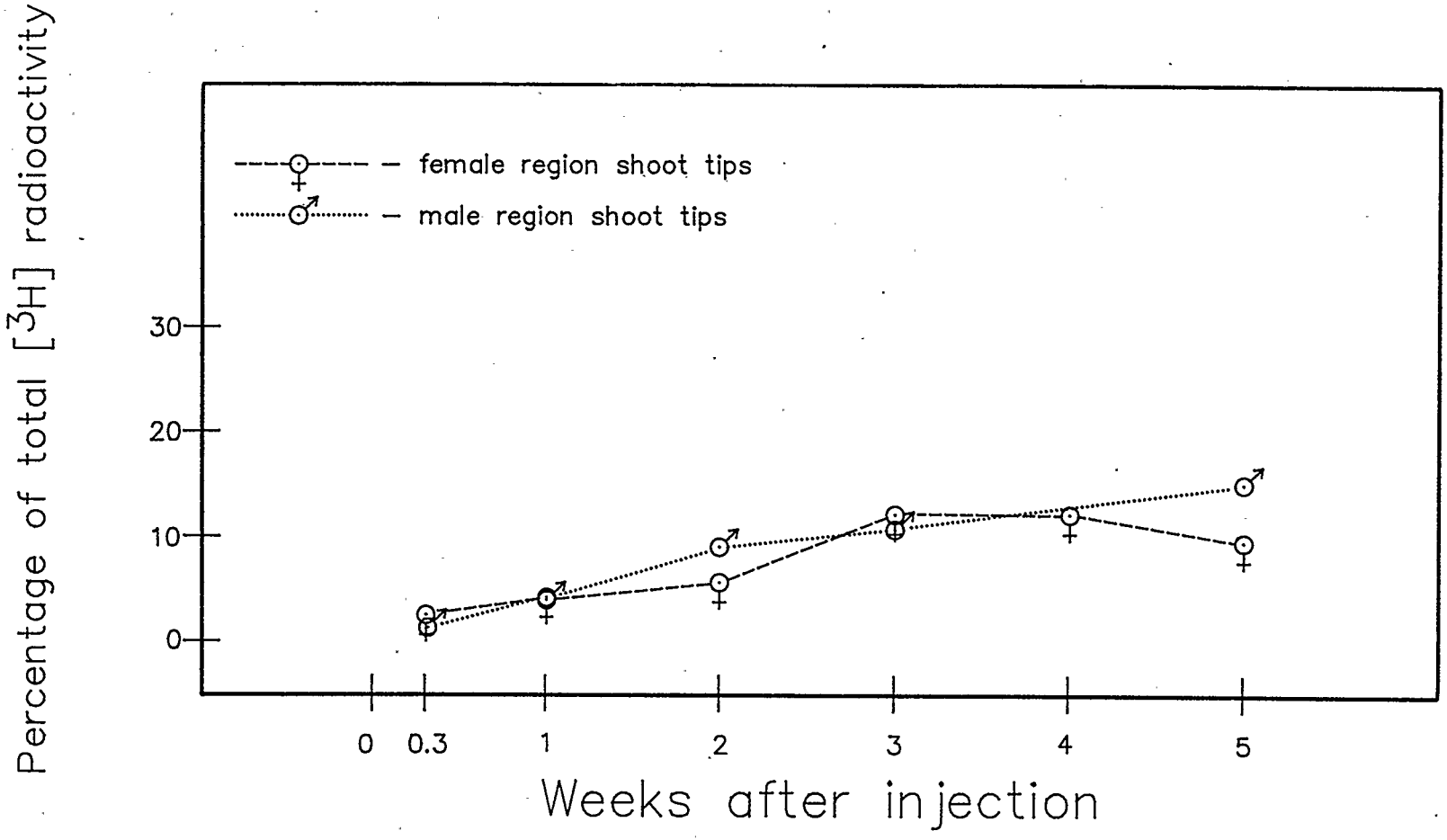

Figure 20. Percentage of total radioactivity (free GA–like plus GA–conjugate–like) present in GA–conjugate–like fraction of GA_3–treated shoot tips over increased time after [^{3}H] GA_3/GA_3 injection.

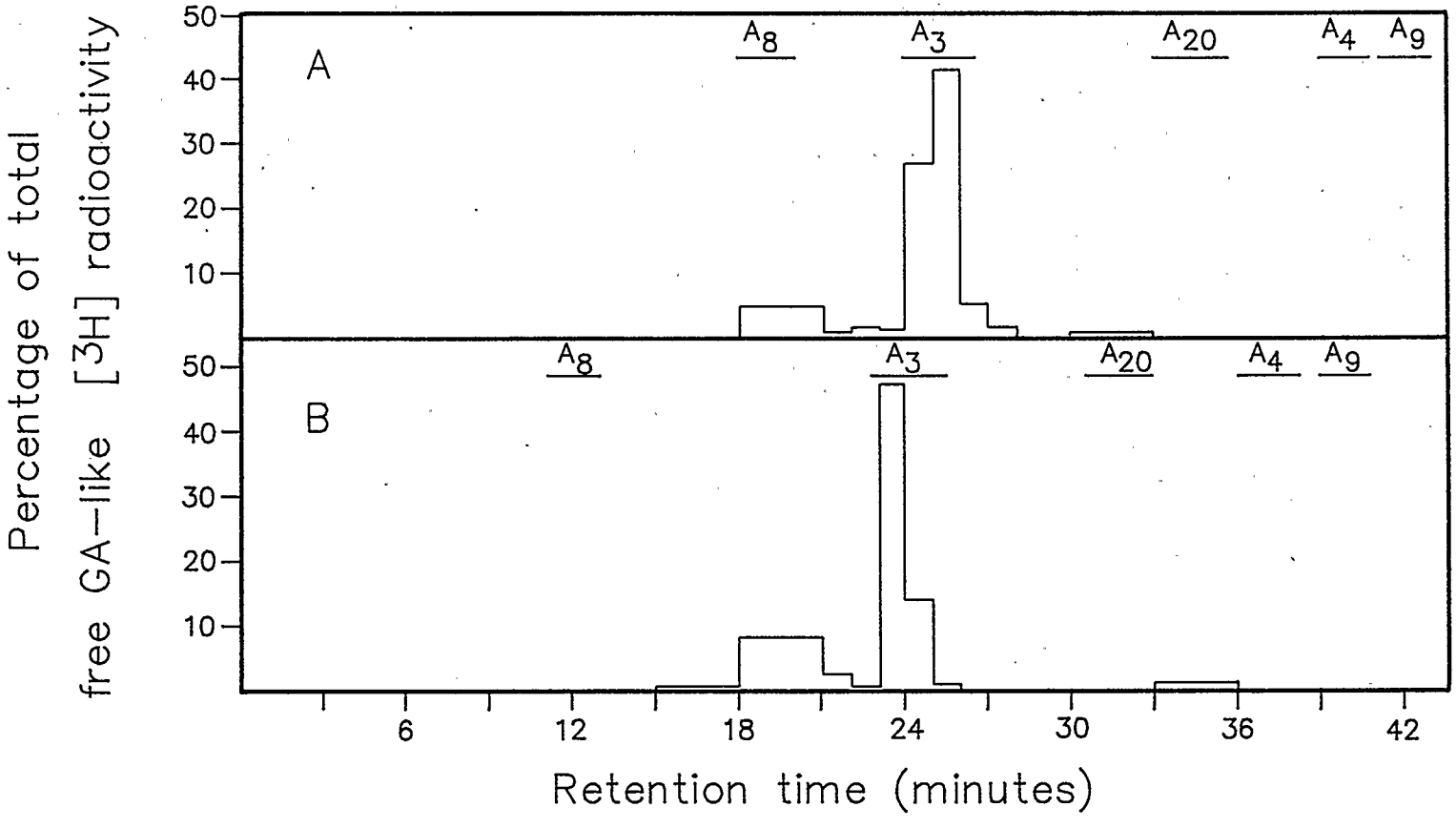

Figure 21. Distribution of percentage of total free GA radioactivity over HPLC fractionation using C18 ubondapack 10–73% MeOH gradient.
A. GA_3–treated female region shoot tips, 2 days after injection.
B. GA_3 –treated female region shoot tips, 4 weeks after injection.
Amounts less than 1% are not shown.

[^{3}H]GA_3 metabolites.

Less polar GA-like HPLC fractions from GA_3-treated shoot tips were bioassayed with Tan-ginbozu dwarf rice seedlings. Significant amounts of GA-like biological activity were present in these samples, particularily near or before the retention time of [^{3}H]GA_{20} for shoot tip samples harvested either two days or one week after GA_3 injection. Considerable biological activity was also seen in fractions between the retention time of [^{3}H]GA_{20} and [^{3}H]GA_4 in shoot tip samples harvested one or two weeks after injection. Lesser amounts of biological activity were present in these same fractions four and five weeks after [^{3}H]GA_3/GA_3 injection.

Losses of less polar GA-like substances from samples separated using the 10-73% MeOH program were not quantified but were estimated to be high and variable (from 50%-90%) based on losses seen in similar HPLC runs with known amounts of [^{3}H]GA_4 and [^{3}H]GA_9 standards. Thus, from these samples it was not possible to quantify, for the various harvests times, amounts of biologically active GA-like substances.

However, additional extracts were made from [^{3}H]GA_3/GA_3-treated female and male region shoot tips harvested one, two and three weeks after GA_3 injection. These were chromatographed using the 64-100% MeOH program, and 60,000 dpm of high specific activity

[^{3}H]GA_9 was added prior to HPLC to allow estimation of losses. The distribution of [^{3}H]GA_3 metabolites in the less polar region of the HPLC spectrum appeared similar to that for the earlier samples chromatographed using the 10-73% MeOH program. However, on this 64-100% gradient, the more polar radioactivity ([^{3}H]GA_3 and its major more polar [^{3}H] metabolites) was not resolved. These less polar HPLC fractions were then bioassayed (on dwarf rice seedlings; Figure 22). The relative heights of peaks (as compared to growth of seedlings given known amounts of GA_3) are representative of one-fiftieth of the biological activity that was present in each fraction. At this stage of chromatography, however, one should not directly compare GA-like biological activity between samples due to different dry weights and also because of varying amounts of [^{3}H] products(s) present in these HPLC fractions. The amount of [^{3}H] associated with a 1/50 aliquot applied to each rice seedling is shown for major biologically active peaks so that the amount of less-polar [^{3}H]GA_3/GA_3 metabolite present in fractions (whether or not biologically active) can be estimated. At the time of [^{3}H]GA_3/GA_3 injection the ratio of [^{3}H]GA_3 to carrier GA_3 was 1 dpm per 700 pg.

The majority of GA-like biological activity was present in fractions near the Rt of the external

Figure 22. Gibberellin-like biological activity in less polar GA-like fractions of GA_3-treated shoot tips. The average leaf sheath length of dwarf rice seedlings (10) is shown in response to application of a 1/50 aliquot of each fraction. Retention times of [^{3}H]GA standards and growth of rice seedlings given known amounts of GA_3 (pg/seedling) are also shown. a, c, e: Male region shoot tips harvested one, two, and three weeks after [^{3}H]GA_3/GA_3 injection respectively. b, d, f: female region shoot tips harvested one, two, and three weeks after [^{3}H]GA_3/GA_3 injection respectively. For large biologically active peaks, the amount of dpm applied to each rice seedling is shown in brackets above the peak. The ratio of [^{3}H]GA_3 to GA_3 at the time of injection to Thuja seedlings was 1 dpm / 700 pg.

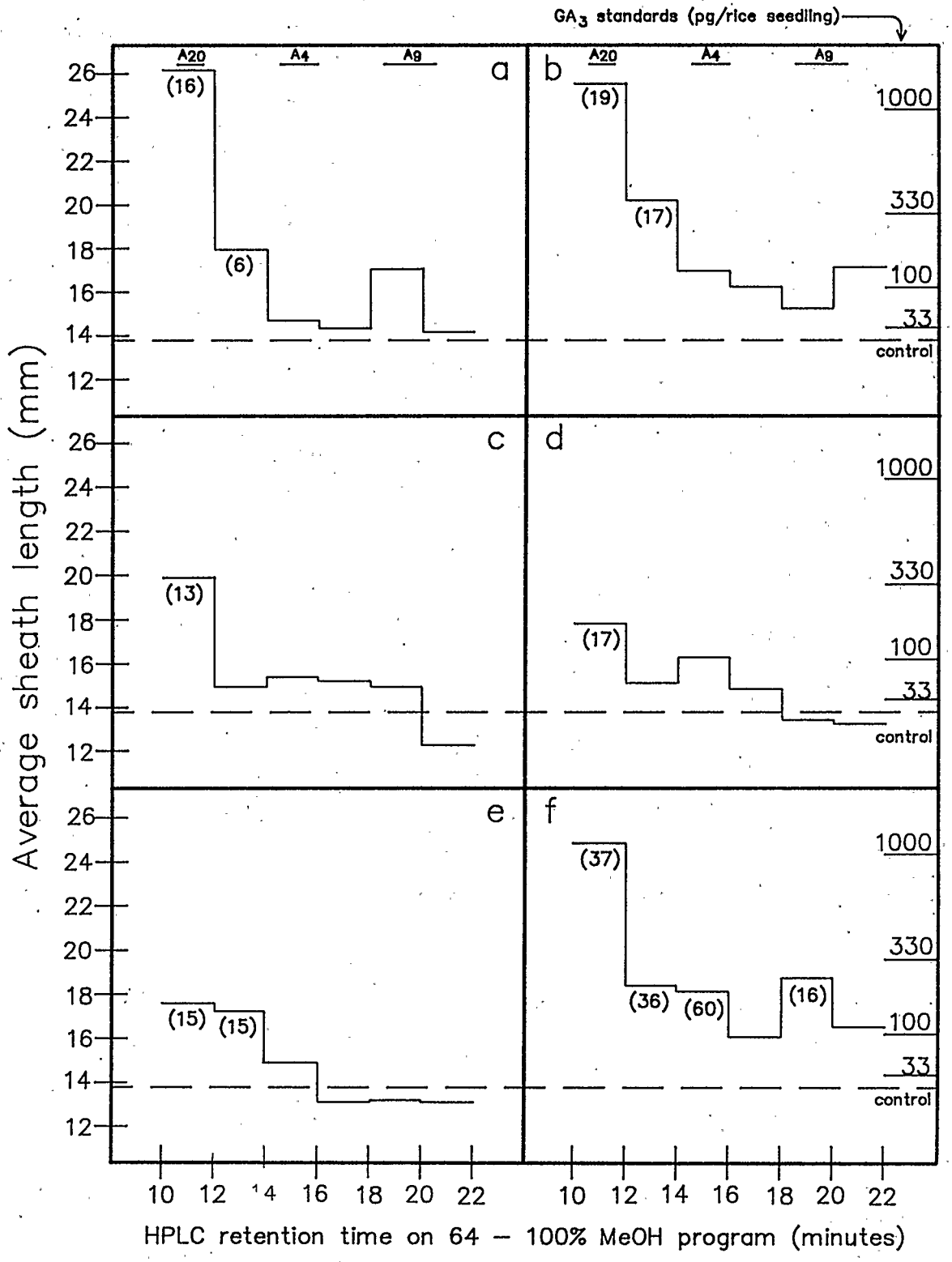
GA3 standards (pg/rice seedling)
A20
A4
A9
a
b
c
d
e
f
(16)
(6)
(19)
(17)
(13)
(17)
(15)
(15)
(37)
(36)
(60)
(16)
1000
330
100
33
control
Average sheath length (mm)
26
24
22
20
18
16
14
12
10
12
14
16
18
20
22
HPLC retention time on 64 – 100% MeOH program (minutes)

Figure 22

standards [^{3}H]GA_{20} and [^{3}H]GA_4. For the fraction at Rt of [^{3}H]GA_{20}, the amount of biological activity in fractions did not seem to be related to the amount of radioactivity present (Figure 22).

The less polar bioactive HPLC fractions from GA_3-treated male and female region shoot tips, harvested one, two, and three weeks after GA_3 injection, were combined such that they contained the same putative GAs (based on HPLC retention times on the 64-100% MeOH program). This was done to get a better estimate of the total biological activity and radioactivity present at each retention time, and to attempt to correlate radioactivity with biological activity. Figure 23 shows that significant radioactivity was present in samples with Rts near those of [^{3}H]GA_{20} and [^{3}H]GA_4, presumably as a result of metabolism of [^{3}H]GA_3 to less polar compounds. The amount of radioactivity present at the Rt of [^{3}H]GA_9 resulting from [^{3}H]GA_3 metabolism could not be determined due to the large amount of high specific activity [^{3}H]GA_9 added (to quantify losses).

The amount of biologically active GA-like substance(s) in the fraction eluting near the Rt of [^{3}H]GA_{20} (min 10-12 on the 64-100% MeOH program) was somewhat less than the sum of that present in these fractions before being combined (Figures 22 and 23). This suggested that bioassay-active inhibitors may be

Figure 23. Total radioactivity (dpm) of [^{3}H] and biological activity in less-polar GA-like HPLC fractions. Extracts of male and female shoot tips one, two, and three weeks after [^{3}H]GA_3/GA_3 (700 ng GA_3/100 dpm) injection to 3-year-old seedlings were combined after HPLC. Biological activities are shown as the average (of 10 plants) leaf sheath length in response to an aliquot of 1/200 of the grouped extracts which represents 2.8 grams dry weight of apices from 3 seedlings. Standard HPLC retention times and average growth responses of rice seedlings to standard amounts of GA_3 (pg/seedlings) are also shown.

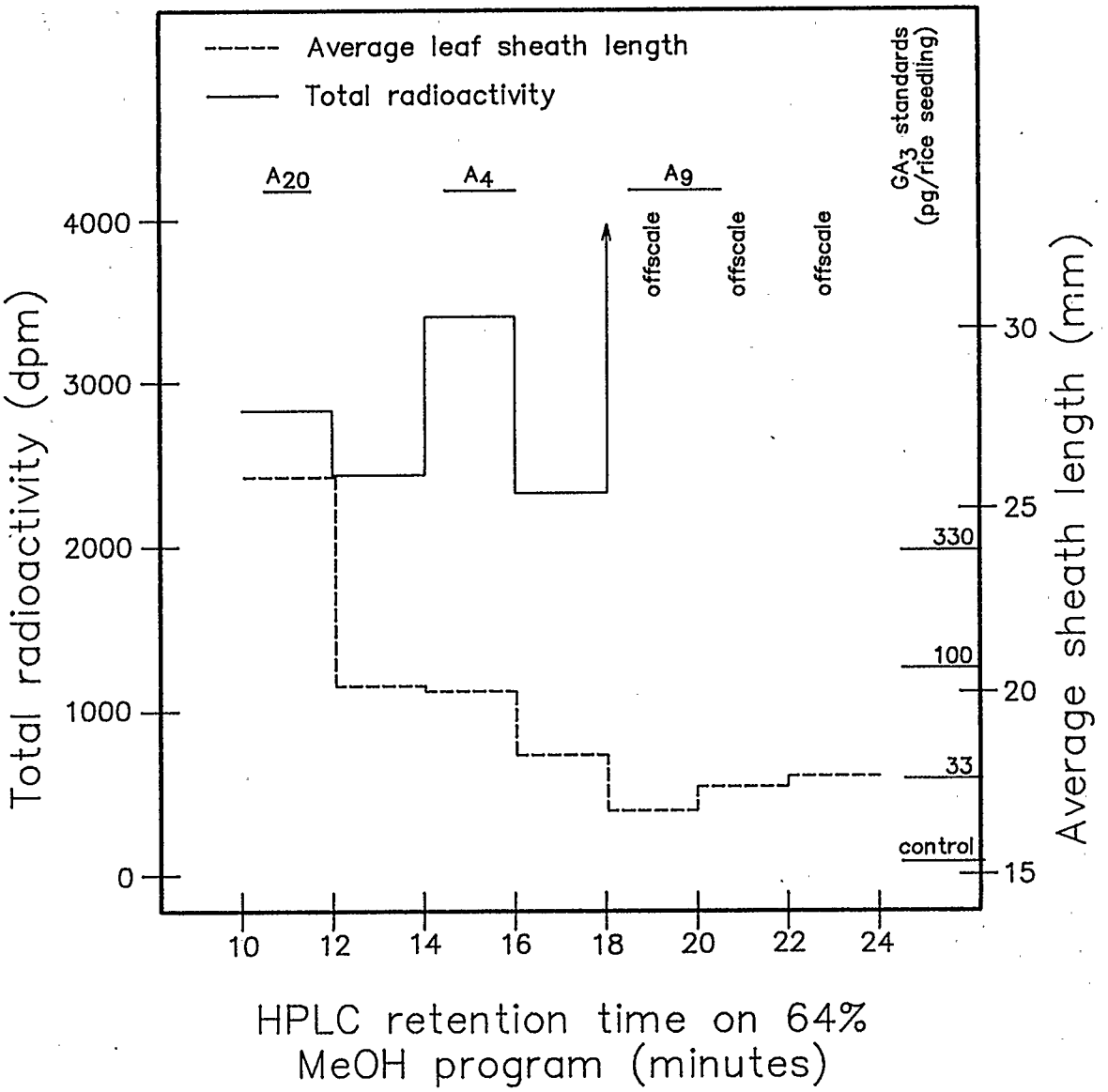
Average leaf sheath length
Total radioactivity
A20
A4
A9
offscale
offscale
offscale
GA3 standards (pg/rice seedling)
330
100
33
control
Total radioactivity (dpm)
Average sheath length (mm)
4000
3000
2000
1000
0
30
25
20
15
10
12
14
16
18
20
22
24
HPLC retention time on 64% MeOH program (minutes)

Figure 23

present in the samples.

A GA_3 Rt HPLC fraction from GA_3-treated female region shoot tips harvested three weeks after GA_3 injection was bioassayed using serial dilutions. Table 5 shows that increasing the dilution used for bioassay decreased the amount of putative inhibitor present. The amount of biological activity calculated to be in the fraction by bioassay (ng GA_3 equivalents) was closest to the amount of biological activity that was expected to be in the fraction (10 dpm = 7 ng GA_3 at the time of $[^3H]GA_3/GA_3$ injection to cedar seedlings) when bioassayed at the highest dilution. The amount of inhibition at a dilution of 1/2000 (the most concentrated) was significant. With this dilution, only 28 percent of the expected GA_3 was calculated to be present in the fraction by bioassay.

Free GAs of control shoot tips of three-year-old seedlings grown in the growth chamber and harvested one, two, or four weeks after $[^3H]GA_3$ (high specific activity) injection, were separated using the 10-73% MeOH HPLC program. A number of less-polar biologically active GA-like substances eluted between the Rts of $[^3H]GA_{20}$ and $[^3H]GA_4$. Quantities of these substances were roughly similiar to those found in parallel HPLC fractions of free GAs from GA_3-treated shoot tips separated on the same gradient. However, without

Table 5. Radioactivity and biological activity (eluting at the Rt of [^{3}H]GA_3) at different bioassay dilutions. The [^{3}H]GA_3/GA_3-like HPLC fraction was from shoot tips of GA_3-treated 3-year-old seedlings (harvested three weeks after injection of 700 ug and 10^6 dpm of GA_3), was purified using two consecutive HPLC programs; the 64% MeOH program, then the 10-73% MeOH program.

Bioassay dilution[a]	dpm/seedling	expected[b] non-[^{3}H]GA_3 (ng/seedling)	GA_3 equivalents by bioassay[c] (ng/seedling)
1/2000	16.3	11.4	3.2
1/4000	8.1	5.7	2.0
1/8000	4.1	2.9	1.6
1/16000	2.0	1.4	1.1

(a) fraction of extract applied to 10 rice seedlings

(b) based on ratio of 700 ng/1000 dpm injected into T. plicata seedlings

(c) determined from growth of rice seedlings in response to standard amounts (ng/seedling) of GA_3 applied

knowing losses it would be difficult to say whether these quantities on per gram dry weight basis would be the same.

Shoot tip samples from non-GA_3-treated plants (mature propagules, three- and one-year-old seedlings) grown in the greenhouse or growth chamber, were harvested during long day (September). Extraction and purification of GA-like substances was done using the procedure outlined in Figure 3 (64-100% MeOH program) and internal [^{3}H]GA_9 was added to follow losses of less polar GAs. Unlike the above-mentioned controls, three-year-old seedling shoot tips, only very small amounts of biologically active GA-like substances were present in all shoot tip samples examined. This may be due to problems and losses encountered during extraction of tissue. Hence, only very rough estimates of biological activity were available. The maximum amount of biologically active GA-like substance in any HPLC fraction was less than 2 ng GA_3 equivalents. The same HPLC fractions from GA_3-treated female region shoot tips always contained 5 to 65 ng (Figure 22). These values were determined by multiplying the amount of GA_3 equivalents applied to each rice seedling by 50 (the bioassay dilution used).

Combined GC-MS was used to attempt to identify the biologically active putative GAs present in the various

shoot tip samples. HPLC fractions from [^{3}H]GA_3/GA_3-treated shoot tips (male and female region) eluting between the [^{3}H]GA_{20} and [^{3}H]GA_4 Rts on the 10-73% MeOH program were combined to give one fraction that contained biologically active GA-like substances. The sample was further purified (as a bulk sample) by silica gel adsorption chromatography. Fifty ng of d_4GA_4 was added, and then the sample was derivatized. The trimethylsilyl ether of the methyl ester (TMSi-ME) of d_4GA_4 was only just detectable by GC-MS-SIM. At the Kovats retention index (KRI) for GA_4 there was a small peak for an ion with a mass:charge ratio of 422 (e.g. the molecular ion of d_4GA_4). Also, at the same KRI there was no peak for the d_4GA_4 characteristic fragmented ion of 288. The poor response of the mass selective detector for ions of the TMSi-ME of d_4GA_4 indicated that the sample was not pure enough for characterization of putative endogenous GAs present in small quantities.

From searches for characteristic ions at the appropriate KRIs for Gibberellins A_{20}, A_{19}, A_{36}, A_{15}, A_4, and A_7 it appeared unlikely that these GAs were present, in appreciable amounts, in the sample. The peaks obtained were either not integrated properly due to wide or irregular peak shapes, or too small to represent measurable quantities of GAs or (where a pair

or more of characteristic ions were monitored for one GA) did not yield the appropriate ratio of characteristic abundances for those ions monitored.

Other samples containing putative GAs were also subjected to GC-MS-SIM only to give the same inconclusive results as with the aforementioned sample.

DISCUSSION AND CONCLUSIONS

Gibberellin A_3-induced conebud production in *Thuja plicata*

Injection was an efficient method of administering exogenous GA to *Thuja* seedlings. A 100 percent conebud induction response in actively growing shoot tips of treated branches was obtained by giving three-year-old seedlings 700 ug GA_3 per branch (Figure 4), even though only roughly five percent of the $[^3H]GA_3/GA_3$ injected was found in the shoot tips (Figure 18). This method gave greater cone production than the spray application method previously used (Ross, 1983) and likely required application of less hormone because only one injection was required compared to several biweekly spray applications.

Injection of the same dose of GA_3 to one-year-old seedlings (approximately the same size as one branch in the three-year-old seedlings) however, resulted in fewer cones being produced from activley growing potentially reproductive shoot tips. It is possible that younger meristematic tissue required more GA_3 to evoke a switch to determinate reproductive growth (Pharis and Kuo, 1977) and that water stress in the three-year-old

seedlings (see Table 1) contributed synergistically to the GA_3-induced cone production (Ross and Pharis, 1985).

Young conebuds were visually identified on three-year-old seedlings five weeks after GA_3 injection (700 ug per branch) and potentially reproductive shoot tips harvested four weeks after injection contained obviously reproductive apices with one or a few pairs of microsporophylls. This agreed with the data published by Owens and Pharis (1971) on GA_3-sprayed Thuja seedlings grown under controlled LD-warm conditions. Anatomical analysis revealed, however, that shoot tip samples collected at any given time after GA_3 injection were not homogeneous in terms of their developmental stages, although conebud development in GA_3-treated shoot tips may have still been more synchronous than that in naturally-induced conebud development.

Seed and pollen conebuds could not be visually distinguished at early stages of development: pollen conebuds were present in the week four and five samples that were expected to contain seed conebuds (short, distal branches). This suggested that the high dosage of GA_3, combined with the uniformly long daylength sequence affected normal sex expression in the trees (Owens and Molder, 1984; Ross, 1983).

The presence of mainly young pollen conebuds in

"seed conebud" samples also indicated that it was unlikely that any biochemical (e.g. protein) differences would be discernable between male and female region shoot tip samples at early stages of development.

[^{35}S] methionine labelling of Thuja plicata shoot tip proteins

Due to the lack of sufficient numbers of replicate samples for many of the experiments below (and mentioned elsewhere), it was not possible to ensure that any data set (whether or not transformed to achieve normal distribution) was in fact normally-distributed. Thus, the statistical analyses should not be taken as conclusive measurements of the variability present in the samples.

In vitro [^{35}S] labelling of Thuja shoot tips was an effective means for examining newly-made shoot tip proteins at different times after GA_3 injection. The method resulted, however, in greatly differing ratios of [^{35}S] labelled protein to total protein for the different samples. This did not necessarily reflect different actual rates of net protein synthesis, but more likely that different degrees of uptake of [^{35}S] methionine into shoot tips had taken place (Figures 9 to 12).

Uptake (and hence also incorporation) of [^{35}S] in shoot tips was enhanced by GA_3 treatment (Figure 11 and Table 3), but not GA_7 treatment (which was not highly florigenic; Tables 2 and 3). This effect of GA_3 supports the notion that GA_3 enhances membrane permeability (Neumann and Janossy, 1977).

Uptake (and hence also incorporation) was not uniform throughout any individual shoot tip incubated in [^{35}S] methionine; rather, more [^{35}S] labelling of proteins took place closest to the cut surface of the shoot tips (Table 3). This indicated that the majority of [^{35}S]-labelled proteins (particularly for GA_7-treated and control shoot tips) were not from the shoot apex itself but from the subtending tissue. The results shown in Table 3 for three kinds of shoot tips also suggest that the differences seen in [^{35}S]-labelled proteins in GA_3- and GA_7-treated shoot tips may have been partly due to the inconsistent degrees of labelling in the shoot tip regions of those samples.

Protein overloading of SDS-PAGE gels for some samples resulted from differential uptake and incorporation of [^{35}S] methionine. Overloading only caused problems in analyzing results when it caused poor resolution of bands or when the lanes were too wide for the laser beam of the densitometer.

Changes in shoot tip proteins during the transition of the vegetative mode to the reproductive mode

The poor resolution of protein bands for many samples and the existence of many 33-38 kDa [^{35}S]-labelled proteins from Thuja shoot tips made it difficult to make conclusive statements about possible changes in proteins during early conebud induction and development due to GA_3 treatment. Gibberellin A_3-treated [^{35}S]-incubated shoot tips harvested at least three weeks after GA_3 injection, almost consistently contained a [^{35}S]-labelled 36 kDa protein, whereas control (non-GA_3-treated) vegetative shoot tips harvested and labelled at the same time, did not (Figure 13). Non-GA_3-treated terminal buds (the terminal never flowers, even when given GA_3) probably did not contain this protein, but did have a [^{35}S]-labelled protein of similiar but lower molecular weight (approx. 35 kDa; Figure 17). [^{35}S]-labelled shoot tip proteins of GA_3-treated terminal shoot tips seemed to contain both proteins although poor resolution made it difficult to determine this conclusively. Thus, the presence of the 36 kDa protein in GA_3-treated terminal (non-flowering) shoot tips would suggest that either this protein was uniformly induced by GA_3 treatment in all tissues, or (if it is assumed that it had some regulatory or

enzymatic role) that the protein was associated with flowering in potentially reproductive shoot tips, but was ineffective in terminal shoot tips that were not predisposed to flower due to the presence or absence of other factors.

Examination of Coomassie blue-stained gels showed equal relative amounts of approximately 36 kDa protein in control (non-GA_3-treated) vegetative shoot tips and in the potentially reproductive GA_3-treated shoot tips (Figure 16). This may or may not have been the same 36 kDa protein that was seen when only [^{35}S]-labelled proteins were considered (Figure 13); for any given molecular weight it is likely that more than one unique protein with a unique role in the cell exists. If this was the same protein as that seen in [^{35}S]-labelled GA_3-treated reproductive shoot tips, it suggests that the turnover rate for its synthesis and breakdown was very much decreased in the control shoot tips compared to the reproductive shoot tips. Further experiments would be required to investigate these possibilities including two-dimensional electrophoretic techniques and possibly in vitro translation of extractable mRNA.

Regardless of the conclusion drawn as to whether or not the 36 kDa protein seen on stained gels (Figure 16) and autoradiograms (Figure 13) was the same protein, it did not appear that the protein played a

structural role in the shoot tips. It was absent from stained gels of naturally-induced well-developed female conebuds. Also, if it was a structural protein associated with microsporophyll development, it should have accumulated in GA_3-treated reproductive shoot tips, but should have been absent in control vegetative shoot tips. The evidence however, does suggest a regulatory role for the protein at the time of microsporophyll initiation and further development.

Additional speculation regarding the role of the 36 kDa protein in early conebud development is not warranted at this stage of research into the topic.

Gibberellin-like substances in GA_3-treated and control shoot tips

It was clear that the $[^3H]GA_3/GA_3$ injected into seedling branches or stems arrived at the shoot tips by translocation very shortly after injection (Figure 19). A gradual metabolism of $[^3H]GA_3/GA_3$ occurred such that less GA_3 and more of its metabolites were present with increased time after injection (Figures 20 and 21).

Analysis of GA-like biologically active substances, however, did not show a gradual increase in biological activity with time after GA_3 injection.

Instead, the results indicated that shortly after GA_3 injection, there was greater measurable GA-like biological activity (in fractions other than the GA_3 fraction) in GA_3-treated samples compared to those harvested at later dates. The fact that there were greater amounts of less-polar GA-like biological activity coincidental with greater amounts of $[^3H]GA_3/GA_3$, but lesser amounts of $[^3H]GA_3/GA_3$ metabolite, does not support the notion of a biologically active less-polar GA_3 metabolite being the inductive agent for flowering. Rather, GA_3 itself or an endogenous less-polar GA (induced by GA_3) could be the inductive agent. If the bioactive endogenous less-polar GA was a predursor to a GA_3-like substance in Thuja, then its level could have risen due to feedback inhibition. Recalling that in other conifers, particularily the Pinaceae, $GA_{4/7}$ application gives much better flowering than does GA_3 application (Ross and Pharis, 1985), the latter hypothesis seems reasonable.

To investigate the GA-mediated process more thoroughly would require determining conclusively whether the increased amount of biologically active less polar GA-like substance(s) seen in GA_3-treated shoot tips, relative to control, non-GA_3-treated vegetative shoot tips, were metabolites of GA_3. Use of higher amounts of high specific activity $[^3H]GA_3$ for injections with

GA_3 would enable more reliable quantification of [^{3}H] metabolites in HPLC fractions. Sequential HPLC on a variety of column types could be used to separate radioactive GA_3 metabolites from the less polar biologically active endogenous GA-like substance(s), if indeed they are separate entities.

Another possible approach would involve the use of cultural treatments on T. plicata seedlings that would promote flowering coupled with injection of high specific activity [^{3}H]GA_3. If T. plicata responds in a manner similar to Cupressus arizonica (Kuo, 1973), or Pinaceae species (Pharis and Ross, 1986), increases in less polar GA-like substances would be found in the flowering shoot tips compared to control (vegetative) shoot tips. These GAs could be correlated with (or separated from) the [^{3}H]GA_3 metabolites, and could be ideally characterized by GC-MS.

Attempts to identify the biologically-active less polar GA-like substance in combined extracts of either GA_3-treated shoot tips or control shoot tips were not successful. This was probably due to the large amounts of contaminating substances present in the samples, not from a lack of GAs beging present. Alternatively, if the GA-like substance is a [^{3}H]GA_3/GA_3 metabolite, identification remains to be done. The mass spectra of known GA_3 breakdown products has not been published

(Pryce, 1973).

GA_3- versus GA_7-treatment of Thuja seedlings

It has now been shown that for many processes affected by GAs, and in many different plant systems, that the different GAs do not function equally in these processes (Pharis and Kuo, 1977; Pharis et al. 1986). Because the less polar GAs are more effective in inducing conebud production in the Pinaceae, whereas the most effective GAs for causing shoot elongation in rice, pea and maize are the C-3, C-13 hydroxylated GA_1 and GA_3 (Phinney, 1984) the responses of Thuja seedlings by elongation and conebud production to GA_3 and GA_7 were compared. Gibberellin A_3-treated seedlings had greater elongation than did GA_7-treated or control seedlings for the first few weeks after injecton (Figure 8). By week four however, controls had elongated more than did either treated group. This suggested that the GAs primarily caused elongation but that when conebud production began to occur, photosynthetic resources may have been used preferentially in the reproductively differentiating shoot tips, rather than in the elongating internodes of the major branches and terminal shoot.

The flowering response of seedlings induced by GA_7

was much lower than for GA_3-treated seedlings. This may have indicated that greater amounts of GA_7 would be required to induce an equal level (to GA_3 treatment) of conebud production. Alternatively, rapid metabolism of GA_7 to more polar GAs (including GA_3, a known metabolite in the fungus, Gibberella) may have occurred before effective levels of GA_7 could reach the site of evocation. Or, uptake of GA_7 into cells or translocation of GA_7 through the phloem may be slower than for GA_3 (the molecular structures of GA_3 and GA_7 are shown in Figure 24). To examine these possibilities properly, [^{3}H]GA_7 would need to be injected with GA_7 so that movement and metabolism of GA_7 sould be followed.

There were insufficient GA_7-induced conebuds to determine whether the two GAs produced different protein profiles from [^{35}S]-labelled shoot tips. The only sample available for the GA_7-treated seedlings did not show a [^{35}S]-labelled 36 kDa protein. However, this could have been due to the particular stage of development that the shoot tips were in at the time of labelling; a GA_3-treated sample harvested at the same time also did not show a [^{35}S]-labelled 36 kDa band (Figure 17).

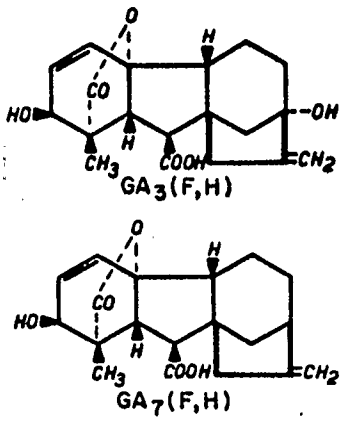

Figure 24. The molecular structures of GA_3 and GA_7.

Conclusions

The use of a whole plant system, Thuja plicata seedlings, did provide tissue that was sufficiently uniform to detect biochemical (protein) differences between vegetative and early flowering shoot tips. This was accomplished through the use of GA_3 injection, and use of seedlings that were young enough to flower minimally without treatment but old enough to flower maximally with treatment.

GA_3-induced conebud development coincided with the appearance of a [^{35}S]-labelled 36 kDa protein that was absent in equivalent, but non-induced vegetative tissue. It was not possible to determine whether this protein was a result of GA_3 treatment or conebud development, or both. Improved electrophoretic techniques and more replicates of the various samples, including naturally-induced conebuds at similiar stages of development, would be required to determine this.

Production of the [^{35}S]-labelled 36 kDa protein was not related to the arrival of GA_3 at the shoot tips but coincided with the time of microsporophyll production, roughly three weeks after GA_3 injection. A Coomassie blue stained 36 kDa protein did not differ in relative intensities between control and flowering shoot tips seven weeks after GA_3 treatment, suggesting

that it was not a structural protein associated with microsporophyll production.

Analysis of GA-like substances showed that floral evocation coincided not only with high levels of exogenously applied GA_3, but also with high levels of a biologically active less polar GA-like substance. This substance decreased in samples harvested at later dates after GA_3 injection. It was suggested that application of large amounts of GA_3 to shoot tips may have caused decreased metabolism of this less-polar substance (by feedback inhibition). However, whether the GA_3 induced flowering *per se*, or whether the unknown substance was causal for flowering, was not determined. Further experiments were suggested to investigate this speculative hypothesis.

GA_7 was not as effective as GA_3 in inducing conebud production in young seedlings of *Thuja plicata*, and it is possible that this was a result of poorer overall uptake of GA_7 into shoot tips, or of rapid GA_7 metabolism, rather than as a result of GA_7 being a less active GA *per se* for conebud induction.

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Appendix 1: Composition of solutions used in Protein Extraction, Gel Electrophoresis and Fixation

Extraction Buffer:

62.5 mM Tris, pH 6.8
2 % SDS
10 % glycerol
5 % 2-mercaptoethanol

Tank Buffer:

0.025 M Tris, pH 8.3
0.192 M glycine
0.1 % SDS

Stacking gel:

4.5 % acrylamide
0.125 M Tris, pH 6.8
0.1 % SDS

Separation Gel: Made with a linear gradient of lower gel solution to upper gel solution.

Lower gel solution:

15 % acrylamide
10 % glycerol
0.37 M tris, pH
0.1 % SDS

Upper Gel Solution:

7 % acrylamide
0.37 M Tris
0.1 % SDS

Gel staining solution:

0.1 % (w/v) R250 Coomassie Blue stain
10 % acetic acid
30 % methanol

Gel Destaining solution:

10 % acetic acid
30 % Methanol

Fixative for Paraffin Embedding series:

2 % formaldehyde
2 % glutaraldehyde
0.05 M phosphate buffer, pH 6.8

Appendix 2: HPLC Conditions Used.

Equipment: Waters Associates Automated gradient controller, U6K injector, and chromatography pumps (M6000A).

Solvents: Glass-distilled water, HPLC grade MeOH, glacial acetic acid, to make 10 % MeOH in 1 % HAc.

Column and program used for optimal separation of polar Gibberellins: Waters Associates C18 uBondapak, 0-5 minutes at 10 % MeOH, 5-35 minutes linear gradient of 10 - 73 % MeOH, 35-50 minutes 73 % MeOH, 50-60 minutes 100 % MeOH. Flow rate 2 mL/min.

Column and program used for optimal separation of non-polar Gibberellins: Waters Associates Radial Pak liquid chromatography cartridge in Radial compression separation unit (Z-module). 0-20 minutes at 64 % MeOH, 20-25 minutes linear gradient of 64-100 % MeOH, 25-40 minutes at 100 % MeOH. Flow rate 1mL/min.

Appendix 3: GC-MS Conditions Used.

Equipment used: Hewlett-Packard 2671G, 9825B printer and controller, 5790A Series Gas chromatograph and Mass selective detector, DB1 column.

Solvent: Dry HPLC-grade Methylene chloride.

Program: 0-25 minutes, linear gradient from 60 °C to 350 °C.