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REVIEW

Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review

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ABSTRACT A number of species of plants produce repertoire of cyanogenic glycosides *via* a common biosynthetic scheme. Cyanogenic glycosides play pivotal roles in organization of chemical defense system in plants and in plant–insect interactions. Several commercial crop plants such as sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*) and barley (*Hordium vulgare*) are cyanogenic and accumulate significant amounts of cyanogenic glycosides. The study of biosynthesis of dhurrin in sorghum has underpinned several early breakthroughs in cyanogenic glycoside researches. Despite great deal of structural diversity in cyanogenic glycosides, almost all of them are believed to be derived from only six different amino acids L-valine, L-isoleucine, L-leucine, L-phenylalanine, or L-tyrosine and cyclopentenyl-glycine (a non protein amino acid). Our knowledge about biosynthesis of cyanogenic glycosides and molecular regulatory processes underlying their biosynthesis has been increased impressively in the past few years. The rapid identification, characterization and cloning of genes encoding enzymes of the cyanogenic glycoside biosynthetic and catabolic pathways from several plants has greatly facilitated our understanding of cyanogenic glycosides biosynthesis and regulation. Today it is known that enzymes of cyanogenic glycoside biosynthetic pathway in sorghum are organized as metabolon most likely to those of other secondary metabolic pathways. Knowledge of state of art of biosynthesis and regulation of cyanogenic glycosides made possible the metabolic engineering of these pathways resulting in development of transgenics of cassava, tobacco, lotus and Arabidopsis with manipulated cyanogenic glycosides content. Simultaneously, many new developments have been witnessed in methods/techniques/ procedures for detection of cyanogenic glycosides in plant samples, foods and foodstuffs. The present review sequentially discusses all of these issues with updated information gathered from the published reports on cyanogenic glycosides.

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More than 2,600 plant species produce myriad of cyanogenic glycosides (CGs), one of the biggest and extensively studied class of plant secondary metabolites (Moller and Conn 1980; Poulton 1990; Conn 1991; Fleming 1999; Moller and Seigler 1999; Vetter 2000; Zagrobelny et al. 2004). Chemically, CGs are defined as glycosides of α-hydroxynitriles; plants store these compounds in vacuoles (Fleming 1999; Vetter 2000). Most of the plant families producing CGs are belong to the Angyospermatophyta and remaining to the Dicotyledonopsida and Monocotyledonopsida (Vetter et al. 2000). Several important crop plants *viz.,* sorghum (*Sorghum bicolor*) (Moller and Conn 1980; Halkier and Moller 1989; 1990), cassava (*Manihot esculenta*) (Andersen et al. 2000), barley (*Hordium vulgare*; Forslund and Johnson 1997; Nielsen et al. 2002) are among others which essentially biosynthesize and accumulate CGs. Several bacteria and a number of animals particularly those feed on cyanogenic plants also contain CGs. [See review by Vetter (2000) for distribution, functions, biosynthesis and analysis of CGs in plants]. Cyanogenic glycosides have great impact on insect biology as well. Zagrobelny et al. (2004) have elaborately discussed biosynthesis and degradation of CGS in insects emphasizing their roles in plant-insect interactions.

Regarding to the biosynthesis of CGs, they are derived from only six amino acids namely L-valine, L-isoleucine, L-leucine, L-phenylalanine, or L-tyrosine and from a non protein amino acid cyclopentenyl-glycine. Cyanogenic glycoside biosynthesis has been broadly studied in many important plants, sorghum (*Sorghum bicolor*; Moller and Conn 1980; Halikar and Moller 1989; 1990; Sibbesen et al. 1994; 1995; Koch et al. 1995; Jones et al. 1999), cassava (*Manihot esculenta*) (Andersen et al. 2000), seaside arrow grass (*Triglochin maritimum*; Nielsen and Moller 1999; 2000) and barley (*Hordeum vulgare*; Nielsen et al. 2002). However, biosynthesis of dhurrin a tyrosine derived CGs found in sor-

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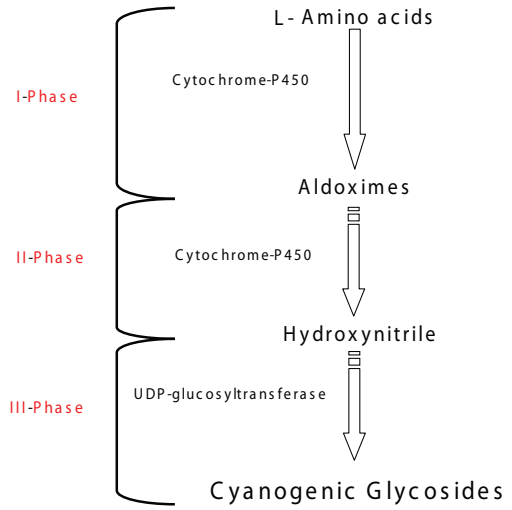

Figure 1. General scheme of the biosynthesis of cyanogenic glycosides in plants.

ghum has been comparatively most elaborately studied among others. Today the knowledge we have about biosynthesis and regulation of CGs in plants has largely gathered from the studies carried out in sorghum. In plants, CGs biosynthetic pathway can be unanimously treated in three steps (Fig. 1). Step- I: A precursor amino acid is converted to aldoxime through two successive N-hydroxylation of amino group of parent amino acid by an enzyme of cytochrome-P450 family. Step- II: aldoxime in turn is converted to cyanohydrin. This reaction is catalyzed by another cytochrome-P450 enzyme. Step- III: cyanohydrins get glycosylated by a soluble enzyme UDP-glucosyltransferase.

In sorghum, two cytochrome enzymes, CYP79A1 and CYP71E1, rcatalyze first and second steps respectively and a UDP-glucosyltransferase catalyzes the final step leading to dhurrin biosynthesis. A recent study has suggested that the above three enzymes are organized as metabolon thereby insuring an efficient channeling of precursors/substrates and intermediates required for biosynthesis of dhurrin in sorghum (Nielsen et al. 2008). Previously, organization of enzymes as metabolon and metabolic channeling has been well documented in biosynthesis of other plant natural products (Jorgensen et al. 2005). In plants and insects, CGs undergoes catabolic processes eventually leading to hydrogen cyanide. Enzymes, β-glucosidases and α-hydroxynitrilases are reported to catalyze catabolism of CGs in plants and insects (Vetter 2000; Zagrobelny et al. 2004).

In recent years, tremendous progress has been made on every aspects of CGs researches, particularly on identification and characterization of genes encoding enzymes of CGs biosynthetic and catabolic pathways rapidly identified and characterized from many cyanogenic plants in past few years (Koch et al. 1995; Hughes et al. 1994; Hasslacher et al. 1996; Trummler and Wajant 1997; Bak et al. 1998; Andersen et al. 2000; Nielsen and Moller 2000; Wang et al. 2004; see Table 2). With these cloned genes, metabolic engineering of pathways leading to CGs has become easier; by using these cDNA, transgenic plants with depleted or enhanced level of CGs have been developed (Bak et al. 2000; Tattersall et al. 2001; Morant et al. 2003, 2007; Siritunga and Sayre 2004a, b; Moller and Bak 2005; Kristensen et al. 2005). Transgenic cyanogens free cassava plant using RNAi technique has been developed (Siritunga and Sayre 2004b). Previously, transgenic cassava plant with negligible cyanogens was developed by selective inhibition of CGs biosynthesis in leaves and roots by antisense expression of CYP79D1/D2 gene fragments (Siritunga and Sayre 2004). Arabidopsis and lotus plants have been successfully engineered by introducing in them an entire dhurrin biosynthetic pathway from sorghum to produce dhurrin (Tattersall et al. 2001; Morant et al. 2003, 2007; Kristensen et al. 2005).

However, a range of articles on almost every aspect of CGs researches are available, information on cloned genes of CGs biosynthesis and catabolism is yet scattered and not available at one place. The main objective of the present article is to provide information on cloned genes of CGs biosynthesis and catabolism. In addition, other aspects of CGs including their occurrence, functions and biosynthesis of CGs derived from six parent amino acids with given emphasis to enzymes, cytochromes P450s and glucosyl transferase have been discussed briefly. Finally, we have included information on technical advancement in the methods of analysis/detection of CGs.

Functions of CGs in plants

Large numbers of CGs are produced in plants to mediate both general and specialized functions. In fact, the primary role of CGs is in organization of chemical defense system in plants and in plant–insect interactions (Zagrobelnya et al. 2004). See review by Zagrobelnya et al. (2004) for detailed description on roles of CGs in plant-insect interactions. CGs have also been described as nitrogen storage compounds (Forslund and Jonsson 1997; Busk and Moller 2002). Moreover, they offer promises as being chemo-taxonomical candidates (Vetter, 2000). For herbivores that are specialists on plants containing CNGs, they serve as phagostimulants (Gleadow and Woodrow 2002). Even, degradation products of CGs such as β-cyanoalanine are reported to serve to deter predators. β-cyanoalanine which possess potent neurotoxin activity is a catabolic product of CGs in some plants (Ressler et al. 1969). These datasets demonstrate that attempts to assign a specific biological role to cyanogenic glucosides may not be meaningful. The function varies dependent on plant species, ecosystem, and abiotic and biotic stress factors. Accordingly, only careful and extensive field trials can decide on

the overall fitness of acyanogenic cassava plants (Jorgensen et al. 2005).

Biosynthesis of CGs

Biosynthesis of CGs have been studied in a number of plants including sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*), seaside arrow grass (*Triglochin maritimum*), and barley (*Hordeum vulgare*; Koch et al. 1995; Jones et al. 1999; Nielsen and Moller, 1999; Andersen et al. 2000; Nielsen et al. 2002; Forslund et al. 2004). Based on these reports all CGs are believed to be biosynthesized from one of the six amino acids, L-valine (L-Val), L-isoleucine (L-Ile), L-leucine (L-Leu), L-phenylalanine (L-Phe) or L-tyrosine (L-Tyr) and cyclopentenyl-glycine (cyclopentenyl-Gly). Several previous studies using labeled amino acids precursors in a number of plants have revealed the tight integration of amino acids with CGs biosynthesis in plants (Vetter 2000). The most elaborately studied CGs is the dhurrin, which is biosynthesized in sorghum plant. Today, all the details surrounding the dhurrin biosynthesis and regulation in sorghum are available including, cloned and characterized genes, intermediates and enzymes involved in its biosynthesis. Two cytochromes-P450s enzymes, CYP79A1 and CYP71E1 and an UDP-glucosyltransferase (UGT85B1) involved in the biosynthesis of the dhurrin in sorghum have been isolated and characterized. Concomitantly with CGs, plants also produce nitrile glucosides but their biosynthesis in plants is poorly understood.

Roles of cytochrome P450senzymes in CGs biosynthesis

Cytochrome-P450s are very well characterized enzymes, ubiquitously appears in many plant and animal species and plays important roles in many biochemical pathways. They have the ability to catalyze a range of biochemical reactions *via* C-hydroxylation and epoxidation, N-and S-oxidations, dehydrations, and O-, N- and S-de-alkylation (Zagrobelny et al. 2004). Many cytochrome-P450s associated with the biosynthesis of terpenoids, volatile phenylpropenes, volatile derived fatty acids, plant hormones (gibberellins), brassinosteroids, ecdysteroids are known (Dudereva et al. 2004; Fischbach and Clardy 2007). Till to date around 800 cytochrome-P450s sequences comprising 53 families are known from plants.

At least two cytochrome P450s enzymes catalyze the first two dedicated steps of CGs biosynthesis. The first committed step of conversion of parent amino acids to aldoximes is catalyzed by cytochrome-P450-I. In the next dedicated step aldoximes are converted into cyanohydrin by action of cytochrome-P450-II. Several cytochrome-P450s enzymes from plants associated with distinct CGs biosynthesis have been isolated and characterized (Koch et al. 1995; Kahn et al. 1997; Bak et al. 1998; Nielsen and Moller, 1999; 2000; Zhang et al. 2003; Andersen et al. 2000; Forslund et al. 2004). One of the first cytochrome-P450s characterized and sequenced was cytochrome-P450tyr from sorghum that catalyzes the conversion of L-tyrosine into Z-p-hydroxy-phenylacetaldoxime the first committed step of the dhurrin biosynthetic pathway. Cytochrome-P450tyr was recognized as the first member of a new family of cytochrome-P450 and given the name CYP79 and now CYP79A1 (Koch et al. 1995; Sibbesen et al. 1995). Also, cytochrome-P450tyr was the first membrane bound multifunctional enzyme isolated from plants. A membrane-bound cytochrome-P450 *N*-hydroxylases type however was known from animals and yeast which had broad substrate specificity catalyzing the hydroxylation of a wide range of xenobiotics. In contrast to cytochrome-P450s *N*-hydroxylases type, cytochrome P450tyr is substrate specific enzyme which hydroxylates only single endogenous substrate, tyrosine (Koch et al. 1995; Sibbesen et al. 1995).

Today several cytochromeP450s are known, two CYP79D1 and CYP79D2 from cassava (Zhang et al. 2003; Andersen et al. 2000) other two CYP79D3 and CYP79D4 from lotus (Forslund et al. 2004) and one CYP79B1 from *Sinapis alba*. The cytochromeP450s, CYP79D1 and CYP79D2 catalyzes the first two committed step in the biosynthesis of linamarin in cassava while CYP79D3 and CYP79D4 are key enzymes in the biosynthesis of cyanogenic glucosides (lotaustralin and linamarin) and glucoside nitrile (rhodenosydes A and D) in lotus plant. The above cytochrome-P450s from sorghum, cassava and lotus are similar in their structure and function. Sequence alignment analysis has revealed homology between lotus's CYP79D3 and CYP79A1 of sorghum and in between CYP79D4 of lotus and CYP73E1 of sorghum (Szczyglowski et al. 1997; Forslund et al. 2004). However, cassava's CYP79D1 and CYP79D2 displayed 54% sequence similarity with CYP79A1 of sorghum. Similarly, CYP79B1 of *S. alba* also showed 54% sequence similarity (with 73% scored similarity) to CYP79A1 of sorghum but CYP79B1 showed 95% sequence similarity to CYP79B2 of Arabidopsis (Bak et al. 1998).

Also, cytochrome-P450s are highly substrate specific and use only one amino acid as a substrate. In a given CGs biosynthetic pathway, the cytochrome P450-I, one, which catalyzes the first committed step comparatively has higher substrate specificity than cytochrome P450-II which has broader substrate specificity. For example, in sorghum the CYP79A1 involved in dhurrin biosynthesis strongly prefers tyrosine as a substrate while the CYP71E1 has broader substrate specificity. The CYP71E1 can broadly hydroxylate the aldoximes derived from phenylalanine, valine and/or isoleucine in addition to those derived from tyrosine (Celenza et al. 2001). The CYP79 of barley, however, is identical to CYP79A1 and CYP79E1 of sorghum but prefers leucine as a substrate and not the tyrosine. An analog of CYP71E1 present in *T. maritima* showed less interest in aldoximes as substrates if derived

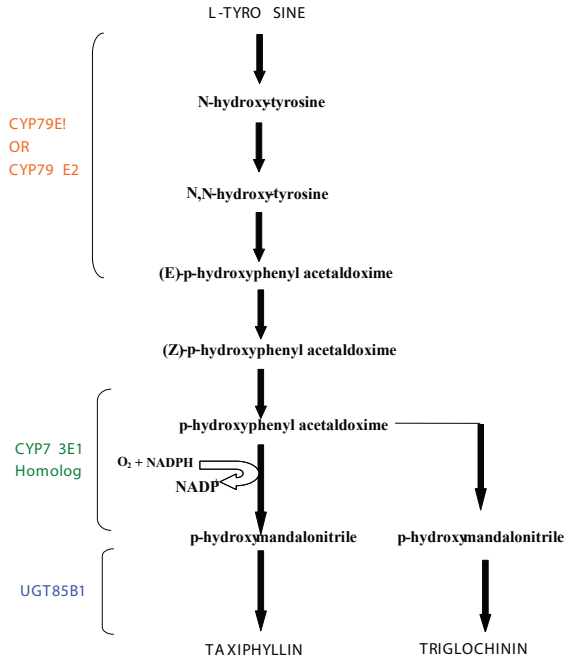

Figure. 2a. Scheme of the biosynthesis of L-tyrosine derived cyanogenic glycoside, dhurrin in *Sorghum bicolor*.

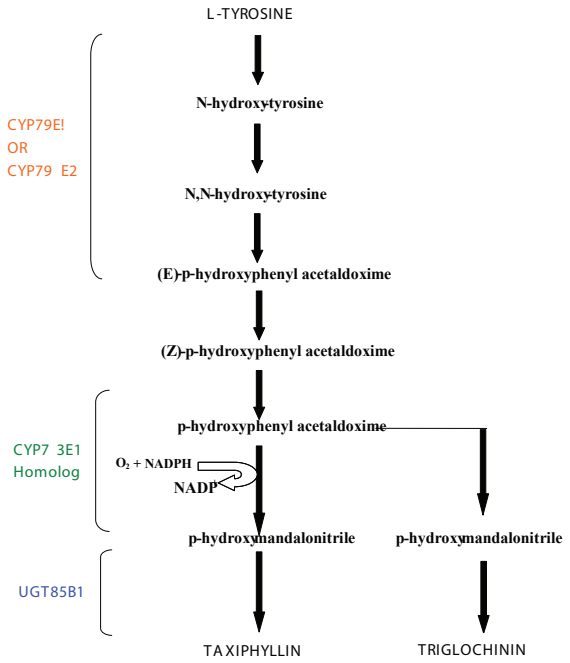

Figure 2b. Scheme of the biosynthesis of L-tyrosine derived cyanogenic glycosides, taxiphyllin and triglochinin in *Triglochin maritime*.

from phenylalanine (*p*-hydroxy-phenylacetonitrile). In *Lotus japonicum,* two cytochrome P450s, CYP79D3 and CYP79D4 almost identical (95%) have shown different choices for the amino acids as substrates, CYP79D3 for L-isoleucine and CYP79D4 for L-valine. In cassava, CYP79D1 preferably chooses L-valine as a substrate and not L-isoleucine a substrate of choice for CYP79D3 of lotus. Several other substrate specific cytochrome-P450s acting upon valine, leucine and tyrosine have been reported from seaside arrow grass (*Triglochin maritima*) and cassava (*Manihot esculenta*; Nielsen and Moller 1999; Andersen et al. 2000).

The huge diversity in CGs structure in plants has been attributed both to the substrate specificity of the first cytochrome-P450s as well as broader substrate specificity of the second cytochrome-P450s. Perhaps, the second step catalyzed by cytochromeP450-II is thought to be a source of diversity in CGs structures. For example cytochrome CYP79A1 in sorghum carried out three consecutive N-hydroxylations of the amino acid tyrosine (Moller and Seigler 1999). However, in barley, hydroxylations of 3-methylbutyro-nitrile intermediate, is carried out by cytochrome CYP71E resulting in hydroxynitiriles corresponding to five different cyano glucosides (Moller and Seigler 1999). The multiple and flexible binding positions of 3-methylbutyro-nitrile in the active site of the CYP71 monooxygenases enables the enzyme to carry out additional hydroxylations at the neighboring carbon atoms as well as to carry out successive hydroxylations at two or three other carbon atoms in followed by dehydration reactions (Nielsen et al. 2002). Recently, Watanabe et al. (2007) have explained the chemo- and regioselectivity of the monooxygenases after investigation of the oxidation of several acyclic monoterpenes by P450 in BM-3 and its mutants. Fischbach and Clardy (2007) have also favoured oxidative modifications catalyzed by four cytochrome P450 monooxygenases for structural diversity of gibberellins. It is plausible that cyanogenic plants might have adopted a similar strategy of diversification through post-aldoxime hydroxylation by cytochromes-P450s resulting in structural diversity in CGs.

Roles of UDP-Glc glucosyltransferase in CGs biosynthesis

UDP-Glc glucosyltransferases catalyzes the third step of the CGs biosynthesis. They are highly regarded enzymes owing to their roles in the biosynthesis of a wide range of primary and secondary metabolites in plants (Vogt and Jones 2000). The basic reaction catalyzed by these enzymes is the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. The CAZy database (Carbohydrates Active Enzymes) provide the continuously updated classification of glycosyltransferases using nucleotide diphospho-sugar, nucleotide monophospho-sugars and sugar phosphates (EC 2.4.1.x) and related proteins into distinct sequence-based families. In cyanogenic plants, glucosyltransferase performs glucosylation of cyanohydrin (final step in the biosynthesis of CGs) to produce distinct CG. A

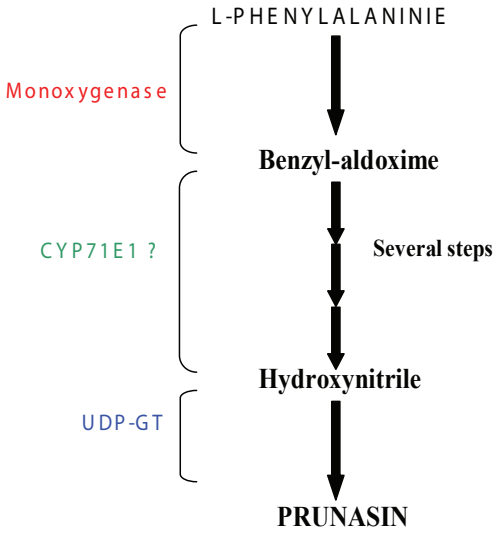

Figure 3. Scheme of biosynthesis of L-phenylalanine derived cyanogenic glycoside, prunasin in *Carica papaya*.

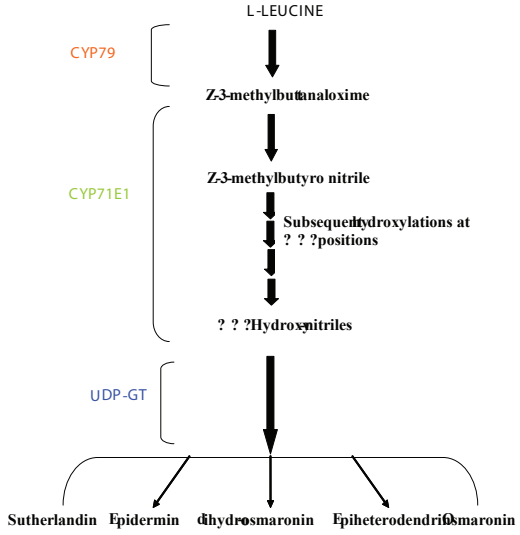

Figure 4. Scheme of the biosynthesis of L-leucine derived cyanogenic glucosides in *Hordium vulgare*.

soluble UDP-Glc-glucosyltransferase (UGT85B1) catalyzing glucosylation of p-hydroxy-mandelonitrile in the final step of dhurrin biosynthesis in sorghum has been isolated and characterized (Jones et al. 1999; Kahn et al. 1999; Thorsoe et al. 2005).

Tyrosine derived CGs

Several CGs *viz.,* dhurrin, triglochinin, taxiphyllin, holocalin and vicianin are derived from L-tyrosine. Study of biosynthesis of dhurrin (p-hydroxy-(S)-mandelonitrile-l-D-glucoside) in *Sorghum bicolor* has provided deeper insight into tyrosine derived CGs (Koch et al. 1995; Sibbesen et al. 1995; Kahn et al. 1997; Bak et al. 1998; Neilsen et al. 2007). A pathway (Fig. 2a) leading to dhurrin biosynthesis in sorghum has been completely elucidated (Sibbesen et al. 1994; 1995; Koch et al. 1995). Biosynthesis of dhurrin starts with the conversion of L-tyrosine into Z-p-hydroxyphenyl acetaldoxime catalyzed by the enzyme CYP79A1. The next enzyme CYP71E1 then catalyzes the conversion of Z-p-hydroxyphenyl acetaldoxime into cyanohydrin, p-hydroxy-mandelonitrile (Koch et al. 1995; Sibbesen et al. 1995; Kahn et al. 1997; Bak et al. 1998). However, a number of unusual and labile intermediates such as N-hydroxyamino acid, an N,N-dihydroxyamino acid, E- and Z-oximes and a cyanohydrin are also produced during dhurrin biosynthesis. These intermediates, except Z-oxime are efficiently channeled for the formation of dhurrin hence could not be trapped (Moller and Conn 1980; Sibbesen et al. 1995; Kahn et al. 1997; Kristensen et al. 2005). Both CYP79A1 and CYP71E1 are membrane bound multi-functional cytochrome P450s and performs six of seven steps in dhurrin synthesis with (Z)-p-hydroxyphenylacetaldoxime as the inter-enzymatic intermediate (Morant et al. 2003). In the final step of the pathway, the labile p-hydroxy-mandelonitrile is stabilized by glucosylation *via* a soluble UDP-glucosyltransferase UGT85B1 to produce dhurrin (Jones et al. 1999; Kahn et al. 1999; Thorsoe et al. 2005; Fig. 2a).

A similar pathway with changed cytochrome-P450s is used by all other plants for the biosynthesis of other tyrosine derived CGs, such as triglochinin and taxiphyllin (Fig. 2b). The pathway branches off from the point of formation of *p*-hydroxy-phenylacetonitrile. The branch point intermediate *p*-hydroxy-phenylacetonitrile leads to taxiphylin and triglinin in *T. maritime*, linustatin and neolinustatin in *Linum usitatissimum*, linamarin and lotaustralin in *Trifolium repens* L. (Nielsen and Moller 1999) and linamarin in *Manihot esculenta* Crantz (Nielsen and Moller 1999; Koch et al. 1992).

Phenylalanne derived CGs

Phenylalanine beside the progenitor of many CGs, also serve as precursor for biosynthesis of secondary metabolites such as flavonoids (Celenza, 2001; Bennett, et al. 1997). Some of the important CGs derived from L-phenylalanine includes prunasin (an aromatic cyanogenic glycoside), acacipetalin, proacacipetalin, 3-hydroxyheterodendrin, sambunigrin, amygdalin, (R)-vicianin, zierin, epilucumin and zierinxylosides. However, only little is known about biosynthesis and regulation of phenylalanine derived CGs in plants except the prunasin biosynthesis in *Carica papya*. *Carica papaya* concomitantly biosynthesizes glucosinolates (benzylglucosinolate) and cyanogenic glucosides (prunasin) using a

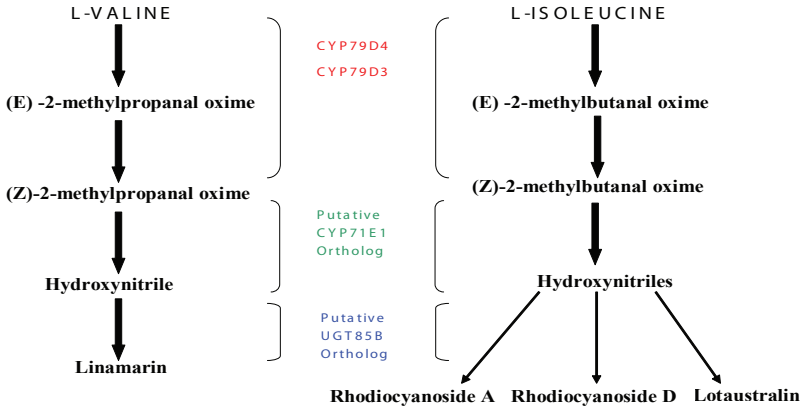

Figure 5. Scheme of the biosynthesis of L-valine and L-isoleucine derived cyanogenic glucosides, linamarin and lotaustralin and the nitrile glucosides, rhodiocyanoside A and D in *Lotus japonicus*.

common biochemical pathway. In *C. papaya*, the cytochrome-P450 (monoxygenase) catalyzes the first dedicated step of conversion of L-phenylalanine into benzylaldoxime in the biosynthesis of prunasin (Bennett et al. 1997; Fig. 3). The cytochrome-P450 monoxygenase is one of the three distinct enzymes (flavoprotein, cytochrome P450 MOs, and peroxidases) involved in the formation of aldoximes from amino acids in plants. Beside *C. papaya*, *Brassica* species have been reported to produce glucosinaolates, similar to those of *C. papaya*. However, our knowledge regarding to the biosynthesis of glucosinolates in plants is very poor but it has become clear that in nitrile glucosides, the hydroxyl and nitrile groups are not linked to the same carbon atom of the aglycone. Also, hydrolysis of nitrile glucosides by β-glucosidases does not result in HCN release (Forslund et al. 2004).

Leucine derived CGs

Leucine derived CGs, sutherlandin, epidermin, osmaronin, dihydro-osmaronin and epiheterodendrin have been reported from the barley (*H. vulgare*) seedlings (Nielsen et al. 2002). In barley, CGs biosynthesis is highly compartmentalized, in one compartment (tissue type) biosynthesis of CGs proceeds; CGs thus produced are accumulated in other compartment and they undergo catabolism in another compartment where the enzyme β-glucosidase start degradation of CGs (Fig. 4). As per the common scheme of CGs biosynthesis, in barley also, the first dedicated step is catalyzed by a CYP79 homolog as a result of which L-leucine is converted into *Z*-3-methylbutanaloxime. The resultant intermediate, *Z*-3-methylbutanaloxime then in two consecutive reactions catalyzed by a CYP71E homolog converted to a cyanohydrin *via* a nitrile compound as an intermediate (Nielsen et al. 2002). In the final step, nitrile is glycosylated by UDP-glucosyl transferase which later produces CGs (Fig. 4). It is reported that in barley CYP71E homolog hydroxylates the 3-methylbutyro-nitrile intermediate at the α-carbon atom.

The multiple and flexible binding positions of 3-methylbutyro-nitrile in the active site of the CYP71 monooxygenase enables the enzyme to carry out additional hydroxylations at the neighboring carbon atoms as well as at two or three other carbon atoms. Hydroxylation together with dehydration reactions provides a mechanism to explain the concomitant generation of hydroxynitiriles corresponding to five different CGs in barley seedlings (Nelsen et al. 2002).

Valine and isoleucine derived CGs

Cyanogenic glycosides, linamarin and lotaustralin found in Lotus species are believed to be biosynthesized from valine and isoleucine (Moller and Seigler 1999; Fig. 5). In most of the species of Lotus, linamarin and lotaustralin are biosynthesized together with nitrile glucosides, such as rhodiocyanoside A, and rhodiocyanoside D (Gebrehiwot and Beuselinck 2001; Andersen et al. 2000). Previous studies with radio labeled valine in Lotus species have revealed that petioles, midrib of the leaf, and the shoot apex are most active in synthesis of linamarin (Bediako et al. 1981). Further studies with radio labeled substrates L-isoleucine and L-valine have indicated that linamarin is derived from L-valine while lotaustralin and nitrile glucosides rhodiocyanoside A, and rhodiocyanoside D are derived from L-isoleucine (Forslund et al. 2004).

Most likely, *Lotus* species also use the common biochemical pathway for the biosynthesis of above CGs and nitrile glucosides. In Lotus species, the first two committed step of conversion of L-valine and L-isoleucine to their corresponding oximes is catalyzed by two cytochrome-P450s enzymes CYP79D3 and CYP79D4, respectively (Forslund et al. 2004; Fig. 5). Unlike others, *Lotus* species use two separate and independent biochemical pathways located in roots and aerial parts for the biosynthesis of CGs. The two pathways has separate CYP79 enzymes which express differentially in roots and aerial parts, CYP79D3 exclusively expressed in aerial parts while CYP79D4 in roots (Forslund et al. 2004).

Also these enzymes differ in catalytic properties that determine the cyanogenic glycosides profiles in lotus. Sequence analysis of amino acids indicated 94% similarity between CYP79D3 and CYP79D4 however little similarity between them was observed in promoter region (Forslund et al. 2004). Despite high degree of sequence similarities, CYP79D3 and CYP79D4 have shown substrate specificity. The CYP79D3 in *L. japonicus* has shown strong affinity for L-isoleucine as substrate while the CYP79D4 preferred L-valine as substrate for biosynthesis of linamarin.

In few cases like in *Lotus* and *Brassica* species in addition to CGs, nitrile glucosides are biosynthesized using a common precursor and a pathway of CGs (Lechtenberg et al. 1996; Nielsen et al. 2002; Forslund et al. 2004). In lotus species, nitrile glucosides namely rhodiocyanoside A and D are derived from the precursor L-isoleucine which is also a precursor for CGs, however, the biosynthesis of this class of compounds is still uncertain. In recent years, biosynthesis of CGs and nitrile glycosides in *L. japonicus* has been extensively studied with an objective to develop it as a model system to understand concomitant biosynthesis of CGs and nitrile glycosides in plants (Handberg and Stougaard, 1992; Asamizu et al. 2000; Nakamura et al. 2002; Perry et al. 2003). Currently, cDNA and genomic sequencing in *L. japonicus* is underway at the Kazusa DNA Research Institute (http://www.kazusa.or.jp/en/database.html) and will be available soon. The genome sequencing may underpin some early breakthrough in CGs and nitrile glucoside biosynthesis and regulation in plants.

Molecular biology and metabolic engineering of CGs biosynthesis

The genes encoding enzymes of CGs biosynthesis and degradation have been rapidly isolated cloned and characterized in the past few years (Table 2). In case of sorghum, all the genes encoding enzymes of dhurrin biosynthesis have been isolated, cloned and characterized (Nielson et al. 2008.). Beside, the list included genes for cytochrome-P450s, UDP-glucosyltransferases, cyanoalanine synthase, hydroxynitrile layases, glucosidases, rhodenases and vicianin hydrolase. The cloned genes (cDNA) are excellent tools in molecular biology and used to gain an understanding of the molecular regulatory processes underlying biosynthetic pathways leading to a variety of products. The whole deep knowledge of regulatory mechanisms is essential for metabolic engineering of the concerned biosynthetic pathway. In a similar way, cDNAs have greatly facilitated our understanding of molecular regulation of CGs biosynthesis as well as the metabolic engineering of the CGs biosynthetic pathways in acyanogenic plants.

Metabolic engineering of biochemical pathways of valuable plant products is rapidly growing for example the modification of flower colors, enhancement of lignin synthesis by down- regulation and production of pharmaceutically useful secondary metabolites. There are reports available on successful metabolic engineering of CGs and development of transgenic plants. The availability of cloned genes (cDNAs) of CGs pathway and deeper knowledge of regulatory process has been indispensable in the metabolic engineering of CGs biosynthesis in commercially valuable plants. In recent years, using metabolic engineering of cytochrome-P450 enzyme, transgenic cassava with depleted CGs, *Arabidopsis thaliana* producing CGs, and *L. japonicus* with altered cyanogenic, cyanoalkenyl or glucosinolate contents have been successfully developed (Tattersall et al. 2001; Morant et al. 2003, 2007; Kristensen et al. 2005).

The major objectives of the metabolic engineering of CGs biosynthesis are to produce CGs free varieties of plants and transgenic plants such as *Arabidopsis* with altered or enhanced production of CGs. With the above objectives, CGs content in cassava, sorghum, barley, lotus, tobacco and Arabidopsis have been manipulated using metabolic engineering (Tattersall et al. 2001; Morant et al. 2003, 2007; Kristensen et al. 2005). Based on the reports on genetic engineering of the CGs, mainly two strategies have been used for engineering of CGs pathways. According to the first strategy, a gene encoding enzyme of CGs pathway along with a promoter is introduced into non-cyanogenic plants. Second strategy utilizes RNAi technique for development of transgenic plants with altered or enhanced levels of cyanogenic glycosides. Jorgensen et al. (2005) have used RNAi technique for developing transgenic cyanogen free cassava plants (*Manihot esculenta* Crantz, cv MCol22). The RNAi was used to block expression of CYP79D1 and CYP79D2 the two key genes encoding the first committed enzymes in linamarin and lotaustralin synthesis. On the other hand, Siritunga and Sayre (2004b) generated transgenic cassava by selective inhibition of CGs biosynthesis in leaves and roots by antisense expression of CYP79D1/D2 gene fragments. Selectively inhibition of these genes in leaves resulted in transgenic plants with largely reduced (60- 94% reduction) linamarin content while the inhibition of same set of genes in roots has drastically reduced (99%) linamarin content in resultant transgenic plants. Previously, transgenic cassava plants were generated by over-expression (13-fold) of hydroxynitrile lyase (HNL) using a double 35S CaMV promoter (Siritunga et al. 2004a). This strategy was based on the fact that over expression of hydroxynitrile lyase (HNL) will accelerate cyanogenesis and cyanide volatilization during food processing thereby reducing cyanogen toxicity in cassava foods. HNL catalyze the conversion of acetone cyanohydrin to cyanide. Unlike previous transgenic cyanogen free cassava, transgenic plants over-expressing HNL in roots retain the herbivore deterrence of cyanogens while providing a safer food product.

Arabidopsis plants are promising in metabolic engineering of CGs. The first transgenic Arabidopsis producing p-hydroxybenzylglucosinolate was developed by introducing

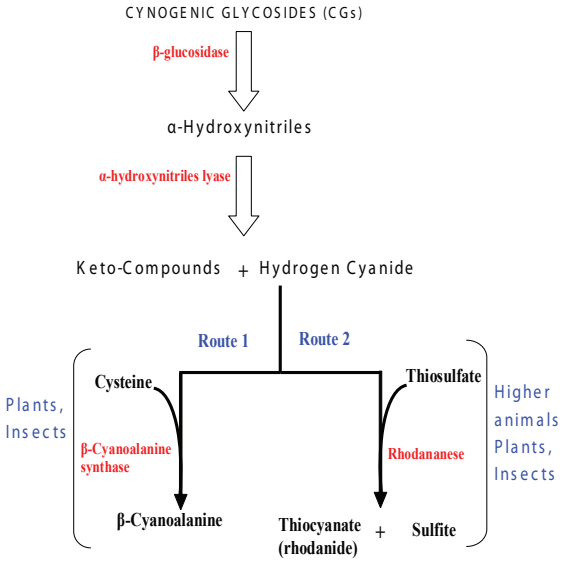

Figure 6. Schematic pathway of catabolism of cyanogenic glycosides.

CYP79A1 from *Sorghum bicolor* (Bak et al. 1999). The ability of CYP79A1 to integrate itself in to cyanogenic glycoside pathway is important for its implications in genetic engineering of CGs (Bak et al. 1999). Later, Bak and associates (2000) have developed transgenic tobacco and Arabidopsis plants expressing two multifunctional cytochrome-P450s, CYP79A1 and CYP71E1 of sorghum. These transformed plants were cyanogenic accumulating metabolites derived from intermediates in dhurrin biosynthesis. As a most recent development in transgenics CGs plants, two transgenic plants *A. thaliana* and *L. japonicus* have been developed by introducing entire dhurrin biosynthetic pathway from sorghum (Morant et al. 2003, 2007; Kristensen et al. 2005). In transgenic *A. thaliana* plant dhurrin content was recorded 4% (w/w) of leaf dry-weight. Ectopic expression of CYP79D2 from cassava (*Manihot esculenta* Crantz.) in *L. japonicus* has been resulted in a 5- to 20-fold increase of linamarin content, whereas the relative amounts of lotaustralin and rhodiocyanoside A/D remained unaltered (Forslund et al. 2004).

Catabolism of CGs

In plants, CGs undergoes catabolic pathways for their complete degradation into hydrogen cyanide (HCN). Enzymes β-glucosidases and α-hydroxynitrile lyases are the most important enzymes of catabolic pathways in plants (Conn 1980; Hosel and Conn 1982; Poulton 1990; Fig. 6). Enzyme β-glucosidase hydrolyzes CGs to the corresponding α-hydroxynitriles, which then dissociates spontaneously into a sugar, a keto- compound, and HCN if pH value is above 6. At lower pH, α-hydroxynitriles did not dissociate spontaneously but an enzyme α-hydroxynitrile lyase may catalyze its dissociation. Hydrogen cyanide, the final product of dissociation of α-hydroxynitriles then detoxified *via* two separate routes (Zagrobelny et al. 2004). The first route involves the formation of β-cyanoalanine from cysteine and is catalyzed by β-cyanoalanine-synthase (Fig. 6). Subsequently, β-cyanoalanine is converted into asparagine (Miller and Conn 1980). This route seems to be most common in plants and possibly in insects also. The second route proceeds by conversion of HCN into thiocyanate and is catalyzed by rhodanese (Bordo and Bork 2002; Fig. 6). Vertebrates predominantly utilizes thiocyanate route, however some plants and insects are also reported to use this route.

Genes encoding some of the enzymes of CGs catabolic-pathways have been cloned and characterized (Table 1). In plants, α-hydroxynitrile lyase are located in the tissues where β-glucosidases are present though their activity is observed in protein bodies (Swain et al. 1992), instead of in chloroplasts or apoplastic space as typically reported for β-glucosidases (Hickel et al. 1996). β-glucosidase and α-hydroxynitrile lyase that begins the cleavage of CNGs are localized in chloroplasts or apoplastic space in plants (Conn 1980; Hosel and Conn 1982; Poulton 1990). β-glucosidase has capability to recognize the aglycone moiety of CGs present within the plant species (Hosel et al. 1987; Hosel and Conn 1982; Nahrstedt 1985), on the other hand, α-hydroxynitrile lyases have shown the activity in protein bodies (Swain et al. 1992).

β-cyanoalanine synthase of mitochondria detoxifies HCN in pyridoxal phosphate (PLP) dependent reaction and produce β-cyanoalanine. The process is beneficial both ways as the detoxification of HCN prevents the mitochondrial degradation from the vulnerable attack of HCN, the resulted detoxification product β-cyanoalanine serve to deter predators (Ressler et al. 1969). Rhodanase is also proposed to be an enzyme involved in cyanide detoxification (Beesley et al. 1985) which is evidenced by high levels of rhodanase activity in 3-day-old etiolated *Sorghum bicolor* seedlings (Miller and Conn 1980). Rhodanese perhaps is not a common enzyme in plants but it is associated with sulfonation of proteins (Bordo and Bork 2002).

Detection of CGs

Several chromatographic procedures have been described for the qualitative and quantitative detection of CGs in plant samples, previously (Brimer et al. 1981; 1983; Brimer and Dalgaard 1984; Brimer and Molgaard 1986; Brimer 1988) (Table 3). Curtis et al. (2002) have described a new method to simultaneously detect cyanide and carbonyl compounds arising from CGs in plants. A portable gas chromatograph housing two detectors using a single carrier gas is employed to measure the carbonyl compound (photoionization detector) and cyanide as its cyanogen chloride derivative (electron capture detector) from the headspace of a plant sample. This method affords in-field, rapid screening of plants to determine

Table 1. Cloned genes encoding enzymes of cyanogenic glycoside biosynthesis and catabolism.

Enzyme	Plant	Accession No.	Size	Protein/Gene	References
Cytochrome P450ox	Sorghum bicolor	AAC39318	531	CYP71E1	Kahn et al. 1997
Cytochrome P450tyr	Sorghum bicolor	AAA85440	558	CYP79	Koch et al. 1995
		Q43135	558	CYP79A1	
Cytochrome P450	Manihot esculenta	AAP57704	511	C15	Zhang et al. 2003
		AF140614	541		Andersen et al. 2000
Putative Cytochrome P450	Lotus japonicus	AAB69644	490		Forslund et al. 2004
Cytochrome P450	*Triglochin maritima*	AAF66544	533	CYP79E2	Nielsen and Moller 2000;
		AAF66543	540	CYP79E1	Nielsen and Moller 1999
A-type cytochromes P450, CYP71E1, CYP98, and CYP99	*Sorghum bicolor*	AAC39318	531	CYP71E1	Bak et al. 1998
		AAC39317	519	CYP99A1	
		AAC39316	512	CYP98A1	
UDP-glucose:p-hydroxymandelonitrile-O-glucosyltransferase	*Sorghum bicolor*	AAF17077	492	UDPGT	Jones et al. 1999
A-hydroxynitrile lyase	*Manihot esculenta*	CAA82334	258	Abhydrolase_1	Hughes et al. 1994
		AAV52632	258	HNL	Wang et al. 2004
		CAA11219	258	HNL4	
		CAA11428	158	HNL 24	
(S)-hydroxynitrile lyase	*Hevea brasiliensis*	AAC49184	257	Hnl	Hasslacher et al. 1996
Hydoxynitrile lyase	*Prunus dulcis*	AAL11514	563	hnl1	Dreveny et al. 2001
		IJU2_B	536	hnl1	
(R)-(+)-mandelonitrile lyase	*Prunus serotina*	P52707	573	MDL3	Hu and Poulton 1999
Rhodanese	*Triticum aestivum*	AAK64575	307	TST	Niu et al. 2002
Rhodanese	*Arabidopsis thaliana*	CAB53639	318	RDH2	Hatzfeld and Saito 2000; Papenbrock and Schmidt 2000
		CAB64716	378	Mst1	
		CAB88023	366	Mst2	
Dhurrinase (β-glucosidase)	*Sorghum bicolor*	AAC49177	565	Glyco_hydro-1	Cicek and Esen 1998; Verdoucq et al. 2004
Dhurrinase (β-glucosidase)	*Zea mays*	AAD09850	563	Glu2	Bandaranayake and Esen1996; Czjzek et al. 2000
Dhurrinase (β-glucosidase)	*Secale cereale*	AAG00614	568	Glyco_hydro-1	Nikus et al. 2003
B-glucosidase	*Prunus avivum*	AAA91166	531	Glyco_hydro-1	Wiersma et al. 1996; Gerardi et al.2001
B-glucosidase (Amygdalin hydrolase)	*Prunus serotina*	AAA93234	553	AH1	Zheng and Poulton1995; Zhou et al. 2002
B-glucosidase	Manihot esculenta	CAA64442	551	Bgl1A	Liddle et al. 1998
B-glucosidase-DIMBOA	*Zea mays*	1E4N_B	512	Glyco_hydro-1	Cicek, and Esen 1999;
		1E4N_A	512	Glyco_hydro-1	Czjzek et al. 2000
B-(1)4-β-glucosidase	*Prevotella ruminicola Bacteria (gram –ve)*	AAA86753	785	cdxA	Wulff-Strobel and Wilson 1995
B-cyanoalanine synthase	*Solenum tuberosum*	BAB18760	351 aa	PCAS-1	Maruyama et al. 2001
		BAB20032	347 aa	PCAS-2	
B-cyanoalanine synthase	*Betula pendula*	AAN86822	352 aa	Beta-CAS	Vahala et al. 2003
Vicianin Hydrolase	*Vicia angustifolia*	ABD03937	509		Ahn et al. 2007
Cyanide hydratase	*Gloeocercospora sorghi*	AAA33353	368	Cht	Wang and Etten 1992
		P32964	368	CHT	
α-acetone cyanohydrin lyase	Linum usitertussimum	CAA70304	422		Trummler and Wajant1997
Prussanin β-glucosidase	*Prunus serotina*	P29265	15		Li et al1992
		P29264	16		
		P29263	14		

cyanogenicity. Simultaneous detection of both the cyanide and the carbonyl compounds allows for confirmation of the presence of CGs and eliminates the problem of false positives often seen in traditional cyanide test kits. This method could be useful for screening cyanogenic foodstuffs to determine suitability for consumption.

A cyanide-specific biosensor has been developed for the detection of CGs in the micro molar concentration in many medicinal and food plants (Keusgen et al. 2004). An immobilized cyanidase has been employed in this biosensor. Under this method, enzymatically formed ammonia is either detected by a potentiometric sensor based on an ammonia electrode or by a pH-sensitive electrolyte/insulator/semiconductor (EIS) layer structure made of Al/p-Si/SiO_2/Si_3N_4 (Keusgen et al. 2004). CGs were also studied using Raman spectroscopy (Thygesen et al. 2004). Surface-enhanced Raman Spectros-

Table 2. Methods for the detection of cyanogenic glycosides in plants.

Methods	Materials	Cyanogenic compounds	Detection limit	Reference
Pyridine-barbituric acid colorimetric procedure	Soybeans and soybean products	Cyanide (HCN)	Microgram	Honig et al. 1983
Direct determination method	Beans and bean paste products	Linamarin	Milligram	Kawamura et al. 1993
Picrate and acid hydrolysis methods	Flax seed and flax seed meal; Bamboo shoots; sorghum leaves	Total cyanide content	Ppm.	Haque and Beabdury 2002
Picrate paper kits	Cassava	Total cyanide content	Microgram	Hidayat et al. 2000
Barbituric acid pyridine, pyridine-pyrazolone and high performance liquid chromatography	Ax seed	Cyanogenic glycosides	Microgram	Kobaisy et al. 1996
Densitometric method	Cyanogenic plant	Small amounts of cyanogenic compounds	Nanogram	Brimer and Molgaard, 1986
Direct determination of cyanides by potentiometric biosensors	Several cyanogenic medicinal and food plants	Cyanogenic glycosides	Micromolar	Keusgen et al. 2004
Chromatographic determination using a porous graphitic carbon column	Almond tree roots	Prunasin and amygdalin		Berenguer Navarro et al. 2002
LC combined with tandem mass spectrometry (LC-MS/MS)	Vitis vinifera L.	Epimers of prunasin and sambunigrin		Franks et al. 2005
Gas chromatographic analysis	Linum usitatissimum L.	Linustatin and neolinustatin	Low- to sub-nanogram	Bacala and Barthet 2007
Gas chromatography-electron capture/photoionization detection	Foodstuffs	Simultaneous determination of cyanide and carbonyl compounds		Curtis et al. 2002
GC/EI-MS or GC/NCI-MS	Passiflora fruits	Mandelonitrile (Prunasin, amygdalin, mandelonitrile rhamnopyranosyl β-D-glucopyranosides, sambunigrin glycosides		Chassagne et al. 1996
Immunoassay using polyclonal antibodies	Pits of fruits and nuts	Amygdalin	Microgram-milligram	Cho et al. 2006

copy (SERS) has been demonstrated to be a more sensitive method for the determination of the cyanogenic potential of plant tissue. The SERS method was optimized by flow injection (FI) using a colloidal gold dispersion as effluent.

Very recently, Cho et al. (2006) have described an enzyme immunoassay method for the detection of high amygdalin content in various seeds and nuts. It utilizes an antiserum reactive to amygdalin for the detection of amygdalin. In fact, there are various other methods available to detect amygdalin in food extracts. Bacala and Barthet, (2007) reported gas chromatographic analysis of the cyanogenic glycosides linustatin and neolinustatin from flaxseed (*Linum usitatissimum* L.) using phenyl- β-D-glucopyranoside as an internal standard. Two quantitative methods direct (using linustatin and neolinustatin external standard curves) or indirect (by use of methyl-α-D-glucopyranoside as a surrogate external standard) were employed for the linustatin and neolinustatin. Limits of detection for all standards were in the low- to sub-nanogram level and were 10-100 times lower than the lower limit of quantification. Thus from the above discussion it is very clear that a broad-spectrum qualitative and quantitative methods are available for the detection of CGs. However, these methods have their own pros and cons, and have a limit of detection of CGs from nano- to milligram level. Furthermore, the principal underneath the detection, suitability and applicability of these methods is markedly varies.

Conclusion

Cyanogenic glycosides (CGs) are abundant in plant kingdom. They are amongst most important components of plant defense systems and mediate interactions of plants with insects. The study of the biosynthesis of CGs is desirable because several commercial and edible crop plants are cyanogenic, particularly sorghum, cassava and barley. Understanding of the CGs biosynthetic pathways and enzymatic steps as well as molecular regulatory process underlying therein is crucial for metabolic engineering of these pathway in order to develop cyanogen free crop plants. Already, transgenic cassava plants with no CGs have been successfully developed through metabolic engineering of key enzymes namely cytochromes-P450s. Similarly, transgenic Arabidopsis thaliana with ability to produce dhurrin, tobacco and Lotus japonicus with altered cyanogenic, cyanoalkenyl or glucosinolate profiles have been successfully generated.

With regard to CGs, genes encoding enzymes involved in their biosynthesis as well as degradation have also been cloned and characterized. Today cloned genes (cDNA) encoding enzymes of the dhurrin biosynthetic pathway from sorghum are available. Our knowledge, however is not limited to the characterization of enzymes but extended to many structural and organizational details including the concept of metabolon formation by the enzymes of cyanogenic glycoside biosynthesis has been uncovered. Also, some progress has been made towards understanding role of sub-cellular compartmentation in regulation of cyanogenic glycosides biosynthesis in plants. Existence of isoforms of cytochrome-P450 represents the compartmentalization of cyanogenic glycosides biosynthesis in plants. Several insect species are dependent on cyanogenic plants for cyanogenic glycoside as their food and live in association with such plants throughout their life cycle. By using the available cDNAs, transgenic plants can now be generated with an altered qualitative and quantitative content of cyanogenic glycosides and the plant-insect interaction could be shattered. Or cyanogenic plants can be made acyanogenic (with no detectable cyanogen glycosides) to deter insects dependent on such cyanogenic plants for cyanogenic glycosides as their food. Cyanogenic glycosides often have toxic effects in humans when consumed along with food and food products. In recent years, several efficient, highly sensitive and rapid method of detection of cyanogenic glycosides in foods and foodstuffs have been developed to determine suitability of these products for human consumptions.

Despite great progress made on every front, biosynthesis, molecular biology and metabolic engineering of cyanogenic glycosides, still we are short of detailed knowledge of the different regulatory mechanisms controlling biosynthesis of cyanogenic glycosides in plants. Also, extensive studies to be carried out to elucidate mechanisms or processes or enzyme characteristics particularly those which favors the formation of metabolon during biosynthesis of cyanogenic glycosides in plants. Another, unsolved but important issue related to cyanogenic glycosides biosynthesis in plants is the concomitant biosynthesis and accumulation of nitrile glucosides about which comparatively very little is known. However, it is expected that complete elucidation of Lotus japanicus genome sequence may provide some important clues to understand biosynthesis of the nitrile glucosides and their biosynthetic relationship with cyanogenic glycosides in plants. Simultaneously, current methods of detection of cyanogenic glycosides should be constantly evaluated to improve their efficiency and sensitivity and newer methods should be developed for detection of cyanogen glycosides in wide variety of plant samples, foods and foodstuffs.

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