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L-PHENYLALANINE AMMONIA LYASE FROM THE LIGNIFIED AND NON-LIGNIFIED REGIONS OF *CUSCUTA CHINENSIS*

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Key Word Index—*Cuscuta chinensis*; Cuscutaceae; L-phenylalanine ammonia lyase; isoenzyme; distribution; lignified and non-lignified regions; *in vivo* decay.

Abstract—L-Phenylalanine ammonia lyase (PAL) was not uniformly distributed along the length of *Cuscuta chinensis* vine but showed alternate zones of high and low activities starting from the tip. The first region of high activity, 0–5 cm from the tip, coincided with high protein and soluble phenolic content. The second zone 17.5–30 cm, coincided with the region of lignification in the secondary xylem. PAL occurs as at least two isoenzymes in the tip and lignified regions and these have been partially purified. The properties of PAL from both the lignified and non-lignified regions could not be distinguished by several criteria including rates of *in vivo* decay following excision of the vine. Some of the properties of the major isoenzyme of PAL from the tip are reported.

INTRODUCTION

L-Phenylalanine ammonia lyase (E.C. 4.3.1.5) (PAL), which catalyses the non-oxidative deamination of L-phenylalanine to *trans*- cinnamic acid is regarded as the first enzyme in the metabolic sequence which leads to cinnamic acids, flavonoids and lignin [1]. The universal presence of low molecular weight phenolics in plant tissues and the restriction of lignification to only some suggests a bifurcation in the modes of utilization of *trans*-cinnamic acid. Circumstantial evidence suggests that control of PAL activity is associated with the formation of different end products. Thus, enhanced PAL activity has been correlated with the onset of lignification in several tissues [2–5]. Besides, in some plant tissues PAL occurs in two isoenzymic forms [6–8] and it has been suggested that these may be subject to control by different end products [7].

Initial studies with the angiosperm plant parasite *Cuscuta chinensis* Lamk. indicated that it was rich in both low molecular weight phenolic compounds and PAL. A distribution study showed that PAL activity was highest both near the tip of the vine where phenolics accumulated maximally and further down where lignification started. *Cuscuta*, therefore, offered itself as good experimental material to study PAL from two regions in the same plant where the end products were different.

RESULTS AND DISCUSSION

Distribution of PAL activity and lignin deposition along the length of Cuscuta *vine*

Cuscuta vine was divided into 2.5 cm long pieces from the tip and each piece further subdivided into two equal parts. The first half was tested for PAL activity by infiltration with ^{14}C-phenylalanine and the second half was examined histochemically for lignin. A zone of high PAL activity (Fig. 1) was found at the tip of the vine (0–5 cm from the apex), followed by a region of low activity (5–15 cm) and a second peak of high activity (15–30 cm from the apex). Beyond 35 cm the activity appeared to rise again but was variable. Since the vine starts to stiffen from about 18 cm from the tip, it seemed possible that this was due to the onset of lignification. This was confirmed by the fact that all sections up to 15 cms from the tip showed no lignin or cambial division activity (Fig. 1, regions A and B). The section at 15 cm (Fig. 1, region C) showed some cambial division activity but was still negative for lignin. However, a positive test for liginin was found in the sections from 17.5 cm onwards (Fig. 1,

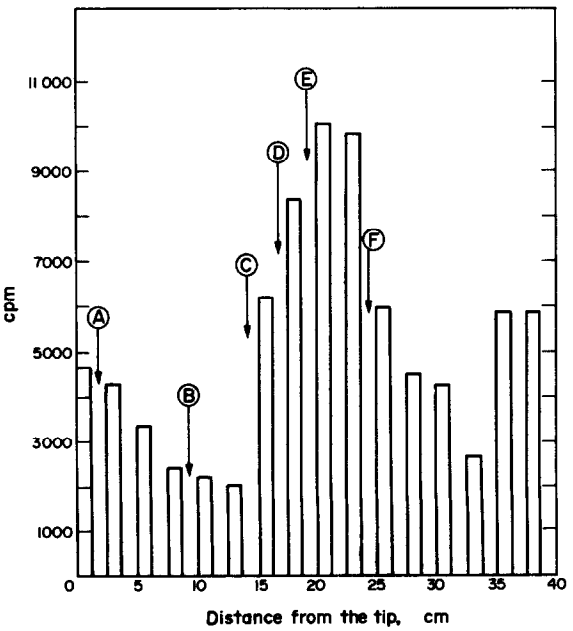

Fig. 1. Distribution of PAL activity along the length of *Cuscuta* vine. A to F indicate regions examined histochemically for lignin.

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Table 1. Distribution of soluble phenolic compounds in *Cuscuta*

Region Distance from tip	Phenolic content (chlorogenic acid equivalent)	
(cm)	(μg/cm of vine)	μg/g (fresh w) of tissue
0–5	60	2420
5–10	22.4	1100
10–17.5	25.6	420
17.5–30	35.2	300

regions D–F). Thus the second peak of PAL activity was clearly correlated with the zone of lignin deposition.

Since no lignin could be demonstrated upto 15 cm from the apex, it was reasonable to surmise that the PAL activity present in the tip leads to the synthesis of soluble phenolics. This was confirmed by analysis of the total concentration of phenolics in regions from the tip (Table 1). It may be noted that there were greater amounts of phenolics in the tip region (0–5 cm) than in any other part of the vine, including the zone of lignification where PAL activity itself was highest.

Specific activity of PAL along the vine

The tip region (0–5 cm) contained the highest amount of protein (values in the histogram bars of Fig. 2) which decreased down the vine to a more or less constant value of half the amount present at the tip. The specific activity of PAL (activity/mg protein) in the different regions of the vine (Fig. 2), shows that the high activity near the tip region was due to its high protein content. Thus the specific activity of PAL in the zone of lignification was about 5 times that in the tip region. Since the growth rate of individual vines of *Cuscuta* range between 5 to 15 cm/day (unpublished results) the peak of PAL activity in the lignified region also 'moves' at this rate. This peak extends over 12 to 15 cm length of the vine and so increase and decay in PAL activity in the lignified region should occur over 1 to 3 days depending on the growth rate.

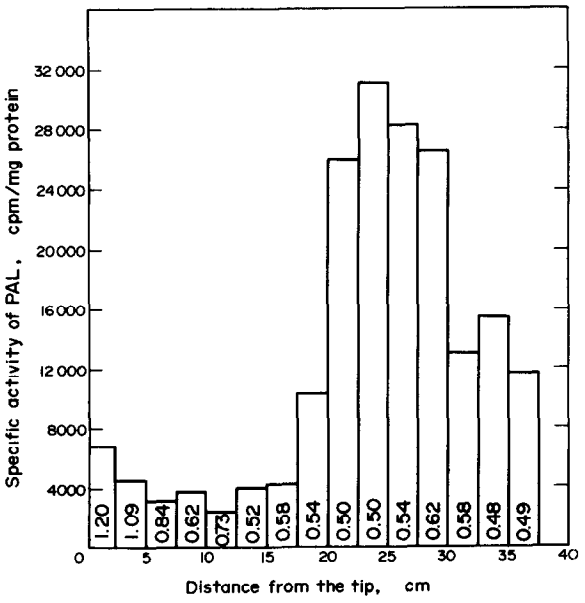

Fig. 2. Specific activity of PAL along the length of *Cuscuta* vine. Numbers in histogram bars denote protein content (mg).

Properties of PAL from the tip and lignified zone of Cuscuta

The increased PAL activity at the zone of lignification as compared to the tip region could be due to one of two possibilities: (a) increased production or activation of the same PAL enzyme(s) as are present at the tip, or (b) formation of new isoenzyme(s) of PAL at the zone of lignification designed specifically to yield precursors of lignin. PAL was, therefore, extracted and purified from acetone powders obtained from pooled (0–5 cm) and lignified (17.5–30 cm) regions of *Cuscuta* vines (Table 2). The higher initial *in vivo* specific activity of PAL from the lignified region (cf Fig. 2) was maintained during extraction and the various steps of purification. The elution pattern of PAL during DEAE-cellulose chromatography, using a sharp gradient was more or less similar for both regions of the stem with a first major peak of PAL activity (PAL I) followed by a smaller shoulder (PAL II) (tip region only shown here, Fig. 3). PAL I from both stem regions eluted as a sharp peak at the same volume, and the activity coincided with a protein peak. PAL II eluted in a more diffuse manner and appeared to be a mixture of components. Moreover, this activity did not coincide with any protein peak in both regions. PAL I and PAL II could be somewhat better separated by using a shallower elution gradient. Even after step VI (Table 2), PAL I from the tip region showed three major and two minor protein bands on acrylamide gel electrophoresis stained with Coomassie blue [9].

Buffer extracts of acetone powers obtained from the tip and lignified regions were also subjected to acrylamide gel electrophoresis. The enzyme from both sources had identical mobilities on acrylamide gels. Percent recovery of activity in the gel after electrophoresis to that applied was 35% for PAL from the tip region and 67% for PAL from the lignified region. With the tip region enzyme only, about 10% of the activity loaded on the gel was recovered in the spacer gel suggesting some *in vitro* aggregation of PAL. This aggregation may have been induced

Table 2. Purification of PAL from tip and lignified regions of *Cuscuta*

Step	Tip region Sp. activity (mUnits/mg protein)	Lignified region Sp. activity (mUnits/mg protein)
I Acetone powder extract (crude enzyme)	2.0	6.6
II Protamine sulfate treatment	2.2	9.13
III Ammonium sulfate fraction (0–50%)	5.6	28.3
IV pH precipitation and heat treatment	12.2	–*
V DEAE-cellulose chromatography†		
PAL I	69.2	106.2
PAL II	39.2	97
VI Sephadex G-100 gel filtration of PAL I	264	–

*Not applicable as no proteins are precipitated during this step.

†Shallow gradient.

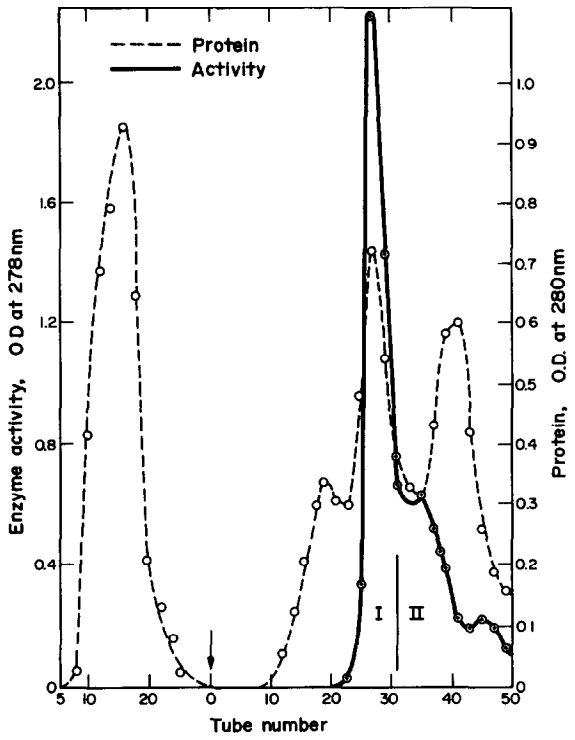

Fig. 3. DEAE-cellulose chromatography of PAL from *Cuscuta* tip (0–5 cm) region. I and II denote PAL I and II isoenzymes. PAL assay by Method A using 0.4 ml of enzyme. Sharp gradient of elution.

by the higher phenolic content of the tip region extract. *In vitro* aggregation of PAL during purification has been observed by other workers [10, 11].

PAL I and PAL II of both regions were obtained in bulk following DEAE-cellulose chromatography (Step V) using shallow elution gradient and their properties were compared. The pH optimum of activity for PAL I from both regions was 8.2 in Tris–HCl buffer (1/2 max. act. pH 6.9 + 9.6). The K_m values were found to be 1×10^{-4} M and $1{\cdot}4 \times 10^{-4}$ M for PAL I from the tip and lignified regions respectively.

Table 3. Effect of phenolic compounds on PAL I and II isolated from tip and lignified regions of *Cuscuta*

	% Activity†			
	PAL I		PAL II	
Compound added*	Tip	Lignified	Tip	Lignified
None	100	100	100	100
t-Cinnamic acid	19	14	19	12
p-Coumaric acid	88	97	92	95
Caffeic acid	81	88	94	87
Ferulic acid	100	99	85	98
Sinapic acid	100	100	75	92
Benzoic acid	80	88	90	81
p-Hydroxybenzoic acid	90	97	100	92
Salicyclic acid	57	42	56	37
Gentisic acid	98	88	92	86
Vanillic acid	97	90	70	93
Syringic acid	100	103	99	91
Catechol	48	35	47	31
Gallic acid	100	101	100	89
Chlorogenic acid	100	96	98	89

*Final concentration = 7×10^{-4} M.
†Method B assay.

Boudet *et al.* [7] described the differential inhibition of the two PAL isoenzymes they isolated from oak leaves by low MW phenolic compounds. However, this was not the case for the *Cuscuta* enzymes (Table 3). *Trans*-cinnamic acid, inhibited both enzymes to about the same extent. Only salicylic acid and catechol inhibited the reaction to over 50% and the inhibition of both PAL I and II from the lignified zone appeared to be somewhat greater than those from the tip region. On the other hand, sinapic and vanillic acids inhibited PAL II of the tip region to a somewhat greater extent than that from the lignified region. All other compounds had either no effect or showed marginal inhibition. The differential inhibitions noted here are of small magnitude (13–23%) and do not, at this stage, warrant a claim that the PAL isoenzyme of both regions are significantly different.

Inactivation of PAL in vivo *following excision of the vine from the host plant*

Initial studies indicated that PAL activity decreased appreciably when excised *Cuscuta* vines were kept at room temperature. A study was therefore made to determine whether there was any differential decay of PAL activity *in vivo* in the tip and lignified regions. The results (Table 4) show that in 9 hr PAL activity in both regions had fallen to about 25% of the original level, and in 24 hr to less than 10% of the original. Light or dark did not appreciably alter these results. When cycloheximide (100 ppm) was added, the fall in 9 hr was the same as without cycloheximide. In 24 hr however cycloheximide seemed to protect the enzyme from the lignified region to a somewhat greater extent than that from the tip region. This delayed and differential effect of cycloheximide of PAL decay was traced to the rate of movement of cycloheximide up the vine. When cycloheximide uptake was aided by partial vacuum infiltration, the decay of PAL activity in the tip region was reduced by the same extent as that in the lignified region. Partial vacuum infiltration of either water or L-phenylalanine showed no difference in decay from control (non-infiltrated) samples. Excised vines kept at 0–4° or deep frozen showed no loss of PAL activity after 24 hr. The similar rates of *in vivo* decay of PAL from both regions again suggests that the same type of PAL enzyme is present in both the tip and lignified regions. Though the

Table 4. *In vivo* inactivation of PAL in the tip and lignified regions of exercised *Cuscuta* vines

Hours after excision of *Cuscuta*	Additives or treatment	% Activity			
		Tip region		Lignified region	
		Dark	Light	Dark	Light
0	—	100	100	100	100
9	—	25	21	25	26
24	—	7.0	11	4.5	4.0
24	Cycloheximide (100 μg/ml)	13	12	29	35
24	Cycloheximide (100 μg/ml) infiltration	24	40	—	—

inactivation of PAL after its induction by light has been reported by several workers, to our knowledge the inactivation of native PAL after the excision of the plant material has so far not been reported. The dramatic drop in PAL activity in the vine of *Cuscuta* upon excision is not due to the onset of senescence and loss of cellular organization as with detached leaves. Indeed during the 24 hr following excision, elongation growth occurs and cycloheximide treatment blocks this growth. Since *Cuscuta* vines in a detached state can survive and grow, the decay of PAL has a physiological significance in preventing the irreversible channelling of a protein amino acid into either secondary storage products or structural strengthening agents.

The inhibition of PAL decay by either cycloheximide or cold treatments suggests the need for protein synthesis in the inactivation process. The protein synthesized may either act directly as a specific proteinaceous inactivator of PAL or as a non-specific protease. Suggestions have been made that light-induced PAL in tissues may be degraded in the dark by enhanced synthesis of proteases [12] or may complex with a specific proteinaceous inactivator [13]. Acid and alkaline proteases have been shown to increase in excised leaves of oats [14]. Whether any similar increase in protease occurred in *Cuscuta* vines following excision was tested by the same procedures [14]. No appreciable change occurred in the levels of both acid and alkaline proteases during the 24 hrs. following excision, ruling out this possibility.

In order to ascertain whether a specific inhibitor of PAL was produced following the excision of *Cuscuta* vines, crude extracts from freshly cut vines (0 hr) and from vines excised 24 hr earlier were prepared. PAL activity was tested in aliquots of 0 hr, 24 hr and a 1:1 mixture of (0 hr + 24 hr) extracts. The PAL activity of the mixture was an average of the other two activities suggesting no specific PAL inhibitor production. However this conclusion would be untenable if inhibitor production was limiting and no excess free inhibitor was present in the 24 hr extract.

The foregoing results indicate that the properties of PAL from the two regions of *Cuscuta*, namely the tip and lignified regions, appear to be similar if not identical in a number of parameters such as elution during DEAE-cellulose chromatography and isoenzymic pattern, mobility on acrylamide disc gel electrophoresis, substrate affinity, pH dependence of activity, effect of several phenolic compounds, *in vivo* decay following excision of vine and its protection by cycloheximide. It is therefore concluded that the same PAL isoenzymes are formed in both the tip and lignified regions, and that the point of control for channelling *trans*-cinnamic acid into the two types of end products, namely soluble phenolics or lignin, lies elsewhere. The enhanced synthesis (and/or activation) of PAL at the beginning of the lignified zone and its subsequent decay should be under the same control system which determined lignification. However, this specific decay of PAL in the lignified zone during normal vine growth is very likely to be under a different control from the general decay of PAL activity which occurs in all regions when the vine is excised.

Further properties of purified PAL I from the tip region of Cuscata

Since PAL from the tip and lignified regions of *Cuscuta* were so similar, further properties of *Cuscuta* PAL was studied using only the purified PAL I of the tip region obtained by Sephadex G-100 gel filtration (step VI in Table 2). This enzyme was about 130-fold purified over the crude acetone powder extract and showed a maximal specific activity of 264 millinuits/mg protein. Following concentration with sucrose, the enzyme could be stored for 6 months at −20° without loss of activity. The enzyme was quite stable between pH 6 and 10 for 30 min at 25–27°. It lost 20% of its original activity at pH 5 and was completely inactivated at pH 4 under the same conditions. The enzyme was specific of L-phenylalanine and DL-*p*-chlorophenylalanine and DL-*p*-fluorophenylalanine were only half as active at 0.4 mM concentration. L-Tyrosine, L-tryptophan and L-histidine were not deaminated. Sodium borohydride inhibited the activity completely (>95%) possibly by reducing the active site as with PAL from other sources [1]. Sodium metabisulfite and potassium cyanide, both at 1 mM concentration, inhibited the enzyme to 31% and 64% respectively. The MW of PAL I was determined from the elution volume *vs*, the log mw plot, and estimated as 280000. PAL from other sources ranges in MW from 230000 and 330000 [1].

EXPERIMENTAL

Materials. Overhanging vines of *Cuscuta chinensis* [15] free of haustoria and growing on *Tecoma stans* Juss. (Bignoniaceae) host were collected locally. For enzyme extraction, the vines were chilled in ice immediately after harvest. Radioactive DL-phenylalanine-1-^{14}C. (sp. act. 48 mCi/mM) and DL-phenylalanine-2-^{14}C (14.7 mCi/mM) were from Radiochemical Centre, Amersham, England.

Infiltration experiments. Vines were subdivided into 2.5 cm pieces from the apex. Each piece was further cut into smaller pieces and vacuum infiltrated with 0.3 ml solution of Tris–HCl (0.1 M; pH 8.2) containing 2-mercaptoethanol (1 mM), unlabelled L-phenylalanine (0.15 μm) and DL-phenylalanine-1-^{14}C (50000 cpm). After incubation for 3 hr at 25°, the suspension was acidified with 0.1 ml of 2N HCl and extracted with 2 ml of toluene. 1 ml of the toluene layer dried with dry Na_2SO_4 was placed in vials containing 5 ml of scintillation fluid (toluene 1000 ml, PPO (2,5-diphenyloxalate) 5 gm) and the radioactivity measured [17]. The reaction product was separately identified as *trans*-cinnamic acid by TLC.

PAL assay. PAL was assayed by two methods. *Method A*: A standard reaction mixture (1 ml) contained L-phenylalanine (4 mM) Tris–HCl (0.1 M; pH 8.2), 2-mercaptoethanol (0.5 mM) and the enzyme. Following incubation at 30° for 60 min the reaction was arrested with 0.2 ml of 2N HCL. The absorbance of *trans*-cinnamic acid in acid pH was measured at 278 nm using a molar extinction value of 20400 [16]. The method was unsuitable with crude enzyme preparations because of interference by phenolics. *Method B*: The reaction mixture was as above except that 0.4 μm of cold L-phenylalanine and either 10^5 cpm of DL-phenylalanine-1-^{14}C or 3×10^5 cpm of DL-phenylalanine-2-^{14}C were used. After incubation ^{14}C-cinnamic and was extracted and determined as described under 'infiltration'.

Histochemical identification of lignin. Cuscuta stem samples were sectioned and stained with phloroglucinol-HCl (1% phloroglucinol in 95% EtOH acidified with HCl) for lignin identification [18].

Estimation of phenolics. Low MW phenolics were extracted with boiling 80% EtOH and filtered [19], the solvent removed under reduced pressure at 45° and the residue dissolved in hot H_2O and filtered. The filtrate was hydrolysed in 2N NaOH under N_2 for 16 hr at r.t., acidified with conc HCl and the acid phenolics extracted 3 × with peroxide-free Et_2O. The residue in the Et_2O extract after removal of the solvent was dissolved in 2 ml of

EtOH and aliquots estimated for phenolics by the method of Swain and Hillis [20] using chlorogenic acid as the reference.

Polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed as described by Davis [21]. A 7.5% gel with separation at pH 8.2 was used.

MW determination. MW of PAL was determined [22] using a Sephadex G-200 column equilibrated with Na–Pi buffer (0.01 M; pH 7.6) containing 2-mercaptoethanol (0.5 mM) and calibrated with catalase (MW 244 000; elution volume 86 ml), γ-globulin (MW 150 000; elution volume-116 ml) and hexokinase (MW 97 000; elution volume—135 ml). Elution volume of PAL I was 81 ml.

Protein estimation. For purified enzyme preparations the procedure of Lowry *et al.* [23] was used. Crude preparations containing phenolic compounds was assayed by the procedure of Potty [24].

PAL purification. Me_2CO powders of *Cuscuta* stem tip (0–5 cm) and lignified (17.5–30 cm) regions were prepared in the presence of 2-mercaptoethanol (2 ml in 200 ml Me_2CO). The powder was stable for several months at −20°. *Step I:* Me_2CO powder (5 gm) was ground in 50 to 75 ml Tris–HCl (0.1 M; pH 8.2) containing 2-mercaptoethanol (0.1 ml/100 buffer). The mixture (0°) was centrifuged (10 min; 22 000 *g*). *Step II:* The supernatant was treated with protamine solphate solution (1 part protamine sulphate to 12 parts protein) dropwise, stirred (15 min) and centrifuged (5 min; 22 000 *g*). *Step III:* The supernatant was taken to 50% saturation with $(NH_4)_2SO_4$, stirred (40 min), and centrifuged (10 min; 12 000 *g*). The ppt. was dissolved in Tris–HCl (0.1 M; pH 8.2) containing 2-mercaptoethanol (0.5 mM) and dialysed against Tris–HCl (1 mM; pH 8.2) with 2-mercaptoethanol (0.5 mM). *Step IV:* The dialysate was treated with an equal volume of citric acid–Na citrate buffer (0.1 M; pH 5.1) and centrifuged (3 min; 33 000 *g*). The supernatant was kept at 50° for 12 min cooled and centrifuged (3 min, 22 000 *g*). The supernatant was brought to pH 7 with 2N NaOH and dialysed against 2-mercaptoethanol (0.5 mM) for 6 hr and stored frozen. *Step V:* The thawed enzyme was passed through DEAE-cellulose (25 × 1.1 cm; PO_4^{3-} form) that had been equilibrated with 2-mercaptoethanol (0.5 mM). Unadsorbed proteins were eluted with 2-mercaptoethanol (0.5 mM). The enzyme was eluted with 200 ml (sharp gradient) or 500 ml (shallow gradient) KPi buffer (pH 6.0) using a linear gradient from 0 to 0.15 M and 3 ml fraction collected. Active fractions were pooled brought to 0.1 mM with respect to Na-EDTA and concentrated in a dialysis bag with solid sucrose at 0°. Enzyme was stored at 0–20°. *Step VI:* The conc enzyme was passed through Sephadex G-100 (90 × 1.7 cm) that had been equilibrated with KPi buffer (10 mM; pH 7.6) containing 2-mercaptoethanol (0.5 mM) and eluted with the same buffer. Enzyme was eluted in the void volume.

PAL inactivation in vivo. *Cuscuta* vines (40 cm) were placed with cut end dipping in ascorbic acid (0.1 mM), with or without cycloheximide (100 μgm/ml) or L-phenylalanine (1 mg/ml). The vines were placed in the dark or in continuous light (0.6 m from 3 × 20 W fluorescent tubes). For experiments requiring facilitated infiltration, the vines were placed in a large desiccator and evacuated briefly before releasing vacuum. At different time intervals (0, 9, 24 hr) vines were removed and the tip (0–5 cm) and lignified (17.5–30 cm) regions separated and assayed for PAL as follows: 0.75 gms of tissue was ground with 10 ml Tris–HCl (0.1 M; pH 8.2) containing 2-mercaptoethanol (0.1 ml/100 ml) and polyvinylpolypyrrolidone (20 mg) and centrifuged (5 min; 12 000 *g*). 1 ml of the supernatant was assayed for PAL by Method B.

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