Leucine-Derived Cyano Glucosides in Barley[1]

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Barley (*Hordeum vulgare*) seedlings contain five cyano glucosides derived from the amino acid L-leucine (Leu). The chemical structure and the relative abundance of the cyano glucosides were investigated by liquid chromatography-mass spectrometry and nuclear magnetic resonance analyses using spring barley cultivars with high, medium, and low cyanide potential. The barley cultivars showed a 10-fold difference in their cyano glucoside content, but the relative content of the individual cyano glucosides remained constant. Epiheterodendrin, the only cyanogenic glucoside present, comprised 12% to 18% of the total content of cyano glucosides. It is proposed that the aglycones of all five cyano glucosides are formed by the initial action of a cytochrome P450 enzyme of the CYP79 family converting L-Leu into *Z*-3-methylbutanal oxime and subsequent action of a less specific CYP71E enzyme converting the oxime into 3-methylbutyro nitrile and mediating subsequent hydroxylations at the α-, as well as β- and γ-, carbon atoms. Presence of cyano glucosides in the barley seedlings was restricted to leaf tissue, with 99% confined to the epidermis cell layers of the leaf blade. Microsomal preparations from epidermal cells were not able to convert L-[^{14}C]Leu into the biosynthetic intermediate, *Z*-3-methylbutanal-oxime. This was only achieved using microsomal preparations from other cell types in the basal leaf segment, demonstrating translocation of the cyano glucosides to the epidermal cell layers after biosynthesis. A β-glucosidase able to degrade epiheterodendrin was detected exclusively in yet a third compartment, the endosperm of the germinating seed. Therefore, in barley, a putative function of cyano glucosides in plant defense is not linked to cyanide release.

Cyanogenesis, i.e. the ability of living cells to release cyanide under certain biotic and/or abiotic stress conditions, is an old trait (Lechtenberg and Nahrstedt, 1999). In most cases, cyanide release reflects cleavage of a cyanogenic glucoside into the corresponding cyanohydrin and Glc by the initial action of a β-glucosidase. Subsequently, the cyanohydrin is cleaved into a ketone or aldehyde and hydrogen cyanide, either catalyzed by an α-hydroxy nitrilase or nonenzymatically. Cyanogenic glucosides have been found in over 3,000 plant species and may exert a role in plant defense reactions (Tattersall et al., 2001).

Cyanogenic glucosides are derived from the amino acids L-Val, L-Ile, L-Leu, L-Phe, or L-Tyr and the nonprotein amino acid cyclopentenyl-Gly. The biosynthetic pathway is initiated by conversion of the amino acid into an aldoxime by a multifunctional P450 monooxygenase belonging to the CYP79 family. A second P450 monooxygenase belonging to the CYP71E family converts the oxime into a cyanohydrin with a nitrile as an intermediate. The cyanohydrin is finally glucosylated to produce the cyanogenic glucoside by a UDP-Glc-glucosyl transferase (for review, see Jones et al., 2000). In sorghum (*Sorghum bicolor*), cyanogenic glucoside synthesis proceeds in the etiolated seedling tip (Halkier and Møller, 1989). In etiolated cassava (*Manihot esculenta* Crantz) seedlings (Koch et al., 1992), cyanogenic glucosides are synthesized in the cotyledons, although a considerable portion accumulate in the root.

Barley (*Hordeum vulgare*) malt (5-d-old seedlings) and primary leaves of 10- to 28-d-old-plants have cyanide potential (Erb et al., 1979; Cook et al., 1990; Ibenthal et al., 1993; Forslund and Jonsson, 1997). A cyanogenic glucoside, epiheterodendrin, thought to be derived from L-Leu (Seigler, 1998), was identified as the source of hydrogen cyanide production. Cyanide release during beer and whiskey production was demonstrated to reflect the action of a yeast (*Saccharomyces cerevisiae*) β-glucosidase on the barley-derived epiheterodendrin (Cook et al., 1990). Hydrogen cyanide release from barley leaf extracts has also been observed (Forslund and Jonsson, 1997). A locus, designated *eph*, has been associated with epiheterodendrin formation using malt cyanide potential as a phenotypic marker. The *eph* locus is placed on barley chromosome 5, at the short arm region 1H (Swantson et al., 1999).

The well-known toxicity of HCN to aerobic organisms has invoked a link between cyanide potential and plant defense systems against herbivores and microorganisms. Thus, cyanogenic glucosides may combat fungi, if the incipient attack causes cleavage of the cyanogenic glucoside to release hydrogen cyanide. Alternatively, the intact cyanogenic glucoside may inhibit fungal growth. In studies of the fungal pathogen *Microcyclus ulei* on leaves of the cyanogenic

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rubber tree (*Hevea brasiliensis*), higher cyanide production was observed during disease development in compatible plant-fungal interactions than in incompatible interactions (Lieberei, 1986; Seigler, 1998).

Analyses of the biotrophic barley powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*; Jørgensen, 1994), revealed a positive correlation between fungal aggressiveness and the cyanide potential of barley leaf tissue (Ibenthal et al., 1993). The content of cyanogenic glucosides and cyanide production in barley during pathogen attack was not investigated (Siegler, 1998). Several different cyano glucosides are present in barley (Ibenthal et al., 1993). Attempts to reveal their chemical structures were based on gas chromatography analyses of trimethylsilyl derivatives. Among the cyano glucosides identified were the β- and γ-cyano glucosides, epidermin and sutherlandin, which have subsequently also been reported in members of the Rosacea (Lechtenberg et al., 1996). A biological function of non-cyanogenic cyano glucosides has not been demonstrated, but a role in nitrogen storage has been suggested (Conn, 1981; Forslund and Jonsson, 1997).

In the present work, the identification and localization of a group of Leu-derived α-, β-, and γ-cyano glucosides in the barley seedling are presented and a simple model for their biosynthesis is proposed. It is demonstrated that the tissue in which they are synthesized is different from the tissue in which they accumulate and that the β-glucosidase required for their degradation is located in yet a third tissue.

RESULTS

Leu-Derived Cyano Glucosides in Barley Leaves

The cyano glucoside profiles of leaf and epidermis tissue of four spring barley cultivars have been studied (Table I). Barley cv Mentor has previously been reported to possess the highest cyanide potential known in barley (1,400 nmol g^{-1} fresh weight; Forslund et al., 1998). Barley cv Pallas has a medium cyanide potential (approximately 400 nmol g^{-1}fresh weight; Forslund et al., 1998), whereas barley cv Emir is a low cyanogenic cultivar as measured in malt as well as in leaves (20 nmol g^{-1} fresh weight; Ibenthal et al., 1993; Forslund et al., 1998). The P-10 line represents a barley cv Pallas near-isogenic line with barley cv Emir as donor, whereas P-01 and P-02 are other near-isogenic lines as described by Kølster et al. (1986).

The presence of Leu-derived cyano glucosides in leaf extracts cannot be monitored by UV absorption. Therefore, the cyano glucoside profiles were determined using liquid chromatography (LC)/mass spectrometry (MS) and specific ion monitoring of the respective $[M + Na]^+$ adduct ions. Specific ion monitoring demonstrated the presence of sutherlandin ($[M + Na]^{+298}$, retention time [r_t] = 3.5 min, Fig. 1A), epidermin ($[M + Na]^{+284}$, r_t = 6.1 min, Fig. 1B), dihydroosmaronin ($[M + Na]^{+284}$, r_t = 10.2 min, Fig. 1B), epiheterodendrin ($[M + Na]^{+284}$, r_t = 14.1 min, Fig. 1B), and osmaronin ($[M + Na]^{+282}$, r_t = 8.7 min, Fig. 1C). The respective *m/z* ion traces are shown as a function of retention time and, when compared with the total ion trace (Fig. 1D), the corresponding peaks are easily recognized. Sutherlandin and epidermin were both isolated by preparative LC and the structures verified by NMR (not shown), whereas epiheterodendrin was verified based on identical mass and retention time compared with an authentic standard. Dihydroosmaronin was assigned based on the well-characterized structures of proposed Leu-derived cyano glucosides in the Rosaceae as published earlier (Lechtenberg et al., 1996). The *m/z* 282 trace displayed one major peak at 8.7 min, assigned as osmaronin, and a 1-min peak at 13.4 min of unknown identity.

Based on the relative intensities of the $[M + Na]^+$ ions (Fig. 1, A–C), the relative abundance of each cyano glucoside was calculated (Table I). Independent of the large difference in cyanide potential among the four cultivars tested, the relative composition of the cyano glucosides remained constant. Thus, epiheterodendrin comprises less than 20% of the total amount of nitrile glucosides in both high and low "cyanogenic" barley cultivars (Table I).

One Biosynthetic Pathway to Generate Five Cyano Glucosides

The data obtained (Fig. 1, A–C; Table I) are most easily explained if the biosynthesis of all the Leu-

Table I. *Correlations between HCN potentials and relative abundance of each of five Leu-derived nitrile glucosides in barley cv Pallas, cv Mentor, cv Emir, and cv P-10*

Cyanide Potential and Relative Abundance of Cyano Glucosides	Barley cv Mentor	Barley cv Pallas	Barley cv Emir	Barley cv P-10
		%		
CN potential in stripped epidermis (nmol g^{-1} fresh wt^{-1})	700–1,270	410–720	50–200	330–510
Cyano glucoside relative abundance				
Sutherlandin	35–41	34–41	41–47	41–44
Epidermin	29–32	31–35	30–33	26–29
Osmaronin	10–11	10–12	8–9	10–11
Dihydro-osmaronin	2–4	2–3	1–2	2–3
Epiheterodendrin	16–19	14–17	13–14	15–17

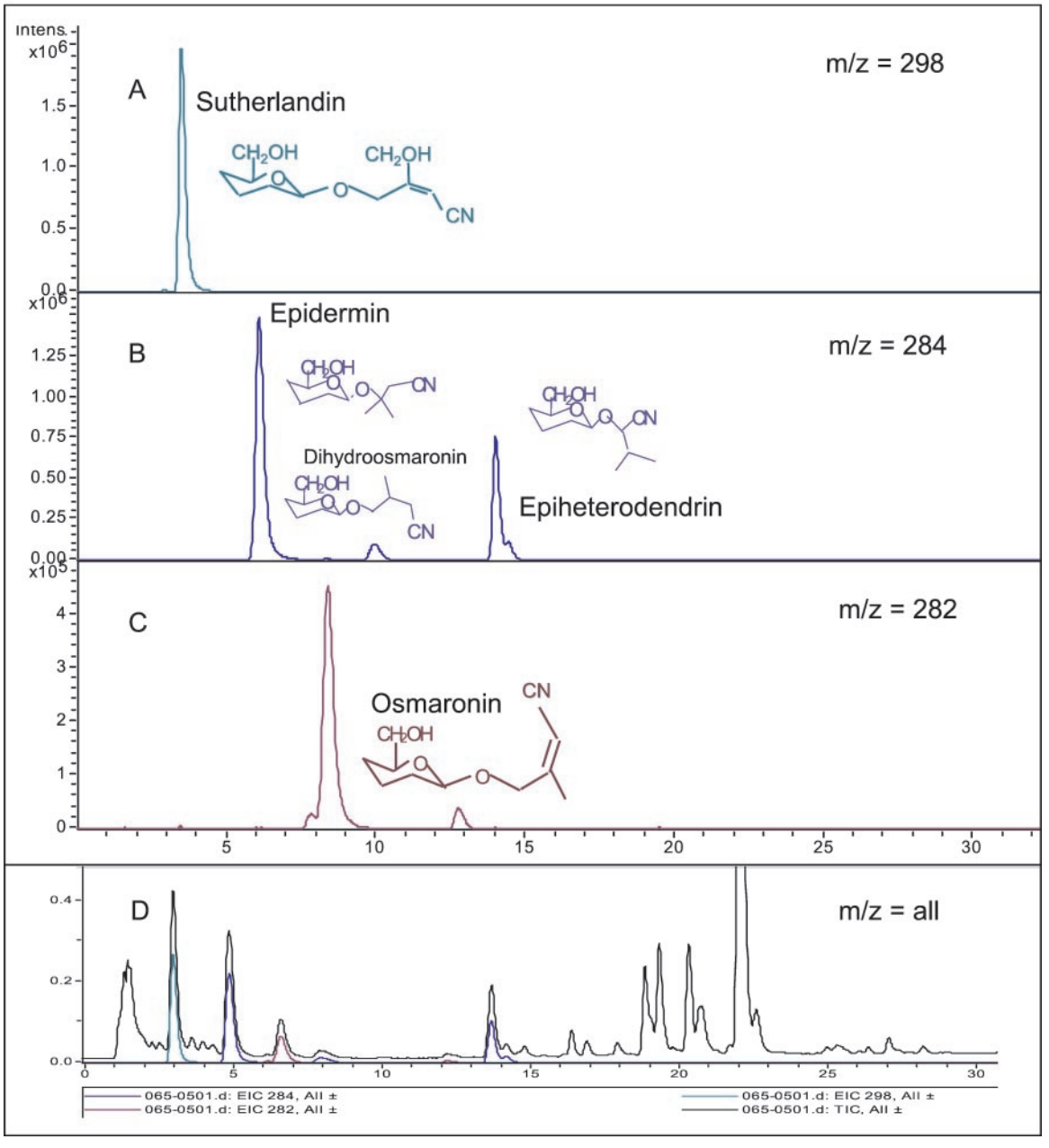

Figure 1. LC-MS profile of Leu-derived nitrile glucosides in the epidermis of barley leaves. Total ion content and extracted ion monitoring of $[M + Na]^+$ adducts were used to determine the relative content of Leu-derived cyano glucosides in methanol extracts of barley cv Mentor.

derived cyano glucosides in barley is catalyzed by the same enzyme system. Generally, the production of cyano glucosides is catalyzed by one CYP79 and one CYP71E enzyme and a soluble UDP-Glc-glucosyl transferase (Jones et al., 2000). In barley, L-Leu is suggested as converted to the *Z*-3-methylbutanal-oxime intermediate by the action of a CYP79 homolog. According to the general biosynthetic scheme for cyanogenic glucosides, the oxime is converted to the corresponding cyanohydrin in two consecutive reactions catalyzed by a CYP71E homolog and with a nitrile compound as an intermediate. It is proposed that the barley CYP71E homolog hydroxylates the 3-methylbutyro-nitrile intermediate at the α-carbon-atom as generally observed. However, 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 to carry out successive hydroxylations at two or three other carbon atoms. In combination with dehydration reactions, this provides a mechanism to explain the concomitant generation of hydroxynitiriles corresponding to five different cyano glucosides. One of

these is a cyanohydrin that is converted to the cyanogenic glucoside, epiheterodendrin. The four remaining aglycones give rise to non-cyanogenic cyano glucosides. A stipulation of the series of hydroxylation events that lead to the production of the five cyano glucosides is shown in Figure 2.

The cyanogenic glucoside epiheterodendrin accounts for less than 20% of the total cyano glucosides and is the result of hydroxylation by CYP71E at carbon atom (B) on the 3-methylbutyro-nitrile and subsequent glucosylation (Table I). Hydroxylation at carbon atom (C) followed by glucosylation results in formation of epidermin. This cyano glucoside comprises about 30% of the Leu-derived cyano glucosides. Hydroxylation at carbon atom (D) produces dihydroosmaronin, the most rare of the cyano glucosides (2%–4%). C-C double bond formation, as observed in osmaronin and sutherlandin, is thought to appear after hydroxylation at either (B) or (C). The hydroxylated aliphatic nitriles may be converted to unsaturated nitriles as a result of a dehydration reaction. The unsaturated nitrile is then amenable to a second hydroxylation at carbon atom (D) to produce a hydroxy nitrile that is finally glucosylated. Such double hydroxylations at either positions (B) and (D) or at (C) and (D) appear responsible for formation of osmaronin, comprising about 10% of the total amounts of cyano glucosides. The formation of sutherlandin, which also contains a C-C double bond, is explained by yet a third hydroxylation at carbon atom (E). Sutherlandin comprises around 40% of the total amount of cyano glucosides. The high relative abundance of double-bond compounds (>50%) suggests that the barley CYP71E homolog catalyzes multiple hydroxylations.

A similar situation with multiple hydroxylations catalyzed by a single active site of a cytochrome P450 enzyme has been observed with CYP79A1 from sorghum, where the formation of small amounts of a nitro compound has been proposed to reflect three consecutive N-hydroxylations of the amino acid Tyr (Møller and Seigler, 1999). Precedence for a biosynthetic route involving several consecutive hydroxylations at the same active site is thus available.

Tissue-Specific Accumulation of the Cyano Glucosides

To determine in which tissues of the barley seedling the cyano glucosides are located, seedlings were dissected into endosperm, scutellum, root, shoot, and coleoptile tissues. Cyano glucosides reached detectable levels 3 d after germination and were exclusively found in shoot tissue (not shown). Thus, leaves, including coleoptiles, are the only vegetative tissues in barley accumulating cyano glucosides. To investigate in more detail the localization of cyano glucosides in the barley leaf, abaxial epidermis strips were peeled off sections of 10-d-old primary leaves of barley cv Mentor and their cyanide potential and content of individual cyano glucosides were determined. The results demonstrate that cyano glucosides accumulate to a high extent in the epidermal cell layer (Table II). To determine whether the cyanide potential found in leaves stripped off their abaxial epidermal cells represented the content of the opposite epidermal cell layer, such leaf material was floated on osmotically adjusted solutions containing cell wall-degrading enzymes to produce protoplasts of the entire mesophyll tissue as observed by release of round-shaped mesophyll protoplasts into the solu-

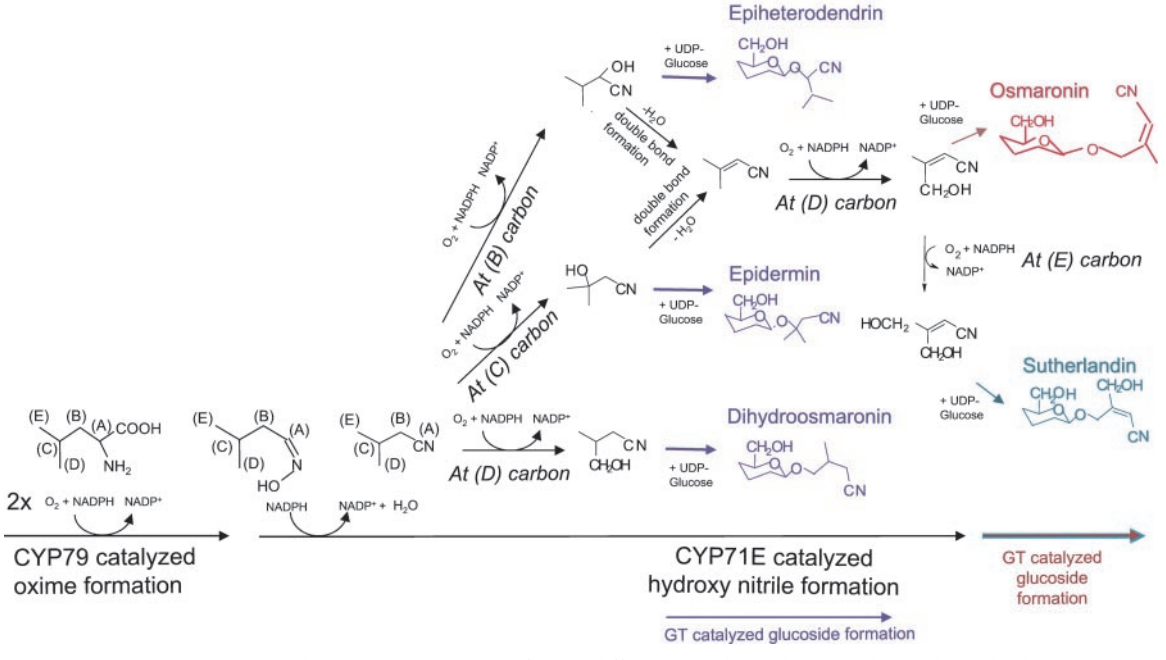

Figure 2. Proposed biosynthetic pathway for the different Leu-derived cyano glucosides in barley.

Table II. *Distribution of cyangenic glucosides in barley tissues*

Tissue	Concentration
	nmol g^{-1} fresh wt^{-1}
Stripped epidermis[a]	~700
Mesophyll[b] with one epidermis layer	~150
Top	~700
Bottom	~400
Coleoptile	~200
Isolated mesphyll protoplasts[c]	~15
Intact, isolated epidermis[d]	~1,300

[a] Stripped abaxial epidermises (approximately 3–5 cm) with vascular tissue. Average fresh wt: 5.5 mg. [b] The leaf tissue from which the "abaxial" epidermis was removed. Area: approximately 3 to 5 cm. Average fresh wt: approximately 50 mg. [c] Mesophyll protoplasts. [d] Structurally fully integrated epidermis sections (6–7 cm).

tion. The procedure leaves the epidermis cell layer intact (Fig. 3A), thus making it possible to obtain a fraction highly enriched in epidermis cells (Fig. 3B). These epidermal cells had very high cyanide potential (Table II). The isolated mesophyll protoplasts remained intact after washing (Fig. 3C) and exhibited low cyanide potential. The relative distribution of the cyanogenic glucosides is shown in Table II and demonstrates that approximately 99% of the cyano glucosides accumulate in the epidermal cell layers of the leaf blade. Unfortunately, the upper 0.5-cm tip of the barley leaf is not amenable to epidermis isolation or protoplast formation. Likewise, it is not possible to isolate abaxial epidermis strips from 3- to 4-d-old leaves.

Site of Biosynthesis at Distance from Sink Tissue

The cytochrome P450 enzymes (CYP79 and CYP71E homologs) catalyzing the conversion of the parent amino acid to the corresponding cyanohydrin are membrane bound (Jones et al., 2000); therefore, these enzyme activities may be studied using microsomal preparations. The intermediates formed are hydrophobic and are easily extracted into ethyl acetate and analyzed by thin-layer chromatography (TLC), followed by detection of radiolabeled signals. When L-[^{14}C]Leu was administered to microsomes prepared from basal segments of barley leaves, radiolabeled E- and Z-3-methylbutanal oxime (Fig. 4, arrows) were formed reflecting the catalytic activity of the barley CYP79 homolog (Fig. 4, lanes 4–6). As expected (Jones et al., 2000), oxime formation was dependent on the presence of NADPH. The accumulation of radiolabeled oxime intermediates was enhanced by the addition of unlabeled Z-2-methylbutanal oxime, the intermediate of the Ile-derived lotaustralin (Fig. 4, lane 6). This is expected because CYP71E enzymes exert broad substrate activity toward oximes (Kahn et al., 1999). The presence of the unlabeled Ile-derived oxime prevents conversion of the radiolabeled Leu-derived oxime into the corresponding cyanohydrin. This greatly facilitates the analytical procedure because the cyanohydrin is labile and dissociates into isobutyraldehyde (Fig. 4, dotted arrow) and hydrogen cyanide, both of which are volatiles that are partly lost during removal of the ethyl acetate solvent and upon drying of the TLC plates. In microsomal fractions prepared from leaf tips (Fig. 4, lanes 1–3) and from 10-d-old isolated epidermal tissue that contain large amounts of cyano glucosides (Fig. 4, lane 7), no metabolism of Leu was

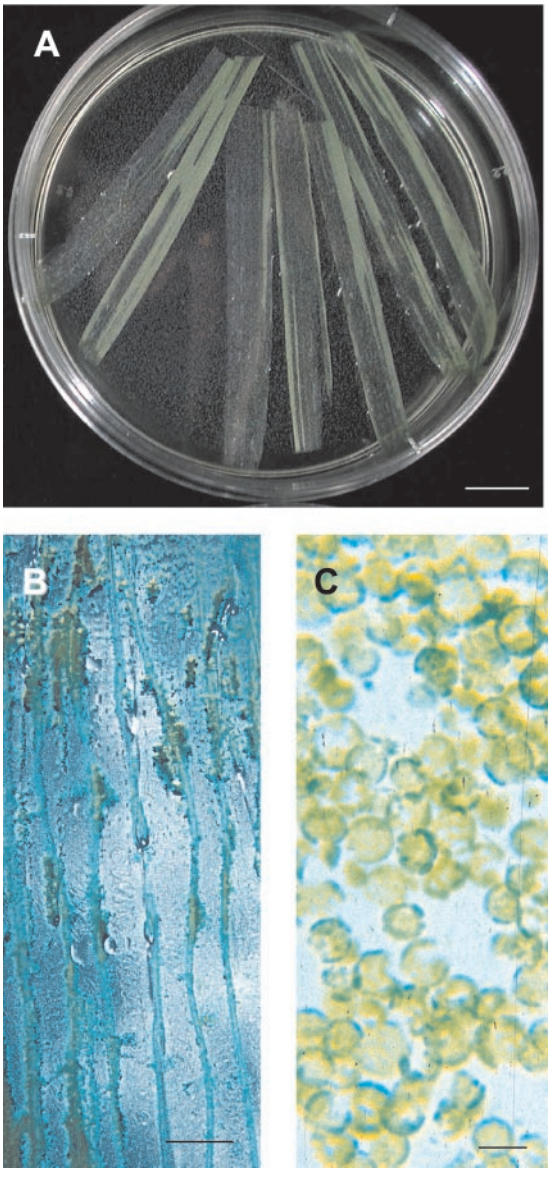

Figure 3. Preparation of mesophyll protoplasts and epidermal cell layer from barley leaves. A, Leaf segments (6–7 cm) after 2 h of incubation on protoplast isolation medium showing the structural integrity and the translucent properties of the epidermis layer due to massive release of mesophyll protoplasts. Bar = 1 cm. B, Preparation of epidermal cell fraction indicating the presence of only few green mesophyll cells. Bar = 100 μM. C, Uniform suspension of round-shaped, green mesophyll protoplasts were obtained after sieving and washing the protoplast suspension from A. Bar = 100 μM.

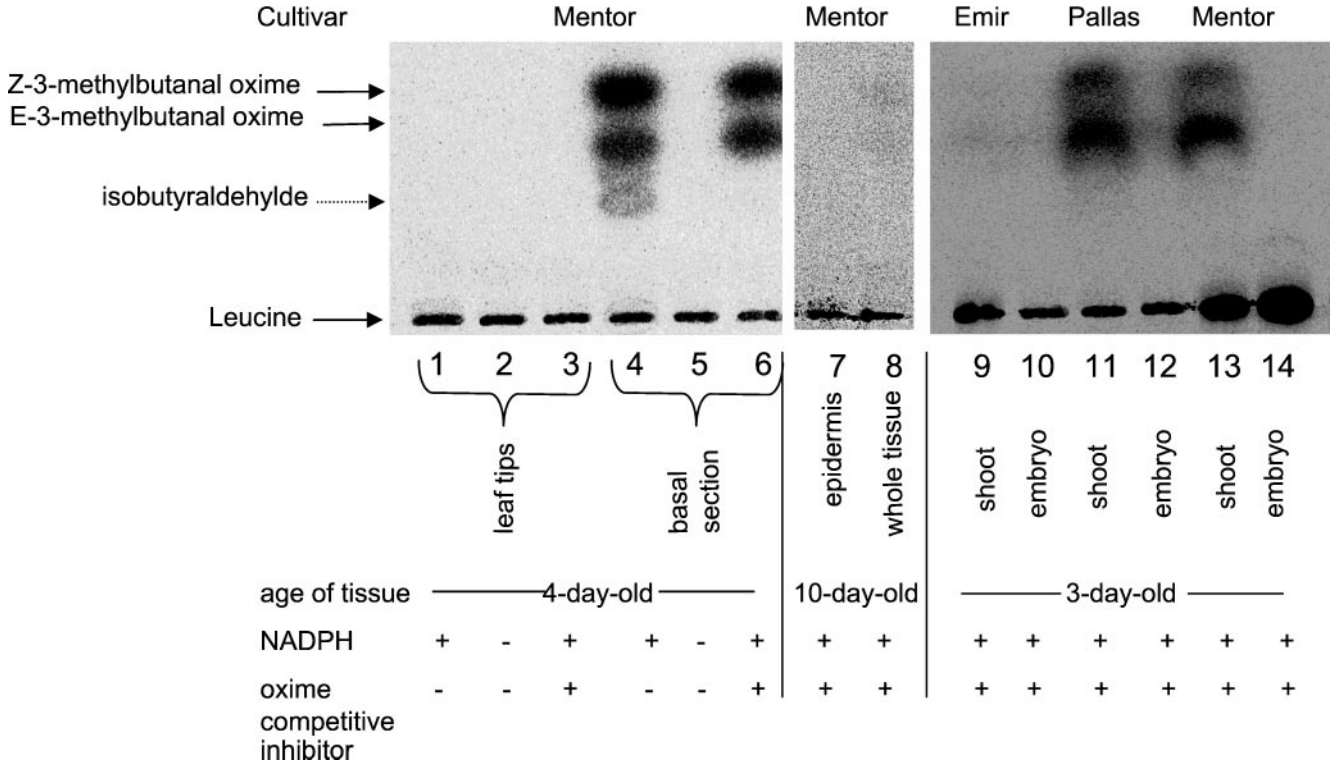

Figure 4. Metabolization of ^{14}C-Leu by barley microsomal fractions. [^{14}C]-Labeled hydrophobic metabolites were visualized after 5 d of exposure of TLC plates using phoshor imaging. Arrows point to radiolabeled E- and Z-oxime intermediates and dotted arrow points to isobutyraldehyde. Four-day-old leaves of barley cv Mentor, tips of leaves (1–3) and basal segments (4–6); 10-d-old leaves of barley cv Mentor, epidermis (7) and a pool of tips and basal segments (8); 3-d-old barley cv Emir (9–10), cv Pallas (lanes 11–12), and cv Mentor (lanes 13–14) shoot and embryo fractions. Samples were incubated with NADPH and competitor (Z-2-methyl-methyl-butanal oxime) as indicated in the figure.

detectable. The metabolic activity in basal segments was always greater in 3- and 4-d-old material compared with 10-d-old material. These analyses used equivalent amounts of plant material from the tip and basal segments of the leaf and of epidermal strips. When the activity in different barley cultivars was compared, the low-cyanide potential barley cv Emir showed greatly reduced activity (Fig. 4, lane 9) compared with the medium and high cyanide potential barley cv Pallas and cv Mentor (Fig. 4, lanes 11 and 13). Metabolic activity was not found in seeds germinated for less than 3 d and no activity was detected in the scutellum of the embryo (Fig. 4, lanes 10, 12, and 14).

Barley Leaves Have Cyanide Potential, But Show No Cyanogenesis during Fungal Attack

The barley powdery mildew fungus infects and grows exclusively in leaf epidermal cell layers, the very same cell layer that contains Leu-derived cyano glucosides. The powdery mildew fungus is an obligate biotroph, and thus nourishes from living host cells. Accordingly, in compatible plant-pathogen interactions, the host cell damage is minute. To determine if any putative β-glucosidase co-occurred with the cyanogenic glucoside epiheterodendrin, the HCN release in mildew-inoculated plants was studied over a 48-h time course. As shown in Table III, no HCN release was detectable in either resistant (barley cv P-02 and cv Mentor) or susceptible (barley cv Pallas and cv P-01) interactions. Hence, the accumulation of epiheterodendrin in plant host tissue was not reflected in release of cyanide as found after fungal attack in the rubber tree-*M. ulei* interaction (Liberei, 1986). To analyze whether the subcellular damage on barley leaf cells during mildew infection were insufficient to induce colocalization of cyanogenic glucosides and β-glucosidase activity and thus to invoke cyanide release, barley tissues of both uninoculated and inoculated leaves were applied directly onto frozen buffer kept in a test tube followed by grinding and sealing of the tube. The samples were allowed to thaw to follow any subsequent HCN release. In the course of a 48-h period, the amount of released HCN corresponded to degradation of less than 10% of the epiheterodendin present in the leaf epidermal tissue. This documents the absence of sufficient amounts of cyanogenic β-glucosidase activity in healthy, as well as mildew-infected, leaf tissue to ensure rapid hydrogen cyanide release from damaged tissue (Table III).

Table III. *Lack of cyanide release from barley leaves after mildew infection and homogenization of leaf tissue*

Tissue	Cyanide Potential before Inoculation	Cyanide Release after Inoculation	Cyanogenesis in Response to Wounding		
			Uninoculated	Inoculated	
	CN^- potential	CN^- release	CN^- release	CN^- release	Ratio[a]
	nmol/three leaves	*nmol/six leaves*	*nmol/three leaves*	*nmol/three leaves*	*%*
Barley					
Barley powdery mildew					
Susceptible, barley cv Pallas	25 (n = 3)	None	1.8	1.2	~5
Susceptible, barley cv P-01	20 (n = 3)	None	1.5	1.9	~10
[b]Resistant, barley cv P-02	20 (n = 3)	None	1.2	2.2	~11
[b]Resistant, barley cv Mentor	50 (n = 3)	None	2.1	2.5	~5

[a] The ratio of cyanide release is calculated as the actual amounts of HCN devided by the cyanide potential in healthy leaves. [b] The mildew isolate A6 is avirulent on both barley cv P-oz and cv Mentor.

Site of Cyanogenic Glucoside Degradation

Crude protein extracts were prepared from different tissues of the barley seedling to determine in which tissues degradation of cyanogenic glucosides may take place. Protein samples were incubated with exogenously added epiheterodendrin because it represents the only cyanogenic glucoside of five cyano glucosides in barley. Due to the β- and γ-glucoside bonds in four of the cyano glucosides, these are non-cyanogenic in contrast to epiheterodendrin, which carries an α-hydroxynitrile group that readily results in HCN release after breakage of the *O*-glucoside bond. The degradation of epiheterodendrin was monitored over an 18-h period using LC-MS and specific ion monitoring $[M + Na]^{+284}$. Barley endosperm tissue was the only tissue that contained an epiheterodendrin-degrading β-glucosidase. Protein extracts obtained from endosperm tissue of 24-h germinated seedlings degraded 80% of the epiheterodendrin within 18 h (Fig. 5). The reaction was linear during the first 4 h (data not shown). Note the absence of background signals of the adduct ion 284 *m/z* at all time points. If the cyano glucosides epidermin and dihydroosmaronin had been endogenous constituents of the endosperm, specific trace signals would have appeared at 6.1 and 10.2 min, respectively. The cyanogenic β-glucosidase was also found to cleave the Tyr-derived cyanogenic glucoside, dhurrin (not shown), thus exhibiting rather broad substrate specificity against cyanogenic glucosides.

DISCUSSION

The present work shows that young barley leaves contain a group of five different cyano glucosides (Fig. 1) stipulated as derived from L-Leu (Lechtenberg, Nahrstedt, 1999; Fig. 2). It is demonstrated that these cyano glucosides are synthesized from L-Leu in

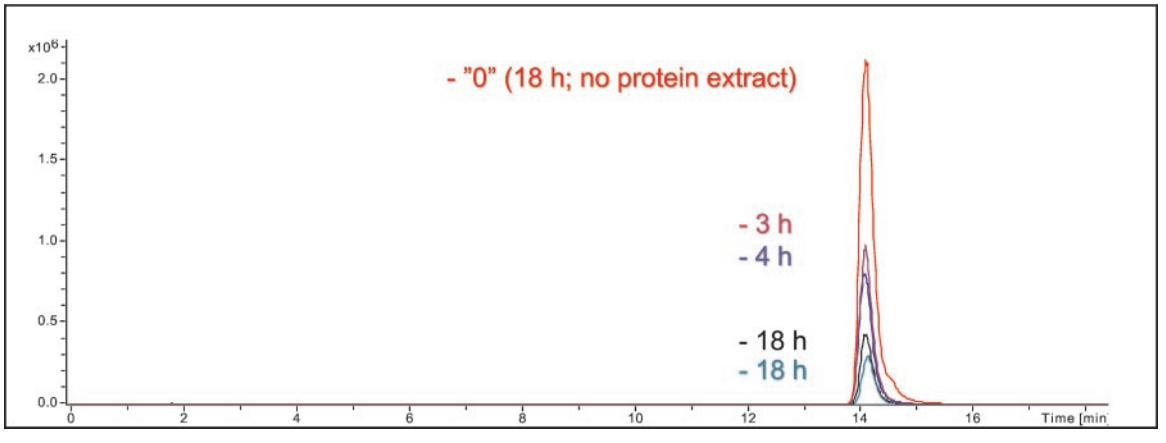

Figure 5. Hydrolysis of epiheterodendrin by endosperm protein crude extracts. Extracted ion chromatogram of $[M + Na]^+$, *m/z* 284, after LC-MS of *n*-pentane-extracted water phases from enzyme assays of barley cv Pallas endosperm crude extracts (24 h of germination) supplied with 10 μmol of epiheterodendrin. Samples showing hydrolysis of epiheterodendrin were incubated for 3, 4, and 18 h (two individual samples) and for 18 h without protein. A linear correlation between amounts of epiheterodendrin as a function of time was found during the first 4 h of incubation as plotted.

the basal leaf segment and transported to leaf epidermal cell layers where they accumulate (Table I and II; Figs. 3 and 4). Neither of these tissues contains a β-glucosidase able to degrade epiheterodendrin. This enzymatic activity is essentially restricted to the endosperm tissue of the germinating barley seed (Fig. 5). Thus, in planta, the cyanide potential of barley leaves is not exploited to release cyanide, e.g. as a fungal defensive mechanism (Table III). Accordingly, a biological function of these secondary metabolites is more likely related to an effect exerted by the intact glucosides, e.g. antifungal activity (Table III).

The absence of a cyanogenic β-glucosidase may reflect a polymorphic inheritance pattern of cyanogenesis as described in white clover (*Trifolium repens*) by Till (1987). In the absence of a cyanogenic β-glucosidase, the formation of α-hydroxynitrile glucosides offers no obvious advantages over non-cyanogenic β-and γ-hydroxynitrile glucosides as defense compounds. Therefore, the selection pressure on the CYP71E to catalyze α-hydroxylations is lost. This offers the possibility for the barley CYP71E to acquire new catalytic functions such as an ability to carry out β- and γ-hydroxylations of the nitrile substrate as stipulated in Figure 2 and as reflected in the cyano glucoside composition (Fig. 1).

Impact of Cyano Glucosides on Fungal Infections

In the rubber tree, attack by the fungal pathogen *M. ulei* resulted in a concomitant release of cyanide. Release of larger amounts of cyanide was observed in compatible plant-fungal interactions as compared with incompatible interactions (Lieberei, 1986; Siegler, 1998). This implies that the fungal pathogen is insensitive to HCN, perhaps due to a cyanide hydratase activity (Osbourn, 1994). Interestingly, previous analyses of barley powdery mildew attack on leaves of malting barley reported less disease symptoms on cultivars with low cyanide potential compared with fungal colonization on cultivars with higher cyanide potential (Ibenthal et al., 1993). This strengthens the possibility of a neutral or even positive effect of cyanogenic glucosides on these fungal pathogens. In the studies of Ibenthal et al. (1993), measurements of the plant cyanide potential was restricted to uninfected leaves. Thus, changes in cyanogenic glucoside synthesis and cyanide production as a result of pathogen attack remained elusive (Siegler, 1998). The additional cyano glucosides present in barley may possess novel, improved functions in plant defense.

One Biosynthetic Pathway for Five Leu-Derived Cyano Glucosides

The CYP71E homolog in barley hydroxylates the aliphatic oxime intermediate, 3-methylbutyronitrile, at different carbon atoms. The highly cyanogenic plant species cassava synthesizes the two cyanogenic glucosides linamarin and lotaustralin derived from Val and Ile, respectively, but does not produce additional non-cyanogenic cyano glucosides (Lykkesfeldt and Møller, 1995). Using microsomal preparations, the cassava CYP71E homolog is able to hydroxylate the α-carbon in aliphatic (Val-, Ile-, and cyclopentenyl-Gly-derived) nitriles as well as aromatic (Phe- and Tyr-derived) nitriles (Koch et al., 1992). Analyses of CYP71E1 from sorghum revealed a more narrow substrate specificity with respect to the nitrile side chain and hydroxylation was again restricted to the α-carbon atom (Kahn et al., 1999; for review, see Jones et al., 2000). Previous studies on cyano glucosides proposed to be derived from L-Leu in species of the family Rosaceae showed the same combination of cyano glucosides as in barley with non-cyanogenic forms constituting the major portion, and in addition, they found two epoxide derivatives (Lechtenberg et al., 1996). These authors concluded that the family Rosaceae is more primitive than the high cyanogenic plants sorghum and cassava, thereby favoring the formation of non-cyanogenic cyano glucosides.

Cyanogenic β-Glucosidase Activity

Cyanogenic β-glucosidase activity in barley was restricted to endosperm tissue of germinating seeds. A database search (http://afmb.cnrs-mrs.fr/cazy/index.html) identified an endosperm-specific barley gene (accession no. L41869) encoding a β-glucosidase (Leah et al., 1995) that groups with the class I cyanogenic β-glucosidases such as dhurrinase I and II from sorghum (Cicek and Esen, 1998). Accordingly, such a β-glucosidase, of which several isoforms have been purified from barley seeds by Hrmova et al. (1996), is a good candidate to be the β-glucosidase able to degrade epiheterodendrin. Thus, the enzyme catalyzing cyanide release in barley is present in yet a third plant compartment and kept at distance from the site for accumulation of cyanogenic glucosides.

In conclusion, we have demonstrated that in young barley seedlings, synthesis and accumulation of cyano glucosides take place in different compartments and that the β-glucosidase required for cyanogenic glucoside degradation is localized in yet a third compartment.

MATERIALS AND METHODS

Plants

Seeds of barley (*Hordeum vulgare*) cv Pallas, cv Mentor, cv Emir, cv P-01, cv P-02, and cv P-10 were obtained from fresh stocks of harvested field material. Seeds were grown at 24°C (16 h light/8 h dark) in 12-cm plastic pots (20 seeds/pot) in soil/vermiculite watered with tap water without nutrient supply.

Cyanide Assays

For cyanide analysis, plant material was harvested directly into liquid nitrogen in Falcon tubes (50 mL). For epidermis analysis, 50 epidermis strips

were collected and for leaf tissue material, sections corresponding to five leaves were combined. After tissue homogenization, liquid nitrogen was allowed to evaporate and the sample was immediately boiled (10 min) in hot 90% (v/v) MeOH (1–5 mL). Residual amounts of solvent were removed by evaporation. After addition of sterile water (0.5 mL) and *n*-pentane (5 mL), the tubes were vortexed vigorously and left (30 min) for complete phase separation. Cell debris accumulating at the interphase was discarded. The aqueous lower phase was transferred to a clean Falcon tube and residual hydrophobic components were removed by extraction of the water phase with *n*-pentane (10 volumes). The water phase was collected and centrifuged (10 min at room temperature) to remove any remains of cell debris. For analyses of cyanide potential, aliquots (10, 50, and 100 μL) were transferred to Eppendorf tubes (Eppendorf Scientific, Westbury, NY), incubated (200 μL total volume in 50 mM MES, pH 6.5) with emulsin (almond β-glucosidase, Sigma, St. Louis), and assayed colorimetrically as described by Forslund and Jonsson (1997).

Metabolization of L-[^{14}C]Leu

Leaf material (0.5 g fresh weight) was homogenized in homogenization buffer (5 mL) composed of 250 mM Suc, 100 mM Tricine (pH 7, 9), 50 mM NaCl, 2 mM EDTA, 2 mM dithiotretiol, and 50 mg of polyvinylpolypyrrolidone using a precooled mortar and pestle. The homogenate was filtered through a nylon cloth and centrifuged (10 min, 12,000 rpm, 4°C). Microsomes were recovered from the supernatant by centrifugation (60 min, 46,000 rpm, 4°C) and resuspended in 50 mM Tris-HCl (50 μL, pH 7.9). Microsomes (approximately 17 μL) were incubated with 0.5 μCi (300 mCi mmol^{-1}) L-[^{14}C]Leu (NEN Life Science Products, Boston; 30 μL) in the presence of either: (a) 1 mM NADPH, (b) no NADPH, or (c) 1 mM NADPH and 3.3 mM *Z*-2-methyl-butanaloxime (30°C, 60 min, moderate shaking [50 rpm]). The reaction mixtures were then extracted with ethyl acetate (2 volumes) and the content of radiolabeled hydrophobic metabolites in the organic phase was analyzed using TLC (Silica gel 60 F254 sheets, Merck, Rahway, NJ) with dichloromethane:ethyl acetate (85:15 [w/v]) as solvent. Radiolabeled components were visualized using a STORM 840 phosphor imager (Molecular Dynamics, Sunnyvale, CA).

Protoplast Formation

Leaf sections (6–7 cm) of 10-d-old seedlings were used. The adaxial epidermis layer was peeled off and the remaining part of the leaf floated (2 h, 30°C) on a solution composed of 1% (w/v) cellulase-RS, 0.1% (w/v) pectolyase Y-23, 0.4 M mannitol, and 0.2 M NaCl (pH 5.8, KOH). The mesophyll protoplasts released were isolated by sieving though a stainless steel net (40-x mesh) and washed in 0.4 M mannitol, 0.2 M NaCl. The epidermis layers were isolated from the top of the sieve. Fractions were subjected to cyanide assays as described above.

Mildew Infection and Cyanide Release

For infection studies, 10 leaves of 10-d-old plants of barley cv Pallas, cv Emir, cv Mentor, and cv P-10 were excised (six leaves per sample) and inoculated with barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) isolate A6 (Nielsen et al., 1999). The A6 isolate is virulent on barley cv Pallas and cv P-01 and avirulent on cv Mentor and cv P-02 (Kølster et al., 1986). The fungal inoculum was kept and maintained on barley cv Pallas plants as described previously (Nielsen et al., 1999). For cyanide analysis, inoculated leaves were kept in sealed tubes (50 mL) for 24 or 48 h. The tubes were fitted with an Eppendorf tube containing 100 μL of NaOH to trap released gaseous HCN. Cyanide was determined colorimetrically as described above.

β-Glucosidase Assays

Seeds were germinated for 1 to 3 d (room temperature, dark) on wetted filter paper in a petri dish (9 cm). For protein extraction, 10 endosperms isolated using forceps were smashed with a hammer and homogenized using a mortar and pestle in 2 mL of 50 mM MES buffer (pH 6.5). The homogenate was transferred to Eppendorf tubes, centrifuged (15,000 rpm, 15 min, room temperature) and supernatant aliquots (10 and 100 μL) were withdrawn avoiding lipid substances accumulating on the surface and incubated (20°C, 18 h) with epiheterodendrin or dhurrin (25 nmol) in 50 mM MES (pH 6.5, total volume of 200 μL). Hydrolysis of epiheterodendrin was monitored by LC-MS of the water phase obtained after *n*-pentane extraction (2 × 10 volumes). Dhurrin hydrolysis was monitored as the formation *p*-hydroxybenzaldehyde and was measured by TLC analysis of EtOAc extracts.

LC-MS

LC-MS (sample volume: 3–15 μL) was carried out using a HP1100 LC coupled to a Bruker Esquire-LC ion trap mass spectrometer (Bruker Instruments, Billerica, MA). An XTerra MS C18 column (3.5 μM, 2.1 × 100 mM, flow rate of 0.2 mL min^{-1}, Waters, Milford, MA) was used. The mobile phases were: A, 0.1% (v/v) HCOOH and 50 μM NaCl; and B, 0.1% (v/v) HCOOH and 80% (v/v) MeCN. The gradient program was: 0 to 2 min, isocratic 3% (v/v) B; 2 to 30 min, linear gradient 3% to 50% (v/v) B; 30 to 35 min, linear gradient 50% to 100% (v/v) B; and 35 to 50 min, isocratic 100% (v/v) B. The mass spectrometer was run in positive ion mode and total ion current and specific $[M + Na]^+$ adduct ions were recorded.

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