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BIOSYNTHESIS OF BENZYLGLUCOSINOLATE, CYANOGENIC GLUCOSIDES AND PHENYLPROPANOIDS IN *CARICA PAPAYA*

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Key Word Index—*Carica papaya*; Caricaceae; pawpaw; biosynthesis; cyanogenic glucosides; benzylglucosinolate; phenylpropanoids.

Abstract—Benzylglucosinolate was detected in all of the tissues of *Carica papaya* (pawpaw). No other glucosinolates were detected in any tissue of *C. papaya*. Previous suggestions that indolyl-3-methylglucosinolate might be present could not be confirmed. The highest concentrations of benzylglucosinolate were found in the youngest leaves, but the compound was also detected in leaf stalks, stem internodes and roots. The presence of benzylglucosinolate in shoots was developmentally regulated—high concentrations in young tissues, declining as they matured. The exception was the stem internodes which maintained relatively constant concentrations. Tap roots had higher glucosinolate content than young roots. Cyanide, specifically released from cyanogenic glucosides, was detected in leaves and roots of *C. papaya*. Cyanide was not detected in comparable glucosinolate-containing tissues from *Brassica napus* (oilseed rape). The cyanide concentrations were highest in the tap roots and young leaves of *C. papaya*, suggesting that cyanogenic glucoside accumulation was also developmentally regulated. NADPH-dependent L-phenylalanine monooxygenase activity was detected in leaves of *C. papaya* catalysing the oxidative decarboxylation of L-phenylalanine. This monooxygenase activity was restricted to leaves, and could not be detected in any other tissues. No other monooxygenase activities were detected, in any tissues, active with any of the amino acids tested. Activity was highest in the young leaves and declined as leaves expanded and matured. This enzyme was significantly inhibited by several cytochrome P450 inhibitors, and to a lesser extent by the flavoprotein-specific inhibitor diphenylene iodonium. No other aromatic amino acids tested were either substrates or inhibitors of this enzyme, suggesting a high degree of substrate specificity. Two other key enzymes involved in the metabolism of L-Phe and L-Phe-derived compounds, phenylalanine-ammonia lyase and peroxidase, were found to be similarly developmentally regulated in tissues of *C. papaya*. Activities were highest in young tissues and declined as the tissues matured. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Very little has been published on the chemical composition or biochemistry of *Carica papaya* (pawpaw) [1–4]. The glucosinolates, distributed among a number of plant families [5, 6], come into contact with cytosolic thioglucosidases (myrosinases) on tissue disruption, leading to the formation of isothiocyanates, nitriles and thiocyanates with fungitoxic and insect behaviour-modifying properties [7]. Benzylglucosinolate (glucotropaeolin) and its breakdown product benzylisothiocyanate, which are major metabolites of the Caricaceae [8–10], may be useful in the chemotaxonomy of related species [10]. Secondary metabolites in dried leaves of *C. papaya* included the expected benzylglucosinolate and also the cyanogenic glucosides prunasin (derived from L-Phe) and tetraphyllin B [11]. However, that paper did not report tissue concentrations for the cyanogenic glucosides, which were apparently very low. *C. papaya* is thus one of the few examples known of a plant containing both glucosinolates and cyanogenic glucosides; *Drypetes* species from the Euphorbiaceae are also reported to contain glucosinolates and cyanogenic glucosides [12].

The biosynthetic pathway(s) for glucosinolates and cyanogenic glucosides are probably dissimilar [7, 13], though both pathways involve conversion of amino acids to aldoximes. Formation of aldoximes for the biosynthesis of aliphatic/alkenyl glucosinolates is catalysed in *Brassica* species by flavoprotein NADPH-dependent monooxygenases (MO) [14–17]. The enzymes involved in the formation of aldoximes for aromatic glucosinolates appears to be determined by the plant species. All *Brassica* species studied contain flavoprotein MOs [17], but *Tropaeolum majus* and *Sin-*

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apis species have a cyt P450 MO [17–19]. In initial studies of *C. papaya* an L-Phe-dependent MO was found [17].

Cytochrome P450-type enzymes are involved in aldoxime-forming reactions in cyanogenic glucoside biosynthesis [20], and it was once thought that they were also involved in the biosynthesis of all glucosinolates [13]. However, it has been shown that the conversion of L-Trp to indole-3-aldoxime, precursor of both indolylglucosinolates and (potentially) IAA, is catalysed by membrane-bound peroxidases [21]. There is no evidence for NADPH-dependent MOs which will metabolize tryptophan to its aldoxime, in any glucosinolate-containing plant species [15, 17, 21]. Thus, to date, three distinct enzyme systems for the formation of aldoximes from amino acids have been discovered in plants: flavoprotein MOs, cytochrome P450 MOs, and peroxidases.

Numerous secondary metabolites are derived from L-Phe, including lignin precursors, the flavonoids and biologically active compounds [23]. There have been several detailed reviews on the metabolism and role of phenylpropanoids in plant resistance [24, 25]. In *C. papaya* an integrated pathway can be imagined for production of the L-Phe-derived secondary metabolites—an L-Phe-dependent MO producing benzylaldoxime (for benzylglucosinolate and prunasin), and PAL catalysing the formation of *t*-cinnamic acid as the precursor for a variety of phenylpropanoid secondary metabolites (Fig. 1).

The aims of this research were to determine the tissue content of glucosinolates and cyanogenic glucosides in pawpaw vegetative tissues, and make an initial investigation of the nature of the enzymes involved in their biosynthesis. The activity and developmental regulation of other L-Phe-dependent enzymes involved in secondary metabolism were also examined for comparison.

RESULTS AND DISCUSSION

Distribution and tissue concentrations of benzylglucosinolate

Benzylglucosinolate was found in significant concentrations in leaves of *C. papaya* (Fig. 2). An earlier suggestion that traces of indolylglucosinolates might be present in *C. papaya* [26] could not be confirmed. The concentration of benzylglucosinolate was greatest in young leaves (30–35 μM), decreasing as leaves expanded and matured. A similar pattern was also found in leaf stalks. This pattern of glucosinolate accumulation has been found in several Crucifer species [17, 27, 28]. The benzylglucosinolate concentrations in the stem internodes varied between 1 and 4 μM, but did not show a clear developmental pattern. Benzylglucosinolate was also found in the roots, with a higher concentration in tap roots than in young roots. No other glucosinolates were detected, in any tissue.

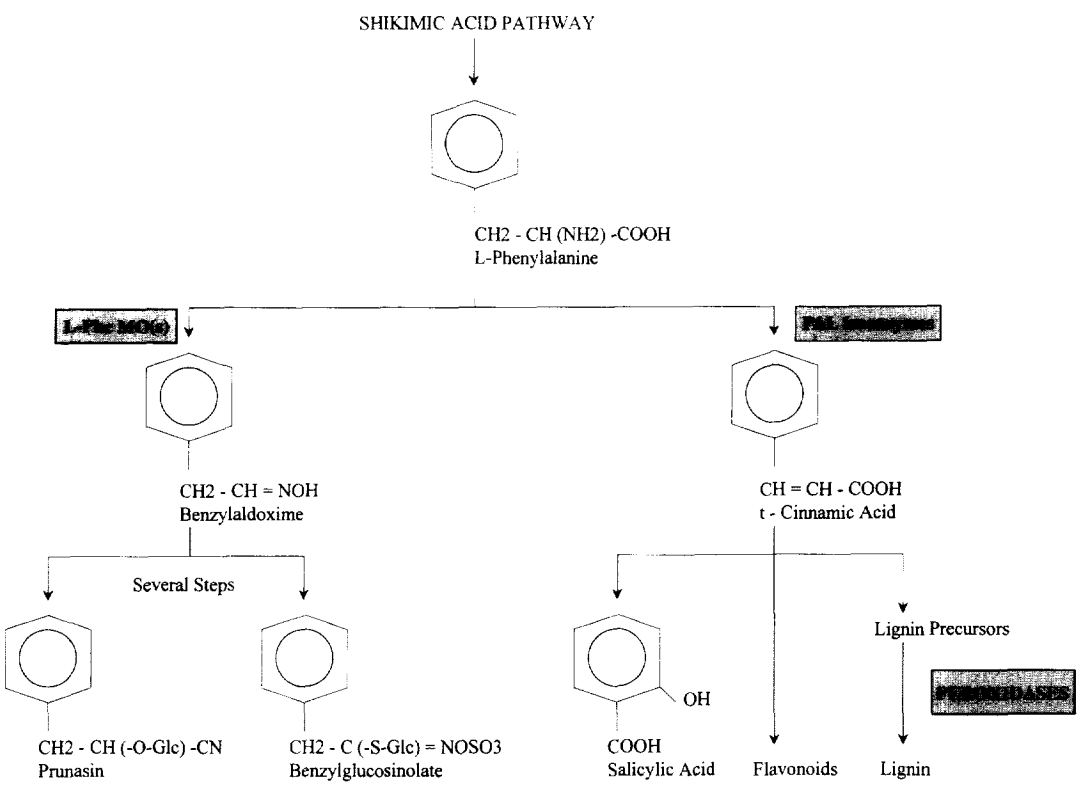

Fig. 1. L-Phe-derived compounds in *Carica papaya*: inter-relationships and key enzymes for the biosynthesis of prunasin, benzylglucosinolate and lignin.

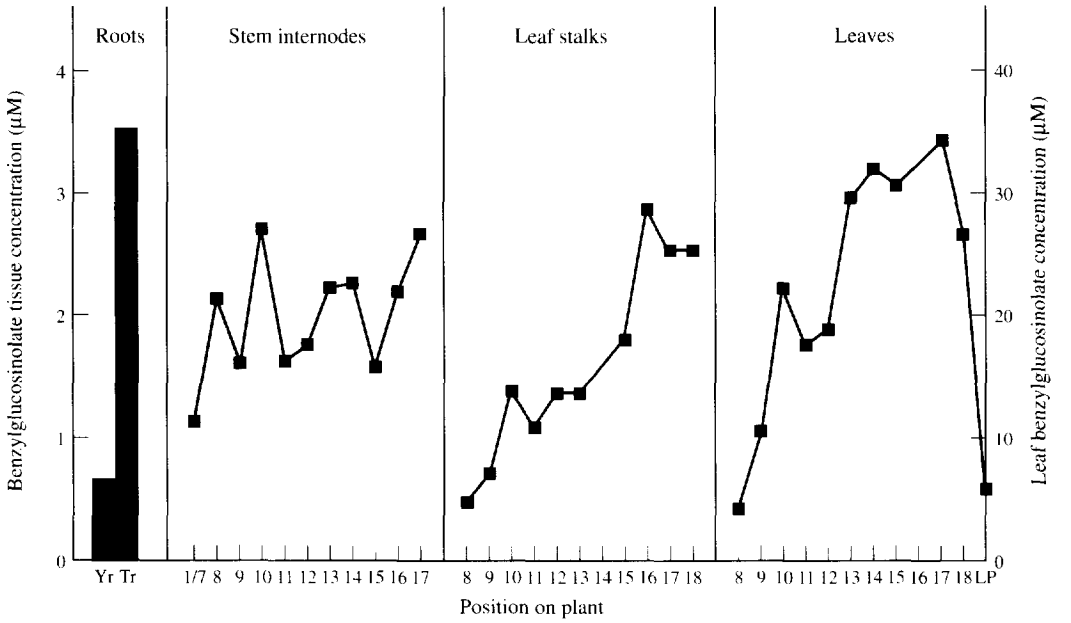

Fig. 2. Benzylglucosinolate concentrations in tissues of *Carica papaya*. The root, stem internodes and leaf stalk concentrations are shown on axis Y1, the leaf concentrations on axis Y2.

Distribution and tissue concentrations of CN⁻ derived from cyanogenic glucosides

Using a cyanogenic glucoside-specific detection method, CN^- was detected in leaves and roots of *C. papaya* (Fig. 3). It was not possible to make accurate determinations of $[CN^-]$ in the leaf stalks or stem internodes because these fibrous tissues homogenised poorly in liquid N_2. The pattern of CN^- distribution was similar to that of benzylglucosinolate. CN^- concentrations were highest in the young leaves and the tap roots, and as the leaves matured the concentration of CN^- declined. The concentration of cyanogenic glucosides was significantly less than that of benzylglucosinolate in leaves, but much higher in roots (especially young roots). Negligible CN^- could be detected in similar tissues from oilseed rape, indicating that the 'cyanide' detected in *C. papaya* tissues did not arise from glucosinolate breakdown products (which could in theory give a positive reading in the CN^- assay).

Activity, distribution and partial characterisation of L-*Phe monooxygenase*

Microsomal NADPH-dependent L-Phe MO activity was found in leaves of *C. papaya* (Fig. 4). There was

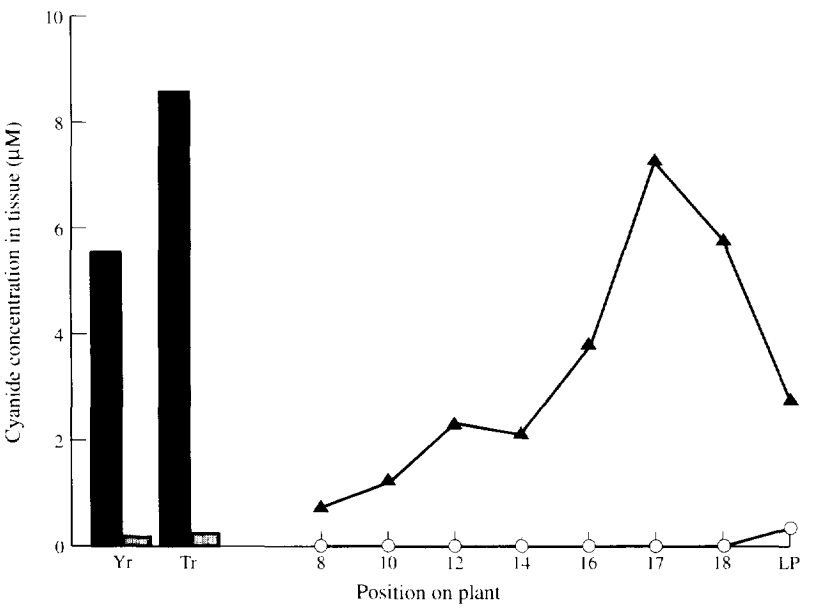

Fig. 3. Cyanogenic glucoside-specific release of cyanide from roots and leaves of *Carica papaya* (solid, ▲) and *Brassica napus* (shaded, ○).

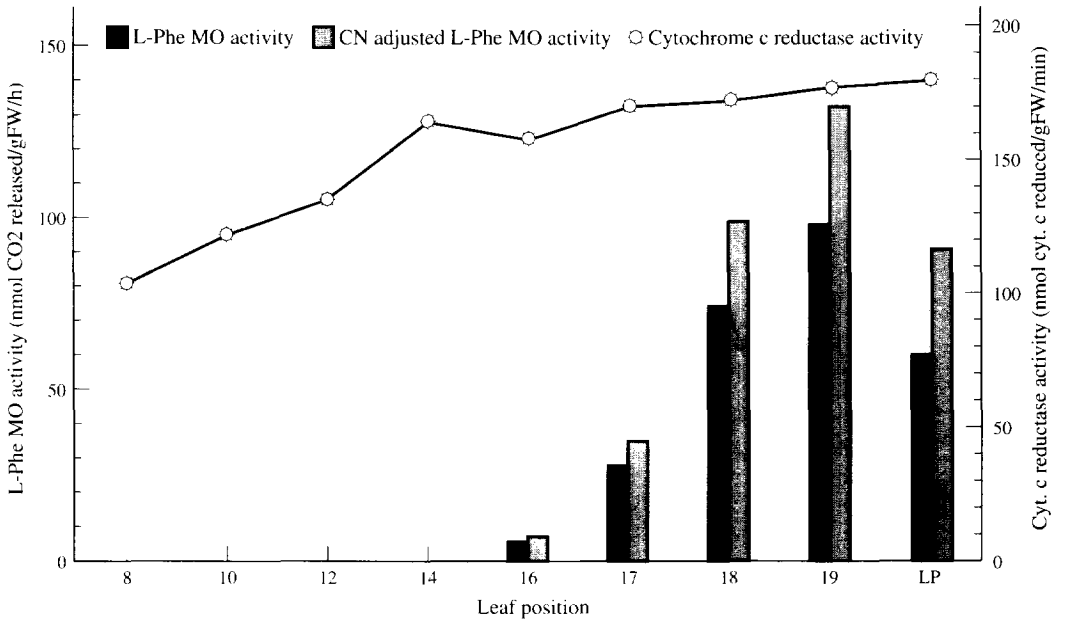

Fig. 4. Microsomal L-Phe MO and cytochrome c reductase activities in leaves of *Carica papaya*. Solid bars show the measured MO activity, and open bars the calculated activity allowing for potential inhibition by cyanide released from cyanogenic glucosides during tissue extraction.

no detectable activity in leaf stalks, stem internodes or root tissues; very low activity was detected in the leaf stalks and internodes of the very youngest leaves (leaf primordia leaf 19, activity <3 pkat g^{-1} fr. wt). There was significant cytochrome c reductase activity in microsomes from all of the tissues analysed, indicating reasonable membrane extraction. No activity could be detected with any of the other 1-^{14}C-amino acids tested, in any tissue. The activity of the L-Phe MO was highest in the youngest leaves, declining as the leaves completed expansion and matured. This pattern of developmental regulation of glucosinolate-biosynthetic MOs has been found in other glucosinolate-containing plants [16]. However, the L-Phe activity was relatively low when compared with the MO activities from glucosinolate-containing *Brassica* species, as has also been found with *Tropaeolum majus* and *Sinapis* species [17].

Transport of cyanogenic glucosides from biosynthetic tissues to other parts of the plant is well established in a variety of species (e.g. cassava [29]), and indeed the capacity to make these compounds appears to be strictly localized. Similar redistribution of glucosinolates has not been found in *Brassica* species (except in the special case of transport from maternal pod wall tissue to filial seed cotyledons [30]), each vegetative tissue synthesising its own spectrum of glucosinolates [27]. Our failure to detect biosynthetic activity in *C. papaya* roots, which nonetheless contain benzylglucosinolate, suggests that in this species glucosinolate transport redistribution between tissues may occur. Root microsomal preparations had active POX and cyt c reductase, so the inability to detect L-Phe MO activity is unlikely to be due to poor membrane extraction or recovery. We cannot, however, exclude the possibility that more specific inhibitors or enzyme inactivators are present in the roots, masking MO activity.

Earlier experiments indicated that the *C. papaya* L-Phe MO could be a flavoprotein [17]. However, the L-Phe MO activity was found to be strongly inhibited by classic cytochrome P450 inhibitors [CN^-, CO and 1-aminobenzotriazole (1-ABT); Table 1]. The flavoprotein-specific inhibitor diphenylene iodonium (DPI) somewhat inhibited the enzyme activity, but not as effectively as it inhibits the *Brassica* MOs [16, 17]. This partial inhibition is not perhaps unexpected, as P450-

Table 1. The effect of potential cofactors, substrates and inhibitors on L-Phe MO activity from young leaves of *Carica papaya*. The concentration of L-Phe in the assay was 20 mM (3 nmol L [1-^{14}C]Phe and 97 nmol unlabelled L-Phe). 100% activity (+NADPH) was 18 pkat g^{-1} fr.wt

Effector	Effector concentration (μM)	Activity (%)
None	—	0
NADH	500	0
NADPH	500	100
N_2	—	93
CO	—	47
CN	250	51
1-ABT	100	55
DPI	250	84
L-Tyr	250	90
L-Trp	250	99
L-HPhe	250	97
L-DHMet	250	96

type enzymes are associated with a flavoprotein (cyt P450 reductase). These results indicate that L-Phe MO activity in *C. papaya* is catalysed by cytochrome P450-type enzyme(s).

The L-Phe MO activity was not significantly affected by the addition of other aliphatic or aromatic amino acids (Table 1), suggesting these other amino acids are not recognised by the active site of the enzyme(s). Such substrate specificity has been found with other MOs involved in glucosinolate biosynthesis [14, 16], and indicates that the activities being measured are not the result of non-specific decarboxylases or other catabolic enzymes.

During the microsome preparation CN^-, released from the cyanogenic glycosides, could have inhibited the L-Phe MO activity. Assuming maximum release of CN^-, and interaction with the enzyme(s), we can calculate a theoretical 'maximum' *in vivo* MO activity based on the measured inhibition of MO activity by exogenous CN^-, and this is also shown in Fig. 4. Although the calculated activities are somewhat higher than those measured *in vitro*, the developmental pattern is not significantly altered. It has also been suggested that isothiocyanates released from glucosinolates can likewise inhibit cytochrome P450-catalysed MO activity [31], and elaborate precautions have been employed to (theoretically) overcome this [18, 19]. Whilst isothiocyanate inhibition during extraction is possible, we have no evidence that it is significant. In pawpaw, as in all other glucosinolate-containing species examined, we have always been able to detect MO activities in appropriate glucosinolate-containing tissues (even in unstressed and un-elicited plants, unlike other reports, e.g. [18, 19]).

Developmental regulation of phenylalanine ammonia-lyase (PAL) and peroxidases (soluble and microsomal)

PAL activity [Fig. 5(a)] was developmentally regulated in all of the *C. papaya* tissues, as found in many other plant species [22]. The activity was highest in the youngest leaves, newly formed stem internodes, and young roots. As the tissues matured PAL activities also decreased. PAL was detected in all of the tissues analysed. Soluble [Fig. 5(b)] and microsomal [Fig. 5(c)] peroxidase (POX) followed very similar developmental patterns. Both soluble and microsomal POX activities were highest in the youngest tissues—leaves, stem internodes and young roots. Soluble POX activities were significantly greater than microsomal POX activities—comparison with cyt c reductase activity suggests that membrane extraction was reasonably good from all tissues, and the changes in microsomal POX were probably not a result of variation in membrane recovery.

Multiple isoforms of both PAL and POX are found in plants. Each isoform may have a specific function and subcellular localization, e.g. plastidic PAL is induced upon fungal attack leading to increased production of phenylpropanoids for lignin formation at sites of infection. Both PAL and POX are involved in defensive chemistry (phytoalexin production and pathogen-induced lignin deposition) and in the normal development of plants. Specific peroxidases have been implicated in both IAA biosynthesis and degradation [21, 32–34]. Peroxidases are also important, in combination with PAL, for the formation of xylem vessels and secondary thickening of cell walls [35]. All of the enzyme activities involving L-Phe or L-Phe metabolites that we assayed appear to be similarly regulated, though we have not investigated isozyme patterns. High enzyme activities in young tissues are not unexpected, as there is a high demand for L-Phe-derived compounds for both normal development and pathogen resistance. Shikimic acid pathway enzymes are also known to be maximally active in young tissues [36, 37].

CONCLUSIONS

Very few plant species have been positively identified as containing both glucosinolates and cyanogenic glucosides. It has been assumed that the two classes of secondary metabolite are mutually exclusive, and that those species apparently containing both were either very exceptional or that there were contaminants in the extracts. The presence of isoleucine- and valine-derived glucosinolates, as well as cyanogenic glucosides, was reported in the genus *Drypetes* [12]. It has been suggested that the production of glucosinolates in these plants arose recently, in evolutionary terms, in an already cyanogenic species [38]. We have confirmed earlier *C. papaya* studies demonstrating that this species accumulates both classes of secondary metabolite.

Glucosinolates have in the past been used as taxonomic markers, based on the assumption that the presence of a particular compound in different plants is indicative of a common evolutionary history. Recent studies with a variety of glucosinolate-containing plants suggest that this is an untenable assumption—three different aldoxime-forming activities, all involved in glucosinolate biosynthesis, have been described [14, 18, 21], differing in their distribution between species [17]. It would seem that these enzyme systems themselves are likely to be better markers than the glucosinolates, and that the capacity to produce glucosinolates has probably arisen more than once in plant evolution. The position of species such as *C. papaya* which make both glucosinolates and cyanogenic glucosides is especially intriguing. Our results suggest a common or closely related biosynthetic system for both classes of metabolite in this species, similar to the aldoxime-forming systems in *Tropaeolum*, and in cyanogenic species such as sorghum and cassava. Despite the presence of some similar glucosinolates in *Brassica* species, these plants use an entirely different biosynthetic system which must have evolved independently.

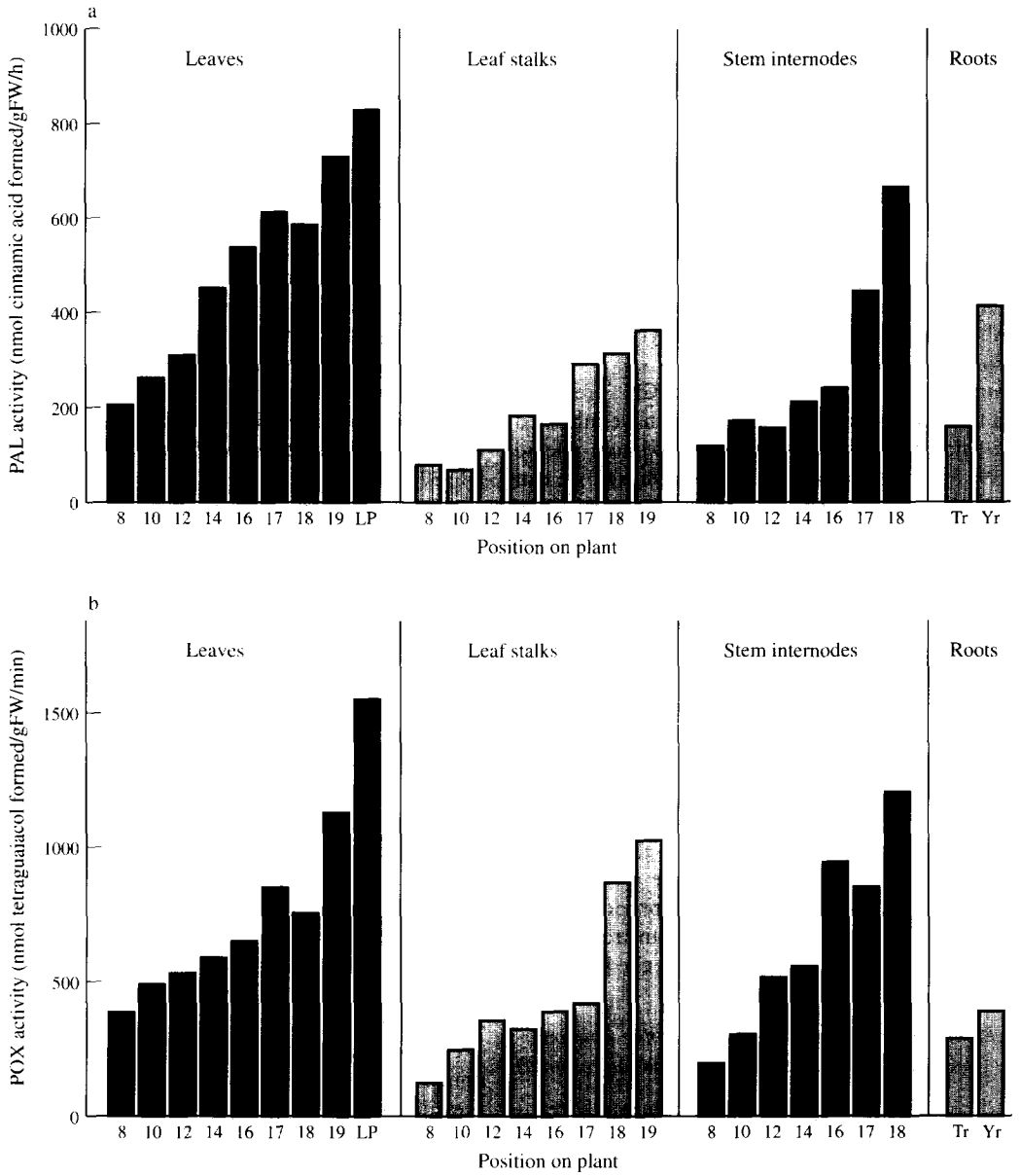

Fig. 5. (a) PAL; (b) soluble POX; and (c) microsomal POX activities in tissues of *Carica papaya*. Cytochrome c reductase activities are included as a comparison with the microsomal POX activities.

EXPERIMENTAL

Chemicals. L-Phenyl[1-^{14}C]alanine was obtained from Sigma, with a specific activity of 59 mCi mmol^{-1} (50 μCi diluted to 12 μCi ml^{-1}). The other 1-^{14}C-amino acids were synthesized using methods previously reported [39]; specific activities of 52 mCi mmol^{-1} diluted to 12 μCi ml^{-1}. Diphenylene iodonium was synthesized using methods reported in ref. [40]. Protein was determined with the BIO-RAD dye-binding system.

Growth and sampling of plant material. Seeds of pawpaw (*Carica papaya*) were obtained from E. W. King (Suffolk Herbs, U.K.). Seeds of oilseed rape (*Brassica napus* cv. Bienvenu) were obtained from Rothamsted stocks, from field grown plants in 1994. The pawpaw plants used in these studies were 118 days old—grown between April and September, in individual pots in compost, in a glasshouse (12/12 hr light/dark—daylight supplemented with a combination of tungsten and fluorescent lamps, 350 μE m^{-2} s^{-1} average PAR, 23/18). Oilseed rape plants were grown under similar conditions, except the average temps were 17/14 day/night.

Glucosinolate extraction and analysis. For the analysis of the pawpaw tissues a minimum of three independent glucosinolate extractions were done, using the methods reported in ref. [28].

Cyanide determination. The extraction and determination of cyanide (specifically released from cyano-

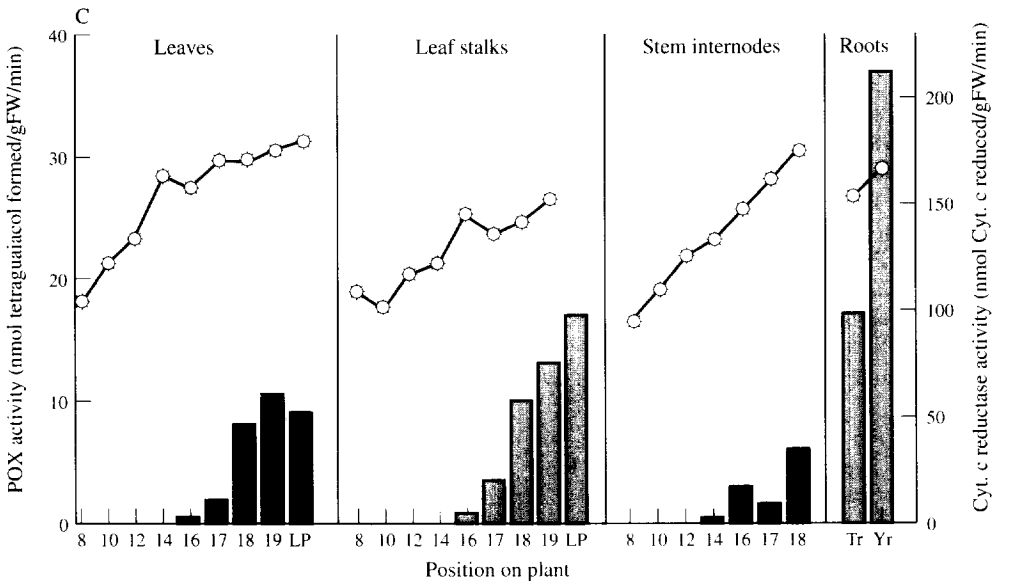

Fig. 5.—*Continued.*

genic glucosides), in tissues from both pawpaw and oilseed rape, used the method in ref. [41]. Final reaction mixts were passed through 2 μM disposable nylon filters, to remove particulate matter, before the A was determined at 585 and 650nm.

Preparation of microsomes. Tissues were homogenized, and microsomes prepd, using previously reported methods [14]. Extraction conditions optimized for both flavoproteins and cytochrome P450-type enzymes were tested [17].

Assay and characterisation of monooxygenase activities. Several 1-^{14}C-amino acids (L-Phe, DL-homophenylalanine, DL-dihomomethionine, DL-trihomomethionine and L-tyrosine; known to be glucosinolate precursors) were tested under assay conditions that were optimal for either flavoproteins or cytochromes P450. The assay involves measuring the NADPH-dependent release of $^{14}CO_2$ from substrates [14, 17]. A minimum of three independent experiments were done to determine the MO activities, using both flavoprotein and cytochromes P450 extraction/resuspension/assay conditions, for all of the samples. The treatment of microsomes with inhibitors/effectors (N_2, CO, CN^-, 1-ABT and DPI) and unlabelled amino acid competitors was performed as previously reported [14, 16, 17].

Cytochrome c reductase assay. A method adapted from that in ref. [42] was used with modifications [17].

Extraction, protein determination, and assays for phenylalanine ammonialyase (PAL) and peroxidases. Soluble and microsomal frs for PAL and POX assays were isolated from *C. papaya* tissues using the microsome extraction protocol described above. The main modifications were the inclusion of 1 mM phenylmethylsulphonyl fluoride in the homogenization buffer and retention of the 100 000 *g* supernatant (for PAL and soluble peroxidases). Microsomal fractions were washed in excess resuspension buffer (10 ml per 2.5 ml of microsomes), re-pelleted at 100 000 *g*, 4 for 1 hr, and resuspended a second time to a final vol. of 2.5 ml. These washed microsome frs were used to determine membrane protein concs, cytochrome c reductase activities, and microsomal peroxidase activities. The 100 000 *g* supernatant was desalted on short columns of Sephadex G25.

PAL and peroxidase assays were based on methods reported in ref. [43]. For PAL, the reaction was initiated by addition of 50 μl of extract to 950 μl of substrate buffer (10 mM L-Phe in 100 mM K borate pH 8.8) and the extracts were incubated for 2 hr at 35 . The A_{290} was recorded and both boiled enzyme and (-) substrate controls were included; a minimum of three replicates for each sample in each of three independent experiments were done. POX assays (both soluble and microsomal) followed tetraguaicol formation from guaicol; the A_{470} was monitored for 5 min and initial rates calcd. The reaction was initiated by the addition of 50 μl of extract to 950 μl of substrate buffer (2.2 mM H_2O_2 in 200 mM KPi pH 5.8). A minimum of three replicates for each sample in each of three independent experiments were assayed.

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