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Soluble Peroxidase Gradients in Lupin Hypocotyls and the Control of the Level of Polarly Transported Indole-3yl-Acetic Acid

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Abstract. The distribution of basic soluble isoperoxidases along the growth gradient of lupin hypocotyl was studied in order to establish the role of these isoenzymes in controlling polarly transported indole-3yl-acetic acid (IAA) levels. The observation that the levels of basic isoperoxidases, which diminish from the young (vascular differentiating) to the older (vascular differentiated) tissues, are related with previously reported IAA oxidation rates in decapitated plants, suggests that these isoenzymes can play a role in the oxidation of IAA during polar transport. The fact that the level of basic isoperoxidases is controlled by IAA in hypocotyl sections harvested from different growth zones is in accordance with the previously described adaptative activation of basic isoperoxidases to IAA content. This adaptative activation of basic isoperoxidases might constitute the basic characteristic of a system of subcellular oscillators, coupled at the cellular level, necessary to generate the supracellular auxin-wave associated with auxin transport.

The involvement of indole-3yl-acetic acid (IAA) in the control of lupin hypocotyl growth has been studied for some years now and has led to the conclusion that only a small number of hypocotyl tissues (the outermost cell layers) are responsive to IAA action (Pearce and Penny 1983). It is believed that cotyledons are the source of IAA to the developing embryonic axis and regulate, together with the activity of the apical meristem, the hypocotyl growth (Elkinawy 1982). In the lupin hypocotyl the regulation of IAA-pool sizes by catabolic processes is an integral part in understanding the hormone action. In order to identify regulatory points in this metabolism, we have investigated the location and the biochemical properties of the enzymatic system responsible for IAA catabolism in this organ (Ros Barceló et al. 1989a, b). This is of special importance since the homeostatic control of IAA pools may involve both a tissue and a cellular compartmentation of the compound, as well as both biosynthetic and catabolic processes regulating its pool size.

The main conclusion of our earlier investigations was that the catabolism of IAA to indole-3yl-methanol in lupin hypocotyls is mediated by B_1 and B_2 basic isoperoxidases (Ros Barceló et al. 1989a). These isoenzymes are found mainly located in the cytoplasm (Ros Barceló et al. 1989b) and restricted, when this was studied at the electron microscope level, to the parenchyma cells associated with the phloem vascular bundles (Ros Barceló et al. 1987).

In this way, our results indicated that this IAA-oxidase system, composed of two basic isoperoxidases, and located in the cytosol of lupin parenchyma cells sheathing the vascular bundles, might be involved in the regulation of the physiologically active pools of IAA during its transport, since both the bulk of IAA and its catabolism are located in the cytoplasm (Sandberg et al. 1990), and polar IAA transport appears to be restricted to parenchyma cells (Jacobs and Gilbert 1983).

With this in mind, the aims of the present work were to study the basic isoperoxidase gradients along the exponentially growing lupin hypocotyl and the nature, if any, of its control by IAA. The role of basic isoperoxidases in controlling polarly transported IAA levels will be reexamined.

Material and Methods

Plant Material

Etiolated lupin (*Lupinus albus* L. cv. multolupa) hypocotyls were grown in darkness until they were in the exponential

growth phase (7 days old) as described previously (Ros Barceló et al. 1989c).

Measurement of Growth and Selection of Growing Zones

The following zones were marked with ink along the axis of hypocotyls of exponentially growing lupin seedlings (9.0 cm): zone I (containing the apical meristem), 0.5 cm starting from the cotyledons; zone II, 2.0 cm beneath zone I; zone V, the most basal 2.0 cm of the hypocotyl; the rest of axis, between zones II and V, was divided into two equal zones, III and IV. The marking was carried out on a population of uniform length. The growth of each zone was expressed as the mean relative elongation for a period of 1 day. Dry weight of each growth zone was determined by heating hypocotyl sections at 65°C until constant weight.

Electron Microscopy

Specimens were prepared from zone I of exponentially growing lupin hypocotyls (Ferrer et al. 1989). The tissues were fixed in 2.5% glutaraldehyde in 0.1 M Hepes-Na buffer, pH 7.4, containing 1% sucrose, for 1.5 h at 4°C. They were washed in the above buffer, and post-fixed for 2.5 h in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2. Pieces were then washed in buffer, and stained in bloc with 2% uranyl acetate for 2 h at 4°C. Embedding was carried out in an Epon-propylene oxide graded series. Ultrathin sections were examined with further staining with uranyl acetate and lead citrate, and photographed with a Zeiss EM 10 electron microscope at 60 kV.

Homogenization and Subcellular Fractionation

Unless otherwise noted, lupin hypocotyl sections harvested from the different growth zones were homogenized with a mortar and pestle in 0.25 M sucrose, 1 mM $Mg(AcO)_2$, and 5 mM Tris-HCl, pH 7.2. Soluble (120,000 g_{max} supernatants) peroxidase fractions were dialyzed against 50 mM Tris-HCl, pH 7.2, for 24 h at 24°C, and chromatographed on a Sephadex G-100 column (30 ml of bed volume), in the presence of 2 M $CaCl_2$, in order to break the conformational isomers present in lupin isoperoxidases (Ros Barceló et al. 1989b). Finally, peroxidase fractions were desalted by dialysis, and stored at −20°C.

Purification of B_1 and B_2 Basic pI Isoperoxidases

Basic soluble isoperoxidases were purified by chromatography on Sephadex G-100, and subsequent ionic exchange chromatography on DEAE-Sephadex A-50, equilibrated with 25 mM Tris-HCl, pH 7.2 (Ros Barceló et al. 1990). The purity of this isoperoxidase fraction was confirmed by isoelectric focusing in 4.0–9.0 pH gradients, showing the presence in this basic fraction of two isoperoxidases (B_1 and B_2) purified 85-fold, with a yield of 61.5% (Ros Barceló et al. 1990).

Biochemical and Zymographic Assays

Guaiacol peroxidase activity and IAA-oxidase activity were determined as reported respectively by Ros Barceló et al. (1989b) and Pedreño et al. (1988). Enzymatic activity, expressed as nkat, was defined as the amount of protein that converts 1 nmol s^{-1} of substrate into product. In order to calculate this, a $\epsilon_{470} = 26.6 \times 10^3\ M^{-1}\ cm^{-1}$ for tetraguaiacol (in the peroxidase assay) and a $\epsilon_{250} = 18.7 \times 10^3\ M^{-1}\ cm^{-1}$ for 3-hydroxymethyl-2-oxindol (in the IAA-oxidase assay) were used.

Polyacrylamide gels were prepared using riboflavin as the photopolymerizing initiating agent (Ros Barceló et al. 1987). Vertical isoelectricfocusing of peroxidases in 4.0–9.0 pH gradients, and visualization with benzidine was performed according to Ros Barceló et al. (1987). Basic isoperoxidase activities were calculated from the integrated recording of the benzidine-stained gels, using a Joyce-Loebl MKII scanner densitometer (Ros Barceló et al. 1989b).

Induction of Peroxidase and IAA-Oxidase Activities by IAA

Induction of guaiacol peroxidase activity and IAA-oxidase activity by IAA was followed in groups (0.30 g) of 2-mm sections harvested from three different growth zones (I, II, and V). These sections were floated on 15 ml of 30 mM Na-phosphate buffer, pH 7.0, containing 0.16 M sucrose and 0.1 mM IAA. The levels of both soluble peroxidase activity and IAA-oxidase activity were determined in the supernatants of the tissue homogenate of a 21,000 g_{max} centrifugation for 20 min. Time points were taken by rinsing a group of sections for 1 min in 10 ml of 30 mM Na-phosphate buffer. Incubations were carried out at 25°C and in darkness. Controls were carried out in the absence of IAA.

The effect of cycloheximide (CHI, Sigma) on the auxin-induced peroxidase activity was tested at 1 μg/ml concentration, with and without CHI preincubation for 2 h prior to the incubation with IAA.

For the measurement of IAA-induced growth of hypocotyl sections, groups (10 sections) of 5-mm segments isolated from growth zones I, II, and V, were incubated for 24 h at 25°C and in darkness in a Petri dish containing 20 ml of a isotonic solution composed of 0.1 mM IAA in 30 mM Na-phosphate buffer, pH 7.0, and 0.16 M sucrose. At the end of the incubation, the increase in segment length was measured under a binocular microscope equipped with a micrometer.

Results

Soluble Peroxidase Gradients in Lupin Hypocotyls

To study the distribution of soluble peroxidase activity along the growth gradient of exponentially growing (7 days old) lupin hypocotyls, five successive regions with different growth potentials were investigated (Fig. 1). Results represented in Fig. 1 reveal that the greatest proportion of soluble peroxidase activity was located in the youngest cells (zone I). We observed a peroxidase gradient which diminishes from the youngest (zone I, growing

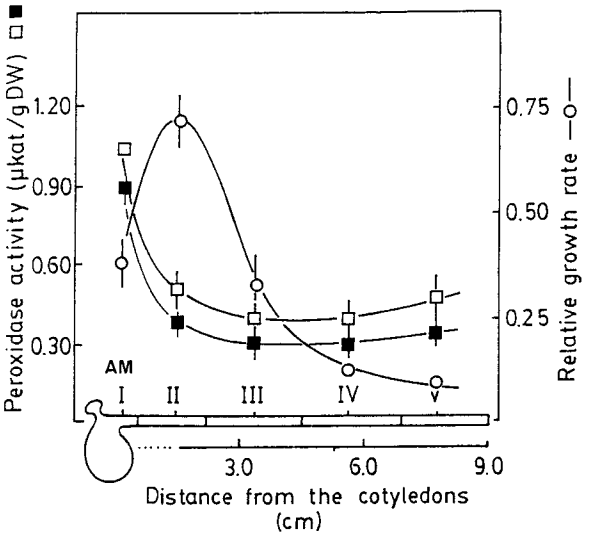

Fig 1. Distribution of total soluble peroxidase activity (□), basic isoperoxidase activity (■), and relative growth (○), along the axis of exponentially growing lupin hypocotyls. Bars indicate SE (N = 3). AM, apical meristem.

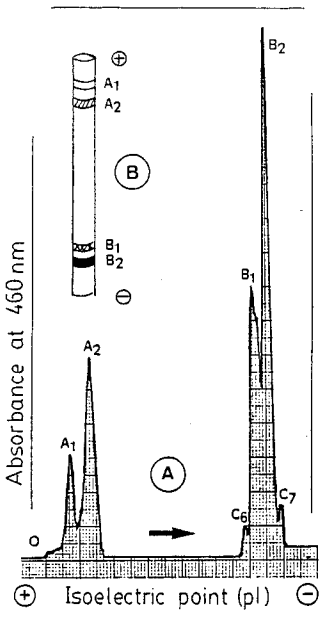

Fig. 2. (**A**) Densitometric recording of the isoenzyme pattern of soluble peroxidase activity extracted from growth zone I of exponentially growing lupin hypocotyls. (**B**) Isoenzyme patterns of soluble peroxidase fractions chromatographed on Sephadex G-100 in the presence of 2 M $CaCl_2$, in order to break the conformational isomery shown by C_6 and C_7 isoperoxidases. Peroxidase was stained with benzidine as substrate. Comparative banding intensity is indicated by the degree of shading. The arrow indicates the direction of protein migration during isoelectric focusing. O, origin.

cells) to the oldest cells (zone V, situated in the basal region of the hypocotyl which hardly showed growth) (Fig. 1). Similar peroxidase gradients were also observed in 5-, 9-, and 12-day-old hypocotyls (data not shown).

Isoelectric focusing and quantification of isoperoxidases in the different peroxidase fractions isolated from the five growth zones show that the increase in the total peroxidase activity (Fig. 1) should be attributed to an enhanced activity of two already present basic isoperoxidases (B_1 and B_2, Fig. 2) rather than to the appearance of additional isoenzymes, which does not take place. Thus, B_1 and B_2 basic isoperoxidases showed a similar gradient to that shown for the total peroxidase activity located in soluble fractions (Fig. 1). It is pointed out that the fastest growing cells (zone II) have ~50% lower basic peroxidase than zone I, even though zone I is slower growing. In this context, lupin hypocotyls showed similar gradients of soluble peroxidase activity, and similar isoenzyme gradients, to that shown by mung bean hypocotyls (Goldberg et al. 1986, 1989), independently of the substrate used to measure and to stain peroxidase activity.

Cytological Characterization of Zone I

Due to the strong localization of basic soluble isoperoxidases in zone I, in comparison with the remaining growth zones (Fig. 1), a cytological study of this growth zone was carried out. The microphotographs shown in Fig. 3 illustrate the presence in this growth zone of the meristematic quiescent center of the hypocotyl (Fig. 3A), and the presence at the level of vascular tissues of a population of highly vacuolated (fusiform) cambial initial cells (Fig. 3B), and the first (Fig. 3C) and later stages (Fig. 3D) of the processes of differentiation of the phloem vascular bundles. Thus, the differentiating phloem elements are almost all located in the most apical growing cells of the hypocotyl, in accordance with reports in a previous study (Ferrer et al. 1989).

These observations allow us to establish that high levels of cytoplasmic basic isoperoxidases are associated with tissues where the early stages of vascular differentiation take place. Such an association, clearly observed through peroxidase histochemistry probes along the growth gradient of the lupin hypocotyl (Ferrer et al. 1990), has also been well documented in graft symbionts during the initial stages of the union formation and phloem development (Feucht and Freitag 1989).

Induction of Peroxidase Activity and IAA-Oxidase Activity by IAA

Due to the relationship existing between the distri-

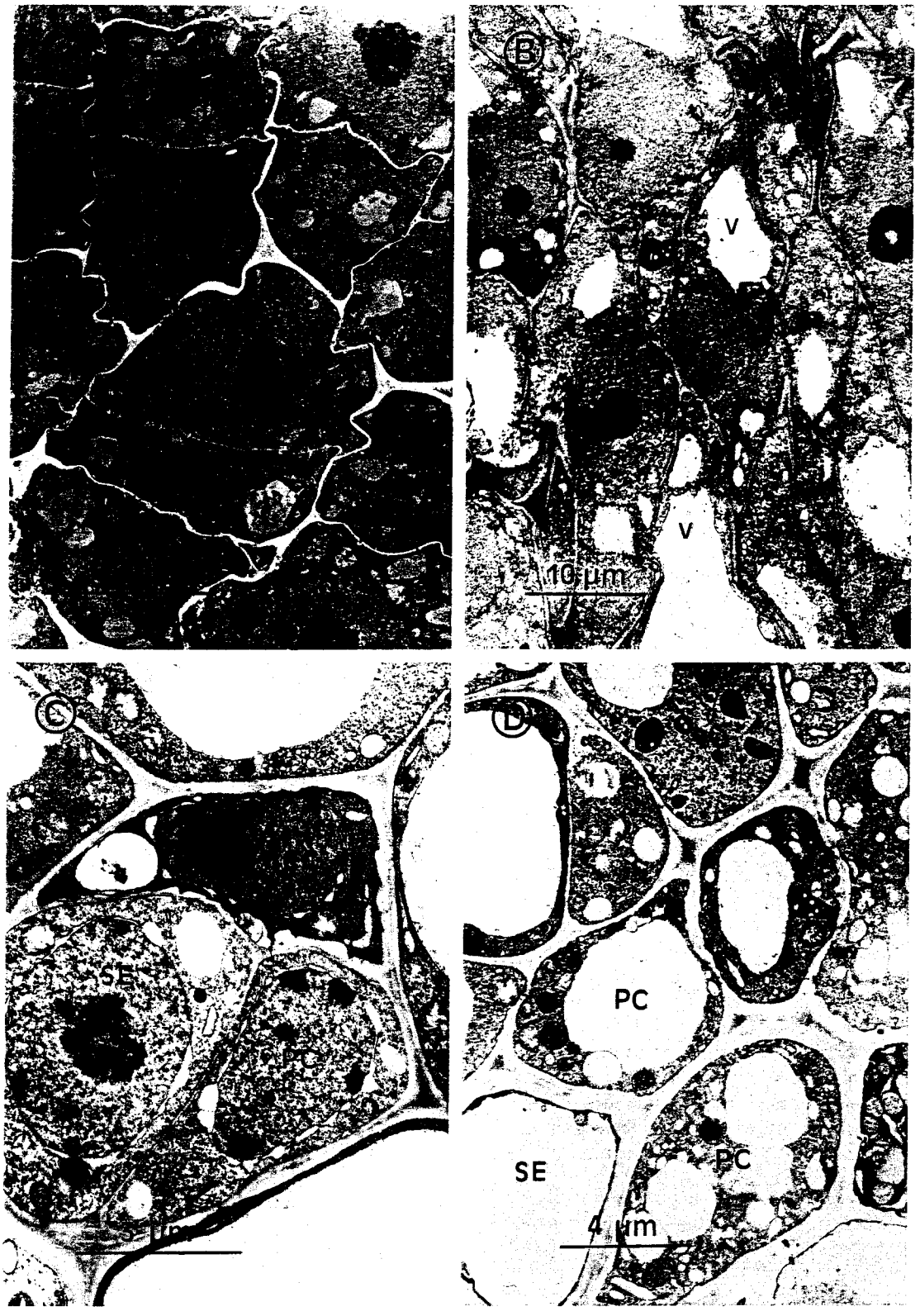

Fig. 3. Detailed view of meristematic (**A**), cambial (**B**), and early (**C**) and later (**D**) stages of the processes of differentiation of the phloem vascular bundles from zone I of the hypocotyl. CC, phloem companion cells; CI, cambial initial cells; MC, meristematic cells; PC, phloem parenchyma cells; SE, sieve element; v, vacuole. Uranyl and lead stain.

bution of IAA (Sánchez-Bravo et al. 1986) and the gradient of soluble peroxidases (Fig. 1) in the hypocotyl, the existence of a hormonal control of peroxidase levels by IAA seems to be probable. In order to check this, lupin hypocotyl sections harvested from growth zone I were incubated with 1×10^{-4} M IAA, and the development of both peroxidase activity and IAA-oxidase activity was followed with time. The results are shown in Fig. 4, and from this figure it can be established that treatment with IAA enhances both peroxidase and IAA-oxidase activities in hypocotyl sections. The effect was transitory, showing a maximum after 3–4 h of incubation, and a later decay with time. Hypocotyl sections harvested from growth zones II and V showed a similar response of both peroxidase activity and IAA-oxidase activity to treatments with IAA (Table 1). The hormonal effect on both enzymatic activities are not correlated with the growth potential of each growth zone (Fig. 1).

The effect of IAA on the enhancement of peroxidase was apparently not due to de novo protein synthesis, since CHI has no effect on the enhancement of both peroxidase and IAA-oxidase activities by IAA, not even if, before the treatment with IAA, hypocotyl sections were preincubated for 2 h with CHI.

Moreover, the enhancement of peroxidase activity after treatment with IAA was not due to the appearance of new isoenzymes, but rather to the increase in activity of the preexisting B_1 and B_2 basic isoenzymes (Table 2).

Dependence of the IAA-Oxidation by Basic Isoperoxidases on Phenols

The ability of the B_1 and B_2 basic isoperoxidases to oxidize IAA was studied in the presence and in the absence of cofactors. The results are shown in Fig. 5 and illustrate that, although in the absence of 2,4-dichlorophenol (2,4-DCP) and $MnCl_2$ these isoenzymes were capable of oxidizing IAA, a strong stimulation of the oxidation was obtained when 2,4-DCP and $MnCl_2$ were introduced as cofactors of this enzymatic system (Fig. 5). This stimulation was characterized by the appearance of a phase of high oxidation rate (phase HR in Fig. 5) after a lag time τ. The lag time is typical of a free radical induction phase, which can be hastened and/or eliminated by substituted phenols and Mn^{2+} (see Pedreño et al. 1988). Similar results have been previously described for the oxidation of IAA by a cell wall-bound peroxidase (Pedreño et al. 1988), and it has been suggested that the phenol (2,4-D) is required for the earlier initiation of the high rate step (phase HR in Fig. 5), where peroxidase apparently works through the peroxidative cycle (Pedreño et al. 1988).

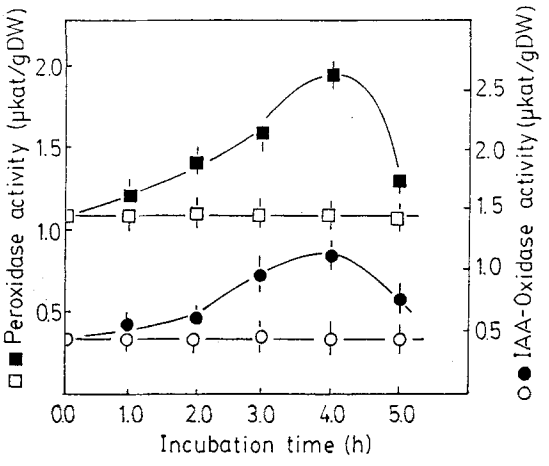

Fig. 4. Time-course of the enhancement of guaiacol-peroxidase (□, ■), and IAA-oxidase (○, ●) activities by treatment with 0.1 mM IAA (closed symbols) of hypocotyls sections harvested from growth zone I. Open symbols, controls. Bars indicate SE (n = 3).

Discussion

The homeostatic control of IAA pools in plants is an integral part of understanding auxin action. In order to identify regulatory points in this metabolism, experimental systems based on the application of exogenous IAA to excised tissue segments are a standard procedure. Recently, however, an alternative approach based on the localization of the auxin catabolism system, both at the cellular and supracellular level, and on the biochemical regulatory properties of that system has been suggested (Ros Barceló et al. 1989b). This is of great importance since the homeostatic control of IAA pools seems to involve a cellular compartmentation of the hormone, as well as both biosynthetic and catabolic processes regulating its pool size (Sandberg et al. 1990).

Local IAA pools in stems are apparently due to the polarly transported auxin (Zajaczkowski et al. 1983). This vectorial transport takes place principally through the phloem parenchyma cells (Jacobs and Gilbert 1983).

In a previous report (Ros Barceló et al. 1990), we have proposed that because the cytosol can act as an anion-trap compartment for the indoleacetate anion (IAA^-) (Goldsmith 1977), and since this compartmentation is the first step in the polar transport of auxins from cell to cell (Goldsmith 1977), the location of the IAA-oxidase system, composed of basic B_1 and B_2 isoperoxidases (Ros Barceló et al. 1989a), in the cytosol of lupin parenchyma cells (Ros Barceló et al. 1987, 1989b) would act as a mechanism for control of the IAA pools in the same

Table 1. Enhancement of peroxidase and IAA-oxidase activity by 0.1 mM IAA in hypocotyl sections harvested from growth zones I, II, and V.

Growth zone	Peroxidase activity (μkat g DW^{-1})		IAA-oxidase activity (μkat g DW^{-1})	
	Control	IAA-treated	Control	IAA-treated
I	1.10 ± 0.11	1.98 ± 0.17	0.44 ± 0.03	1.13 ± 0.11
II	0.55 ± 0.06	0.87 ± 0.09	0.21 ± 0.02	0.33 ± 0.02
V	0.53 ± 0.05	1.00 ± 0.09	0.22 ± 0.02	0.42 ± 0.05

Data on enzymatic activities were taken for 4 h of incubation and are given as the means ± SE (N = 3).

Table 2. Enhancement of basic isoperoxidase activities by 0.1 mM IAA in hypocotyl sections harvested from growth zones I, II and V.

Growth zone	Basic isoperoxidase activity (μkat g DW^{-1})		IAA-induced growth (μm day^{-1})
	Control	IAA-treated	
I	0.71 ± 0.06	1.58 ± 0.12	301 ± 40
II	0.34 ± 0.03	0.58 ± 0.05	610 ± 50
V	0.32 ± 0.01	0.70 ± 0.06	90 ± 20

Data for isoperoxidase activities were taken after 4 h of incubation and the responsiveness to growth for each section was measured after 24 h. Data are given as means ± SE (N = 3).

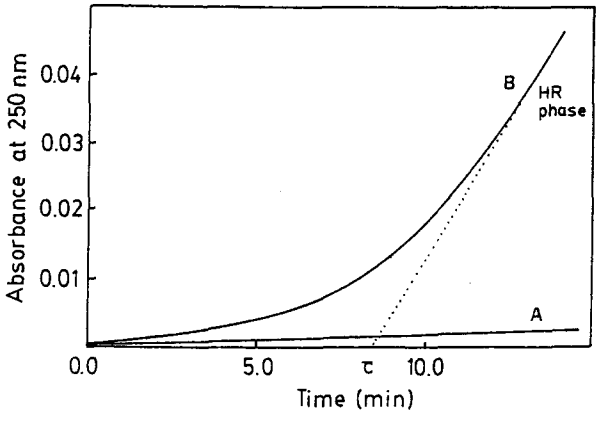

Fig. 5. Time-course of the oxidation of IAA by basic soluble isoperoxidases in the absence (A) and in the presence (B) of 0.1 mM 2,4-DCP and 0.1 mM $MnCl_2$. Phase HR, phase of high rate; τ, time lag calculated by extrapolation of the straight part of the progress curve as far as the abscissa.

subcellular compartment where IAA is accumulated during its polar transport.

In order to check this, basic isoperoxidase gradients were studied along the growth gradient of the lupin hypocotyl, and the result showed (Fig. 1) that the greater proportion of soluble basic isoperoxidases was located in the youngest cells, showing a gradient that diminishes from the youngest to the oldest cells (Fig. 1). The gradient is similar to that found for in vivo IAA oxidation rates in 10-days-old plants (Sánchez-Bravo et al. 1988). Besides this, maximum basic isoperoxidase levels were reached in the hypocotyl growth zone I containing the differentiating vascular elements (Fig. 3C–D).

Due to the relationship existing between the distribution of IAA (Sánchez-Bravo et al. 1986), and the gradient of soluble basic peroxidases (Fig. 1) in the lupin hypocotyl, the existence of a direct hormonal control of basic isoperoxidases by IAA is rather probable. In this context, the activity of basic isoperoxidases is rapidly enhanced by treatment of hypocotyl sections with IAA (Table 2), this effect not being abolished by preincubation with CHI. This fact, and the observation that IAA-induced peroxidase synthesis in ribosomes takes place after a lag period of about 5 h (Miassod et al. 1970), suggests that protein synthesis is not required in the enhancement of basic isoperoxidase activities by treatments with IAA. Thus, this rapid elicitation (activation) of basic isoperoxidases by IAA, frequently reported in the literature (Gaspar et al. 1985), must be due to the activation of preexisting inactive isoenzymes and, in this way, to be included within the primary responses of lupin hypocotyls to auxins.

In the elucidation of the mechanism of hormone action in plants, it is important to establish which responses are primary effects of the hormone and which are secondary. At the level of protein activation, we might expect primary responses to hormones to be rapid and direct.

In this respect, the basic isoperoxidase activation responses which we have presented in this study were consistent with primary responses to auxins. In this study, no attempts were made to determine what role the activation of basic isoperoxidases might have played in the auxin-induced growth but it was, however, apparent that the activation responses observed were not specific to a developmental stage. Thus, the activation of basic isoperoxidases can be detected in elongating or basal non-elongating sections of excised hypocotyls (Table 2).

On the other hand, and although we observe equivalent activation responses in a variety of organ sections that ultimately respond quite differently to applied auxins (Table 2), we do not believe that this is inconsistent with auxin action in plants. These results might simply indicate that all the organ sections are capable of responding to applied auxins. At this point, there is no a priori reason to expect primary auxin responses to differ in the various organ sections of the plant. In fact, it has been shown that the primary hormone response is the same in organ parts that are programmed, or determined, to ultimately respond to the hormone by totally different growth responses (Hagen and Guilfoyle 1985).

Thus, this adaptative activation of basic isoperoxidases to IAA levels, first described by Galston and Dalberg (1954), and a probable inactivation of basic isoperoxidases during catalysis (Acosta et al. 1988, Nakajima and Yamazaki 1980), together with the existence of a continuous and vectorial IAA transport (supply) system in the plant, provide the essential features of a self-contained mechanism for production of rhythmicity in auxin level in the plant. These basic characteristics of the system, together with the ability of peroxidase to create sustained oscillations of substrate levels through peroxidase–oxidase cycles (Fed'kina et al. 1984), might constitute the molecular basis of a system of subcellular oscillators, coupled at the cellular level, necessary to generate the supracellular auxin-wave associated with auxin transport (Wodzicki and Wodzicki 1981). The inhibition of the IAA-oxidase activity by well-known auxin transport inhibitors recently reported by Beffa et al. (1990) supports our hypothesis.

Production of rhythmicity in the auxin level by this mechanism may explain the out-of-phase distributions of auxin (Sánchez-Bravo et al. 1986) and basic isoperoxidases (similar to that shown in Fig. 1) found in the youngest (from 5–8 cm of length) hypocotyls.

Finally, according this model, monophenols, which are cofactors of the IAA oxidation by basic isoperoxidases (Fig. 5), might advance the transitions between the oxidative and peroxidative cycles of the enzyme (Pedreño et al. 1988).

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