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Journal of Experimental Botany, Vol. 46, No. 288, pp. 731–741, July 1995

Journal of Experimental Botany

REVIEW ARTICLE

Cyanogenesis in cassava (*Manihot esculenta* Crantz)

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Received 29 September 1994; Accepted 28 February 1995

Abstract

Cassava is the most agronomically important of the cyanogenic crops. Linamarin, the predominant cyanogenic glycoside in cassava, can accumulate to concentrations as high as 500 mg kg^{-1} fresh weight in roots and to higher levels in leaves. Recently, the pathway of linamarin synthesis and the cellular site of linamarin storage have been determined. In addition, the cyanogenic enzymes, linamarase and hydroxynitrile lyase, have been characterized and their genes cloned. These results, as well as studies on the organ- and tissue-specific localization of linamarase and hydroxynitrile lyase, allow us to propose models for the regulation of cyanogenesis in cassava. There remain, however, many unanswered questions regarding the tissue-specific synthesis, transport, and accumulation of cyanogenic glycosides. The resolution of these questions will facilitate the development of food processing, biochemical and transgenic plant approaches to reducing the cyanogen content of cassava foods.

Key words: Cyanide, cyanogenic glycosides, linamarin, cyanogens.

Introduction

Cyanide is toxic to most living organisms due to its ability to bind to the metal (Fe, Zn and Cu) functional groups or ligands of many enzymes. Examples of cyanide-inhibited processes include the inhibition of oxygen reduction in the respiratory electron transfer chain, the inhibition of plastocyanin reduction in photosynthesis and the inhibition of catalase activity. While it seems counter-intuitive that plants would produce and metabolize cyanide it is apparent that all plants surveyed to date are capable of cyanide metabolism (Miller and Conn, 1980; Esashi *et al.*, 1981; Peiser *et al.*, 1984; Kakes and Hakvoort, 1992). Plants produce cyanide as a by-product of ethylene synthesis. Furthermore, cyanide may regulate development or seed germination in some plants and affect the alternate respiratory pathway (Esashi *et al.*, 1981; Peiser *et al.*, 1984). In addition, cyanide can be assimilated by the ubiquitous plant enzymes, β-cyanoalanine synthase and rhodanese.

The magnitude of cyanogen and/or cyanide metabolism varies greatly between different plant species. Whereas most plants produce small amounts of cyanide associated with ethylene production, between 3–12 000 plant species produce sufficient quantities of cyanogenic compounds that they may function as translocatable forms of reduced nitrogen or as chemical defence molecules against herbivores (Kakes, 1990; Poulton, 1990). Several economically important plants are highly cyanogenic, including white clover, flax, almonds, sorghum, wild lima bean, rubber tree, and cassava. The most agronomically important of the cyanogenic food crops, however, is cassava (*Manihot esculenta* Crantz). Over 153 million tons of cassava root are harvested annually in the tropical regions of the world and cassava ranks tenth among all crops in world-wide production. It is the major source of calories for peoples living in sub-Saharan Africa (Cock, 1985; Best and Hargrave, 1994). Due to the presence of cyanogenic glycosides cassava is potentially toxic to human consumers; however, cassava has many desirable agronomic traits including the ability to grow on acid soils, drought resistance, herbivore resistance, and food security. Furthermore, cassava roots are an important insurance crop for subsistence farmers throughout the tropics. They can remain in the soil for up to three years before harvesting and the presence of cyanogens protects them from herbivory and theft by vandals (Arias and Bellotti, 1984; Bellotti and Arias, 1993; Cock, 1985; Balagopalan *et al.*, 1988).

All tissues of cassava, with the exception of seeds,

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contain copious quantities of the cyanogenic glycosides, linamarin and lesser amounts of lotaustralin. The leaves and the peel (cortex) of cassava roots contain the highest quantities of cyanogenic glycosides (Cock, 1985; Balagopalan *et al.*, 1988; Brimer and Dalgaard, 1984; Bradbury and Egan, 1992). In addition, there are cultivar-dependent differences in root cyanogenic glycoside contents (Cock, 1985; Wheatley *et al.*, 1993). Most cassava cultivars have root cyanogenic glycoside contents which are less than 100 mg kg^{-1} fresh weight, but there are many 'bitter' cultivars which contain up to 500 mg kg^{-1} root fresh weight (Wheatley *et al.*, 1993). Significantly, no acyanogenic cassava cultivars have been identified, and only one member of the genus (*M. gracilis*) (L.J.C.P. Carvalho, Washington University, personal communication) has been reported to be acyanogenic.

The presence of cyanogens in poorly processed cassava food products can cause health problems for people that subsist on cassava-based diets (Cock, 1985; Tylleskar *et al.*, 1992; Rosling *et al.*, 1993). The cyanogens (linamarin, lotaustralin, and their respective aglycones or α-hydroxynitriles) are converted to cyanide in the body by generalized β-glycosidases and elevated temperatures and pH (discussed below). Significantly, free cyanide has not been found in processed cassava foods due to its volatility (Tylleskar *et al.*, 1992; Rosling *et al.*, 1993). The health disorders associated with subsistence on a high cyanogen-containing diet include (1) hyperthyroidism, resulting from thiocyanate interference in iodine metabolism, (2) tropical ataxic neuropathy, a neurological disorder, and (3) konzo, a rapid and permanent paralysis (Osuntokun, 1981; Tylleskar *et al.*, 1992; Rosling *et al.*, 1993). The onset of these disorders can be gradual or immediate and is dependent on the state of health and nutrition of the consumer and the quantity of cyanogen consumed. A contributing factor to cyanide-induced disease is the amount of cysteine in the diet since sulphur-containing amino acids are required for cyanide detoxification as thiocyanates (Tylleskar *et al.*, 1992; Rosling *et al.*, 1993). It is apparent, however, that adequately processed cassava and the use of low cyanogenic cultivars can reduce the cyanogen content of cassava foods to non-toxic levels. Ironically, certain societies prefer high cyanogenic cultivars. The basis for these preferences is not fully understood, although high cyanogenic cultivars may offer improved food security (D. Dufour, University of Colorado, personal communication).

Research on the cyanogens of cassava has focused largely on the biochemistry and physiology of linamarin synthesis and metabolism. This is because linamarin accounts for 95% of the total cyanogenic glycoside present in intact cassava tissues (Cock, 1985; Balagopalan *et al.*, 1988). The precursors and intermediates of the linamarin synthesis pathway have been characterized, however, the enzymology of linamarin synthesis is not well characterized. As demonstrated by Conn's group, the precursor for the first dedicated step in linamarin synthesis is valine (Butler and Conn, 1964; Nartey, 1968; Cutler and Conn, 1981). Recently, Møller's group has demonstrated that the conversion of valine to acetone cyanohydrin, the non-glycosylated form of linamarin, is catalysed by NADPH-dependent cytochrome P450 (s) (Koch *et al.*, 1992). The initial step is the *N*-hydroxylation of valine followed by the formation of 2-methyl-propanal oxime and its dehydration to yield 2-methylpropionitrile. The addition of oxygen to the nitrile forms the unstable intermediate, acetone cyanohydrin, which is then glycosylated by a soluble UDPG-glucosyltransferase to form linamarin (Hahlbrock and Conn, 1971; Koch *et al.*, 1992; Bokanga *et al.*, 1993). As demonstrated by Møller's group, the initial reactions take place on membrane (microsomal) fractions (Koch *et al.*, 1992). More recently, it has been suggested that acetone cyanohydrin is probably synthesized by enzymes localized on the tonoplast membrane. Using isolated vacuoles, McMahon and Sayre (1995) demonstrated that valine was converted to linamarin in an NADPH-dependent reaction. These results suggest that the cytochrome P450s involved in the initial biosynthetic steps are localized on the tonoplast and that the soluble UDP-glucosyltransferase is presumably vacuolar (Gruhnert *et al.*, 1994; Mederacke *et al.*, 1995). A vacuolar site of linamarin synthesis is supported also by the observation that vacuoles contain more than sufficient quantities of linamarin to account for that present in intact cells (White *et al.*, 1994). A vacuolar site for cyanogenic glycoside storage has also been demonstrated in *Sorghum* and *Hevea* (Saunders and Conn, 1978; Gruhnert *et al.*, 1994).

In contrast to linamarin synthesis, the enzymology of cyanide production from linamarin is well characterized. The generation of cyanide from linamarin is a two-step process involving the initial deglycosylation of linamarin and the cleavage of acetone cyanohydrin to form cyanide and acetone (Fig. 1). These steps are catalysed by two enzymes, a β-glucosidase (linamarase) and an α-hydroxynitrile lyase (HNL). In the following sections, we discuss the biochemistry of cyanogenesis in cassava, the physiological basis for varietal differences in root linamarin content, and future trends in cassava cyanogenesis research.

Linamarase

The first step in the conversion of linamarin to cyanide is the deglycosylation or hydrolysis of linamarin by linamarase to form acetone cyanohydrin and glucose (Fig. 1). Since acetone cyanohydrin may spontaneously or enzymatically decompose to cyanide and acetone, it has generally been assumed that linamarase activity is the rate-limiting step in cyanogenesis (however, see section

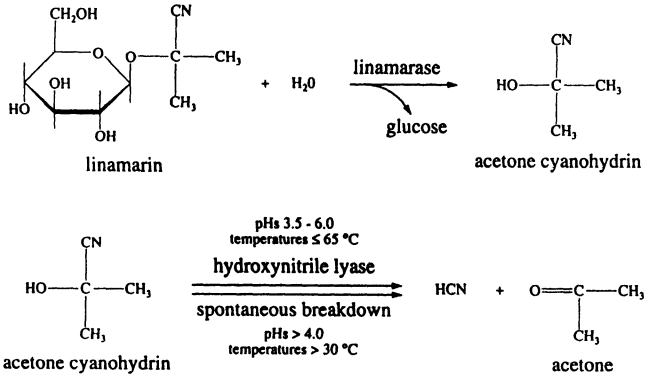

Fig. 1. Cyanogenesis from linamarin.

on HNL, below). As a result, the biochemistry of linamarase has received much attention. Linamarase was initially purified from cassava by Cooke *et al.* (1978). They achieved a 350-fold increase in linamarase specific activity following fractionation of whole leaf extracts, but apparently did not achieve a homogeneous preparation. In 1988, Eksittikul and Chulavatnatol purified cassava linamarase to apparent homogeneity using crude extracts from various plant organs. Three different isoforms (63 kDa) of the enzyme, having isoelectric points (*pI*s) of 2.9, 3.3 and 4.3, were identified (Table 1). Significantly, the three isoforms differ in their tissue specific localization. The $pI = 4.3$ isoform was the most abundant form present in petioles and stems, but equivalent amounts of all three forms were present in root tissue. Kinetic studies indicated that there was also a 10-fold difference in the specific activities of the three isoforms. Consistent with the low levels of linamarase in roots, the isoform ($pI = 4.3$) having the highest specific activity was least abundant in this tissue (Mkpong *et al.*, 1990). Each isoform, however, had substantially lower specific activity than linamarases isolated from other plants (Table 1) (Eksittikul and Chulavatnatol, 1988; Yeoh, 1988).

In 1990, Mkpong *et al.* reinvestigated the properties of the cassava linamarase. They developed a rapid four-step procedure for isolation of β-glucosidase activity based on a low pH (3.5) extraction of whole leaves. Most leaf proteins were denatured and precipitated by the low pH extraction buffer, however, linamarase remained soluble. A 100-fold enrichment in β-glucosidase activity was achieved, yielding a protein having a molecular weight (65 kDa), identical to the linamarase isolated by Eksittikul and Chulavatnatol (1988). Three different isoforms, with *pI*s similar to those observed by Eksittikul and Chulavatnatol (1988), were detected on isoelectric focusing gels using the fluorogenic substrate 4-methylumbelliferyl β-D-glucopyranoside. The kinetic properties of the mixed isoform linamarase isolated by Mkpong *et al.* (1990) differed, however, from linamarase isolated by Eksittikul and Chulavatnatol (1988). The enzyme isolated by Mkpong *et al.* (1990) had a specific activity of 29.4 mmol linamarin mg^{-1} protein h^{-1}, 8-fold higher than the enzyme(s) isolated by Eksittikul and Chulavatnatol (1988). This difference in activity may be attributed to differences in (1) the assay temperature (Eksittikul and Chulavatnatol (1988) used lower temperatures (37 °C) than Mkpong *et al.* (1990) who used optimal assay temperatures (55 °C)), (2) the assay procedure (Eksittikul and Chulavatnatol (1988) measured volatile cyanide production, whereas Mkpong *et al.* (1990) measured glucose production), or (3) the presence of inhibitors of linamarase activity (Koch *et al.*, 1992; Bokanga *et al.*, 1993).

As shown in Table 1, the pH (6–7) and temperature (55 °C) optima for cassava linamarase reported by various investigators are in agreement. The high temperature optimum of cassava linamarase is similar to that of

Table 1. *Kinetic properties of linamarases from different species*

See references for experimental details. (a) = Cooke *et al.*, 1987, (b) = Eksittikul and Chulavatnatol, 1988, (c) = Yeoh, 1988, (d) = Mkpong *et al.*, 1990, (e) = Selmar *et al.*, 1987, (f) = Itoh-Nashida *et al.*, 1987. ND = not determined.

Plant	V_{max} (mmol mg^{-1} protein h^{-1})	K_m (mM)	Temperature optimum (°C)	pH optimum
Manihot esculenta (a)	0.095	1.45	ND	6
Manihot esculenta (b)				
$pI = 4.3$	3.02	0.57	ND	7.0
$pI = 3.3$	0.71	ND	ND	ND
$pI = 2.9$	0.39	ND	ND	ND
Manihot esculenta (c)				
Leaf	0.54[a]	2.08	55	6–7.3
Root peel	0.18[a]	2.34	55	6–7.3
Root cortex	0.01[a]	3.93	55	6–7.3
Manihot esculenta (d)	29.4[b]	1.9	55	7.0
Hevea brasilensis (e)	11.6	7.6	62	5.6
Phaseolus lunatus (f)	14.6	5.56	ND	5.1–6

[a] = Partially purified.
[b] = Measured at 55 °C.

Hevea (62 °C) which also belongs to the Euphorbiaceae (Table 1). As might be expected for an enzyme exhibiting a high temperature optimum, cassava linamarase has been shown to be extremely stable. Mkpong and Sayre have isolated active enzyme from exfoliated (3 months) and desiccated leaves (unpublished results). Recent evidence suggests that the unusual stability and kinetic properties of cassava linamarase may be associated with post-translational modifications of the protein. As demonstrated by Hughes *et al.* (1992), cassava linamarase is glycosylated. Digestion of linamarase with endoglycosidase H, which cleaves M5, M8, and M9 type high-mannose and hybrid structures, reduces its molecular weight from 70 to 65 kDa. In order to determine whether glycosylation of the enzyme affected its kinetic properties, McMahon and Sayre digested purified cassava linamarase with endoglycosidase H, purified the fully deglycosylated form of linamarase by exclusion from a concanavalin A column, and measured its activity. They observed an 80% loss (average of three experiments) in *p*-nitrophenol-β-D-glucopyranoside-dependent β-glucosidase activity for the deglycosylated form relative to the non-deglycosylated form of the enzyme (McMahon and Sayre, unpublished results). The deglycosylated enzyme, however, exhibited no further loss in activity following 1 h heat treatment (60 °C). This was in contrast to the native enzyme which lost 16% of its activity following a 1 h incubation at 60 °C versus incubation at 25 °C. These results suggest that the state of linamarase glycosylation affects the activity of the enzyme. Furthermore, the extent of glycosylation may account for the different kinetic properties of the three linamarase isoforms. As demonstrated by Eksittikul and Chulavatnatol (1988), all three isoforms have essentially identical amino acid compositions suggesting that post-translational modifications (glycosylation) could account for the variation in kinetic and isoelectric properties of the three isoforms.

All β-glucosidases, including linamarase, can be classified on the basis of their substrate specificity, amino acid sequences, and stereospecificity of hydrolysis of the β-glucosidic bond. The linamarases of some plants have such broad substrate specificities that they are more accurately designated generalized β-glucosidase(s) rather than linamarase(s) (Selmar *et al.*, 1987). For example, the 64 kDa β-glucosidase of *Hevea brasiliensis* is apparently the only β-glucosidase present in the plant and will hydrolyse a variety of β-linked glycones and aglycones (Table 2) (Selmar *et al.*, 1987; Tull *et al.*, 1991; Yeoh and Yeoh, 1994). Most plants, however, have multiple β-glucosidases with narrow substrate specificities. As shown in Table 2, the cassava 65 kDa β-glucosidase has broad substrate specificity, but not as broad as the *Hevea* linamarase (Cooke *et al.*, 1978; Selmar *et al.*, 1987; Eksittikul and Chulavatnatol, 1988; Yeoh, 1988; Mkpong *et al.*, 1990). Cassava linamarase will hydrolyse prunasin and several non-physiological monoglycosidic substrates (ρ-nitrophenol-β-D-glucopyranoside), but will not hydrolyse the cyanogenic diglycosides amygdalin or linustatin. The implications of this substrate specificity are discussed below in the context of cyanogen transport.

A significant increase in our understanding of the structure and function of cassava linamarase was achieved with the isolation and sequencing of a cassava linamarase cDNA clone (Hughes *et al.*, 1992). The linamarase cDNA encodes a protein of 532 residues, having a predicted molecular weight of 65 kDa. Sequence analysis has indicated that the cassava protein has 43% amino acid sequence identity with white clover linamarase, but is most similar to the β-glucosidase of *Agrobacterium tumefaciens*. Typical of BGA-type β-glucosidases, cassava linamarase has a conserved β-glucosidase active site domain (residues 410–415; YVTENG in the linamarase sequence) located at the C-terminus of the protein (Grabnitz *et al.*, 1991; Tull *et al.*, 1991; Hughes *et al.*, 1992). Additional important protein structural motifs include an *N*-terminal, hydrophobic signal sequence, and five putative *N*-asparagine glycosylation sites (NX(ST)) located in the C-terminal third of the protein (Hughes *et al.*, 1992). *N*-terminal protein sequencing of cassava linamarase has subsequently demonstrated that the first 17 amino acids are removed from the initial translation product (Mkpong and Sayre, unpublished results) as predicted by Hughes *et al.*, (1992).

The presence of an amino-terminal signal sequence and *N*-asparagine glycosylation sites is consistent with the subcellular targeting of the mature enzyme. Previously, it had been shown that the linamarases of white clover and wild lima bean are apoplastic (Kakes, 1985; Frehner and Conn, 1987). Using the assay system developed by Frehner and Conn (1987), Kurzhals *et al.* (1989) demonstrated that about 40–60% of the cassava linamarase was removed from whole leaves in apoplast extracts. Subsequently, White *et al.* (1994) and Grunhert *et al.* (1994) demonstrated that apoplastic forms of the enzyme accounted for the majority (50–80%) of the leaf linamarase activity. The apoplastic localization of cassava linamarase was also confirmed by Mkpong *et al.* (1990) using antibodies generated against cassava linamarase. Using immuno-gold localization techniques, they demonstrated that cassava linamarase was localized in the cell wall. The cell wall, however, is not the only apparent site of linamarase localization. Using linamarase antisense riboprobes, Pancoro *et al.* (1992) demonstrated that linamarase messenger RNA was localized in laticifer cells. Enzyme activity assays also suggested that linamarase was enriched in the latex relative to whole leaf extracts. It was apparent from the results of Pancoro *et al.* (1992), however, that laticifers were not the only site of linamarase localization. They observed that varietal differences in whole leaf linamarase activity were not correlated with

Table 2. *Substrate specificities of cassava linamarase and other β-glucosidases*

Relative activities are expressed as a percentage of the rate for linamarin in each column. ND = not determined. Rates were not determined at the same temperatures, pHs or substrate concentrations for the different experiments. See original references for experimental details. (a) = Mkpong *et al.*, 1990, (b) = Eksittikul and Chulavatanatol, 1988, (c) = Yeoh and Yeoh, 1994, (d) = Selmar *et al.*, 1987. NP = nitrophenol.

Substrate	Cassava linamarase			β-glucosidases	
	(a)	(b)	(c)	*M. glaziovi* (c)	*H. brasiliensis* (d)
Linamarin	100	100	100	100	100
Lotaustralin	ND	ND	ND	ND	52
Linustatin	ND	ND	ND	ND	0
Neolinustatin	ND	ND	ND	ND	0
Prunasin	65	ND	3.3	0	0.3
Amygdalin	0	ND	0	ND	0
Cellobiose	1.1	ND	0	ND	0
*p*NPβGlucoside (c)	65	441	611	72	153
*o*NPβGlucoside	ND	ND	ND	ND	583
*p*NPβGalactoside	ND	ND	22	3.6	6.0
*o*NPβGalactoside	19	ND	ND	ND	9.2
*p*NPβMannoside	ND	ND	22	3.6	2.0
*p*NPβFucoside	ND	459	611	78	ND

latex linamarase activity. For example, the whole leaf linamarase activity of cassava cultivar MFij4 was demonstrated 7-fold higher than that of cultivar MNgal, however, the relative activity of MFij4 latex linamarase was 13% lower than that of MNgal. Overall, these results suggest that latex has elevated linamarase activity, but probably accounts for only a fraction of the total leaf linamarase activity. The linamarase present in cell walls undoubtedly accounts for the majority of whole leaf linamarase activity.

The distribution of linamarase activity and linamarin varies between different organs and tissues of the same plant. Leaves may have 3–50-fold higher levels of linamarase activity than do roots. Significantly, this range in values is not due to variations in leaf linamarase activity but apparently due to varietal variations in root linamarase activity (Cooke *et al.*, 1978; Yeoh, 1988; Mkpong *et al.*, 1990). In addition, there are tissue specific differences in linamarase levels within the same plant organ. The root peel (the outer 2–5 mm of the root cortex) may have a 15-fold higher linamarase activity (protein basis) than the inner root parenchyma (Yeoh, 1988). While the linamarin content of leaves is similar regardless of cultivar type (low or high cyanogenic cultivars) the root linamarin content may vary by 50-fold (Mkpong *et al.*, 1990; Bradbury *et al.*, 1991). Furthermore the distribution of linamarin in the roots is not uniform. The root peel typically has a higher (12-fold) linamarin concentration than the inner parenchyma (Bradbury *et al.*, 1991; Balagopalan *et al.*, 1988).

The elevated levels of linamarin and linamarase activity in root peels may affect the resistance to certain root herbivores (Arias and Bellotti, 1984; Bellotti and Arias, 1993). As noted by Bellotti and co-workers, the youngest instar generations of the burrowing bug, *Cyrtomenus bergi*, are more sensitive to feeding on high cyanogenic roots than low cyanogenic roots. Furthermore, the young instar generations are more susceptible to poisoning than are the adult generations. The basis for this developmental difference in cyanogen sensitivity may reside in the length of the insect's feeding stylet (Bellotti and Arias, 1993). The feeding stylets of the younger instar generations are unable to pass through the root peel (cortex) to the less toxic phloem parenchyma. In contrast, the longer stylets of the adults pass through the toxic peel, resulting in lower mortality when feeding on high cyanogenic cassava. Interestingly, both young and adult instars survive on low cyanogenic cultivars indicating that high cyanogenic glycoside levels effectively reduce herbivory in cassava, particularly by root surface feeders.

Hydroxynitrile lyase

The final step of cyanogenesis from linamarin is the breakdown of acetone cyanohydrin to cyanide and acetone (Fig. 1). This reaction can occur spontaneously at temperatures greater than 35 °C or at pHs greater than 4.0. Acetone cyanohydrin can also be converted to cyanide and acetone by hydroxynitrile lyase (HNL) (Carvalho, 1981; White and Sayre, 1993; White *et al.*, 1994; Hughes *et al.*, 1994). Undoubtedly, both the spontaneous and the enzyme catalysed decomposition of acetone cyanohydrin affect the cyanogen content of cassava food products. As shown by Rosling and co-workers, fermentation at pHs ($\leqslant 4.0$) which inhibit the spontaneous decomposition of acetone cyanohydrin, result in a more toxic food product (Tylleskar *et al.*, 1992). Processing procedures which include incubation at elevated pHs ($\geqslant 5.0$) or a final heat treatment following linamarin hydrolysis facilitate the decomposition of acetone cyanohydrin and thereby reduce the toxicity of the food product.

The plant hydroxynitrile lyases are a diverse group of

enzymes. Based on their cofactor requirements HNLs can be subdivided into two broad classes, those that contain flavins (FAD) and those that do not (Table 3). The flavin-containing HNLs are restricted to the Rosaceae and have substantially higher specific activities and lower K_m's (for the α-hydroxynitrile) than do non-flavin HNLs. In addition, flavin-containing HNLs are glycosylated, unlike most non-flavin HNLs (Carvalho, 1981; Yemm and Poulton, 1986; Xu *et al.*, 1986; Kukori and Conn, 1989; Selmar *et al.*, 1989; Wajant and Mundry, 1993; Hughes *et al.*, 1994).

Cassava HNL was first characterized by Carvalho (1981) following its isolation from an acetone leaf extract using a seven-step purification procedure. The cassava enzyme has a subunit molecular weight of 28.5 kDa, but may form various homo-oligomeric complexes as a function of ionic strength and protein concentration (Carvalho, 1981). In 1994, Hughes *et al.* reported that the native enzyme was a homo-trimer, an uncommon oligomeric state for a soluble enzyme. In addition, HNL was reported to have unusual substrate-dependent rate kinetics (Carvalho, 1981). In contrast to HNLs from other organisms (Cutler and Conn, 1981), cassava HNL has non-Michaelis–Menten rate kinetics (Carvalho, 1981; Hughes *et al.*, 1994). The cassava HNL has been reported to have a specific activity of 2.1 mmol mg^{-1} protein h^{-1}, but a very high K_m (500 mM) for acetone cyanohydrin (Carvalho, 1981; Hughes *et al.*, 1994). The unusual rate kinetics could be modelled using an allosteric regulation model, however, the high K_m in relation to specific activity is perplexing and implies a low catalytic efficiency.

Recently, cassava HNL was isolated and characterized by White *et al.* (1994). In contrast to other investigators (Carvalho, 1981; Hughes *et al.*, 1994), White *et al.* (1994) used apoplast extracts as the starting material for their purification. Cassava apoplast extracts were preferred for HNL isolation since they have a 10-fold higher specific activity than acetone extracts of whole leaves (Carvalho, 1981; Hughes *et al.*, 1994) (Table 3). The apoplastic HNL was isolated via a four-step procedure including size exclusion, hydroxyapatite, and concanavalin A chromatography. This rapid and more gentle isolation procedure gave an enzyme having a 10-fold higher specific activity than the cassava HNL isolated from acetone extracts by Hughes *et al.* (1994) (Table 3). The reason(s) for the apparent differences in specific activities for HNLs isolated by the different procedures is not known, however, the higher activities measured by White *et al.* (1994) could not be attributed to the spontaneous decomposition of acetone cyanohydrin since they subtracted the spontaneous rate from the total rate of cyanide generation. These precautions also allowed White *et al.* (1994), to determine the optimal pH for HNL activity. As shown in Table 3, the optimal pH (5.5) for cassava HNL activity is identical to that for HNLs from other species. Similar to linamarase, HNL has been shown to be a very stable enzyme with little or no loss in enzyme activity following one month's storage at 4 °C (Hughes *et al.*, 1994; White *et al.*, 1994).

The presence of HNL in apoplast extracts implies that HNL may be localized in cell walls similar to linamarase. Based on the intensity of Coomassie blue-stained proteins separated on SDS–PAGE gels, it was demonstrated that cassava leaf apoplast extracts had approximately equal amounts of HNL and linamarase (White *et al.*, 1994). HNL and linamarase each accounted for approximately 30% of the total apoplastic protein extract. In addition, apoplast extracts have an 8-fold higher HNL activity

Table 3. *Kinetic and structural properties of hydroxynitrile lyases from select species*

References: *Hevea brasiliensis* (Selmar *et al.*, 1989); *Linum usitatissimum* (Cutler and Conn, 1981); *Sorghum bicolor* (Wajant and Mundry, 1983); *Ximenia americana* (Kukori and Conn, 1989); *Manihot esculenta* (Hughes *et al.*, 1994; White *et al.*, 1994); *Prunus lyonii* (Xu *et al.*, 1986); *Prunus serotina* (Yemm and Poulton, 1986).

Species	pH optimum	K_m (mM)	V_{max}[a]	Subunit size (kDa)	Native enzyme (kDa)	*pI*	Glycosylated	Flavin component
Non-Rosaceae								
Hevea brasiliensis	5.5	0.7	1.3	—	—	—	—	—
Linum usitatissimum	5.5	2.5	2.0	42	82	4.5–4.8	no	no
Sorghum bicolor	—	—	—	$\alpha=22$ $\beta=33$	$2\alpha2\beta$	—	yes	—
Ximenia americana	5.5	0.28	27.2	36.5	36.5	—	yes	no
Manihot esculenta (Hughes *et al.*, 1994)	3.5–5.4	500	2.1	28.5	92	4.1–4.6	no	no
Manihot esculenta (White *et al.*, 1994)	5.5	—	24	28.5	—	—	no	no
Rosaceae								
Prunus lyonii	5.5	0.09	6.6×10^3	59	59	4.75	yes	yes
Prunus serotina	6–7	0.17	—	57–59	57–59	4.6	yes	yes

[a] mmol mg^{-1} protein h^{-1}.

than whole leaf extracts. These results suggest that both HNL and linamarase are present in cell walls and can act in series to produce cyanide from linamarin.

As previously indicated, the hydroxynitrile lyases are a diverse group of enzymes (Table 3). There are a variety of subunit sizes and glycosylation patterns. In addition, some forms of the enzyme contain flavin and some do not. In 1994, Hughes *et al.* (1994) reported the sequence of a cassava HNL cDNA clone. Not surprisingly, the predicted amino acid sequence of the cassava HNL had no similarity to any other known HNL genes or proteins, including the HNL from *Prunus serotina* (Cheng and Poulton, 1993). In addition, cassava HNL apparently does not have a proteolytically processed amino-terminal signal sequence nor is it glycosylated (Hughes *et al.*, 1994). The absence of an apparent targeting sequence is not unusual, however, for low molecular weight cell wall proteins (Li *et al.*, 1993). In summary, it is probable that the whole leaf activities and localization of HNL are similar to linamarase and that HNL acts in series with linamarase to produce cyanide.

Cultivar-dependent differences in cyanogen accumulation

The physiological basis for cultivar-dependent differences in root linamarin content remains one of the more perplexing and controversial aspects of cyanogenesis in cassava. Several lines of evidence suggest that the linamarin present in roots is synthesized in shoots. Stem-girdling experiments have indicated that linamarin is transported from the shoot to the root (De Bruijn, 1973). Similarly, studies on the cyanogen content of roots harvested from grafts (root–shoot) between low and high cyanogenic potential cultivars suggest that the linamarin content of roots may be determined in part by contributions from the shoot (Makame *et al.*, 1987). In addition, ^{14}C-valine labelling studies with germinated seedlings have indicated that primary roots do not synthesize linamarin (Koch *et al.*, 1992).

The apparent mechanism by which linamarin could be transported from the shoot to the root, however, is unknown. The presence of linamarase in cell walls would preclude the apoplastic transport of linamarin since it presumably would be cyanogenic. Therefore, other mechanisms for cyanogen transport must be considered. Two alternative mechanisms for cyanogen transport in plants have been proposed by Selmar and co-workers (Selmar, 1993; Selmar *et al.*, 1988). According to their model, linamarin could be transported either symplastically via the phloem or apoplastically as the non-hydrolysable glucoside of linamarin, linustatin. A linustatin pathway of apoplastic cyanogen transport apparently operates in *Hevea* and may also occur in cassava seedlings (Selmar, 1994; Selmar *et al.*, 1988; Koch *et al.*, 1992). In *Hevea*, linamarin is converted to linustatin at the source site and transported apoplastically to the sink. At the sink site linustatin is deglycosylated either by a 'simultaneous' diglycosidase, which produces gentiobiose and acetone cyanohydrin, or it is sequentially deglycosylated to linamarin and then acetone cyanohydrin. In the simultaneous diglycosidase pathway, acetone cyanohydrin is either converted to cyanide and acetone or re-glycosylated by a UDP-glucosyltransferase to form linamarin. Any cyanide produced is then re-assimilated by β-cyanoalanine synthase and subsequently hydrated to form asparagine (Nartey, 1968). In the sequential deglycosylation pathway, linamarin may be stored at the sink or hydrolysed to acetone cyanohydrin. Any cyanide produced from acetone cyanohydrin decomposition would be reassimilated to produce asparagine. Since linamarin is an intermediate of the sequential diglycosidase pathway it is more likely that this pathway is involved in cyanogen redistribution throughout the plant than in redistribution of nitrogen for amino acid and protein synthesis (simultaneous pathway).

The simultaneous linustatin diglycosidase pathway apparently plays an important role in nitrogen transport during *Hevea* seed germination. In germinating *Hevea* seeds the seedling linustatin diglycosidase and β-cyanoalanine synthase activities increase co-ordinately with the mobilization of linustatin from seed reserves. Furthermore, the mobilization of linustatin is associated with (1) a reduction in endosperm linamarin levels, (2) the occurrence of linustatin in the seedling phloem, and (3) a reduction in the seed linustatin diglucosidase and β-cyanoalanine synthase enzyme activities (Selmar *et al.*, 1988, Selmar, 1993). These results indicate that *Hevea* endosperm linamarin functions as a mobilizable form of nitrogen for the growing seedling (Selmar, 1993).

Evidence in support of the operation of a cyanogen nitrogen transport pathway in germinating cassava seedlings is less compelling. Analyses of the cyanogen and free amino acid content of cassava seeds indicated that there is no linamarin and little free valine available for linamarin synthesis (Nartey, 1968). Cassava seeds apparently store reduced nitrogen in the form of free asparagine and proline (Nartey, 1968). Furthermore, growing seedlings apparently accumulate asparagine and glutamine rather than linamarin. These results suggest that asparagine and not linamarin is the major transportable form of nitrogen in germinating cassava seedlings (Nartey, 1968). After germination, however, cassava seedlings synthesize both linamarin and linustatin, suggesting the operation of a linustatin nitrogen transport system at later stages in development (Koch *et al.*, 1992; Selmar, 1994). Consistent with the temporal operation of a linustatin nitrogen transport system, Nartey (1968) demonstrated that cassava seedlings could assimilate cyanide into amino

acids. Cassava seedlings exposed to gaseous $H^{14}CN$ assimilated nitrogen into asparagine, apparently via β-cyanoalanine synthase and hydrase activity. The demonstration that β-cyanoalanine synthase was the primary mechanism of cyanide assimilation was supported by the fact that over 80% of the assimilated label was present in asparagine and that 97% of that label was in the C4 position. These results indicate that $H^{14}CN$ probably reacts with a C3 acceptor (e.g. cysteine) in a reaction catalysed by β-cyanoalanine synthase. At present, however, the relative age-dependent contributions of asparagine endosperm reserves and *de novo* linamarin/linustatin synthesis to nitrogen transport in germinating cassava seedlings has not been resolved.

In contrast to germinating cassava seedlings, there is less evidence to support the operation of a linustatin cyanogen transport system in mature cassava. Linustatin has been detected in mature cassava, but the quantities are exceedingly low (Selmar, 1994). In fact, several investigators have been unable to detect linustatin in mature cassava tissues either by analysis of whole plant extracts using HPLC chromatography and a refractive index detector or by analyses (thin layer chromatography/radiography) of extracts obtained from ^{14}C-valine labelled (48 h) cassava leaves and petioles (Bainbridge *et al.*, 1993; White *et al.*, 1994; McMahon and Sayre, 1993). There also are conflicting reports on the presence of linustatin and linamarin in phloem exudates (foliar droplets). Pereira and Splittstoesser (1987) reported that foliar droplets had no linamarin and were composed mostly of fructans. Similarly, Selmar (1994) was unable to detect linamarin in phloem exudates, but did detect linustatin (no values given). In contrast, Calatayud *et al.* (1994) reported that foliar droplets contain linamarin at concentrations (1.5 mg g^{-1} dw) equivalent to those found in whole leaves, results which suggested that linamarin could be transported symplastically.

The overriding concern, however, must be whether cyanogen transport from leaves to roots is necessary to account for the varietal differences observed in root cyanogen content. In order to characterize the biochemical factors which determine the steady-state linamarin content of roots, McMahon and Sayre (1995) reinvestigated the ability of leaves and roots to synthesize linamarin and/or linustatin from ^{14}C-valine. They demonstrated that intact leaves were capable of synthesizing linamarin, however, no linustatin was detected in either leaf or petiolar (transported) extracts (following 48 h incubation with ^{14}C-valine) regardless of cultivar (high or low cyanogen) type. Interestingly, linamarin was detected in petiolar extracts suggesting that it could be transported from the leaves, however, ^{14}C-valine was detected in petiolar extracts as well. Therefore, it could not be determined whether the labelled linamarin present in the petiolar extracts was transported from the leaves or newly synthesized in the petiole. The inability to detect linustatin in any extract, however, implied that differential linustatin transport probably does not account for cultivar-dependent differences in root cyanogenic potentials. In order to determine whether cultivar-dependent differences in root linamarin content were associated with differences in leaf linamarin synthesis rates, whole leaves were labelled with ^{14}C-valine and rates of linamarin synthesis were determined. These studies indicated that linamarin synthesis rates may vary in a cultivar- and age-dependent manner. Leaves of similar position (fifth from apex) of young (49 d from germination) high cyanogenic cultivars synthesized linamarin at twice the rates of leaves from low cyanogenic cultivars (White *et al.*, 1994). As the plants aged, however, leaves of similar position on older plants (83-d-old plants) did not synthesize linamarin from externally applied ^{14}C-valine. In order to determine whether these results were due to experimental artifacts or reflected the true physiological state of the plant isolated leaf protoplasts from young and old plants were labelled with ^{14}C-valine and linamarin synthesis rates determined. In contrast to intact leaves, leaf protoplasts from young or old plants, and from low or high cyanogenic cultivars all had similar rates of linamarin synthesis (McMahon and Sayre, 1995). These results suggest that differences in valine loading or metabolism in intact leaves may account for the observed age- and cultivar-dependent differences in linamarin synthesis in intact leaves.

Unfortunately, these results do not resolve the question of what is the physiological and biochemical basis for varietal differences in root linamarin content. As previously indicated, it had been generally assumed that roots did not synthesize linamarin and that linamarin present in roots was transported from the top of the plant. In order to characterize the parameters which determine root linamarin content more fully it was necessary to reinvestigate the possibility that secondary roots could synthesize linamarin from ^{14}C-valine. Using secondary roots from low- and high-cyanogenic cultivars of young plants (49-d-old) McMahon and Sayre (1995) demonstrated that roots were capable of synthesizing linamarin at rates (fresh weight basis) equivalent to leaves of high cyanogenic cultivars (White *et al.*, 1994). Similar to intact leaves, however, intact secondary roots of older plants (8 months) synthesized linamarin at 10-fold lower rates than young plants (1.5 months). It was apparent from these studies, however, that both roots and leaves were capable of synthesizing linamarin. Whether there are age-dependent differences in root linamarin synthesis is less well resolved (see Cutler and Conn, 1981). To date, protoplasts have not been isolated from cassava roots for ^{14}C-valine labelling studies. Overall, it is apparent that both shoots and roots can synthesize linamarin, however,

the factors which determine the steady-state linamarin content of roots remain to be identified.

Cyanide metabolism in cassava

As indicated in the introduction, all plants surveyed to date have been shown to contain β-cyanoalanine synthase activity, presumably to metabolize free cyanide. While cyanide metabolism in cassava has not been extensively analysed, it has been demonstrated that all tissues have the capability to metabolize cyanide. This conclusion is based on the observation that all tissues have high levels of β-cyanoalanine synthase activity, incorporate free cyanide into C4 compounds (presumably by β-cyanoalanine synthase), and have rhodanese activity which catalyses the formation of thiocyanate from CN^- and $S_2O_3^{2-}$ (Blumenthal *et al.*, 1968; Kakes and Hakvoort, 1992; Nambisan, 1993). At present, the evidence suggests that any free cyanide is incorporated into asparagine via the concerted activities of β-cyanoalanine synthase and β-cyanoalanine hydrase (Castric *et al.*, 1972). While the levels of free cyanide production in intact cyanogenic plants remains to be determined it is not likely to be great.

A model summarizing our current understanding of cyanogenesis in a cassava leaf mesophyll cell is shown in Fig. 2. As noted, linamarin is synthesized and accumulates in the vacuole. Following rupture of the cell, the linamarin is converted to cyanide by the linamarase and HNL present in the cell wall. At present, cyanogen transport and tissue specific differences in cyanide metabolism are not sufficiently characterized to model cyanogen metabolism at the whole plant level.

Future directions

There are both practical issues as well as basic biochemical and physiological aspects of cyanogenesis which need to be addressed in cassava. The accumulated evidence indicates that cassava is a highly productive and safe crop which can be grown under poor agronomic conditions (Cock, 1985). Furthermore, efficient processing of cassava can eliminate cyanogens in food products. Cyanogen removal is facilitated by practices which facilitate linamarin hydrolysis. This can be achieved by soaking and grinding the tissue (Cock, 1985), by adding exogenous linamarase (Padmaja *et al.*, 1993), by fermentation with linamarin metabolizing bacteria (Legras *et al.*, 1990), and possibly by producing transgenic plants which overexpress linamarase. Processing techniques which facilitate the access of linamarin to linamarase (e.g. cell wall dissolution or maceration) also enhance cyanogen turnover (Andrew Westby, London, personal communication). Acetone cyanohydrin levels also can be reduced by elevating the pH or temperature of the cassava processing procedure. In addition, processes which lead to increased

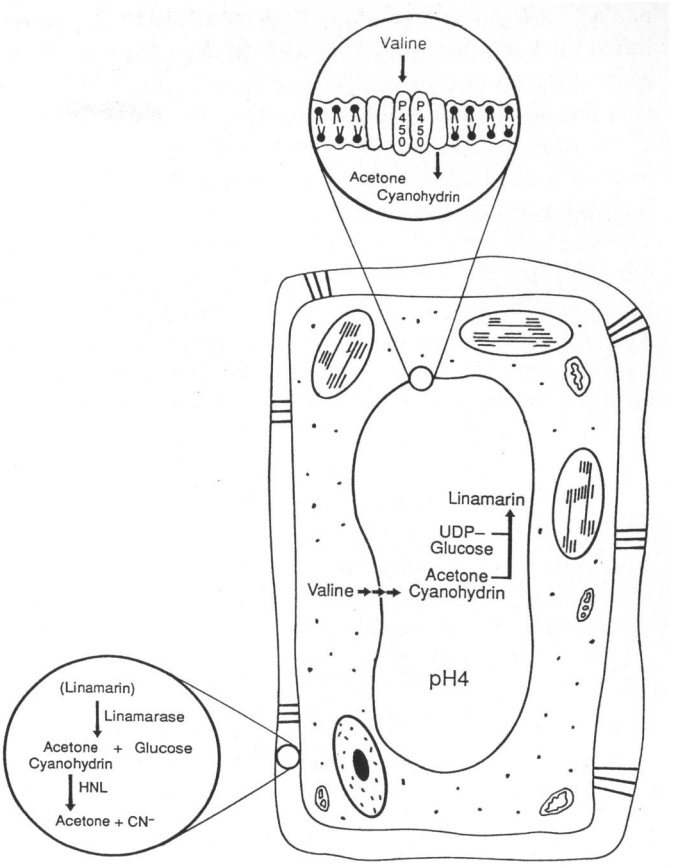

Fig. 2. A model for linamarin synthesis and cyanide production following rupture of a cassava leaf mesophyll cell.

HNL activity during cassava processing could reduce the cyanogen content. It is now apparent that a combination of practices or strategies which facilitate both linamarin hydrolysis and the elimination of acetone cyanohydrin are necessary to facilitate cyanogen removal. It is also apparent that the functions of cyanogens in cassava are not well resolved. It is not clear whether the cyanogenic glycosides present in leaves and roots serve the same purpose. Are they nitrogen storage reserves, plant defence chemicals, or both? What determines the steady-state linamarin content of leaves, shoots, and roots? What is the enzymology of linamarin synthesis? These and other questions are currently being addressed by both biochemical and molecular genetic approaches. Tissue specific labelling studies with linamarin precursors will help quantify the contributions of cyanogen synthesis from the various organs to root linamarin levels. Labelling studies may also reveal the form(s) by which linamarin is transported between tissues. Biochemical studies will lead to the isolation and localization of the linamarin synthesizing enzymes. In addition, transgenic plants, which are capable of synthesizing linamarin in only the roots or shoots, can be used to determine the contributions of different tissues to steady-state root linamarin

levels. Ultimately, the relationships between cyanogen synthesis, turnover and transport, and cyanide assimilation will need to be characterized before the functions of cyanogenic glycosides in cassava are fully understood.

Acknowledgements

The original research reported in this paper was supported by the U.S. Agency for International Development and the Rockefeller Foundation to RTS. We thank Diana Arias-Garzon for critical reading of the manuscript, Jeffrey Rodriguez for laboratory assistance and James B. White for illustrations. In addition, we thank Drs M. Bokanga, M. Hughes, P. Kakes, B. Møller, J. Streeter, and D. Selmar for useful discussions.

References

Arias B, Bellotti AC. 1984. *Aspectos ecologicos y de manejo de* Cyrtomenus bergi *Froeschner, chinche de la viruela en el cultivo de la yuca* Manihot esculenta *Crantz*. Cali, Colombia: CIAT Publication.

Bainbridge Z, Southwell K, Poulter NH. 1993. Development of HPLC techniques for cyanogenesis in cassava. In: Roca WM, Thro AM, eds. *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, Cartegena, Colombia*, 25–28 *August* 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, 400–5.

Balagopalan C, Padmaja G, Nanda S, Morthy S. (eds) 1988. *Cassava in food, feed and industry*. Boca Raton, Florida: CRC Press.

Bellotti AC, Arias B. 1993. The possible role of HCN on the biology and feeding behavior of the cassava burrowing bug (*Cyrtomenus bergi* Froeschner). In: Roca WM, Thro AM, eds. *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, Cartegena, Colombia*, 25–28 *August* 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, 406–9.

Best R, Hargrove TR. 1994. *Cassava: The latest facts about an ancient crop*. Cali, Colombia: CIAT Publication.

Blumenthal SG, Hendrickson HR, Abrol YP, Conn EE. 1968. Cyanide metabolism in higher plants. The formation of β-cyanoalanine. *Journal of Biological Chemistry* **243,** 5302–7.

Bokanga M, Halkier B, Møller B. 1993. Studies on the biosynthesis of cyanogenic glucosides in cassava. In: Roca WM, Thro AM, eds. *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, Cartegena, Colombia*, 25–28 *August* 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, 418–23.

Bradbury JH, Egan SV, Lynch MJ. 1991. Analysis of cyanide in cassava using acid hydrolysis of cyanogenic glycosides. *Journal of the Science of Food and Agriculture* **55,** 277–90.

Bradbury JH, Egan SV. 1992. Rapid screening assay of cyanide content of cassava. *Phytochemical Analysis* **3,** 91–4.

Brimer L, Dalgaard L. 1984. Cyanogenic glycosides and cyanohydrins in plant tissues—qualitative and quantitative determination by enzymatic post-column cleavage and electrochemical detection, after separation by HPLC. *Journal of Chromatography* **303,** 77–88.

Butler GW, Conn EE. 1964. Biosynthesis of the cyanogenic glycosides linamarin and lotaustralin. *Journal of Biological Chemistry* **239,** 1674–9.

Carvalho JPCC. 1981. Studies on the aliphatic ketone cyanohydrin hydroxynitrile lyase from *Manihot esculenta* Crantz. PhD thesis, University of California, Davis.

Calatayud PA, Rahbe Y, Delobel B, Khuong-Huu F, Tertuliano M, Le Ru B. 1994. Influence of secondary compounds in the phloem sap of cassava on expression of antibiosis towards the mealybug *Phenacoccus manihoti*. *Entomology Experimental Applications* **72,** 47–57.

Castric PA, Farnden KF, Conn EE. 1972. Cyanide metabolism in higher plants. 5. The formation of asparagine from β-cyanoalanine. *Archives of Biochemistry and Biophysics* **152,** 62–9.

Cheng I-P, Poulton JE. 1993. Cloning of a cDNA of *Prunus serotina* (*R*)-(+)-mandelonitrile lyase and identification of a putative FAD-binding site. *Plant Cell Physiology* **34,** 1139–43.

Cock JH. 1985. *Cassava: new potential for a neglected crop*. London: Westfield Press. 191pp.

Cooke RD, Blake GG, Battershill JM. 1978. Purification of cassava linamarase. *Phytochemistry* **17,** 381–3.

Cutler AJ, Conn EE. 1981. The biosynthesis of cyanogenic glucosides in *Linum usitatissimum* (Linen flax) *in vitro*. *Archives of Biochemistry and Biophysics* **212,** 468–74.

De Bruijn GH. 1973. The cyanogenic character of cassava (*Manihot esculenta*). In: Nestel B, MacIntyre R, eds. *Chronic cassava toxicity*. Proceedings of an Interdisciplinary Workshop, London. IDRC, Ottowa, IDRC-010e.

Eksittikul T, Chulavatnatol M. 1988. Characterization of cyanogenic β-glucosidase (linamarase) from cassava (*Manihot esculenta* Crantz). *Archives of Biochemistry and Biophysics* **266,** 263–9.

Esashi Y, Sakai Y, Ushizawa R. 1981. Cyanide-sensitive and cyanide-resistant respiration in the germination of cocklebur seeds. *Plant Physiology* **67,** 503–8.

Frehner M, Conn EE. 1987. The linamarin β-glucosidase in Costa Rican wild lima beans (*Phaseolus lunatus* L.) is apoplastic. *Plant Physiology* **84,** 1296–1300.

Grabnitz F, Seiss M, Rucknagel KP, Staudenbauer WL. 1991. Structure of the β-glucosidase gene *bgl*A of *Clostridium thermocellum*. *European Journal of Biochemistry* **200,** 301–9.

Gruhnert CH, Biehl B, Selmar D. 1994. Compartmentalization of cyanogenic glucosides and their degrading enzymes. *Planta* **195,** 36–42.

Hahlbrock K, Conn EE. 1971. Evidence for the formation of linamarin and lotaustralin in flax seedlings by the same glucosyltransferase. *Phytochemistry* **10,** 1019–23.

Hughes J, Carvalho FJP, De C, Hughes MA. 1994. Purification, characterization, and cloning of α-hydroxynitrile lyase from cassava (*Manihot esculenta* Crantz). *Archives of Biochemistry and Biophysics* **311,** 496–502.

Hughes MA, Brown K, Pancoro A, Murray BS, Pxtoby E, Hughes J. 1992. A molecular and biochemical analysis of the structure of the cyanogenic β-glucoside (linamarase) from cassava (*Manihot esculenta* Crantz). *Archives of Biochemistry and Biophysics* **295,** 273–9.

Itoh-Nashida T, Hiraiwa M, Uda Y. 1987. Purification and properties of β-D-glucosidase (linamarase) from the butter bean, *Phaseolus lunatus*. *Journal of Biochemistry* **101,** 847–54.

Kakes P. 1985. Linamarase and other β-glucosidases are present in the cell walls of *Trifolium repens* L. leaves. *Planta* **166,** 156–60.

Kakes P. 1990. Properties and functions of the cyanogenic system in higher plants. *Euphytica* **48,** 25–43.

Kakes P, Hakvoort H. 1992. Is there rhodanese in plants? *Phytochemistry* **31,** 1501–5.

Koch B, Nielsen VS, Halkier BA, Olsen CE, Møller BL. 1992. The biosynthesis of cyanogenic glycosides in seedlings of

cassava (*Manihot esculenta* Crantz). *Archives of Biochemistry and Biophysics* **292,** 141–50.

Kukori GW, Conn EE. 1989. Mandelonitrile lyase from *Ximenia americana* L.: stereospecificity and lack of a flavin prosthetic group. *Proceedings of the National Academy of Sciences, USA* **86,** 6978–81.

Kurzhals C, Grutzmacher H, Selmar D, Biehl B. 1989. Linustatin, the linamarin-glycoside protected against cleavage by apoplastic linamarase. *Planta Medica* **55,** 673 (abstract).

Legras JL, Jory M, Arnaud A, Galzy P. 1990. Detoxification of cassava pulp using *Brevibacterium* sp. R312. *Applied Microbiology and Biotechnology* **33,** 529–33.

Li Z-C, Durachko DM, Cosgrove DJ. 1993. An oat coleoptile wall protein that induces cell wall extension *in vitro* and that is antigenically related to a similar protein from cucumber hypocotyls. *Planta* **191,** 349–56.

Makame M, Akoroda MO, Hahn SK. 1987. Effects of reciprocal stem grafts on cyanide translocation in cassava. *Journal of Agricultural Science, Cambridge* **109,** 605–8.

McMahon JM, Sayre RT. 1993. Differential biosynthesis and transport of linamarin in high- and low-cyanide cultivars of cassava (*Manihot esculenta* Crantz). In: Roca WM, Thro AM, eds. *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, Cartegena, Colombia,* 25–28 *August* 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, 376–8.

McMahon JM, Sayre RT. 1995. Regulation of cyanogenesis in cassava (*Manihot esculenta* Crantz). In: Roca WM, Thro AM, eds. *Proceedings of the second international scientific meeting of the Cassava Biotechnology Network, Bogor, Indonesia* (in press).

Mederacke H, Selmar D, Biehl B. 1995. Glucosyltransferases of the cyanogenic plant cassava (*Manihot esculenta*). *Journal of Applied Botany* (in press).

Miller JM, Conn EE. 1980. Metabolism of hydrogen cyanide by higher plants. *Plant Physiology* **65,** 1199–1202.

Mkpong OE, Yan H, Chism G, Sayre RT. 1990. Purification, characterization, and localization of linamarase in cassava. *Plant Physiology* **93,** 176–81.

Nambisan B. 1993. Cyanogenesis in cassava. In: Roca WM, Thro AM, eds. *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, Cartegena, Colombia,* 25–28 *August* 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, 424–7.

Nartey F. 1968. Studies on cassava, *Manihot utilissima* Pohl. *Phytochemistry* **7,** 1307–12.

Osuntokun BO. 1981. Cassava diet, chronic cyanide intoxification and neuropathy in Nigerian Africans. *World Review of Nutrition and Dietetics* **36,** 141–73.

Padmaja G, George M, Moorthy SN. 1993. Detoxification of cassava during fermentation with a mixed culture inoculum. *Journal of the Science of Food and Agriculture* **63,** 473–81.

Pancoro A, Hughes MA. 1992. *In situ* localization of cyanogenic-glucosidase (linamarase) gene expression in leaves of cassava (*Manihot esculenta* Crantz) using non-isotopic riboprobes. *The Plant Journal* **2,** 821–7.

Peiser G, Wang TT, Hoffman NE, Yang SF, Lui HW, Walsh CT. 1984. Formation of cyanide from carbon of ACC during its conversion to ethylene. *Proceedings of the National Academy of Sciences, USA* **81,** 3059–62.

Pereira JF, Splittstoesser WE. 1987. Exudate from cassava leaves. *Agricultural Ecosystems and Environment* **18,** 191–4.

Poulton JE. 1990. Cyanogenesis in plants. *Plant Physiology* **94,** 401–5.

Rosling H, Mlingi N, Tylleskar T, Banea M. 1993. Causal mechanisms behind human diseases induced by cyanide exposure from cassava. In: Roca WM, Thro AM, eds. *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, Cartegena, Colombia,* 25–28 *August* 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, 366–75.

Saunders JA, Conn EE. 1978. Presence of the cyanogenic glycoside dhurrin in isolated vacuoles from *Sorghum*. *Plant Physiology* **61,** 154–7.

Selmar D. 1993. Transport of cyanogenic glycosides: linustatin uptake by *Hevea* cotyledons. *Planta* **191,** 191–9.

Selmar D. 1994. Translocation of cyanogenic glycosides in cassava. *Acta Horticulturae* **375,** 61–8.

Selmar D, Lieberei R, Biehl B. 1988. Mobilization and utilization of cyanogenic glycosides. *Plant Physiology* **86,** 711–16.

Selmar D, Lieberei R, Biehl B, Conn EE. 1989. α-hydroxynitrile lyase in *Hevea brasiliensis* and its significance for rapid cyanogenesis. *Physiologia Plantarum* **75,** 97–101.

Selmar D, Lieberei R, Biehl B, Voigt J. 1987. *Hevea* linamarase—a non-specific β-glycosidase. *Plant Physiology* **83,** 557–63.

Tull D, Withers SG, Gilkes NR, Kilburn DG, Warren RAJ, Aebersold R. 1991. Glutamic acid 274 is the nucleophile in the active site of a 'retaining' exoglucanase from *Cellulomonas fimi*. *Journal of Biological Chemistry* **266,** 15621–5.

Tylleskar T, Cooke RD, Banea M, Poulter NH, Bikangi N, Rosling H. 1992. Cassava cyanogens and konzo, an upper motoneuron disease found in Africa. *Lancet* **339,** 208–11.

Wajant H, Mundry K-W. 1993. Hydroxynitrile lyase from *Sorghum bicolor*: a glycoprotein heterodimer. *Plant Science* **89,** 127–33.

Wheatley CC, Orrego JI, Sanchez T, Granados E. 1993. Quality evaluation of the cassava core collection at CIAT. In: Roca WM, Thro AM, eds. *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, Cartegena, Colombia,* 25–28 *August* 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, 255–64.

White WLB, McMahon JM, Sayre RT. 1994. Regulation of cyanogenesis in cassava. *Acta Horticulturae* **375,** 69–78.

White WLB, Sayre RT. 1993. Partial purification and characterization of hydroxynitrile lyase from cassava. In: Roca WM, Thro AM, eds. *Proceedings of the first international scientific meeting of the Cassava Biotechnology Network, Cartegena, Colombia,* 25–28 *August* 1992. Cali, Colombia: Centro Internacional de Agricultura Tropical, 379–83.

Xu LL, Singh BK, Conn EE. 1986. Purification and characterization of mandelonitrile lyase from *Prunus lyonii*. *Archives of Biochemistry and Biophysics* **250,** 322–8.

Yemm RS, Poulton JE. 1986. Isolation and characterization of multiple forms of mandelonitrile lyase from mature black cherry (*Prunus serotina*) seeds. *Archives of Biochemistry and Biophysics* **247,** 440–5.

Yeoh H-H. 1988. Kinetic properties of β-glucosidase from cassava. *Phytochemistry* **28,** 721–4.

Yeoh H-H, Yeoh Y-C. 1994. Some properties of β-glucosidases from tropical plant species. *Phytochemistry* **35,** 1391–3.