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PHENYLALANINE AMMONIA-LYASE: REGULATION OF ITS INDUCTION, AND ITS ROLE IN PLANT DEVELOPMENT

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Abstract—The properties of phenylalanine ammonia-lyase from higher plants and its position in phenylpropanoid metabolism are briefly reviewed Emphasis is then placed on the biological circumstances in which this enzyme is produced The mechanisms by which active enzyme appears and the techniques used to investigate this process are considered The various enzyme-inducing stimuli are individually examined and product modulation of lyase formation is reviewed The contribution of phenylalanine ammonia-lyase to experimental work on plant development, particularly xylogenesis and lignification in tissue culture, is then discussed in the wider context of the influence of plant growth regulators and synthesis of macromolecules during these processes

INTRODUCTION

In the twenty-two years since phenylalanine ammonia-lyase (PAL, EC 4 3 1 5) was discovered in barley seedlings by Koukol and Conn [1], it has become the most studied enzyme concerned with secondary metabolism in plants [2] Literature on PAL, especially relating to its enzymology, has recently been reviewed by Hanson and Havir [3, 4] In this article more emphasis is placed on biological aspects regulation of the induction of PAL by various stimuli and its role in plant development

MAIN CHARACTERISTICS OF THE ENZYME

PAL catalyses the elimination of ammonia and the *pro*-3S hydrogen from L-phenylalanine to form *trans*-cinnamate (Fig 1), this being the first committed step for biosynthesis of the phenylpropanoid skeleton in higher plants [4] Preparations from monocotyledons and fungi often show tyrosine ammonia-lyase (TAL) activity [2] PAL is sensitive to inhibition by its product, *trans*-cinnamate [1, 2] The active site, of which there may be two per tetramer [3], probably contains a dehydroalanyl residue [5]

PAL may display negative cooperativity with respect to L-phenylalanine [6], giving rise to two apparent Michaelis constants The enzyme from parsley seems fairly typical of that from higher plants with $K_m^L = 0\,3 \times 10^{-4}$ M, $K_m^H = 2\,4 \times 10^{-4}$ M, oligomer molecular weight = 330 000, subunit molecular weight = 83 000 [7] These properties have been extensively tabulated by Camm and Towers [2] and Hanson and Havir [4], together with references to purification procedures Preparations of PAL may possess only one Michaelis constant [8] The molecular explanation of the phenomenon interpretable as negative cooperativity must wait until the pure enzyme is available in greater quantities At present, the occurrence of copurified isoenzymes with differing K_m values cannot be ruled out [Bolwell, G P, personal communication]

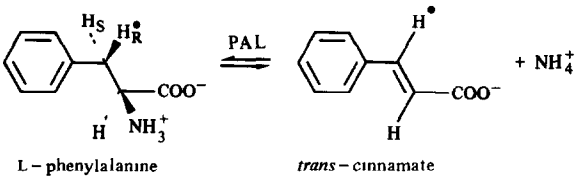

Fig 1 The deamination of L-phenylalanine catalysed by PAL

PAL is not an easy enzyme to purify The initial cell extract is taken through at least five stages beginning with an ammonium sulphate fractionation, followed by anion exchange chromatography, gel filtration, and sometimes hydroxylapatite chromatography [7, 9] The final step, preparative polyacrylamide gel electrophoresis (PAGE), usually results in yields of less than 10% with about a 400-fold increase in specific activity Analytical PAGE, although revealing essentially a homogeneous preparation under non-denaturing conditions, generally resolves several additional minor bands of protein in the presence of sodium dodecyl sulphate (SDS) This led Havir [9] to estimate that her preparation was 80–85% pure PAL The enzyme is subject to changes in properties during purification procedures [4]

Abbreviations 2,4-D, 2,4-dichlorophenoxyacetic acid, IAA, indoleacetic acid, L-AOPP, L-α-amino-oxy-β-phenylpropanoic acid, NAA, 1-naphthylacetic acid, PAGE, polyacrylamide gel electrophoresis, PAL, phenylalanine ammonia-lyase, Pfr, active form of phytochrome (far-red absorbing), SDS, sodium dodecyl sulphate, TAL, tyrosine ammonia-lyase

METABOLIC ROLE AND LOCATION OF PAL

The carbon skeletons deaminated by PAL may reach a wide variety of destinations as shown in Fig 2 [2–4] (For recent reviews on the various pathways, see contributions to the volume edited by Conn [10]) The ultimate range of products is dependent on the type and developmental stage of the tissue, and the environmental stimulus provided [11, 12] Compartmentation of the relevant enzymes at the cellular and subcellular level is an important factor in determining which branches of a pathway predominate In mustard cotyledons, light-induced PAL is regulated differently in the upper and lower epidermis, forming either anthocyanins or flavonols [12] In pea leaves, synthesis of anthocyanins and flavonoid glycosides is localised in the epidermis, whereas lignin synthesis takes place in the remaining tissues [13] Different isoenzymes of 4-coumarate CoA-ligase have been implicated in the formation of either lignin in stems or flavonoids in buds [14] The variation in substrate specificity between these isoenzymes seems to play a pivotal role in determining which particular pathway follows the general phenylpropanoid conversions [11, 15]

The subcellular location of PAL is mainly cytoplasmic [16], although it may also be associated with some membranous organelles [reviewed 17] Where the enzyme occurs in various forms, differences in properties and metabolic end products have been correlated with differences in subcellular location [4] Some PAL may be loosely associated [4] with the membrane-bound cinnamate hydroxylase or benzoate synthase systems shown in Fig 2, because cinnamate formed from phenylalanine *in situ* is metabolically channelled towards subsequent conversions in preference to exogenous cinnamate This was shown by Czichi and Kindl [18, 19] who fed [^{3}H]-phenylalanine and [^{14}C]-cinnamate to microsomal membranes and found that the $^3H/^{14}C$ ratio was increased in the *p*-coumaric acid produced Stafford [20] discusses the concept of loosely associated multi-enzyme complexes in phenylpropanoid metabolism

REGULATION OF THE LEVELS OF PAL ACTIVITY

PAL seems to be extraordinarily sensitive to the physiological state of the plant [2] Changes in activity can occur during growth, or they may follow traumatic or pathological events, dilution of suspension cultures or the action of light Far more work has been done on the physiological factors influencing PAL levels than on the enzymology and molecular properties of the protein Many combinations of stimuli and biological systems have been investigated These have been tabulated by Hanson and Havir [4] The main findings are outlined below for light and pathological effects Induction of PAL by dilution of suspension cultured cells and by plant growth regulators is then considered in greater detail, since these stimuli have not received as much attention from reviewers

A few general points need to be made regarding the evidence for PAL level as a regulator of phenylpropanoid metabolism, and the mechanisms by which the level may change An increase in PAL activity has not always been correlated with the production of a specific phenylpropanoid compound [2] Where a correlation has been shown, in only a few instances has the accumulation of a specific product been exactly related to the integrated values of PAL activity with time (implying that end-product inhibition is not significant) Such investigations by research groups at Freiburg [11, 21] have shown that PAL is the rate-limiting enzyme in flavonoid glycoside biosynthesis in response to irradiation but not dilution of cell cultures

Kinetic regulation of PAL probably occurs *in vivo* to provide the fine tuning of pool sizes in the short term Negative cooperativity with respect to L-phenylalanine would tend to minimize the effect of fluctuations in the phenylalanine pool on phenylpropanoid metabolism It renders the binding of substrate by the enzyme more sensitive to inhibition by relative increases in the concentration of the product, cinnamic acid [22] The physiological significance of product inhibition of PAL catalysis

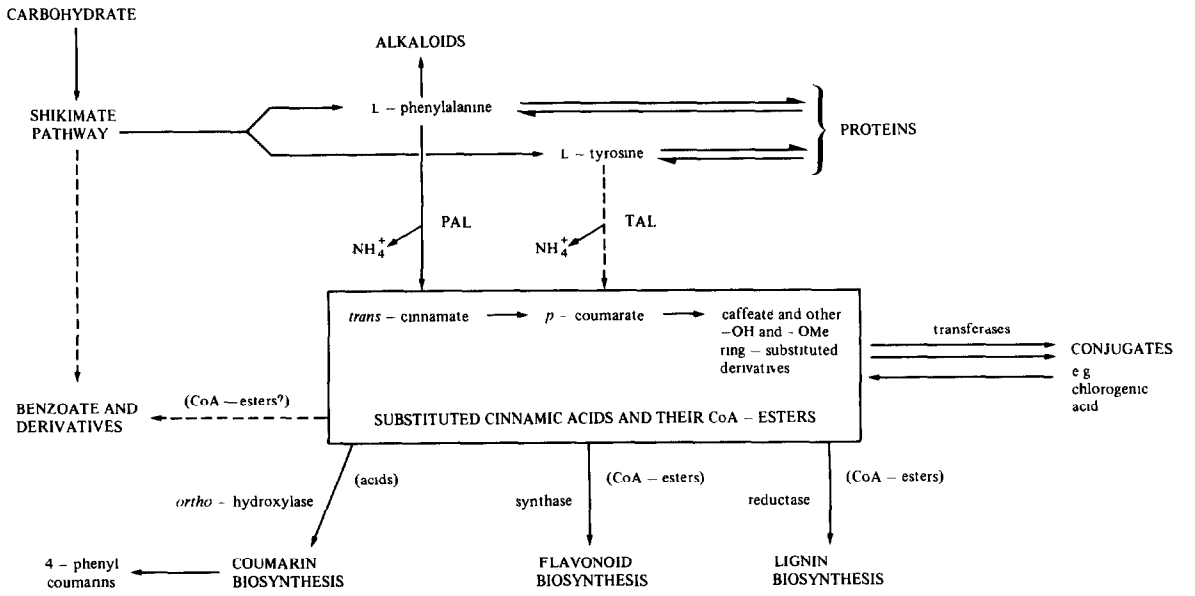

Fig 2 General layout of phenylpropanoid metabolism (after [2–4])

depends on local concentrations and compartmentation Kinetic inhibition by cinnamate may be negligible *in vivo* since metabolic channelling within membranes (see above) might minimise the free concentration of this toxic compound [23] The end-products of lignin and flavonoid biosynthesis are likely to be completely separated from most enzymes *in vivo* [11]

To explain the magnitude and timing of PAL induction, there must be changes in the number of catalytically competent enzyme molecules, rather than large variations in the catalytic efficiency of a fixed amount of already active PAL Production of active enzyme requires transcription and translation of the PAL gene, with the attendant post-transcriptional and post-translational processing that occurs in eucaryotes The latter must occur in the case of PAL if dehydroalanine forms part of the active site [5] The mechanism of formation of the dehydroalanyl prosthetic group has been speculated upon [4] If the carbohydrate reported to be associated with PAL by Havir [24] is covalently attached, then this post-translational modification may be physiologically important It is not known whether limited proteolysis of a PAL precursor also takes place In addition to production of PAL activity, its removal by inactivation or degradation must be postulated to explain many observations This degradation mechanism might be specific to PAL or common to many other proteins

In the systems outlined below the controlling step of PAL induction has usually been claimed to be either transcription or post-translational activation The group of hypotheses based on the former postulate that the stimulus initiates transcription of the PAL gene, with post-transcriptional processing and translation of the PAL-mRNA following Transcription of the PAL gene resulting in *de novo* synthesis of PAL-mRNA has not always been conclusively demonstrated, and a post-transcriptional event might be rate limiting The possibility exists that stored PAL-mRNA is released from a ribonucleoprotein particle on receipt of the stimulus, and initiation by ribosomes then takes place in the usual way The PAL inactivation mechanism is either said to be constantly active (resulting in first-order decay of PAL activity and hence a net fall in PAL activity when synthesis stops), or the rate of inactivation itself is said to vary The latter set of explanations depend on post-translational activation of accumulated PAL precursor on receipt of the stimulus An alternative to activation by prosthetic group formation in variants of this idea is activation by dissociation of a (labile) proteinaceous inhibitor—and inactivation by re-association with (newly synthesized) inhibitor Feedback modulation by cinnamate may impinge on the process of PAL production and removal

Investigations are directed towards establishing which event is the rate-limiting step in the appearance of enzyme activity in a system, and hence the point at which a perceived stimulus interacts with and could control the process It must be emphasized that the demonstration of an event as the rate-limiting step in production of active PAL in one system remains only that Such a result cannot be extrapolated to exclude the occurrence of other stages in the process (e g post-translational activation may still be an essential step even though synthesis is rate-limiting), nor can it be universally applied to other combinations of stimuli and biological systems (The problems of interpretation of experimental results to pinpoint a locus of control of a pool of assayable enzyme, including PAL, have been discussed in detail by Schopfer [25] In reviewing limitations of methodology, he cautions that the ability of a particular experimental technique to produce unequivocal results depends strongly on the nature of the control mechanism to be elucidated)

Density labelling has been very useful in studies on the kinetics of PAL induction The method is independent of the specific activity of label in the amino acid pools from which enzyme is synthesized Lamb and Rubery [26] point out that "the interpretation of comparative density labelling experiments in terms of the identification of the biochemical site of action of a stimulus leading to increased extractable enzyme activity is not possible unless a definitive mechanism for the turnover of the enzyme has first been established" This is because "only the rate of density labelling of catalytically active enzyme molecules is directly determined"—the labelling of other putative inactive forms (precursor or breakdown product) can only be inferred

Immunochemical techniques should enable such inactive forms to be detected, if there are sufficient antigenic determinants common to active and inactive PAL for binding to a polyclonal antiserum (raised against the purified enzyme) Two possible approaches may be adopted, quantitating either total amounts present or rates of synthesis of PAL antigenic material throughout the time-course of induction of enzyme activity (Experiments on immunotitration of enzyme activity belong to the first type) If, in the rising phase of PAL enzyme activity, the time-course for the total amount of PAL protein could be shown to correspond exactly with the profile of enzyme activity, one might conclude that post-translational activation (if it occurred!) was very rapid compared with the time for *de novo* synthesis of the pro-enzyme In the absence of such a clear cut result, interpretation of the data would be complicated by the nature of the inactive form both pro-enzyme (not yet activated) and breakdown product (enzyme which had been active and was then inactivated prior to degradation) would not be distinguished by the antibodies

The second immunochemical approach measures the rate of synthesis of PAL antigenic material by applying short pulses of radioactive amino acids *in vivo* followed by extraction, immunoprecipitation and denaturing electrophoresis (SDS) to enable the radioactivity incorporated into PAL protein (subunits) to be measured This direct kinetic means of establishing the rate-limiting step in PAL formation has generally been adopted in preference to quantitation of total pool sizes It offers the sensitivity of radioactive detection methods and, although also depending on antiserum specificity, may be less prone to doubts about the nature of any inactive form because only protein synthesized during the relatively short radioactive pulse is detected To relate the rate of synthesis of PAL protein measured above to the appearance of active enzyme requires estimates of the rate of increase of the latter to be made from the time course of PAL activity Only when there is exact correspondence between these two parameters throughout the time course, can the clear cut inference be made that synthesis and not activation is the rate limiting step in the appearance of enzyme activity As with investigations on the mechanisms of enzymic catalysis, a more complex kinetic scheme can always be invoked to explain a set of data The rate-limiting step may change from one stage of the response to another—the early stages require the most sensitive assays

Kinetic modelling [27] is essential for analysis of the results of radiolabelling/immunoprecipitation experiments, and has also been performed with data from density labelling work Changes in enzyme activity (E) throughout the time course of PAL induction may be described by $(dE/dt) = k_s - k_d\ E$, where (dE/dt) is the rate of change of enzyme activity with respect to time (t), k_s is the zero order rate constant of formation of active enzyme, and k_d is the first order rate constant of disappearance of enzyme activity In general, E, k_s and k_d will be functions of t, but in most [11, 25]—though not all [28]—cases k_d is held not to be so (Labelling in short pulses enables small increments of t to be considered so that k_s is effectively invariant for the duration of each pulse) In the terminology of this analysis, the question of the rate-limiting step in production of active enzyme becomes the question of the meaning of k_s As an hypothesis, k_s can be related *a priori* to 'rate of synthesis' or 'rate of activation', and both these contradictory conclusions have been drawn for light-induced PAL in mustard cotyledons [25] The purpose of such modelling is to make quantitative predictions (e g of $k_s(t)$ calculated from $E(t)$) as an aid to testing hypotheses (in this example by comparison of such predicted values with directly measured rates of synthesis) Where data are available, they support the interpretation of k_s as the rate constant for *de novo* synthesis in most cases, with synthesis thus being a function of the stimulus parameter (e g Pfr [25]) and time

Light

Of all the stimuli examined, the greatest research effort has been expended on the mechanism by which light of various qualities (white, red/far red or blue/UV) may induce PAL [2] This is to be expected given the profound influence of light on plant development generally, further discussion of which is beyond the scope of this review White light was first shown to induce PAL in potato tuber slices by Zucker [29, reviewed 30] The major product is chlorogenic acid [29, 31] It has been shown by D_2O density labelling [28] that the peak in PAL activity results from modulation of both the rate constants for enzyme synthesis and for removal of active enzyme

The earlier work on phytochrome involvement in PAL responses (induction of PAL by red light and suppression of this response by far-red light) is discussed by Camm and Towers [2] In etiolated mustard seedlings, there is much dispute over the rate-limiting step in active PAL formation at which phytochrome exerts control Schopfer and co-workers have evidence for *de novo* synthesis of PAL with a constant first-order decay rate [32, 33, reviewed 25] Activation of accumulated PAL precursor is claimed to be the critical step by Smith [34, 35, reviewed 36] The problems of quantitating PAL molecules lie at the centre of this dispute—other techniques in addition to D_2O density labelling may be necessary Schopfer [25] has summarized work on mustard and parsley "one has to state that there is clear evidence for a control of PAL *de novo* synthesis by Pfr It must be re-emphasized, however, that this statement is valid only within the limits set by the experimental data, which in no way exclude that a change of *de novo* synthesis is the consequence of, or accompanied by, post-translational Pfr effects"

Parsley cell suspension cultures show an UV-dependent PAL response resulting in flavonoid formation [37] (They may also show the typical phytochrome-mediated induction described above, provided they have been 'activated' with UV light [11] Density labelling of parsley cells provided clear cut results indicating control of *de novo* synthesis of PAL by Pfr [38]) It has been clearly shown by radiolabelling and immunoprecipitation that regulation of the availability of PAL-mRNA is enough to account for PAL induction, and that the enzyme is subject to a constant degradation rate in this system [39–42, reviewed· 11] This implies that transcription (or a post-transcriptional processing step) is influenced by the UV receptor A preparation enriched in PAL-mRNA has been prepared from these cells and some of its properties examined [43]

Blue light induces PAL activity in gherkin seedlings [44] This system provided evidence based on density labelling studies [45], for activation of a stored form of PAL by dissociation of an inhibitor protein [4, 46] Gupta and Acton [8] caution that phenolic compound/lipoprotein complexes could account for some observations of such inhibitory effects

Pathological effects excision, wounding and infection

Excision and the resultant wounding induce increases in PAL activity in many plant tissues [2] In potato tuber slices hydroxycinnamic acid esters (such as chlorogenic acid) are produced [31], and the mechanism of the response seems to parallel that for light on this tissue [4] By returning excised discs to the tuber, Smith and Rubery [47] showed that it was not tissue damage alone that induced PAL but the removal of tissue to a different environment Sweet potato tuber slices have also provided evidence for modulation of both synthesis and degradation of PAL following excision [48, 49] Ethylene has been implicated in these PAL responses amid some conflicting results [2, 50] There is evidence for antagonism between ethylene and CO_2 in the control of PAL appearance [50, and references therein]

PAL is also induced in response to infection by plant pathogens Viruses, such as tobacco mosaic virus [51], may cause *de novo* synthesis of PAL Most research has concentrated on PAL induction following infection by fungi, or exposure to polysaccharides derived from them [11] The responses of the host involve synthesis of lignin-like materials and anti-microbial phytoalexins, which are isoflavonoid derivatives in the Leguminosae [52] Both the rates of *de novo* synthesis and removal of PAL (estimated by density labelling) are varied in *Phaseolus vulgaris* cell cultures forming phaseollin in response to natural or unnatural elicitors [52] Immunochemical techniques also provide evidence for *de novo* synthesis of PAL (with possible variation of the rate of degradation) in immature pea pods inoculated with macroconidia of *Fusarium solani* [53] The rapidly occurring increases in PAL activity are usually large (but transient), and it now seems that *de novo* synthesis of PAL is the general rule in response to fungi or their elicitors [54] Induction of PAL activity "is more efficient at high elicitor concentrations", which may reflect the operation of some additional post-translational control [55] Borner and Grisebach [56] showed that PAL formation was a specific response to the race of *Phytophthora megasperma* f sp *glycinea* used to infect soybean hypocotyls, rather than a simple wound response The integrated curves of PAL activity corresponded with those for glyceollin accumulation during the first 12 hr of infection Phaseollin production and

increased PAL activity in bean tissue culture may occur in the absence of fungal or chemical inducers, and be influenced by the level of artificial auxins supplied [57]

For further details of PAL induction associated with responses to plant disease, particularly phytoalexin accumulation in *Phaseolus vulgaris*, the reader is referred to two recent reviews [52, 54]

Dilution of suspension cultured cells

When parsley cell suspension cultures in the late linear phase of growth are transferred to fresh medium [58] or to water [59], large increases in PAL activity take place There is a lag period of 2–3 hr before a peak in activity at 12–15 hr after dilution of the culture, followed by a rapid decline In addition to PAL, the other two enzymes of general phenylpropanoid metabolism, cinnamate 4-hydroxylase and 4-coumarate CoA-ligase, are also induced However, the enzymes specific to flavonoid biosynthesis are not induced unless a period of illumination is also provided Neither flavonoids nor lignin are produced on dilution in darkness, but there is evidence for production of substituted cinnamate esters [11] The activities of enzymes of primary metabolism do not increase, nor does a general stimulation of protein synthesis form part of the mechanism As far as the appearance of PAL activity is concerned, *Phaseolus vulgaris* cells behave very similarly to parsley cells when subcultured into fresh medium [60] or into water [Jones, D H, unpublished results]

It has been shown that the changes in enzyme activity in diluted parsley cells may be accounted for by corresponding changes in the amount of enzyme-specific polysomal mRNA [61] This is the same mechanism as occurs for light induced PAL in this system (considered above), and again the rate of PAL degradation remains constant throughout the response The implication of these findings is that PAL is under transcriptional control by light and/or dilution in parsley cells However, the isolation of polysomal mRNA, *in vitro* translation and immunoprecipitation of the radioactive product quantifies the recruitment of the PAL-mRNA into polysomes and is not a direct measure of transcription *per se* The appearance of the PAL-mRNA might be due to its release from ribonucleoprotein particles or the operation of some other post-transcriptional processing step The only direct evidence for involvement of transcription is the actinomycin D sensitivity of both the dilution [59] and light responses [39, 62, 63] This criticism might have been partly offset if total RNA (i e extracted by phenol to deproteinise RNA from both ribonucleoproteins and polysomes) had also been assayed by cell-free translation, and an increase in PAL-mRNA shown However, to demonstrate *de novo* PAL-mRNA synthesis rigorously, incorporation of labelled uridine into the RNA species that hybridizes to a specific cDNA probe for the PAL-mRNA must be demonstrated This remains one of the longer term objectives of such work Immunologically identical molecular species of PAL protein (as indicated by double-diffusion analysis) are induced by dilution and/or irradiation Betz and Hahlbrock [64] have shown that tryptic peptides obtained from PAL induced by either stimulus are identical, indicating that the same mRNA is involved in their synthesis

The exact nature of the stimulus which the cells receive on subculture is not known Substrate induction by exogenous phenylalanine does not occur [59], this would be expected from the similarity of the response in fresh medium and distilled water The size of the PAL peak depends on the degree of dilution of the cells subculturing to lower initial cell densities results in higher induced PAL activity In order to determine whether the induction was due to a decrease in cell density *per se*, Hahlbrock and Schroder [59] reduced the density of an actively growing culture by removal of cells without transfer to fresh medium No induction resulted, implying that the stimulus depends on a decrease in concentration of a compound of cellular origin on subculturing The nature of this compound was not investigated further in parsley, though the possibility was noted that a reduction in intracellular hydroxycinnamic acid concentration might be responsible for derepression of PAL synthesis [65] One approach to this problem is to test the effect of subculturing cells into media in which cultures have previously been growing In order to eliminate the possibility of a kinetic inhibition of PAL catalysis, appropriate controls (and preferably direct quantitation of PAL molecules) should be undertaken in addition to measurement of enzyme activity In French bean [66] and potato cells [67] spent medium could inhibit the increase in PAL activity, though to a variable and not always complete extent Gilliatt [67] also showed that distilled water washings from cells contained inhibitory activity Hydroxycinnamic acids may be responsible for the inhibition, but it could also be explained by the presence of a macromolecular factor It was noted [67] that a gaseous factor might be involved The concentration of dissolved CO_2 could be the critical parameter carbon dioxide is essential for initiation of growth in cultured sycamore cells [68] and its absence on subculturing might be involved in PAL induction It has been speculated that increased availability of oxygen on dilution of late linear phase cultures may give a general boost to oxidative phosphorylation and hence protein synthesis [Bellini, E, personal communication] Whatever the effector, which particular proteins are synthesized might depend on the previous history of the cells

Besides the first three enzymes of phenylpropanoid metabolism [11], shikimate dehydrogenase, *O*-methyltransferases and peroxidase are also induced on subculture of tobacco cells [69] Enzymes of nitrate and nitrite metabolism also increase [listed in 59] Subculture caused a rapid stimulation of polysome formation and increased the translatable levels of a small group of (unidentified) mRNAs in French bean and soybean cells [70] Thus PAL is not the only enzyme to respond in this way The shock of subculture to cells in suspension may be comparable in its effects with the trauma following excision of intact plant tissues

Plant growth regulators

In comparison with the stimuli reviewed above, little is known of the mechanisms by which plant growth regulators may influence the level of PAL activity *in vivo* The wider issue of the involvement of plant growth regulators and PAL in xylogenesis will be dealt with later This section, which is included here for completeness, concentrates on changes in PAL activity following alterations in exogenous supplies of growth regulators and examines the specificity of such effects

A decrease in exogenous auxin* and an increase in exogenous cytokinin supplied to tissue cultures results in an increase in PAL activity within a few days Haddon and Northcote [71] observed that dark-grown bean (*Phaseolus vulgaris*) callus maintained on a solid medium containing 2 mg l^{-1} [9.05×10^{-6} M] 2,4-dichlorophenoxyacetic acid (2,4-D), developed increased PAL activity from 6 days after transfer to medium containing 1 mg l^{-1} [5.40×10^{-6} M] 1-naphthylacetic acid (NAA) and 0.2 mg l^{-1} [0.93×10^{-6} M] kinetin Dark-grown bean cells in liquid suspension culture show a similar response from 4 days onwards Bevan and Northcote [72] have shown that NAA is required at an optimal concentration of 1 mg l^{-1} [5.40×10^{-6} M] at least 2 days before the resultant increase in PAL activity Kinetin need not be present until within 24 hr before the increase in activity when a graded response to concentration may occur in the range 0.1 mg l^{-1} [0.46×10^{-6} M] to 0.5 mg l^{-1} [2.32×10^{-6} M] The inhibitory effect of 2 mg l^{-1} [1.07×10^{-5} M] NAA on PAL induction was overcome by an increased concentration of kinetin These timing effects indicate that auxin and cytokinin may influence different stages of the process of PAL production

The PAL response of suspension cultured bean cells to exogenous growth regulators has been investigated by Jones and Northcote [73] Inhibitors of transcription (actinomycin D) and translation (D-2-[4-methyl-2,6-dinitroanilino]-*N*-methylpropanamide) were applied in optimised doses to dark-grown cells exposed to increased cytokinin and decreased auxin concentrations The rising phase of enzyme activity was prevented indicating that both transcription and translation are obligatory During the falling phase of PAL activity, superinduction by actinomycin D, but not by the propanamide derivative, suggests that the availability of PAL-mRNA for translation is a regulatory factor in this response (Explanations of actinomycin D superinduction and its relationship to *de novo* synthesis of enzyme are discussed in [73]) Although inhibitors are relatively crude experimental tools [25], the above findings are a first indication that the mechanism of PAL induction by growth regulators may parallel that for irradiation and dilution of suspension cultured cells (see above)

Dixon and Fuller [57] have also observed an increase in specific activity of PAL on lowering the auxin concentration of light-grown suspension cultured bean cells 2,4-D at concentrations of 2×10^{-5} M and above was inhibitory to cell growth, phaseollin production and PAL activity In dark-grown suspension cultures of Paul's Scarlet Rose, concentrations of 2,4-D greater than 5×10^{-6} M are inhibitory to the normal development of PAL activity in linear growth phase and polyphenol accumulation is repressed [74] Actinomycin D at 1 mg l^{-1} strongly inhibits the rising phase of PAL activity indicating that transcription is required for this response Some inhibition was also found with cycloheximide Kuboi and Yamada [69] decreased the exogenous auxin and increased exogenous cytokinin in tobacco suspension cultures grown in darkness by transferring 10 day old cells from medium containing 1×10^{-6} M 2,4-D to medium containing 1×10^{-6} M 6-benzyladenine These cells behave similarly to bean a PAL peak at 12 days occurs only in the presence of exogenous cytokinin

*Throughout this review the term 'auxin' may be taken to include artificial and naturally occurring substances

In parsley cell suspension cultures [63], the inducibility of PAL by light is maximal during the late linear phase of growth when exhaustion of nitrate from the medium occurs Havir [9] has grown soybean cell suspension cultures on a high nitrogen medium which results in a PAL peak 8–10 days after inoculation when the cells are in stationary phase Nitrate was not limiting when this peak of enzyme activity was reached However, the auxin/cytokinin induced increase in PAL activity in bean cells cannot be an artefactual nitrate depletion or late growth phase effect, since it is specific to one set of hormone conditions and occurs during the exponential phase of growth (see Figs 4 and 5 of [73]) Earlier evidence for the specificity of this stimulus was obtained by Bevan [75] who showed that bean cells growing in medium containing either 2 mg l^{-1} [9.05×10^{-6} M] 2,4-D, 2 mg l^{-1} [1.07×10^{-5} M] NAA, or 2 mg l^{-1} [9.05×10^{-6} M] 2,4-D plus 20% (v/v) coconut milk did not induce PAL over a period of 10 days The same cell line produced a PAL peak at 4–6 days after subculture when grown in a medium containing 1 mg l^{-1} [5.40×10^{-6} M] NAA and 0.2 mg l^{-1} [0.93×10^{-6} M] kinetin Since all these media had the same initial nitrate content, the higher concentration of auxin in the former cultures must have been responsible for the non-appearance of a PAL peak This hormone-dependent (or high auxin-repressible) PAL peak is quite distinct from the dilution peak (see previous section) in timing and shape

Gibberellic and abscisic acids can also influence the appearance of PAL activity Haddon and Northcote [76] showed that the PAL peak which occurred in bean callus after lowering the ratio of auxin to cytokinin could be inhibited by abscisic acid and delayed by addition of gibberellic acid In pea and other plants gibberellic acid has been reported to promote PAL activity and lignification [77, 78] Fry [79] found that gibberellic acid (GA_3) repressed PAL activity in *Spinacia* suspensions Heinzmann and Seitz [80] reported that gibberellic acid repressed anthocyanin synthesis and *de novo* synthesis of PAL in *Daucus* suspension cultures Some other reports are listed in ref [2]

Product modulation of PAL production

There exists much experimental evidence that cinnamate and hydroxycinnamate derivatives may alter the level of many PAL responses [4] Lamb [81] working on potato tuber discs used inhibitors of nucleic acid and protein biosynthesis to investigate the modulation of PAL activity by light, and by exogenous cinnamate and *p*-coumarate Photomodulation of enzyme levels occurred by a relatively slow, actinomycin D sensitive mechanism implying that light stimulates transcription of PAL-mRNA In contrast, modulation of enzyme activity by pathway intermediates occurred by a rapid, post-transcriptional mechanism Perhaps cinnamate could affect processing or translation of PAL-mRNA? Smith and Rubery [47] working with the same tissue interpreted an oscillation in PAL activity on transfer of host tuber incubated discs to a dark environment as being due to metabolite feedback regulation of PAL formation The transition to an oscillatory time course of PAL activity that follows transfer of such discs from *in situ* pre-incubation to air appears to be accompanied by the development of enzyme activity becoming desensitized to repression by exogenous cinnamate [50]

Excision of pea epicotyl tissue results in a transient

increase in PAL activity Exogenous supplies of pathway intermediates inhibit the initial development of PAL activity and, if added at the time of high enzyme levels, cause a rapid decrease in enzyme activity [82] By using density labelling it was shown that cinnamate both inhibited *de novo* synthesis and stimulated the removal of pre-existing enzyme This dual effect did not seem to be due to general cytotoxicity of cinnamate since labelling of acid phosphatase was unaffected As in potato tuber discs, the effects of cinnamate may be post-transcriptional

Modulation by cinnamate of both the rate constants for synthesis and removal of PAL can explain the effects of D-phenylalanine [4] or L-α-amino-oxy-β-phenylpropanoic acid (L-AOPP) on PAL levels *in vivo* L-AOPP is a powerful competitive inhibitor of PAL which enhances the level of enzyme activity over that normally occurring in the later stages of an increase in PAL, e g by light in gherkin hypocotyl [83] This might occur by L-AOPP inhibiting endogenous generation of cinnamate, thus breaking the feedback loop normally restricting synthesis and enhancing degradation of PAL Such an explanation may be extended to include the effects of L-AOPP or D-phenylalanine on the pathway of chlorogenic acid biosynthesis [84] (The term 'superinduction' has been applied to enhancement of already increasing PAL levels by L-AOPP *in vivo* [83] This phenomenon seems to be distinct from actinomycin D superinduction [73], which consists of a reversal of decline in PAL activity The mechanism proposed to explain the effects of L-AOPP *in vivo* depends on cinnamate modulation of a post-transcriptional stage of PAL formation However, the mRNA-competition hypothesis which could account for actinomycin D superinduction [73] depends essentially on the timing of inhibition of transcription by this antibiotic)

In addition to density labelling studies [82], immunoprecipitation has also been used to demonstrate *de novo* synthesis of PAL following addition of L-AOPP to an anthocyanin-producing culture of carrot cells [85] It was shown that phenylalanine accumulation occurred when extractable PAL activity was dramatically enhanced by L-AOPP treatment The next two enzymes of (general) phenylpropanoid metabolism were not affected by L-AOPP suggesting independent regulation of PAL during this inhibitor effect

However, some tissues do not respond to exogenous L-AOPP or cinnamate [4, 86] In this context, accurate measurement of PAL activity depends on removal of L-AOPP from cell extracts, so that kinetic inhibition does not produce artefacts L-AOPP may be tightly bound to PAL from some plants—soybean [86] and French bean [Jones, D H and Northcote, D H , in preparation]—such that dialysis against buffer prior to PAL assay may not be sufficient to enable the true PAL activity to be measured This problem might not be immediately obvious, especially if stopped enzyme assays are used The conditions necessary for dissociation of the enzyme-ligand complex *in vitro* have been investigated [Jones, D H and Northcote, D H , in preparation]

PAL IN THE CONTEXT OF DEVELOPMENT

Plant growth regulators and differentiation

In this review the terms 'plant growth regulators' and 'plant hormones' are used interchangeably, largely in keeping with most current literature Fosket [87] gives a good account of the 'conventional' view in which the concentration-dependency of plant hormone effects in cultured tissues is seen as evidence of their regulatory role in morphogenesis Trewavas [88, 89] challenges the classical hormone concept in plants, focussing attention on receptor level as the important variable determining the concentration of growth substance–receptor complexes required for a response Thus, the sensitivity of the tissue to growth substances would reflect receptor level and be subject to developmental control

The variable results obtained with different tissues when exogenously applied hormones are used to induce xylogenesis [90] may indicate variations in concentration of growth substances already present, or in their compartmentation or transport, or the competence of different cells to respond may vary Accurate assay of endogenous growth substance levels by physicochemical methods should therefore form an essential part of future investigations Synchronized suspension cultures would greatly simplify investigations on the cytodifferentiation relationships between plant growth regulator action and the cell cycle, though in most cases synchrony remains an elusive goal [91] Moss protonema (though not of higher plant origin [1]) is a promising system for discerning growth regulator actions on morphogenesis, especially by genetic approaches [reviewed 92]

Critical variables for the initiation of cytodifferentiation are some combination of auxin and cytokinin [reviewed 87, 90] It has been well established since the work of Skoog and Miller [93] that root initiation in callus cultures is promoted by high exogenous auxin levels, and bud formation by cytokinin Formation of xylem and phloem in callus tissue was demonstrated using an agar wedge as source of auxin and sucrose [94, 95] Involvement of sugars was investigated by Jeffs and Northcote [96] who reported that organised xylem in vascular nodules resulted only from the presence of a disaccharide, such as sucrose, with an α-glycosyl group at the non-reducing end A comparative study of IAA and sucrose effects in six different species was undertaken by Aloni [97] The differentiation of nodules of xylem and phloem may follow changes in the ratio of auxin to cytokinin [98] The order of xylem and phloem development in callus may be the reverse of that found during vascularisation in the intact plant [71] Xylogenesis has been very extensively reviewed by Roberts [90]

It may be that requirement for a certain concentration of auxin rather than cytokinin is more fundamental for xylogenesis [87] and an increase in PAL activity in bean cells In support of this contention, root initiation and not shoot formation is generally observed if a bean culture forms organised tissue (e g [71]) Secondly, Bevan and Northcote [72] showed that lowered exogenous auxin was an earlier requirement than increased exogenous cytokinin (which is not absolutely required) for a rise in PAL activity in bean cell suspension culture (see earlier for details) The variability of different cell lines, and loss of morphogenetic potential and capability for PAL induction in these cells after prolonged subculture [60], could be due to changes in their receptor complement and/or biosynthetic capacity for endogenous growth factor production There was no evidence that selective proliferation of clones of non-inducible cells was responsible for the loss of these capabilities

Synthesis of macromolecules in specializing cells

The regulation of the expression of specific genes in eucaryotes has been studied, for reasons of technical expediency, using systems which make abundant proteins The most famous examples in animal systems include ovalbumin production in chick oviduct, globin synthesis in erythropoietic tissue, immunoglobulin production, vitellogenin synthesis in *Xenopus* liver, silk fibroin and lens crystallin In plants, the large subunit of ribulose bisphosphate carboxylase comprises up to 50% of the soluble protein of leaf cells The expression of this gene has been most intensively studied (as befits the most abundant protein in the world! [99]) The genes for leghaemoglobin, α-amylase and seed storage proteins have been shown to be both qualitatively and quantitatively regulated (e g references listed in [100]) In future we may be able to add the major phloem proteins to this list [101] The elegance of much of this work and the pace at which new information becomes available may have tended to obscure the perception that these proteins (and by implication their genetic regulation) are exceptional DNA/RNA hybridization experiments indicate that 30 000 structural genes are required to programme early sea urchin development [102], and 60 000 diverse structural genes (11% of single-copy DNA, 4 6% of genome) are expressed during the life-cycle in an entire tobacco plant [100] Most of these must code for minor proteins generally present in very small quantities compared with those listed above

The simpler and more plastic nature of plant development does not appear to be due to any deviation from the basic molecular biology of gene control in multicellular eucaryotes, as has been shown by recent work [references given in 100] Thus these considerations apply equally to plants and animals However, animal cells are largely constructed of protein (with mineralisation in bones and teeth), whereas plant cells remain relatively unspecialised internally, the extracellular wall being the site of ultrastructural differentiation Since the wall is composed mainly of polysaccharides (with lignification of some mature elements) [103], this must serve to emphasize that most differentiation in plants depends on the activity of genes coding for minor proteins The macromolecular manifestations of cell wall development are not directly encoded in this DNA, but are the 'downstream' product of the metabolic pathways involved These processes have been extensively described [104, 105], and possible kinetic controls affecting the enzymes have been examined *in vitro*

Hybridization experiments give an overall view without identification of the rare-class messenger RNAs produced Any detailed understanding depends on techniques to quantitate their translation products However, Kamalay and Goldberg [100] have claimed that, in common with sea urchin and mouse, "post-transcriptional processing and/or selection mechanisms may operate to regulate the sequence composition of each specific rare-class polysomal mRNA sequence set in tobacco" This conclusion arose from hybridization experiments with nuclear RNA, which revealed that structural genes not utilized for the production of functional (i e polysomal) mRNAs in a given organ were nevertheless transcribed, and were represented by transcripts in the hnRNA It remains to be seen whether the processing of these constitutively transcribed RNAs is altered by environmental effectors such as growth regulators

"For all (plant) hormone responses RNA synthesis is required in the medium to long term, and in time all hormones cause changes in RNA metabolism, often in all (RNA) species although only changes in the minor species seem to be indispensable in hormone action" [106] Most studies have been performed on the general regulation of (non-specific) mRNA and protein synthesis, such as indications that auxin can influence RNA polymerase II dependent transcription [106] Dudley and Northcote [107] detected differences in translatable mRNAs from bean cells which had been exposed to reduced exogenous auxin and increased cytokinin mRNA from cells exposed to these conditions coded for a relatively large amount of a few polypeptides, whereas that from cells grown on maintenance medium coded for a large number of polypeptides all present in fairly equal amounts Subculture of cell suspensions caused a rapid increase in the translatable levels of a small group of mRNAs, and within 2 hr auxin modulated the levels of certain messages following this primary subculture stimulus [108, see also 109]

The main examples of studies on specific gene products are described below Verma *et al* [110] showed by cell free translation and immunoprecipitation that cellulase mRNA increased as a result of auxin (2,4-D) treatment of pea epicotyls The results suggested that in addition to transcriptional effects, translation of the enzyme might be affected by auxin *in vivo* Jacobsen [106] describes work showing that changes in α-amylase synthesis induced by gibberellin in barley aleurone can be attributed to the effect of the hormone on the level of this mRNA A short lag (2–3 hr at 25°) between appearance of amylase-mRNA and enzyme activity indicated that translational controls were not operative A general effect of gibberellin in stimulating rRNA and poly(A) + RNA synthesis in castor bean seeds has been reported [111] No specific stimulation of synthesis of a particular mRNA species was found, indicating that increased isocitrate lyase activity could be accounted for by an overall increase in RNA and hence protein synthesis according to a predetermined pattern during germination [112] General stimulatory effects seem to account for the influence of cytokinins on transcription of all RNA species [106, 113]

It is by now obvious that the use of the word 'induction' cannot be taken to have the same literal mechanistic meaning in pro- and eucaryotic systems It has long been evident that eucaryotes do not have polycistronic mRNAs, nor are there any reports of contiguous structural and control genes—one of the basic features of the classical bacterial operon [114] More recently, the discovery of 'split genes' (including plants, e g [115]) and the importance of post-transcriptional events (see above) have further widened the evolutionary gap, making the ideas of Davidson and Britten [116, and references therein] a more appropriate conceptual framework

The role of PAL in differentiation and xylogenesis

Less than 0 1% of total cellular protein can be accounted for by PAL, even after allowing for activity losses during isolation Gupta and Acton [8], using enzyme maximally induced by far-red light in mustard cotyledons, reported that a 200-fold purified preparation of the lyase constituted about 0 01% soluble cell protein Despite the very small amount present, the effect of PAL on development can be dramatic Amrhein and Hollander [117]

blocked PAL activity *in vivo* using L-AOPP anthocyanin formation in developing flowers was inhibited (with little interference in their normal development) resulting in completely white petals The accumulation of phenylpropanoids (especially anthocyanins and other flavonoids) during cell and tissue differentiation has recently been reviewed [118]

The pathway of lignin synthesis has been extensively described [11, 15], including its biological aspects [90] An increase in lignin content during differentiation of tissue cultures has been shown by chemical analysis [95] This correlated with an increased xylose/arabinose ratio in *Phaseolus vulgaris* callus induced to form xylem and phloem by an agar wedge containing IAA and sucrose Rubery and Northcote [119] showed, for a number of species, that differentiating xylem tissue not only contained high PAL activities but could convert phenylalanine to lignin PAL could not be detected in non-lignifying tissues of these plants A supply of precursors from cambium may not be obligatory, since protein degradation by autolysis of xylem cell contents might furnish the phenylalanine required Radioactively labelled phenylalanine or cinnamic acid was incorporated into lignin in the tissues with high PAL activity no specific organelles were involved in labelling the lignified secondary thickening [120] Changes in PAL activity in soybean callus cultures and *Coleus* internode slices induced to form xylem followed the same time course as the formation of lignified elements [121] Some other examples, including correlations of PAL activity with synthesis of other polyphenols, are given by Camm and Towers [2]

The specificity of exogenous growth regulator effects on PAL activity in cultured cells has already been discussed These increases in PAL activity in bean cells were correlated with formation of nodules containing xylem and phloem on solid [71, 76, 122] and liquid media [60] PAL activity fell when the rate of nodule formation decreased PAL activity was more directly correlated with xylogenesis and nodule formation than with the formation of soluble phenols In addition, the decline in morphogenetic capability with repeated subculture was accompanied by a decline in induced PAL activity, although soluble phenol formation remained high [122] The rise in PAL activity was therefore taken to reflect increased provision of lignin precursors during xylogenesis on induction medium

Variation of auxin concentration can lead to xylem and root formation in many tissue cultures and can result in increased PAL activity (see above for details) Where xylogenesis and lignification are observed, PAL must have been active However, a hormone-dependent increase in PAL does not imply that morphogenesis is also occurring since the processes can be separately observed as in the following examples Gilliatt [67] found that transfer of solid callus or suspension cultures of potato to a hormonal induction medium resulted in an increase in PAL activity *which was not associated with tissue differentiation* Repeated maintenance of bean cells on medium supplemented with 2,4-D alone leads to a gradual loss both in xylem element formation and inducibility of PAL (on transfer to an induction medium with reduced auxin and increased cytokinin) [60] When cells are maintained on medium containing 2,4-D and 20% coconut milk, the gradual decline in both parameters occurs much more slowly (The diploidy of *Phaseolus vulgaris* cells was maintained by inclusion of coconut milk to 20% (v/v) in the growth medium—low ploidy correlated with morphogenetic capability [123]) Coconut milk medium was therefore routinely used in this laboratory to maintain cells in liquid culture [73] Under conditions where xylem element formation and PAL response gradually decline. the rate of loss of morphogenetic potential is always greater than the rate of loss of PAL response, so that a hormone-dependent PAL peak may be observed without associated xylogenesis The similarity of PAL induction in tobacco and bean cell suspensions has already been noted Kuboi and Yamada [69] observed an increase in activity of enzymes along the pathway from shikimic acid to lignin, correlated with tracheary element differentiation They found that large cell aggregates formed more xylem elements than small aggregates Large clumps also showed higher net activities for the enzymes of lignin synthesis, except for PAL and peroxidase which increased little with increasing clump size Differentiated tracheary elements are only found within cell aggregates in bean suspension culture

A more simple system for investigating cytodifferentiation following changes in exogenous growth substances may be single cells isolated from the mesophyll of *Zinnia elegans* [124] Under such special circumstances cell division is not obligatory for cytodifferentiation tracheary elements formed without an intervening mitosis by the fourth day of culture PAL and wall-bound peroxidase activity have been correlated with this tracheary element differentiation [125] This system is even further removed from the intact plant than most, since cell aggregates are not involved Roberts [90] points out that in callus cultures it takes far longer for vascular nodules to become established (e g [95]) than for tracheary elements to differentiate (e g [126])

The quantitative relationship of increased PAL activity to lignin deposition has not been thoroughly investigated One of the major problems is reliable quantitation of such an inert polymer as lignin, often necessitating harsh degradation conditions, e g alkaline nitrobenzene oxidation [95] It seems inherently unlikely that PAL alone could exert effective control over the lignin pathway, as implied by the results on tobacco cell aggregates quoted above [69] The role of isoenzymes of 4-coumarate CoA-ligase in preferentially directing phenylpropanoid units toward lignin, flavonoid glycoside or cinnamate ester synthesis [11] has been mentioned earlier In addition to this overall control on product range, the later enzymes in the pathway could become rate-limiting during the process of lignin deposition

In conclusion, PAL is the first committed step of phenylpropanoid synthesis but the cinnamate produced during xylogenesis is not absolutely committed to lignin synthesis Lignification is the terminal phase of xylogenesis, and it is unlikely that lignification *per se* has any direct bearing on the early events occurring during the initiation of cytodifferentiation [90] In the normal course of differentiation, both in the intact plant and in callus or suspension culture, PAL activity appears as part of a complex predetermined sequence of enzyme synthesis (e g PAL appears concomitantly with xylan synthetase, but later than arabinan synthetase [127, 91], thus correlating with enhanced hemicellulose production during formation of secondary wall, following suppression of pectin synthesis associated with formation of the primary wall Greatly increased incorporation *in vivo* of radio-

active precursors into xylan or arabinan was shown to occur at the time of increases in activity of the respective enzymes [128]—compare xylose/arabinose ratio discussed above [95] Following repeated subculture, loss of PAL inducibility occurs at about the same time as loss of xylan synthetase inducibility, but there is no loss in inducibility of arabinan synthetase, which is associated with cell division rather than differentiation [Bolwell, G P, personal communication]) Growth regulators are probably essential cofactors enabling (if not initiating) the expression of these minor gene products, and so integrating metabolite transport and interactions between cells

The growth-regulator dependent PAL response of cultured cells occurring without morphogenesis represents the last part of the xylogenesis sequence taken out of its normal biological context If PAL induction still occurs with the same timing and magnitude under these conditions as in developing tissues, then there is no *a priori* reason to suppose that the basic controls on production of this enzyme are any different from those in the intact plant However, the early stages of the sequence and the presence of many contiguous cells might influence subsequent PAL formation This is the inevitable compromise when cell culture is used to dissect a biological system to make biochemical experiments possible

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