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THE INFLUENCE OF GIBBERELLIC ACID AND ABSCISIC ACID ON CELL AND TISSUE DIFFERENTIATION OF BEAN CALLUS

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SUMMARY

Bean callus was induced to form roots (tissue differentiation) and vascular nodules (cell differentiation) by lowering the ratio of auxin to cytokinin in the growth medium. Both types of differentiation were inhibited by the addition of abscisic acid (at concentrations greater than 1 μM) to induction medium. Initiation of differentiation was inhibited, but its subsequent development was not, and the inhibition was not affected by the addition of gibberellic acid. Addition of gibberellic acid (GA) alone to induction medium stimulated tissue differentiation, although cell differentiation was unaffected (30 μM GA) or inhibited (45 μM GA) and its onset was delayed at both concentrations. Root initiation was also stimulated by gibberellic acid (0·1–45 μM) at an auxin-to-kinin ratio 10 times that normally optimal for cell differentiation.

The phenylalanine ammonia lyase (PAL) activity of the calluses was closely correlated with the amount of cell differentiation which had occurred, and measurement of this confirmed that gibberellic acid delayed the initiation of cell differentiation. The increase and subsequent decline of PAL and $\beta 1 \rightarrow 3$ glucan synthetase activities, normally induced by transfer to induction medium, was abolished by abscisic acid. Addition of gibberellic acid did not affect the $\beta 1 \rightarrow 3$ glucan synthetase activity.

INTRODUCTION

Skoog & Miller (1957) showed that differentiation in tobacco callus was controlled by the ratio of auxin to cytokinin supplied in the growth medium, and many workers have since found that a disorganized callus can be induced to form vascular tissue or recognizable organs by adjusting the relative concentrations of these 2 types of growth hormone (Halperin, 1969; Torrey, 1963). We have shown that lowering the auxin-to-kinin ratio of the growth medium of bean (*Phaseolus vulgaris* L.) callus induces the formation of nodules containing xylem and phloem (Haddon & Northcote, 1975) and that during this differentiation the activities of marker enzymes used for the formation of xylem and phloem (phenylalanine ammonia lyase, PAL and UDP glucose:$\beta 1 \rightarrow 3$ glucan glycosyl transferase, respectively) increase and then fall to a level approximately twice that observed in undifferentiated callus.

The hormones gibberellic acid (GA) and abscisic acid (ABA) are also known to affect growth of the plant and to interact with each other (Bewley & Fountain, 1972) as well as with auxins and kinins (Osborne, 1965; Seth & Wareing, 1967) in various hormonal responses.

The effect of gibberellic acid on organogenesis in tobacco callus has been investi-

gated. Thorpe & Meier (1973) confirmed the findings of Murashige (1961), that gibberellic acid inhibited the formation of shoot initials, although their subsequent development was not repressed. Abscisic acid inhibited growth but partially relieved the inhibition of differentiation induced by gibberellic acid. Engelke, Hamzi & Skoog (1973), however, found that gibberellic acid stimulated differentiation in the presence of cytokinin, and the ratio of gibberellic acid to kinin affected the morphology of the induced shoots.

Hillman (1970) showed that bud outgrowth from the stems of decapitated *Phaseolus* plants could be controlled by the application of hormones to the cut surface. He found that auxin partially inhibited growth, while auxin, kinin and abscisic acid were required for maximum inhibition. Gibberellic acid overcame the inhibition in both cases.

Since we have developed a sensitive quantitative method for the estimation of the course of differentiation, we have used the activities of PAL and glucosyl transferase, as well as histological techniques, to examine the early and longer term effects of gibberellic and abscisic acid on callus grown on a routine maintenance medium and on a medium which would normally induce differentiation (Haddon & Northcote, 1975).

MATERIALS AND METHODS

Materials

Analytical grade chemicals and glass-distilled water were used whenever possible. UDP-[U-^{14}C]glucose was obtained from the Radiochemical Centre, Amersham, Bucks; gibberellic acid (GA3) from B.D.H. Ltd, Poole, Dorset; racemic abscisic acid from Calbiochem, San Diego, California; Special Agar Noble from Difco Laboratories, Detroit, Michigan; and sterile containers for media from Sterilin Ltd, Richmond, Surrey.

Media

Chemically defined media containing the salts and vitamins of Gamborg, Miller & Ojima (1968) were used. Maintenance medium was supplemented with 2 mg/l. 2-4-dichlorophenoxyacetic acid (2:4D) and 2 % sucrose, while induction medium contained 1 mg/l. naphthyleneacetic acid (NAA), 0·2 mg/l. kinetin (furfuryl aminopurine) and 3 % sucrose. Media were solidified by the addition of 1 % agar before sterilization by autoclaving at 103·4 kN m^{-2} for 30 min. Gibberellic acid and abscisic acid solutions were sterile-filtered and added as required to liquid medium before dispensing 20-ml portions into sterile containers. All transfers were made under sterile conditions.

Isolation and growth of callus

Callus was obtained from the hypocotyl of *Phaseolus vulgaris* var. Canadian Wonder and maintained as previously described (Haddon & Northcote, 1975). Callus was used in the experiments after 3 transfers to fresh maintenance medium.

Assay methods

PAL was extracted from the callus by grinding with 0·1 M borate buffer pH 8·8. The 20000 g supernatant was used to assay PAL activity spectrophotometrically (Zucker, 1965). The glycosyl transferase activities were obtained by measuring the incorporation of radioactivity from UDP-[U-^{14}C]-D-glucose into material insoluble in boiling water (Haddon &

Northcote, 1975). Since the concentration of UDP glucose used was that which favours $\beta 1 \rightarrow 3$ glucan formation in both this species (Musolan, Ordin & Kindinger, 1975) and the related *P. aureus* (Chambers & Elbein, 1970), and since neither our previous studies (Haddon & Northcote, 1975) nor an analysis of the polysaccharide formed from radioactive UDP-glucose by callus after 21 d in culture showed the formation of linkages other than $1 \rightarrow 3$, the total incorporation was used as an indication of callose synthetase activity.

Unless otherwise stated, data represent the mean of 2 determinations on samples of the same strain of callus, and the experiment has been repeated with similar results on at least one other strain of callus. Where significant differences are claimed there was no overlap between values obtained for the callus incubated on the 2 different media.

RESULTS

Growth and differentiation of callus

The growth rate of the callus was similar on maintenance and induction media. Addition of gibberellic or abscisic acid at concentrations up to 45 μM to either medium did not alter the growth rate. Callus grown on maintenance medium was creamy-coloured and friable, while transfer to induction medium resulted in a slight browning of the callus, the formation of hard lumps and in many cases the initiation of roots which were visible after 12–15 days. Addition of gibberellic acid (30 or 45 μM) to induction medium increased the proportion of samples showing root initiation and also the number of roots from each sample. Addition of abscisic acid (30 or 45 μM) to induction medium in the presence or absence of an equimolar amount of gibberellin inhibited both the browning of the callus and root formation. Microscopic examination of callus transferred to induction medium showed nodules containing both xylem and phloem, as well as scattered xylem elements (Haddon & Northcote, 1975), while nodules formed in callus grown on induction medium containing gibberellic acid had a similar appearance.

Time course of differentiation

The concentrations of nodules in callus at various times after subculture are shown in Fig. 1A. The course of induction is similar to that previously reported (Haddon & Northcote, 1975). Addition of abscisic acid to the induction medium abolished the increase in nodule concentration normally observed, while addition of gibberellic acid without abscisic acid slightly increased the time lag before nodules were formed. At concentrations of 30 μM of gibberellic acid, the final concentration of nodules was equal to that in callus grown on unsupplemented induction medium, but at 45 μM the concentration was decreased. The time at which root initiation was first observed was not affected by the addition of gibberellic acid.

Time course of PAL activity

The PAL activity of the various cultures is shown in Fig. 1B. Activity in callus grown on maintenance medium remained fairly constant, whereas that of callus on induction medium increased 9 d after transfer, reached a maximum after 12 d and then fell to a constant level above that on maintenance medium. Addition of gibberellic acid (30 or 45 μM) resulted in an increased lag time, PAL activity rising only after

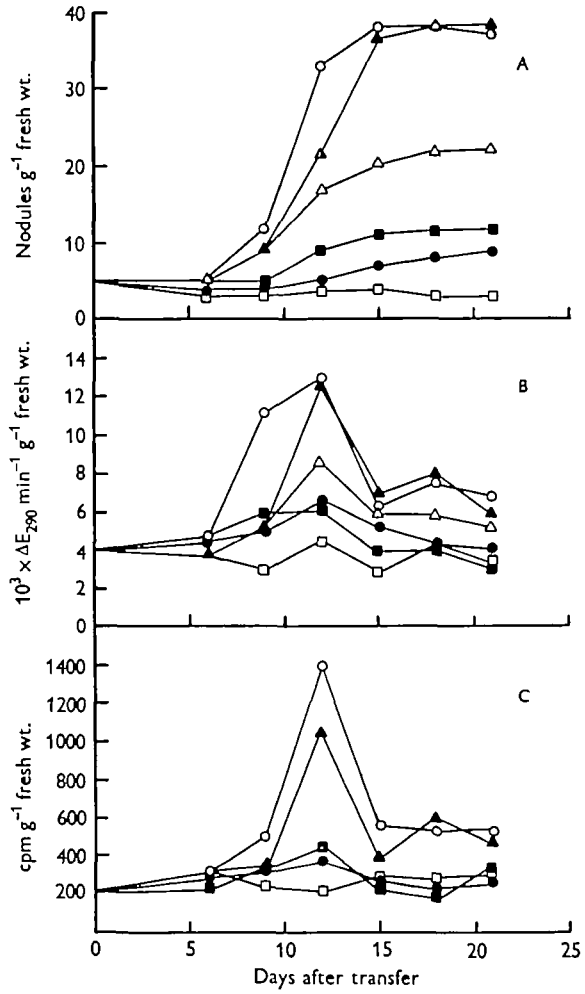

Fig. 1. The time course of cell differentiation of callus transferred from maintenance medium to: maintenance medium (—□—□—); induction medium (—○—○—); induction medium plus 45 μM GA (—△—△—); induction medium plus 30 μM GA (—▲—▲—); induction medium plus 30 μM ABA (—●—●—); and induction medium plus 30 μM GA plus 30 μM ABA (—■—■—). Fig. 1A, nodule formation; B, PAL activity; C, β1 → 3 glucan synthetase activity.

12 d of culture. After 12 d PAL activities on induction medium were the same in the presence and absence of 30 μM gibberellic acid, but at 45 μM gibberellic acid, PAL activity was consistently about 20% below this value, although still well above that measured in callus grown on maintenance medium. Addition of abscisic acid (30 or 45 μM) abolished almost all of the increase in PAL activity and by the end of the 21-d culture period the PAL activity of these calluses was the same as that of callus grown on maintenance medium. Addition of gibberellic acid in the presence of abscisic acid did not stimulate any increase in PAL activity.

Time course of glucan synthetase activity

The glucan synthetase activities of the various cultures are shown in Fig. 1 C. Transfer of callus to induction medium resulted in a marked increase in activity after 12 d, followed by a decline, but addition of abscisic acid (30 μM) abolished the stimulation. Activity on induction medium containing gibberellic acid (30 μM) was greater than that on maintenance medium or induction medium containing abscisic acid at times later than 12 d after transfer.

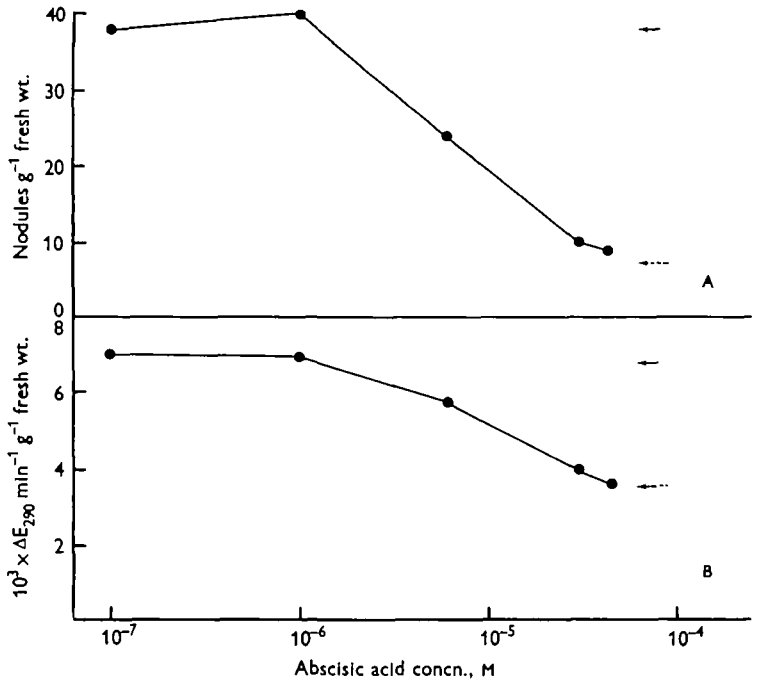

Fig. 2. The effect of ABA concentration on cell differentiation. The calluses were transferred from maintenance medium to induction medium plus various concentrations of ABA. Fig. 2A, nodule concentration and B, PAL activity, 21 days after transfer. The solid arrow indicates the value obtained for callus transferred to induction medium without ABA and the dotted arrow indicates the value obtained for callus transferred to maintenance medium without ABA.

Effect of different concentrations of abscisic acid on differentiation

Abscisic acid 0·1, 1, 6, 30 and 45 μM was added to induction medium and PAL activity and nodule concentration estimated 21 d after transfer to these media. The results are shown in Fig. 2 A, B. Root initiation at the same frequency as on induction medium was observed with 0·1 and 1 μM abscisic acid, but no root initiation was observed at higher concentrations. Nodule concentrations and PAL activity were the same as on induction medium with 0·1 and 1 μM abscisic acid, and the same as on maintenance medium with 30 and 45 μM abscisic acid, while intermediate values were observed at 6 μM.

Effect of gibberellic acid and abscisic acid added to media other than induction medium

Addition of gibberellic acid or abscisic acid (30 or 45 μM) to maintenance medium did not affect background nodule concentration or PAL activity 21 d after transfer. Replacement of the 6 μM NAA in induction medium by gibberellic acid (30 μM) inhibited callus growth, but replacement of the kinetin (1 μM) by gibberellin (30 or 45 μM) resulted in root initiation. Increased nodule concentration (35 nodules/g) and PAL activity ($7{\cdot}5 \times 10^{-3}$ E_{290} min^{-1} g^{-1}) were observed at the lower concentration. The effect of replacing the kinetin by lower concentrations of gibberellic acid (0·1, 1·0 and 6·0 μM) was also investigated. Root initiation was observed at all the gibberellic acid concentrations tested; 1 μM gibberellic acid gave the greatest number of roots per callus. No roots were formed when both gibberellic acid and kinetin were omitted from the medium.

Time of exposure required for abscisic acid-induced inhibition of differentiation

Callus transferred to induction medium after 3 d of growth on induction medium containing 30 μM abscisic acid had the same PAL activity and nodule concentration as callus which had been grown in the absence of abscisic acid. Growth on induction medium plus abscisic acid for 9 d prevented any differentiation or increased PAL activity when the callus was subsequently transferred to induction medium for 21 d. Intermediate values were observed when the transfer was made after 6 d. Transfer of callus from induction medium to induction medium containing abscisic acid inhibited differentiation and PAL activation unless callus was grown on induction medium for at least 9 d before transfer. If the transfer was made after 9 d, root formation was also not initiated. The inhibition of cell and tissue differentiation was complete in callus transferred to medium containing abscisic acid after 3 d on abscisic acid-free medium; and partial if the transfer was made after 6 d.

DISCUSSION

It is convenient to consider the process of differentiation in tissue cultures from two interrelated aspects; first the division and differentiation of cells to form recognizable cell types (cell differentiation) scattered throughout the callus and in nodules (Wetmore & Rier, 1963), and secondly the coordinated division and development of a group of cells to produce a definite organ (tissue differentiation). We have developed a method for following the course of the cell differentiation, since this is accompanied by an induction of increased activities of enzymes required for the synthesis of the characteristic material in either xylem or phloem cells. There is no corresponding sensitive method for estimating the course of tissue differentiation, but at a late stage the frequency of root formation is a useful indication of the amount of organized development which has been induced.

The response of callus to the application of plant growth substances will be modified by the presence of unknown quantities of endogenous hormones synthesized by the callus or carried over from previous media. We have shown that the application of

auxin and kinin in a ratio of 5:1 induced the formation both of vascular tissue (cell differentiation) and of roots (tissue differentiation) (Haddon & Northcote, 1975), while application of higher ratios abolished this differentiation.

The two aspects of differentiation were not normally separated by the application of auxins and kinins, except that the potential for root initiation was lost at an earlier stage of subculture in maintenance medium (fourth subculture) than was nodule formation (sixth subculture) (Haddon & Northcote, 1975). Application of gibberellic acid to callus, however, had two different effects, the promotion of root initiation, and a variable effect upon nodule formation. Organized development was stimulated by gibberellic acid at concentrations of 30 and 45 μM in the presence of a suitable ratio of auxin to kinin. It also stimulated root formation in the absence of kinetin at concentrations of 0·1–30 μM. Thus in the presence of gibberellic acid ratios of auxin to kinetin of about 50, which did not normally induce any differentiation, did stimulate formation of roots. The induction of nodules under these conditions was variable and could not be reliably estimated.

The effect of gibberellic acid upon the formation of scattered vascular elements, rather than the induction of organized roots, depended upon the concentrations used. On transfer to induction medium containing gibberellic acid there was an increased time lag before nodules were formed and this time lag was also apparent when the more sensitive indicator, PAL activity was measured. Gibberellic acid at a concentration of 45 μM inhibited the amount of cell differentiation as well as delaying its initiation.

The effect of gibberellic acid on *P. vulgaris* callus can be compared with that of gibberellin and auxin on xylogenesis in sycamore and *Phaseolus multiflorus* stems (Wareing, Hanney & Digby, 1964), where indolyl acetic acid stimulated the formation of scattered xylem elements, gibberellin stimulated organized cambial division, and both were required for normal xylem formation. In bean callus auxin and gibberellin were again required for maximum organized development, with scattered elements being differentiated in the absence of gibberellic acid, although some organized development could still occur, probably because of endogenous gibberellic acid formation. At high concentrations of gibberellic acid, when the ratio of the exogenous applied hormones (gibberellic acid:auxin) to the callus was approximately 10:1, some inhibition of cell differentiation occurred. This was not observed with the experiments of Wareing *et al.* (1964) on stems, but the ratios they applied were approximately 1:1.

Abscisic acid had an inhibitory effect on both cell and tissue differentiation, and abolished the formation of nodules and roots even in the presence of gibberellic acid. The induction of both enzymes used as markers for xylem and phloem formation was also inhibited.

Abscisic acid inhibition was observed only at concentrations greater than 1 μM (racemic mixture $\pm$ABA). The amount of PAL activity in the callus followed closely the extent of differentiation that occurred and it again emphasized the value of the use of PAL activity as a sensitive method for detecting cell differentiation. Abscisic acid inhibition of differentiation appeared complete and irreversible after 9 d of culture on

induction medium containing 30 μM abscisic acid. Part of the irreversibility may have been due to a carry over of the growth factor into the fresh medium, although the weight of callus was less than 10% of the weight of the medium so that the abscisic acid carried over must have been diluted to a non-inhibitory or only partially inhibitory concentration. Once differentiation had been initiated, between 6 and 9 d after transfer to induction medium, subsequent transfer to abscisic acid did not inhibit normal development and the concentration of nodules reached its normal maximum after 21 d. Roots were not normally visible after 9 d on induction medium, but appeared subsequent to this and, when transfer to abscisic acid was made after 9 d, they were still formed, so that abscisic acid inhibited the initiation rather than the subsequent development of cell and tissue differentiation.

Although our results seem to be directly opposed to those of Thorpe & Meier (1973) who used tobacco callus, the inhibitory effect of abscisic acid on bean callus was more consistent with its action as a growth inhibitor (Milborrow, 1974). The stimulatory effect of gibberellic acid that we found on tissue differentiation was consistent with the results of Engelke *et al.* (1973). There seems to be little correlation between the observations using whole tissue and those with callus, since abscisic acid inhibited rooting of bean stems only in the presence of gibberellin (Krelle & Libbert, 1969) and buds that were stimulated to develop by gibberellic acid were inhibited by abscisic acid. The influence of endogenous growth factors, their transport and destruction, in experiments using intact plants is unknown, and it is likely that these are important in any response to an applied hormone which could alter the relative amounts of the growth factors within the tissue. Although endogenous growth factors are present in callus tissue, the variations caused by their transport from one part of the tissue to another are considerably reduced.

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