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Review

β-Glucosidases as detonators of plant chemical defense

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ABSTRACT

Some plant secondary metabolites are classified as phytoanticipins. When plant tissue in which they are present is disrupted, the phytoanticipins are bio-activated by the action of β-glucosidases. These binary systems – two sets of components that when separated are relatively inert – provide plants with an immediate chemical defense against protruding herbivores and pathogens. This review provides an update on our knowledge of the β-glucosidases involved in activation of the four major classes of phytoanticipins: cyanogenic glucosides, benzoxazinoid glucosides, avenacosides and glucosinolates. New aspects of the role of specific proteins that either control oligomerization of the β-glucosidases or modulate their product specificity are discussed in an evolutionary perspective.

Contents

Abbreviations: BGAF, β-glucosidase aggregating factor; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; ESM, epithiospecifier modifying protein; ESP, epithiospecifier protein; BxGlc, benzoxazinoid glucoside; MBP, myrosinase binding protein; MyAP, myrosinase associated protein; TFP, thiocyanate-forming protein.

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1. Introduction

β-Glucosidases that belong to the family 1 glycoside hydrolases (http://www.cazy.org/fam/GH1.html) catalyze the hydrolysis of the β-glucosidic bond between two carbohydrate moieties or a carbohydrate and an aglucone moiety. In plants, β-glucosidases play important roles in diverse aspects of plant physiology, e.g. (1) formation of intermediates in cell wall lignification (Dharmawardhana et al., 1995; Escamilla-Trevino et al., 2006), (2) cell wall degradation in endosperm during germination (Leah et al., 1995) and (3) activation of phytohormones (Kristoffersen et al., 2000; Lee et al., 2006) and (4) activation of chemical defense compounds (Nisius, 1988; Poulton, 1990; Jones et al., 2000; Halkier and Gershenzon, 2006; Suzuki et al., 2006). β-Glucosidases are receiving increased attention due to their use in biotechnological and industrial applications; examples are their importance in aroma formation in tea, wine and fruit juice (Mizutani et al., 2002; Maicas and Mateo, 2005; Fia et al., 2005) and in engineering microorganisms for use in biomass conversion as β-glucosidase constitutes an important part of the cellulase complex (Van Rensburg et al., 1998; Den Haan et al., 2007).

Plants, unlike animals, are sessile organisms that cannot flee their predators. Through the course of evolution, plants have become nature's organic chemists *par excellence*, and collectively synthesize a plethora of secondary metabolites to defend themselves against herbivores and microorganisms and adapt to different types of abiotic environmental stresses. Traditionally, plant defense compounds are grouped into preformed defense compounds (phytoanticipins) forming the first chemical barrier to herbivore and pathogen attack and defense compounds synthesized in response to herbivore or pathogen attack (phytoalexins; Vanetten et al., 1994).

Many plant defense compounds are stored in a non-active glucosylated form to chemically stabilize and increase the solubility of the defense compound, to render it suitable for storage in the vacuole, and to protect the plant from the toxic effects of its own defense system (Jones and Vogt, 2001). Upon cell disruption, caused for example by a chewing insect, the defense compounds are bioactivated via hydrolysis of the glucosidic linkage catalyzed by β-glucosidases. In intact plant tissue, the β-glucosidases are stored separately from the substrates. This two-component system, of which each of the individual components is chemically inert, provides plants with an immediate chemical defense against attacking herbivores and pathogens.

This review summarizes the four most well characterized preformed two-component defense systems: cyanogenic glucosides, benzoxazinoid glucosides, avenacosides and glucosinolates, with emphasis on the β-glucosidases responsible for their bio-activation, followed by an overview of proteins regulating β-glucosidase oligomerization and product profile.

2. β-Glucosidases as a bio-activating component in plant defense

2.1. β-Glucosidase reaction mechanism

β-Glucosidases belonging to the family 1 glycoside hydrolases catalyze the hydrolysis of the glucosidic bond between the anomeric carbon (C1 of the glucose) and the glucosidic oxygen by a mechanism in which the anomeric configuration of the glucose is retained (Davies and Henrissat, 1995). The catalytic mechanism is illustrated in Fig. 1. Two conserved glutamic acid residues serve as a catalytic nucleophile and a general acid/base catalyst, respectively. In retaining β-glucosidases, the catalytic glutamic acid residues are situated on opposite sides of the β-glucosidic bond of the docked substrate at a distance of ~5.5 Å (Davies and Henrissat, 1995). As the initial step in catalysis, the nucleophile performs a nucleophilic attack at the anomeric carbon, which results in formation of a glucose–enzyme intermediate. In this process, aglucone departure is facilitated by protonation of the glucosidic oxygen by the acid catalyst. During the second catalytic step (deglucosylation), a water molecule is activated by the catalytic base to serve as a nucleophile for hydrolysis of the glucosidic bond and release of the glucose (http://www.cazy.org/fam/ghf_INV_RET.html; Davies and Henrissat, 1995).

Under suitable conditions, β-glucosidases can perform a transglucosylation in which the covalently bound glucose in the enzyme–glucose intermediate is transferred to an alcohol or a second sugar group. Even though this reaction has not been observed to occur *in vivo*, β-glucosidases such as cassava (*Manihot esculenta*) linamarase (β-glucosidase specific for the cyanogenic glucosides linamarin and lotaustralin) are used for industrial production of alkyl β-glucosides and show great industrial application potentials (Svasti et al., 2003; Hommalai et al., 2005).

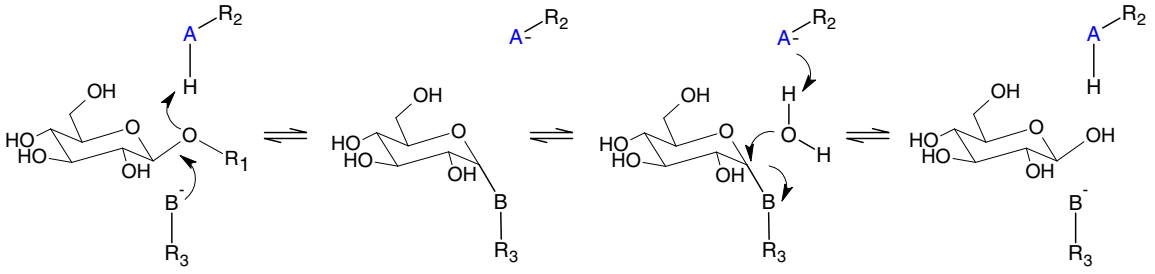

Fig. 1. Reaction mechanism of retaining β-glucosidases. A glutamic acid residue in the conserved TFNEP motif (Davies and Henrissat, 1995) serves as a general acid/base catalyst (AH) while a glutamic acid residue in the I/VTENG motif (Davies and Henrissat, 1995) serves as a nucleophile (B). The reaction cycle is specified in the text.

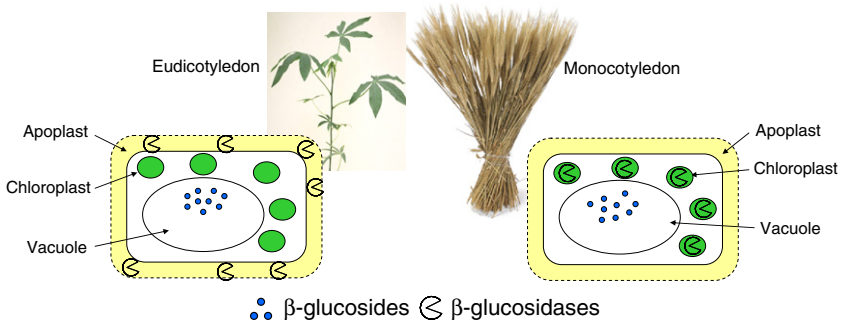

Fig. 2. Compartmentalization of β-glucosidases and their substrates in eudicotyledenous and monocotyledenous plants. Simplified schematic representations of plant cells are shown to illustrate the subcellular localization of the glucosylated defense compounds and the corresponding β-glucosidases. In eudicotyledenous and monocotyledenous plants, the phytoanticipins are stored in the vacuole. The bio-activating β-glucosidases in eudicotyledons are glycoproteins that are stored apoplastically or intracellularly in protein bodies (exemplified here by an apoplast location which is most often reported). In monocotyledons, the β-glucosidases are unglycosylated and plastid-localized (exemplified here by chloroplasts). The plants shown are the eudicotyledenous *Manihot esculenta* and the monocotyledenous wheat that accumulate cyanogenic glucosides and benzoxazinoid glucosides, respectively.

2.2. Compartmentalization of glucosylated defense compounds and their bio-activating β-glucosidases

The biological activity of the defense compounds presented in this review is attributed to their hydrolysis products, and therefore separation of glucosides and β-glucosidases into different (sub)cellular compartments in intact tissue is a critical feature of the two-component defense systems. As illustrated in Fig. 2, the glucosides are stored in the vacuole (Saunders et al., 1977; Saunders and Conn, 1978; Oba et al., 1981; Kesselmeier and Urban, 1983; Gruhnert et al., 1994) while the subcellular localization of the generally abundantly expressed β-glucosidases differ in monocotyledenous and eudicotyledenous plants. Monocotyledenous β-glucosidases contain an N-terminal transit peptide resulting in localization to plastids (Thayer and Conn, 1981; Nisius, 1988; Esen and Stetler, 1993; Gusmayer et al., 1994). In eudicotyledons, the β-glucosidases contain an N-terminal signal peptide that results in direction through the secretory pathway via the endoplasmatic reticulum and in co-translational glycosylation (Hughes et al., 1992; Li et al., 1992; Burmeister et al., 1997; Cairns et al., 2000; Ahn et al., 2007). Their final localization might be either apoplastic or intracellular in protein bodies (Oba et al., 1981; Kakes, 1985; Frehner and Conn, 1987; Swain et al., 1992; Poulton and Li, 1994). In spite of the high degree of amino acid sequence identity and structural conservation between the monocotyledenous and eudicotyledenous β-glucosidases (Fig. 3), the monocotyledenous β-glucosidases are active in a non-glycosylated form whereas glycosylation is essential for the activity of eudicotyledenous β-glucosidases (Zhou et al., 2002). The lack of activity of non-glycosylated eudicotyledenous β-glucosidases is probably due to glycosylation dependant protein stability as evidenced by the failure to express active eudicotyledenous β-glucosidases on a non-glycosylated form in *Escherichia coli* (Zhou et al., 2002) or tobacco (Wei et al., 2004). Upon fusion to a stabilizing fusion protein, however, active non-glycosylated eudicotyledenous β-glucosidases have been expressed in *E. coli* (Keresztessy et al., 1996; Suzuki et al., 2006).

In some plants, the substrate and the bio-activator are additionally separated at the cellular level. In seedlings of the monocotyledenous crop sorghum (*Sorghum bicolor* Moench), the cyanogenic glucoside dhurrin is stored exclusively in the epidermal cell layer, while the corresponding β-glucosidases, the dhurrinases, are stored in the chloroplasts in the bundle sheath cells (Kojima et al., 1979; Thayer and Conn, 1981). The separate compartmentalization provides the plant with a two-component defense system, in which each separate component is chemically inert. Upon tissue disruption, the glucosides come into contact with the degrading β-glucosidases resulting in an immediate release of toxic defense compounds.

Accumulation of glucosylated defense compounds and expression of their degrading β-glucosidases is typically developmentally regulated. Often, the highest amount of the two components is found in seedlings and young plant parts in order to protect the plant from herbivore and pathogen attack at this fragile stage (Sue et al., 2000a,b; Forslund et al., 2004).

2.3. Phylogenetic relationship and structural similarity between β-glucosidases involved in plant defense

The β-glucosidases involved in hydrolysis of defense compounds upon tissue disruption form three distinct clusters in the phylogenetic tree shown in Fig. 3A: eudicotyledenous *O*-β-glucosidases, dicotyledenous *S*- β-glucosidases (myrosinases) and monocotyledenous *O*-β-glucosidases. The functions of the β-glucosidases included in the phylogenetic tree are described in the sections below. Although separated into three different clusters, these β-glucosidases show a high degree of amino acid sequence identities and very similar overall tertiary structures. This is illustrated by white clover (*Trifolium repens*) linamarase and sorghum dhurrinase 1 that share 43% identity at the amino acid level and whose known overall structural folds (Barrett et al., 1995; Verdoucq et al., 2004) are almost identical as illustrated by superimposition of the monomer backbone structures in Fig. 3B.

2.4. The cyanide bomb: Cyanogenic glucosides and cyanogenic β-glucosidases

Cyanogenic glucosides (Fig. 4A) are amino acid derived phytoanticipins found in more than 2650 different plant species from ferns and gymnosperms to monocotyledenous and eudicotyledenous angiosperms (Bak et al., 2006). Upon tissue disruption, the cyanogenic glucosides are hydrolyzed to yield an unstable aglucone, which either spontaneously or enzymatically degrades into a ketone or an aldehyde and toxic HCN (Conn, 1980; Poulton, 1990; Møller and Seigler, 1999; Morant et al., 2003). HCN blocks cellular respiration via inactivation of the mitochondrial cytochrome oxidase (Nelson, 2006). Cyanogenic glucosides are present in a disproportionate high amount of crops (Jones, 1998) suggesting a role of cyanogenic glucosides as important natural pesticides, i.e. a trait that has been favored during domestication of modern crop plants. The natural pesticide properties of cyanogenic glucosides are exploited by using cyanogenic plants for biofumigation purposes, e.g. in order to suppress nematode activities (Widmer, 2000;

Widmer and Abawi, 2002). While in most crops, the cyanogenic glucoside content is negligible in the plant parts ingested by the consumer, the tuberous roots of cassava accumulate high levels of the cyanogenic glucosides linamarin and lotaustralin (Bokanga, 1994). Cassava is one of the world's most important root crops and constitutes the major staple food in many third world countries. Careful processing to remove the cyanogenic glucosides results in concomitant loss of vitamins and minerals (Maziya-Dixon et al., in press) thus further compromising the nutritional value of this important starch source. Consumption of improperly processed cassava may constitute a health problem in rural areas of sub-saharan African countries like the Democratic Republic of Congo where cassava derived products provide a high percentage of the daily calory intake. In severe cases this may result in acute cyanide intoxication and in chronic paralytic diseases such as konzo and tropical ataxic neuropathy (Oluwole et al., 2000; Ernesto

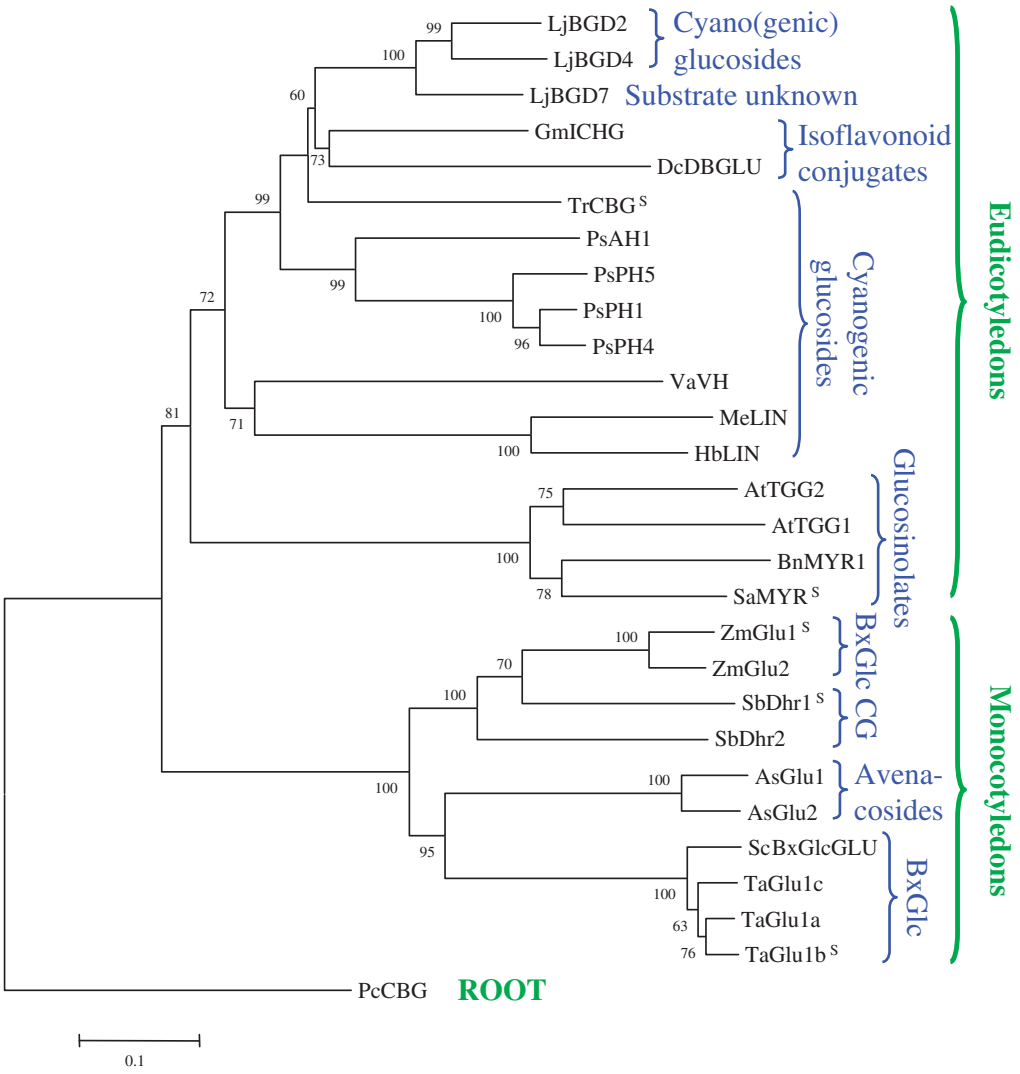

Fig. 3. Phylogenetic analysis and structural conservation of β-glucosidases involved in plant defense. (A) Neighbor-Joining phylogenetic tree including a selection of cyanogenic glucoside and isoflavonoid conjugate hydrolyzing β-glucosidases from eudicotyledons, glucosinolate-degrading myrosinases (Capparales) and β-glucosidases involved in bio-activation of defense compounds in monocotyledons. The defense compounds bio-activated by the β-glucosidases are indicated in blue (CG = cyanogenic glucosides). "S" indicates enzymes for which the crystal structures have been solved. LjBGD2, LjBGD4 and LjBGD7: *L. japonicus* β-glucosidases (Morant et al., 2008). TrCBG: White clover (*Trifolium repens*) linamarase (Barrett et al., 1995). GmICHG: Soybean (*Glycine max*) isoflavone conjugate-hydrolyzing β-glucosidase (Suzuki et al., 2006). DcBDGLU: Thai rosewood (*Dalbergia cochinchinensis* Pierre) dalcochinase (Cairns et al., 2000). PsAH1, PH1, PH4 and PH5: Black cherry (*Prunus serotina*) amygdalin hydrolase and prunasin hydrolase isozymes (Kuroki and Poulton, 1987; Zheng and Poulton, 1995; Zhou et al., 2002). VaVH: *Vicia angustifolia* vicianin hydrolase (Ahn et al., 2007). MeLIN: Cassava (*Manihot esculenta* Crantz) linamarase (Hughes et al., 1992; Keresztessy et al., 2001). HbLIN: Rubber tree (*Hevea brasiliensis*) linamarase (Selmar et al., 1987). AtTGG1 and AtTGG2: *Arabidopsis thaliana* myrosinases (Barth and Jander, 2006). SaMYR: White mustard (*Sinapis alba*) myrosinase (Burmeister et al., 1997). BnMYR1: oilseed rape (*Brassica napus*) myrosinase (Chen and Halkier, 1999). ZmGlu1 and ZmGlu2: Maize (*Zea mays*) DIMBOAGlc β-glucosidases (Czjzek et al., 2001). SbDhr1 and SbDhr2: Sorghum (*Sorghum bicolor* Moench) dhurrinases (Hosel et al., 1987; Verdoucq et al., 2004). AsGlu1 and AsGlu2: Oat (*Avena sativa*) avenacosidases (Gusmayer et al., 1994; Kim et al., 2000). ScBxGlcGLU: Rye (*Secale cereale*) DIBOAGlc β-glucosidase (Nikus et al., 2003). TaGlu1a, b and c: Wheat (*Triticum aestivum* L.) DIMBOAGlc β-glucosidases (Sue et al., 2006). PcCBG: Lodgepole pine (*Pinus contorta*) coniferin β-glucosidase (Dharmawardhana et al., 1995; Dharmawardhana et al., 1999). The phylogenetic tree was rooted using PcCBG as outgroup. The bootstrapped Neighbor-Joining tree was built in MEGA4.0 (Tamura et al., 2007). The tree was bootstrapped with 1000 iterations (node cutoff value 50%). The underlying amino acid sequences in fastA format and the multiple alignment can be assessed at http://www.p450.kvl.dk/VintherMorant_etal_Figure3A_FASTA.tfa and http://www.p450.kvl.dk/VintherMorant_etal_Figure3A_Alignment.pdf. For the bootstrap analysis 1000 trials were performed, and the bootstrap values are shown as in%. Bootstrap node values below 50% are not shown. (B) Cartoon representations of the overall structures of monomers of white clover linamarase and sorghum dhurrinase 1, for which the three dimensional structures have been solved at high resolution (Barrett et al., 1995; Verdoucq et al., 2004). Superimposition of the two monomers illustrates the high degree of structural conservation between eudicotyledenous and monocotyledenous β-glucosidases involved in bio-activation of plant defense metabolites. The figure was constructed using the PyMOL molecular visualization system (http://pymol.sourceforge.net/).

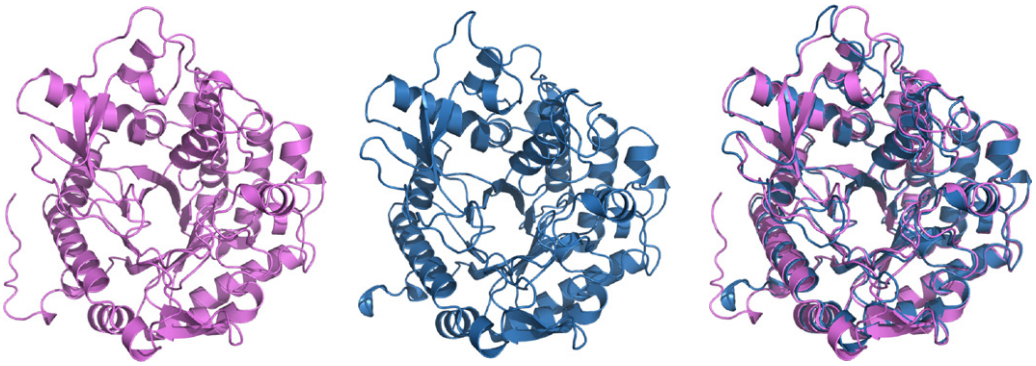

Fig 3. *(continued)*

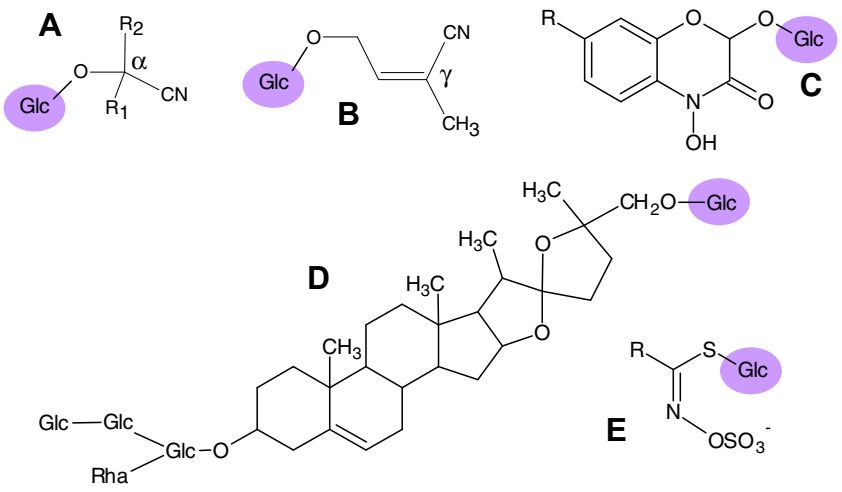

Fig. 4. Chemical structures of the β-glucosidic defense compounds presented. (A) General structure of cyanogenic glucosides, which are β-glucosides of α-hydroxynitriles. (B) Non-cyanogenic glucosides, exemplified here by rhodiocyanoside A, are β-glucosides of β- or γ-hydroxynitriles. (C) DIMBOAGlc, the main benzoxazinoid glucoside found in maize and wheat. (D) Avenacoside B, a saponin accumulated in oat leaves. (E) General structure of glucosinolates that are β-thioglucosides with structures related to those of cyanogenic glucosides. The glucose moieties cleaved off by the specific β-glucosidases to yield the biologically active compounds are highlighted in purple. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2002). While the cyanogenic glucoside/linamarase system may pose a health problem for cassava consumers, the very same cassava linamarin/linamarase system holds promise in cancer treatment either by injection of cells transfected with the gene encoding linamarase (Cortes et al., 1998) or direct linamarase delivery via nanoparticles (Link et al., 2006) into murine tumors followed by treatment with linamarin. The hydrolysis of linamarin results in cell death and effectively eradicates tumors without toxic side effects *in vivo* (Cortes et al., 1998; Link et al., 2006).

The biosynthesis and bio-activation of cyanogenic glucosides has been the focus of much research. Cyanogenic glucosides are synthesized from valine, isoleucine, leucine, phenylalanine, tyrosine and from the non-protein amino acid 2-cyclopentenyl-glycine. The genes and enzymes of the entire cyanogenic glucoside biosynthetic pathway are known from sorghum, which produces the tyrosine-derived dhurrin at up to 30% dry weight in etiolated seedling tips (Saunders et al., 1977; Halkier and Møller, 1989). The amino acid is converted into the cyanogenic glucoside via the concerted action of two cytochromes P450, CYP79A1 and CYP71E1, and a UDPG-glucosyltransferase, UGT85B1 (Halkier and Møller, 1990; Sibbesen et al., 1994; Fig. 5; Halkier et al., 1995; Sibbesen et al., 1995; Kahn et al., 1997; Bak et al., 1998a; Jones et al., 1999; Hansen et al., 2003; Thorsøe et al., 2005). The biosynthetic enzymes are supposedly organized into a multienzyme complex, a metabolon, to facilitate channeling and prevent release of toxic intermediates (Jørgensen et al., 2005; Nielsen et al., 2008). Due to its unique genetic simplicity, the cyanogenic glucoside pathway has a pioneering status in plant metabolic engineering (Bak et al., 1999; Kristensen et al., 2005; Morant et al., 2007), and the transfer of the entire dhurrin pathway to the non-cyanogenic model plant *Arabidopsis thaliana* unequivocally demonstrated the ability of cyanogenic glucosides to deter herbivores (Tattersall et al., 2001). Cyanogenic β-glucosidases have been characterized from a wide variety of cyanogenic plants including sorghum, cassava, white clover, rubber tree (*Hevea brasiliensis*), black cherry (*Prunus serotina*), flax (*Linum ussitatissimum*) and *Lotus japonicus* (Fan and Conn, 1985; Kuroki and Poulton, 1986; Hosel et al., 1987; Selmar et al., 1987; Kuroki and Poulton, 1987; Pocsi et al., 1989; Mkpong et al., 1990; Morant et al., in press). In the phylogenetic tree (Fig. 3A), the β-glucosidases known from the literature to hydrolyze cyanogenic glucosides form separated clades in monocotyledons and eudicotyledons. This argues that the ability to hydrolyze cyanogenic glucosides has evolved independently in monocotyledons and eudicotyledons. Two or more β-glucosidase

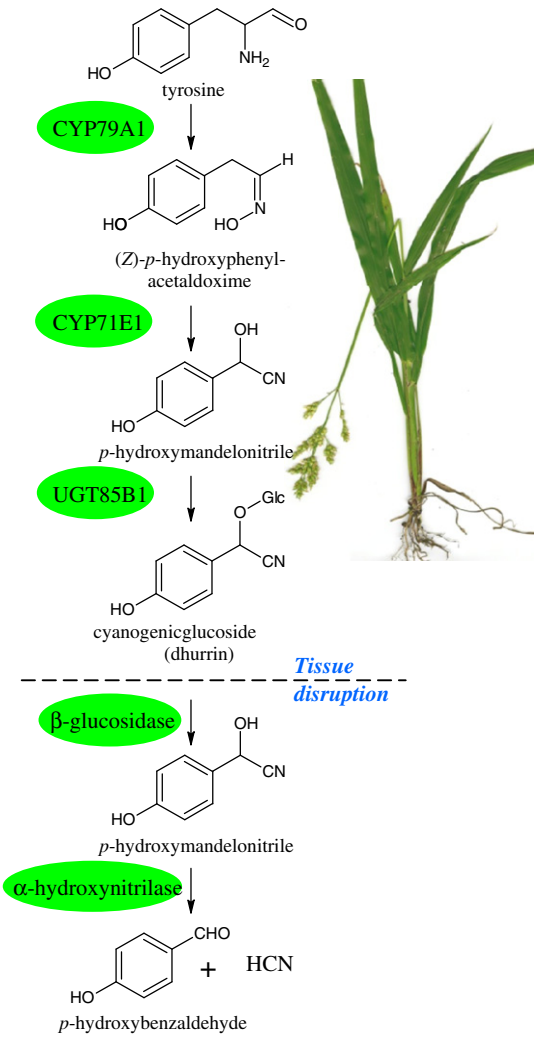

Fig. 5. Biosynthesis and bio-activation of cyanogenic glucosides. Cyanogenic glucosides are synthesized from an amino acid by the consecutive action of two cytochromes P450 and a UDPG glucosyltransferase. The entire pathway is known from sorghum (represented in the picture; Halkier et al., 1995; Sibbesen et al., 1995; Bak et al., 1998a; Jones et al., 1999; Sibbesen et al., 1994) that accumulates the tyrosine-derived dhurrin. Upon tissue disruption, the cyanogenic glucosides are hydrolyzed by β-glucosidase to yield an unstable aglucone that either spontaneously or enzymatically decomposes to yield a ketone or an aldehyde and toxic HCN.

isozymes with apparently similar substrate profiles are often found in one cyanogenic plant species. Examples include sorghum dhurrinase 1 and dhurrinase 2 (Fig. 3A) that are 72% identical at the amino acid level and show different developmental regulation (Hosel et al., 1987; Cicek and Esen, 1998), *L. japonicus* BGD2, BGD4 and BGD7 (Fig. 3A) that are ~85% identical at the amino acid level, show organ specific expression with *BGD2* and *BGD4* expressed in aerial tissue and *BGD7* expressed in roots, and for which nearly identical substrate profiles have been described for BGD2 and BGD4 (Morant et al., in press), and black cherry amygdalin and prunasin hydrolases for which several isozymes with high sequence identity and similar substrate specificities are known (Kuroki and Poulton, 1986, 1987; Li et al., 1992). Even though the origin of the multiple amygdalin and prunasin hydrolase isozymes found in black cherry may in part be due to the polyploidity of this plant, the presence of two or more β-glucosidase isozymes that are differentially expressed suggests that cyanogenic glucoside breakdown is regulated according to developmental stage and in a tissue specific manner. Hence it appears that plant β-glucosidases constitute a highly fine-tuned system, where even subtle differences in biochemical activity, localization or expression are important for bio-activation of defense compounds in response to diverse biotic stresses at different stages of plant development.

Based on the phylogenetic analysis (Fig. 3A), isoflavonoid glucoside β-glucosidases in legumes appear to have evolved from the β-glucosidases involved in cleavage of cyanogenic glucosides (Chuankhayan et al., 2007a; Morant et al., in press). The observed ability of *L. japonicus* BGD2 and BGD4 (Morant et al., in press) and almond β-glucosidase to hydrolyse daidzin and the ability of the isoflavonoid glucoside cleaving β-glucosidase from thai rosewood (*Dalbergia cochinchinensis* Pierre) to hydrolyze cyanogenic glucosides as well as isoflavonoid glucosides is in agreement with this hypothesis (Chuankhayan et al., 2007b).

Cyanogenic glucosides are β-glucosides of α-hydroxynitriles. β-Glucosides of β- and γ-hydroxynitriles belong to a structurally related group, the non-cyanogenic hydroxynitrilealk(en)yl glucosides (Bjarnholt et al., 2008). Hydrolysis of the non-cyanogenic glucosides yields a stable aglucone, that in contrast to the α-hydroxynitrile does not dissociate to release toxic HCN. β-Glucosides of β- and γ-hydroxynitriles derived from isoleucine are known as rhodiocyanosides (Fig. 4B) and always co-occur with the isoleucine-derived cyanogenic glucoside lotaustralin (Forslund et al., 2004; Bjarnholt et al., in press; Bjarnholt and Møller, in press). The co-occurrence strongly suggests that rhodiocyanosides are synthesized via the same biosynthetic pathway as lotaustralin. This is supported by the fact that CYP79D3 and CYP79D4 both catalyze the first and rate limiting step in cyanogenic glucoside and rhodiocyanoside biosynthesis in *L. japonicus* (Forslund et al., 2004; Morant et al., 2007). In leaves of *L. japonicus*, the rhodiocyanosides are efficiently hydrolyzed upon tissue disruption by the cyanogenic β-glucosidases likewise responsible for cyanogenic glucoside hydrolysis (Morant et al., in press). The co-localization of rhodiocyanosides and cyanogenic glucosides in *L. japonicus* (Forslund et al., 2004) and their parallel hydrolysis upon tissue disruption suggests that rhodiocyanosides like cyanogenic glucosides are defense compounds, although this remains to be shown experimentally. A parallel scenario is observed in barley (*Hordeum vulgare*) which accumulates the cyanogenic glucoside epiheterodendrin and four non-cyanogenic hydroxynitrilealk(en)yl glucosides, all derived from leucine (Nielsen et al., 2002). In agreement with our suggestion, the β-glucosides of α-, β- and γ-hydroxynitriles have previously been proposed to be synthesized via the same biosynthetic pathway (Møller and Seigler, 1999; Nielsen et al., 2002).

In addition to their role in plant defense, cyanogenic glucosides have been proposed to serve as nitrogen storage compounds (Selmar et al., 1988; Sánchez-Pérez et al., 2008). Recently, biochemical evidence for mobilization of nitrogen from dhurrin in sorghum has been presented that does not involve the liberation of HCN and hence circumvents the need for detoxification of HCN *in planta* (Jenrich et al., 2008). According to this catabolic pathway, the activity of dhurrinase is modified by the interaction with a yet unknown protein co-factor to yield *p*-hydroxyphenylacetonitrile instead of *p*-hydroxymandelonitrile. *p*-Hydroxyphenylacetonitrile is subsequently converted into *p*-hydroxyphenylacetic acid and free ammonia by the action of the sorghum heteromeric nitrilase, NIT4B2/4A (Jenrich et al., 2008). The presence of nitrilase orthologs in other species within Poaceae (Kriechbaumer et al., 2007) provides evidence that cyanogenic glucosides may serve as storage compounds of reduced nitrogen offering the possibility to adjust imbalances in the provision of amino acids for primary metabolism, e.g. in rapidly developing tissues during seed germination or seed/fruit development. This may be a general mechanism operating in cyanogenic Poaceae species. The strict distinction between primary and secondary metabolism is thus vanishing.

The occurrence of cyanogenic glucosides is not restricted to the plant kingdom. Some specialist herbivores are able to *de novo* synthesize cyanogenic glucosides or to sequester the glucosides obtained from their food plants for use in their own defense against predators (Zagrobelny et al., 2004). As an example, *Zygaena* species are able to *de novo* synthesize linamarin and lotaustralin, the very same cyanogenic glucosides found in their food plants. The cyanogenic glucosides have been shown to play several intimate roles in *Zygaena*, e.g. by being transferred as nuptial gifts during the mating process (Zagrobelny et al., 2004, 2007a,b).

2.5. Benzoxazinoid glucosides

The benzoxazinoids (also referred to as hydroxamic acids) DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) and DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; Figs. 4C and 6) are defense chemicals widely distributed within Poaceae (Niemeyer, 1988) and have also been found in a number of eudicotyledenous plant species (summarized in Baumeler et al., 2000). Benzoxazinoids have been implicated in resistance of maize against insects, fungi and bacteria (Niemeyer, 1988) and maize mutants deficient in DIMBOA biosynthesis are compromized with respect to their disease resistance (Hamilton, 1964; Frey et al., 1997). DIMBOA is highly effective in conferring resistance to phloem feeding aphids (Givovich et al., 1994; Givovich and Niemeyer, 1995; Cambier et al., 2001) as evidenced by the failure to establish a population of *Metopolophium dirhodum* aphids on young maize seedlings (Cambier et al., 2001). DIMBOA specifically blocks growth of *Agrobacterium tumefaciens* and the presence of benzoxazinoids has been suggested as one of the main factors explaining the recalcitrant behavior of monocotyledons to *Agrobacterium*-mediated transformation (Sahi et al., 1990). Besides their importance as natural pesticides, benzoxazinoids and their degradation products show significant allelopathic effects (Burgos and Talbert, 2000). Hence from an agricultural point of view, benzoxazinoid synthesizing crops are interesting not only because of their strong resistance to herbivores and pathogens, but also for their potential as cover crops to control weeds and in crop rotation schemes to reduce nematode infestation (Fig. 7).

In planta, the benzoxazinoids are found on their glucosylated form (BxGlc). The genes and enzymes of the entire biosynthetic pathway of DIBOAGlc and DIMBOAGlc have been isolated and characterized in maize (Fig. 6; Frey et al., 1997, 2003; von Rad et al., 2001; Jonczyk et al., 2008) and some from wheat (Nomura et al., 2002). In addition, the DIBOA biosynthetic pathway has been shown to proceed via identical enzymatic steps in maize and rye (Glawischnig et al., 1999), which suggests that this biosynthetic pathway is identical in maize, wheat and rye. The biosynthetic pathway branches off from the tryptophan biosynthetic pathway at the point of indole, synthesized from indole-3-glycerol phosphate via the action of a tryptophan synthase α homologue denoted BX1. The indole is converted into DIBOA via four consecutive hydroxylations catalyzed by four cytochromes P450 belonging to the CYP71C subfamily (BX2–BX5; Frey et al., 1997). BX6 and BX7 catalyze the conversion of DIBOA into DIMBOA (Frey et al., 2003; Jonczyk et al., 2008), and glucosylation by glucosyltransferases (BX8 and BX9) yields the non-toxic BxGlcs (von Rad et al., 2001). The genes encoding BX1 to X5 are located in a single cluster in the maize genome (Glawischnig et al., 1999) and most of the *CYP71C* genes found in the allohexaploid wheat are located on the chromosome group 5 (Nomura et al., 2002). The four *CYP71C* genes (*Bx2–Bx5*) are thus most likely results of a gene duplication event preceding the speciation of maize, wheat and rye. In spite of their high sequence similarity and the fact that all four CYP71C enzymes catalyze hydroxylations, each CYP71C is highly specific for a particular hydroxylation step in the DIBOA pathway (Glawischnig et al., 1999). Hence, each CYP71C enzyme has evolved to possess a new and specific catalytic function, which renders the set of genes involved in the BxGlc pathway a unique system for studying the evolutionary processes behind recruitment of new functions in multigene families.

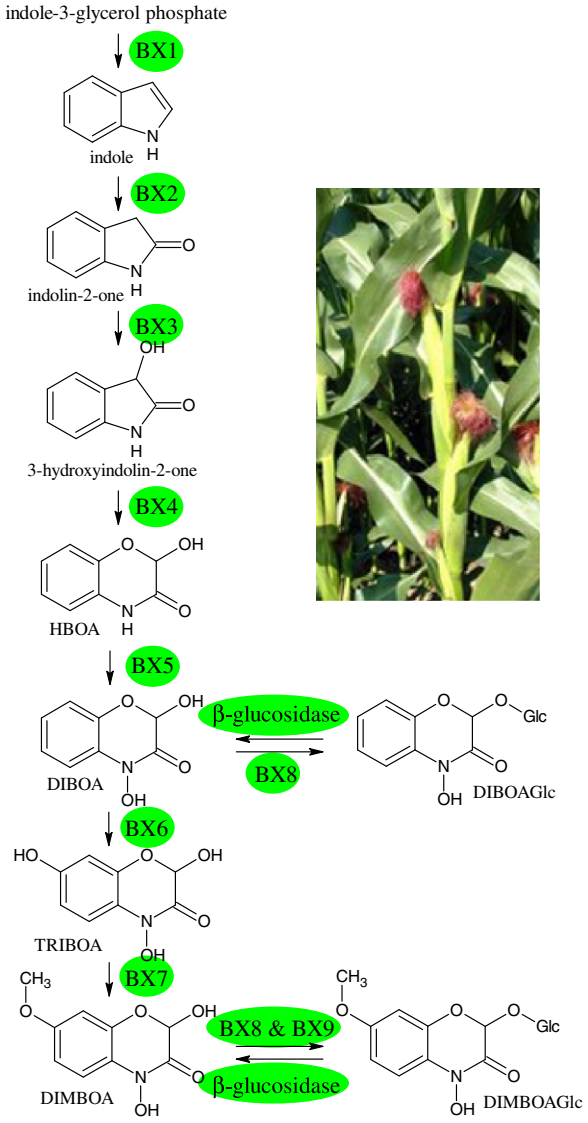

Fig. 6. Biosynthesis and bio-activation of benzoxazinoid glucosides. In maize (shown in the picture), a tryptophan synthase α homologue (BX1) and four cytochromes P450 belonging to the CYP71C family (Frey et al., 1997) convert indole-3-glycerol phosphate into DIBOA, which by a hydroxylation (catalyzed by a 2-oxoglutarate-dependant dioxygenase, BX6) and a methylation (catalyzed by an *O*-methyltransferase, BX7) is further converted into DIMBOA (Frey et al., 2003). Two glucosyltransferases glucosylate DIBOA and DIMBOA into the corresponding β-glucosides (von Rad et al., 2001). An identical pathway has been described in wheat (Nomura et al., 2002). Upon tissue disruption, DIBOAGlc and DIMBOAGlc are hydrolyzed by β-glucosidases (ZmGlu1 and ZmGlu2 in maize (Esen, 1992; Cicek and Esen, 1999) to yield glucose and the bio-active benzoxazinoids.

Upon tissue disruption, the BxGlcs are hydrolyzed by β-glucosidases to yield the toxic aglucones (Fig. 6), which decompose spontaneously to yield formic acid and benzoxazolinones (Niemeyer, 1988). β-Glucosidases responsible for the bio-activation of DIBOAGlc and DIMBOAGlc have been isolated and characterized from maize (Esen, 1992; Cicek and Esen, 1999), wheat (Sue et al., 2000b) and rye (Sue et al., 2000a; Nikus et al., 2003). The β-glucosidase substrate specificities (Oikawa et al., 1999; Sue et al., 2000a,b) reflect the predominant BxGlc present in the respective plants:

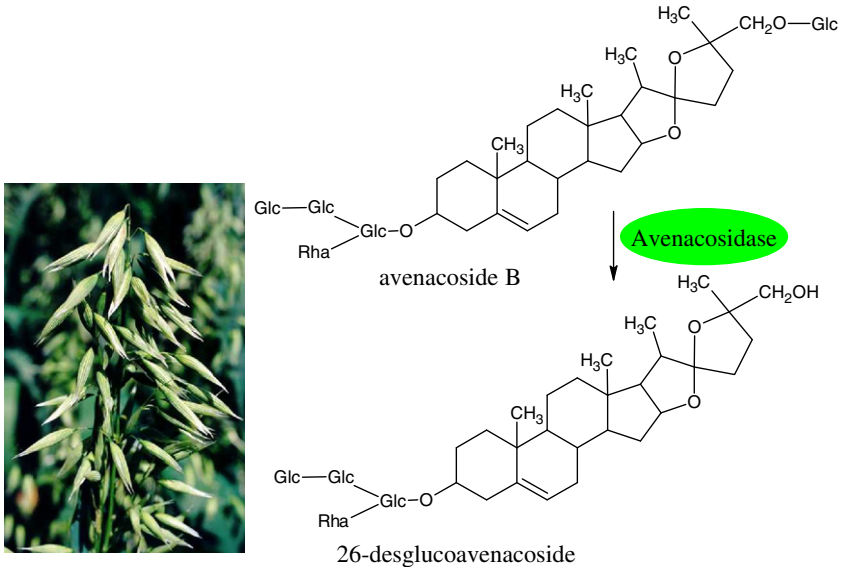

Fig. 7. Bio-activation of avenacoside B by hydrolysis of the C26 β-glucosidic bond. Upon tissue disruption in oat (shown in the picture) leaves, avenacosides A and B (molecules shown) are deglucosylated at the C26 position by avenacosidase to yield biologically active 26-desglucoavenacosides.

DIMBOAGlc in maize and wheat, and DIBOAGlc in rye (Niemeyer, 1988). Two β-glucosidase isozymes, ZmGlu1 and ZmGlu2 with 90% identity at the amino acid level, have been cloned from maize (Cicek and Esen, 1999) and three isozymes, TaGlu1a,b and c, sharing ~95% amino acid identity have been cloned from wheat (Sue et al., 2006). The isozymes are all specific for DIMBOAGlc, but are differentially regulated at the transcriptional level (Cicek and Esen, 1999; Sue et al., 2006) as also observed for the cyanogenic β-glucosidases. This suggests that the plant is able to fine tune the activity of the BxGlc/β-glucosidase system in response to i.e. developmental stages and imposed biotic stresses. While only one BxGlc β-glucosidase has been cloned from rye (Nikus et al., 2003), the purification scheme for the rye BxGlc β-glucosidase indicated the presence of several isoforms in rye (Sue et al., 2000a). Whereas the three different parental genomes that constitute the allohexaploid wheat might be the origin of the three DIMBOAGlc β-glucosidases identified in this plant, the hexaploid nature of wheat most likely does not account for the maintenance of more than one β-glucosidase isozyme because two or more β-glucosidase isozymes apparently also exist in maize and rye, which are both diploid.

The three-dimensional structures for ZmGlu1 and TaGlu1b (59% amino acid sequence identity) have been solved at high resolution (Czjzek et al., 2001; Sue et al., 2006) and ZmGlu1 has been used as a model for extensive studies of the aglucone specificity determining residues of β-glucosidases (Czjzek et al., 2000, 2001; Cicek et al., 2000; Verdoucq et al., 2003). ZmGlu1 and TaGlu1b show 40% identity to white clover linamarase at the amino acid level, and 72% and 58% identity to sorghum dhurrinase 1, respectively. The overall structures of ZmGlu1 and TaGlu1b are highly similar to linamarase and dhurrinase 1 (Czjzek et al., 2001; Sue et al., 2006) confirming the high degree of structural conservation observed for the β-glucosidases.

A maize β-glucosidase with 100% amino acid identity to ZmGlu1, denoted Zm-p60.1, has been characterized at the biochemical and structural level (Brzobohaty et al., 1993; Rotrekl et al., 1999; Zouhar et al., 2001) in parallel with ZmGlu1. In contrast to ZmGlu1, Zm-p60.1 has been assigned as the β-glucosidase responsible for release of free cytokinins in maize meristems (Brzobohaty et al., 1993; Kristoffersen et al., 2000) and referred to as an allozyme (an isozyme whose synthesis is controlled by codominant alleles of one gene) of ZmGlu1 (Zouhar et al., 2001). However, the fact that ZmGlu1 and Zm-p60.1 are 100% identical at the amino acid – and nucleotide levels, suggests that the same enzyme is responsible for bio-activation of DIMBOAGlc and cytokinin glucosides in maize, or alternatively that the enzyme is only involved in one of these physiological processes *in vivo* while able to cleave both substrates *in vitro*.

2.6. The oat avenacoside/avenacosidase two-component system

Oat (*Avena sativa*) is one cereal that does not accumulate BxGlcs. Instead, oat accumulates saponins which are wide spread defense compounds found in many plant species, although oat represents the only saponin accumulating cereal (Osbourn, 2003). Saponins possess detergent-like properties (Osbourn, 1996) and have been proposed to exert their antibiotic effect by insertion and complex formation with sterols in cellular membranes leading to formation of pores and membrane disruption (Morrissey and Osbourn, 1999). Saponins are mainly characterized as antimicrobial although insecticidal effects have also been reported (Weissenberg et al., 1998). Apart from their importance as preformed plant defense compounds, different saponins have important pharmocological properties and are exploited for production of medicine, adjuvants and hormone biosynthesis precursors (Francis et al., 2002; Osbourn, 2003). In particular, saponins have received increasing attention due to their potential use as potent anticancer agents (Hanausek et al., 2001; Raju and Bird, 2007). *In planta*, saponins are found either in their active monodesmosidic form (glycosylated at the C3 position) or in their non-active bisdesmosidic forms (glycosylated at the C3 and the C26 or C28 positions; Osbourn, 1996). How plants are able to store active saponins and prevent them from exerting their membrane disrupting effects *in planta* is unknown.

Oat accumulates two forms of saponins. Roots contain avenasides, which are triterpenoids stored directly in their active, monodesmosidic form. Avenaside deficient *sad* mutants show significantly increased disease susceptibility (Papadopoulou et al., 1999), which substantiates the classification of plant saponins as defense compounds. Oat leaves accumulate non-active bisdesmosidic saponins, avenacosides, that need to be bio-activated by a specific β-glucosidase in order to exert their biocidal effects (Nisius, 1988). Only little is known about the genes and enzymes involved in biosynthesis of the structurally complex saponins. The collection of avenacin deficient *sad* mutants generated in the diploid oat

species *Avena strigosa* shows that genes located at more than seven different loci are involved in the biosynthesis of avenacin (Papadopoulou et al., 1999). Of these, the *sad1* locus has been shown to encode an oxidosqualene cyclase that catalyzes the first committed step in avenacin biosynthesis (Haralampidis et al., 2001). However, out of 10 different avenacin deficient mutants, only one (*sad9*) had a simultaneous reduction in leaf avenacoside B levels, showing that the two forms of oat saponins are synthesized by two largely different biosynthetic pathways (Papadopoulou et al., 1999). The oat leaf avenacosides are glycosylated at the C3 and C26 positions (Fig. 4D). The glycosylation at the C3 position consists of a trisaccharide (a rhamnose and two glucose moieties) in the case of avenacoside A or a tetrasaccharide (a rhamnose and three glucose moieties) in the case of avenacoside B (Fig. 4D). The single glucose moiety linked to C26 by an *O*-β-glucosidic bond renders the saponin biologically inactive. Upon tissue disruption, this β-glucosidic bond is immediately hydrolyzed by a specific β-glucosidase to yield the active 26-desglucoavenacoside (Kesselmeier, 1982), which has been shown to possess antifungal activity (Nisius, 1988). The β-glucosidases responsible for avenacoside bio-activation, named avenacosidases after their substrate, are specific for the β-glucosidic bond at the C26 position leaving the β-glucosidic bonds linking the glucose residues at the C3 position intact (Nisius, 1988). The avenacosidases form defined large structures known as the stromacentre in oat plastids (Nisius, 1988). The particular multimerization pattern of the avenacosidases is discussed in a later section of this review. Two genes encoding isozymes of avenacosidase, AsGlu1 and AsGlu2 (88% amino acid sequence identity; Fig. 3A), have been cloned from oat (Gusmayer et al., 1994; Kim et al., 2000). As observed for the BxGlc β-glucosidase isozymes found in maize and wheat, the avenacosidase isozymes are differentially expressed (Kim and Kim, 1998), which supports the suggestion that plants are able to carefully regulate these two-component defense systems.

The saccharide linked to the C3 position of saponins is essential for the antimicrobial activity (Simons et al., 2006). Fungi that show pathogenic activity toward oat leaves posses α-rhamnosidase and β-glucosidase activities that detoxify 26-desglucoavenacosides by sequential hydrolysis of the sugar moieties at the C3 position (Wubben et al., 1996; Morrissey et al., 2000). Interestingly, a fungal pathogen of tomato, which like oat accumulates saponins as a chemical antimicrobial defense, exerts its pathogenic properties through a double mechanism in which the tomato saponin α-tomatine is first detoxified by hydrolysis of a glucose moiety by a β-glucosidase, and induced plant defense responses are subsequently suppressed by the action of the very same saponin hydrolysis product (Bouarab et al., 2002).

2.7. The mustard oil bomb: the glucosinolate–myrosinase system

Glucosinolates (Fig. 4E) are amino acid derived secondary metabolites found almost exclusively in the order Capparales, including oilseed rape, mustard, vegetables such as broccoli, cauliflower and cabbage and the model plant Arabidopsis (Halkier and Gershenzon, 2006). Glucosinolates and their biologically active hydrolysis products are of interest to the consumer due to their pungent taste and flavor and as potent inhibitors of carcinogenesis. The latter effect is due to the formation of hydrolysis products of the alkylisothiocyanate type, in particular sulforaphane, which mediates induction of detoxification enzymes that act as chemo protectors against cancer (Nestle, 1997; Fahey et al., 1997). The glucosinolates play important roles in agriculture owing to the toxic effects of their breakdown products on most insects and herbivores (Lambrix et al., 2001; Kliebenstein et al., 2002; Agrawal and Kurashige, 2003; Lazzeri et al., 2004) and due to their use as natural biofumigants (Brown and Morra, 1995; Lazzeri et al., 2004; Zasada and Ferris, 2004). Glucosinolates serve as attractants and feeding stimuli for some, often highly specialized herbivores (Gabrys and Tjallingii, 2002; Mewis et al., 2002). As observed for specialist herbivores feeding on cyanogenic plants, some specialized insects are able to sequester glucosinolates present in their food plants for use in their own defense against predators (Müller et al., 2001; Müller et al., 2002).

Glucosinolates are structurally and biosynthetically related to cyanogenic glucosides (Figs. 4 and 8) and are thought to have evolved from a cyanogenic glucoside predisposition (Poulton and Møller, 1993; Bak et al., 1998b; Rask et al., 2000; Halkier and Gershenzon, 2006). They are synthesized from the amino acids alanine, isoleucine, valine, tryptophan, tyrosine, phenylalanine and methionine and from chain elongated derivatives of the latter two amino acids (Halkier and Gershenzon, 2006) via a biosynthetic pathway whose initial steps correspond to those of cyanogenic glucoside biosynthesis (Ettlinger and Kjær, 1968; Bak et al., 1998b; Halkier and Gershenzon, 2006). Further secondary modifications of the core structure significantly increase the number of structurally different glucosinolates found in nature. Hydrolysis of glucosinolates results in release of glucose and an unstable aglucone, which spontaneously decomposes into different compounds of various toxicity including isothiocyanates (mustard oils), nitriles, epithionitriles and thiocyanates (Rask et al., 2000; Bones and Rossiter, 2006) depending on the reaction conditions (e.g. pH) and the nature of the glucosinolate side chain. The sugar moiety in glucosinolates is linked to the aglucone via an *S*-β-glucosidic bond (Fig. 4E). The subgroup of β-glucosidases cleaving glucosinolates is therefore β-thioglucoside glucohydrolases, commonly refered to as myrosinases. In myrosinases, the glutamic acid residue that acts as a general acid/base catalyst (Fig. 1) is replaced by a glutamine residue resulting in a TFN**Q**P motif instead of TFN**E**P (Burmeister et al., 1997). The lack of the general acid/base catalyst in myrosinases is compensated for in part by the glucosinolate aglucone being an excellent leaving group making protonation of the glucosidic sulfur atom by the acid catalyst unnecessary (Burmeister et al., 1997), and in part by incorporation of ascorbate within the active site, supposedly mediating activation of the water molecule (activated by the general base catalyst in *O*-β-glucosidases; Fig. 1) needed for hydrolysis of the β-glucosidic bond of the glucose–enzyme intermediate and release of the glucose (Burmeister et al., 2000) (Fig. 9).

Myrosinases are generally present in several differentially regulated isoforms within a given plant species (Lenman et al., 1993; Xue et al., 1993; Rask et al., 2000; Eriksson et al., 2001; Xu et al., 2004). In Arabidopsis, six genes encoding myrosinases (TGG) are found, including two pseudogenes (Zhang et al., 2002; Xu et al., 2004; Barth and Jander, 2006). Of these, TGG1 and TGG2 are responsible for myrosinase activity in green tissues as exemplified by the lack of myrosinase activity in leaves of *tgg1 tgg2* double mutants (Barth and Jander, 2006). The latter has significant effects on herbivores as exemplified by increased weight gains observed in two species of Lepidoptera feeding on the *tgg1 tgg2* mutant as compared to wild-type Arabidopsis (Barth and Jander, 2006), and further substantiates the importance of the bioactivation process for plant-insect interactions. White mustard (*Sinapis alba*) and oilseed rape (*Brassica napus*) contain at least 10 and 20 different isoforms of myrosinase, respectively (Xue et al., 1992; Rask et al., 2000; Eriksson et al., 2001). Like other dicotyledenous β-glucosidases, the myrosinases contain an N-terminal signal peptide directing the protein through the secretory pathway with resultant protein glycosylation.

As generally observed for the two-component defense systems, myrosinase and its substrate glucosinolates are compartmentalized at the cellular and subcellular level (Kelly et al., 1998). Myrosinases are mainly localized in specific myrosin cells (Thangstad et al.,

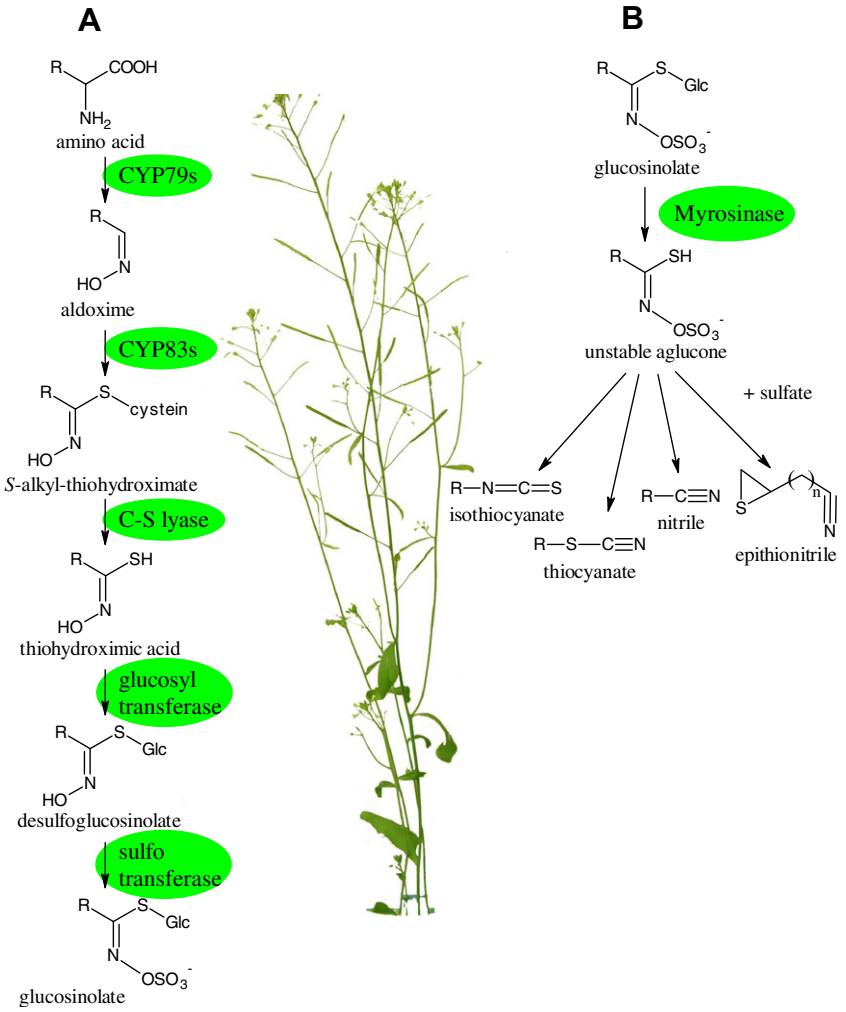

Fig. 8. Biosynthesis and bio-activation of glucosinolates. (A) Glucosinolates are synthesized from amino acids (or their chain elongated derivatives) via the action of two cytochromes P450, a C-S lyase, a glucosyl transferase and a sulfo transferase (Halkier and Gershenzon, 2006). The glucosinolate biosynthetic pathway has the CYP79 catalyzed synthesis of an aldoxime from the amino acid and further catalysis by a cytochrome P450 belonging to the CYP71/CYP83 family in common with the biosynthetic pathway for cyanogenic glucosides (Fig. 5). The glucosinolate core structure may be subject to further secondary modifications such as acylation or oxidation of the R-group. (B) Upon cell disruption, glucosinolates are hydrolyzed by myrosinases (β-thioglucoside glucohydrolases). The unstable aglucone spontaneously decomposes to yield a range of different products depending on the nature of the glucosinolate side chain and the reaction conditions. The glucosinolate accumulating Arabidopsis is illustrated in the picture.

1990; Höglund et al., 1991; Andreasson et al., 2001). In embryos of mustard greens (*Brassica juncea*), the glucosinolate sinigrin is found in vacuoles of non-myrosin cells (Kelly et al., 1998) while the glucosinolates accumulate in specific S-cells in Arabidopsis (Koroleva et al., 2000). At the subcellular level, myrosinases have been localized to vacuoles known as myrosin grains (Thangstad et al., 1991; Höglund et al., 1992; Andreasson et al., 2001) and recently myrosin grains have been shown to form a continuous reticular system denoted the myrosin body (Andreasson et al., 2001). The very specific yet different compartmentalization in Arabidopsis and *Brassica* species suggests that the glucosinolate/myrosinase system might have developed to fulfill partly different functions in Arabidopsis and *Brassica* species (Andreasson et al., 2001).

Herbivores have developed different strategies to counteract the presence of glucosinolates. The diamondback moth (*Plutella xylostella*; Ratzka et al., 2002) and the desert locust (*Schistocerca gregaria*; Falk and Gershenson, 2007) "disarm the mustard oil bomb" by expressing a glucosinolate desulfatase in their guts. Upon ingestion of glucosinolate containing plant material, the enzyme rapidly desulfates the glucosinolates to yield desulfo-glucosinolates. Desulfo-glucosinolates cannot be hydrolyzed by the myrosinases and as a result no toxic degradation products are formed enabling the insects to feed on these glucosinolate containing plants (Ratzka et al., 2002; Falk and Gershenson, 2007).

2.8. Examples of two-component systems missing one component

Leaves of barley seedlings accumulate the cyanogenic glucoside epiheterodendrin and four non-cyanogenic (β- and γ-) hydroxynitrile glucosides (Nielsen et al., 2002). While barley leaves are not cyanogenic due to the lack of a co-located cyanogenic β-glucosidase (Nielsen et al., 2002), a β-glucosidase able to hydrolyze the cyanogenic- and non-cyanogenic glucosides in barley is present in germinating seeds (Nielsen et al., 2002, 2006). The cyanogenic activity of the seed β-glucosidase most likely reflects broad substrate specificity of a β-glucosidase whose physiological function is the degradation of cellobiose during cell wall degradation in the seed during germination (Leah et al., 1995; Nielsen et al., 2006). A specific leaf β-glucosidase able to degrade cyano glucosides has most probably been lost and hence the physiological significance of the presence of the α-, β- and γ-hydroxynitrile glucosides in leaves of barley seedlings remains unclear.

In white clover (*Trifolium repens*) and common bird's foot trefoil (*Lotus corniculatus*), cyanogenesis exists as a polymorphic trait

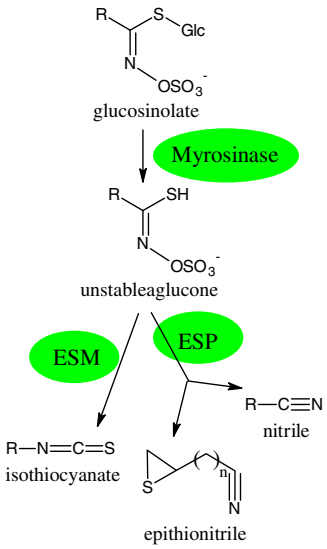

Fig. 9. Controlled diversion of glucosinolate breakdown products toward epithionitrile (and nitrile) by epithiospecifier protein (ESP) or isothiocyanate by epithiospecifier modifier protein (ESM). ESM directs aglucone decomposition toward isothiocyanates from aliphatic and aromatic glucosinolates (Zhang et al., 2006), while ESP favors the production of epithionitriles over isothiocyanates from aglucones of alkenyl glucosinolates and, in the case of Arabidopsis ESP, production of simple nitriles from other glucosinolate aglucones (Bernardi et al., 2000; Foo et al., 2000; Lambrix et al., 2001).

(Hughes, 1991; Gebrehiwot and Beuselinck, 2001; Olsen et al., 2007, in press). Populations of white clover comprise cyanogenic plants in which cyanogenic glucosides as well as linamarase (the cyanogenic β-glucosidase) are present. In other plants, one or both components are absent. The polymorphic trait is supposedly retained in populations of white clover by the action of opposing selective forces such as cyanogenesis-dependent reduced herbivory or cyanide intoxication as a result of frost-induced cell damage giving an evolutionary disadvantage of cyanogenesis in cold climates where plant tissue exposed to frost is disrupted causing cyanogenesis to occur with resultant exposure of the plant to the toxic effects of its own chemical defense system; Till (1987). In addition, the growth rate has been shown to be higher in low cyanogen versus high cyanogen varieties of the same plant species (Gleadow and Woodrow, 2002; Goodger et al., 2004).

The preponderance of specialist or generalist herbivores comprises two opposing selective forces on glucosinolate content in black mustard (*Brassica nigra*; Lankau, 2007). Because defense compounds of many plants act as attractants or feeding stimuli for specialist herbivores, the opposing selective forces posed by specialist and generalist herbivores might be a general mechanism contributing to maintenance of defense systems as a polymorphic trait.

3. Regulation of β-glucosidase oligomerization, activity and product formation

3.1. β-Glucosidase oligomerization

The tertiary structure of plant β-glucosidases belonging to the family 1 glycoside hydrolases is highly conserved (Fig. 3B) as are the active site amino acids involved in glucone binding (Barrett et al., 1995; Burmeister et al., 1997; Czjzek et al., 2001). The amino acids that define the aglucone binding pocket are highly variable, albeit found at conserved positions in the active site (Czjzek et al., 2000; Cicek et al., 2000; Czjzek et al., 2001; Verdoucq et al., 2003). In contrast, the regions involved in oligomerization vary greatly among β-glucosidases (Czjzek et al., 2001). This is reflected in the diversity of quarternary structures observed for plant β-glucosidases. Different cyanogenic β-glucosidases exist in monomeric, dimeric, tetrameric and oligomeric forms: Black cherry amygdalin hydrolases and some prunasin hydrolase isoforms are isolated as active monomers (Kuroki and Poulton, 1986, 1987; Li et al., 1992), while active white clover linamarase and *L. japonicus* cyanogenic β-glucosidases are recovered as dimers (Hughes and Dunn, 1982; Barrett et al., 1995; Morant et al., in press). Sorghum dhurrinase 1 exists *in planta* as a tetramer, but dissociates into dimers upon isolation without loss of activity, whereas sorghum dhurrinase 2 is isolated in its tetrameric form (Hosel et al., 1987). Rubber tree linamarase exists in multiple oligomeric forms (Selmar et al., 1987) and flax linamarase appears to be a decamer (Fan and Conn, 1985). It is not known to what extent the specific oligomerization influences the activity of these β-glucosidases.

In maize, dimerization stabilized by disulfide bridge formation is necessary for ZmGlu1/p60.1 activity (Rotrekl et al., 1999; Zouhar et al., 2001). White mustard myrosinase is a homodimer like the white clover linamarase (Burmeister et al., 1997) but the orientation of the subunits within these two dimers is different (Burmeister et al., 1997). Wheat TaGlu1a and TaGlu1b form homo- and hetero-hexamers and in contrast to the above mentioned β-glucosidases, hexamerization is a prerequisite for activity (Sue et al., 2006). Whether the seven different combinations of homo- and hetero-hexamers of the two isozymes display different activity and substrate specificities remains to be resolved.

The oat avenacosidase exists in two isoforms with different tissue distribution (Kim and Kim, 1998) and kinetic properties (Kim et al., 1996). Type I is a homomultimer of AsGlu1 and type II is a heteromultimer of AsGlu1 and AsGlu2 (Kim and Kim, 1998). The presence of the AsGlu1 monomer is critical for multimerization. Thus AsGlu1 alone or in combination with AsGlu2 forms multimers, while AsGlu2 alone forms only dimers (Kim et al., 2000). The type I avenacosidase displays an intriguing quarternary protein structure by formation of multimeric hollow fibrillae comprised of AsGlu1 monomers whose active sites line the interior tunnel (Kim et al., 2005). The particular quarternary structure has been suggested to allow discrimination of avenacosides from other β-glucoside substrates in oat (Kim et al., 2005) and enzyme activity increases with multimerization (Gusmayer et al., 1994). The detailed impact of hetero-multimer formation and of the fibrillar conformation on *in vivo* activity and substrate specificity remains to be clarified.

The multitude of different oligomerization forms and differential orientation of the monomers within the oligomers in spite of the high degree of primary, secondary and tertiary structure conservation among the β-glucosidases suggests a functional, yet unresolved, importance of oligomerization, and that the β-glucosidases have evolved to form specific quarternary structures after speciation to fulfill defined functions or allow specific regulation of bioactivation of defense compounds in a particular plant species.

3.2. β-Glucosidase binding proteins: β-glucosidase aggregating factors and myrosinase binding proteins

Specific proteins have been shown to interact with β-glucosidases resulting in formation of large insoluble aggregates. The most well characterized examples are the maize β-glucosidase aggregating factor (BGAF) and the myrosinase binding proteins (MBP).

BGAF is a 35 kDa protein (Esen and Blanchard, 2000) present in some maize genotypes in which the BxGlc β-glucosidases are found as large insoluble aggregates upon extraction (Esen and Cokmus, 1990; Esen and Blanchard, 2000). BGAF binds specifically to ZmGlu1 and ZmGlu2, but not to the highly similar sorghum dhurrinase 1 (Blanchard et al., 2001). BGAF is induced by jasmonic acid

and contains two distinct protein domains: A jacalin-related lectin domain and a disease response or "dirigent protein" domain (Kittur et al., 2007a). BGAF homologues are found only within Poaceae. A BGAF cloned from sorghum with 67% amino acid sequence identity to the maize orthologue binds neither sorghum nor maize β-glucosidases (Kittur et al., 2007b). The physiological significance of BGAF remains unknown. BGAF binding and aggregation has no apparent effect on β-glucosidase activity (Esen and Blanchard, 2000) but β-glucosidase aggregation upon tissue disruption has been suggested to play a role in concentrating the β-glucosidase activity to the wounding site and in protecting BGAF and/or β-glucosidase from proteolytic degradation by proteases secreted by the attacking insect (Blanchard et al., 2001; Kittur et al., 2007a). The fact that lectins have been implicated in plant defense (Peumans and Van Damme, 1995) and that BGAF possesses lectin activity even when bound to β-glucosidase prompted Kittur et al. (2007a) to suggest that insect ingestion of the BGAF/ β-glucosidase complex would lead to binding of glycoproteins in the oral cavity or the peritrophic matrix of the insect and result in a local burst of DIMBOA or damage of the peritrophic matrix. The restriction of BGAF to some maize genotypes and the finding that sorghum BGAF does not bind β-glucosidase suggests that BGAF-mediated aggregation of β-glucosidases upon cellular disruption is not a general defense mechanism in Poaceae. A BGAF cloned from wheat was shown to be highly similar to maize BGAF, but whether this BGAF binds one or more wheat β-glucosidases was not examined (Wang and Ma, 2005) and hence it is not known whether the β-glucosidase aggregating activity is unique to maize BGAF or wide spread in Poaceae.

A similar scheme of β-glucosidase aggregation mediated by a protein-binding partner has been observed in some glucosinolate accumulating plants. White mustard and oilseed rape express numerous myrosinases divided into three subfamilies (Lenman et al., 1990; Lenman et al., 1993; Rask et al., 2000; Eriksson et al., 2001). Whereas myrosinases belonging to one subfamily (MA) are always isolated in a soluble dimer form, the remaining myrosinases belonging to the MB and MC subfamilies are recovered in the insoluble fraction in large complexes with MBPs (Lenman et al., 1990; Taipalensuu et al., 1996; Geshi and Brandt, 1998; Eriksson et al., 2001). This demonstrates that MBP binds specifically to a subgroup of myrosinases. MBP is necessary for myrosinase complex formation (Eriksson et al., 2002), but like the BGAF/ β-glucosidase system, MBP is not necessary for myrosinase activity (Chen and Halkier, 1999; Eriksson et al., 2002), and antisense mediated knock-down of MBP accumulation in oilseed rape had no effect on the feeding behavior of the flea beetles and fungal pathogens tested (Eriksson et al., 2002). The expression of MBP is developmentally regulated (Geshi and Brandt, 1998), and interestingly MBP and myrosinase are compartmentalized at the cellular level in developing seeds of oilseed rape (Andreasson et al., 2001; Eriksson et al., 2002) and become co-localized at later growth stages (Andreasson et al., 2001). This suggests different functions for MBP-myrosinase complex formation at different stages of development. MBPs are a family of 30–110 kDa proteins (Falk et al., 1995) containing jacalin-type lectin domains (Taipalensuu et al., 1997b; Geshi and Brandt, 1998). MBP expression is wound inducible and jasmonic acid inducible (Taipalensuu et al., 1997b,c; Geshi and Brandt, 1998). These characteristics correspond to those known for BGAF, which implies a common physiological role for MBP and BGAF. The lectin properties of MBP have been suggested to be responsible for complex formation through binding of the myrosinase glycoproteins (Taipalensuu et al., 1997b; Geshi and Brandt, 1998) but this cannot be the case for BGAF binding to β-glucosidase in maize, because the monocotyledenous β-glucosidases are not glycosylated. While the MBP *in vivo* function remains unknown, the physiological roles proposed for MBP correspond to those suggested for BGAF.

Another group of proteins unrelated to MBP co-precipitate with the MBP-myrosinase complexes in oilseed rape (Taipalensuu et al., 1996). These proteins have been assigned as myrosinase associated proteins (MyAP); they are wound and jasmonate inducible glycoproteins (Taipalensuu et al., 1996; Taipalensuu et al., 1997a; Andreasson et al., 1999), and like MBP their physiological function is unknown. Proposed functions for MyAPs include cleavage of acyl groups from acylated glucosinolates to make them available for myrosinase hydrolysis (Taipalensuu et al., 1997a), regulation of myrosinase stability, activity or specificity (Taipalensuu et al., 1996) or regulation of the nature of the glucosinolate breakdown products (Taipalensuu et al., 1996).

In Arabidopsis, a β-glucosidase/β-glucosidase binding protein system exists, that deserves mentioning in spite of the apparent lack of relation to any of the two-component systems presented in this review. Although the physiological substrate of the Arabidopsis β-glucosidase PYK10 remains unknown, this β-glucosidase is of interest in the context of defense related β-glucosidase activity mediated by binding to other proteins. PYK10 was first reported to be a root and hypocotyl specific myrosinase (Nitz et al., 2001). However, the fact that no biochemical activity towards glucosinolates was shown and that PYK10 has two catalytic glutamates instead of the myrosinase glutamic acid/glutamine pair strongly suggests that PYK10 is not a myrosinase. PYK10 was later shown to have *O*-β-glucosidase activity toward artificial *O*-β-glucosidic substrates (Matsushima et al., 2003a, 2004) and to be the main component of an organelle called the ER body found in cotyledons, hypocotyls and roots of Arabidopsis (Matsushima et al., 2003b). In analogy to the myrosinase/MBP and ZmGlu/BGAF systems, PYK10 forms large insoluble complexes and co-precipitates with PBP1, a 35 kDa protein with jacalin-type lectin repeats also found in MBP and BGAF (Nagano et al., 2005). In contrast to the systems described above, however, PYK10 is able to form large complexes in the absence of PBP1, and PBP1 increases PYK10 activity (Nagano et al., 2005), while there is no evidence of BGAF and MBP affecting the activity of their binding partners. The physiological function of PYK10 and PBP1 is unknown, but the fact that ER bodies are induced by wounding and methyl jasmonate in rosette leaves and that PYK10 and PBP1 have separate subcellular locations allowing PYK10 activation upon tissue disruption suggests a role in defense against herbivores. Curiously, the wound induced ER bodies do not contain PYK10 (Matsushima et al., 2003a). Matsushima et al. (2003a) suggested that the induced ER bodies might instead accumulate BGL1, a β-glucosidase with 70% amino acid sequence identity to PYK10 whose expression in rosette leaves is induced by insect feeding and jasmonic acid (Stotz et al., 2000). Identification of the PYK10 *in vivo* substrate would aid in elucidation of the physiological function of PYK10 and PBP1 and might unravel the existence of an as yet uncharacterized two-component defense system.

3.3. Regulation of myrosinase hydrolysis products

The epithiospecifier protein (ESP) found in some glucosinolate accumulating plants (Foo et al., 2000; Lambrix et al., 2001) alters the final outcome of the myrosinase-catalyzed hydrolysis of glucosinolates without having any enzymatic activity on intact glucosinolates. ESP favors the production of epithionitriles over isothiocyanates from aglucones of alkenyl glucosinolates and, in the case of Arabidopsis ESP, production of simple nitriles from other aglucones of glucosinolates (Lambrix et al., 2001). While ESP co-elutes with myrosinase during protein purification (Bernardi et al., 2000), ESP is apparently not myrosinase specific (Foo et al., 2000) suggesting that ESP is not directly associated with myrosinase and that the presence of myrosinase is required for production of the glucosinolate aglucone, not for interaction with ESP in order for ESP to regulate the product profile. This is sup-

ported by the work by Burow et al. (2006) who were unable to detect direct association between myrosinase and ESP. The reaction mechanism of ESP remains unknown, but biochemical studies suggest that ESP possesses enzymatic activity and is not merely a cofactor for myrosinase (Burow et al., 2006). A 37 kDa Arabidopsis ESP is present in Landsberg (L*er*) and absent in Columbia (Col) ecotypes of Arabidopsis in correlation with the preferential accumulation of nitriles in L*er* and isothiocyanates in Col (Lambrix et al., 2001; Burow et al., 2007b). The generalist herbivore *Trichoplusia ni* showed significant preference for plants producing (epithio) nitriles over the more toxic isothiocyanates (Lambrix et al., 2001). Lambrix et al. (2001) suggested that regulation of isothiocyanate to nitrile ratios could be important in the adaption of cruciferous plants to the opposing selective forces presented by generalist and specialist herbivores.

A prime example of specialist herbivore adaptation to the defense system of their food plants is the nitrile specifier protein mediated diversion of glucosinolate product formation from toxic isothiocyanates to nitriles observed in larvae of the specialist insect cabbage white butterfly (*Pieris rapae*; Wittstock et al., 2004). Nitrile specifier protein favors simple nitrile formation from all glucosinolates tested by a scheme similar to the plants' own ESP system (Burow et al., 2006), although no sequence similarity exists between ESP and the 73 kDa insect functional homologue (Wittstock et al., 2004).

Recently, an epithiospecifier modifier protein (ESM) was described from Arabidopsis and shown to have the opposite profile of ESP (Zhang et al., 2006). ESM is present in Col and not in L*er* and favors the production of isothiocyanates over nitriles upon hydrolysis of aliphatic and aromatic glucosinolates (Zhang et al., 2006). In parallel with the results of Lambrix et al. (2001), Zhang et al. (2006) showed that the glucosinolate hydrolysis profile determined by ESM influenced *T. ni* herbivory. Unlike ESP, Arabidopsis ESM is annotated as a MyAP. The study by Zhang et al. (2006) thus demonstrates a physiological function of this MyAP in determination of the glucosinolate hydrolysis product profile.

In addition to ESP and ESM, a third determinant of the glucosinolate hydrolysis product profile is the thiocyanate-forming protein (TFP). TFP has been characterized from garden cress (*Lepidium sativum*; Burow et al., 2007a). Like ESP and ESM, TFP controls the nature of the glucosinolate hydrolysis product in a myrosinase dependent manner without showing catalytic activity toward the glucosinolate itself. TFP shares ~65% amino acid sequence identity with known ESPs, but compared to the ESPs, TFP favors the formation of several different hydrolysis products depending on the nature of the glucosinolate (thiocyanates and simple nitriles from benzylglucosinolates and simple nitriles and epithionitriles from aliphatic glucosinolates; Burow et al., 2007a). In addition, TFP shows an organ specific activity profile (Burow et al., 2007a). TFP thus constitutes a mechanism for control of glucosinolate hydrolysis product profile that is regulated at several different levels.

Several findings suggest that one physiological function of MBP could be regulation of the glucosinolate hydrolysis product via specific protein complex formation involving either ESP, ESM or TFP in addition to myrosinase. First, ESP is associated with myrosinase (Bernardi et al., 2000) without being myrosinase specific (Foo et al., 2000). Second, Arabidopsis ESM is a MyAP (Zhang et al., 2006), and third, ESP proximity to myrosinase is required for ESP activity although no stable interaction could be detected between the two enzymes (Burow et al., 2006). In this case, MBP could be responsible for formation of a metabolon-like multiprotein complex in which the determinants of product profile (ESP, ESM and TFP) are kept in immediate proximity of the myrosinases and the unstable aglucones produced. TFP shares up to 55% amino acid sequence indentity with Arabidopsis MBPs (Burow et al., 2007a) and hence might mediate both myrosinase aggregation and control of product formation. These schemes would allow a multidimensional system for highly fine tuned regulation of glucosinolate hydrolysis product profile in response to various biotic stresses.

4. Conclusions

This review has focused on cyanogenic glucosides, benzoxazinoid glucosides, avenacosides and glucosinolates and the corresponding β-glucosidases as these are the two-component plant defense systems that have received the most attention in the literature and for which the bio-activating β-glucosidases have been characterized in detail. Additional two-component defense systems for which both substrate and β-glucosidase have been studied to some extent include glucosylated isoflavones from the leguminous soybean (*Glycine max*; Hsieh and Graham, 2001; Suzuki et al., 2006) and thai rosewood (Cairns et al., 2000), coumarin glucosides from sweet clover (*Melilotus alba* Desr.; Oba et al., 1981) and hydrojuglone β-glucoside from walnut (*Juglans regia* L.; Durroux et al., 1998). The vast number of unexamined plant species in nature is bound to comprise numerous as yet uncharacterized two-component β-glucoside/β-glucosidase defense systems with a yet undiscovered potential for agricultural, industrial and medicinal applications.

The current chemical defense systems active in plants are multifaceted traits and are the outcome of millions of years of a complex evolutionary arms race between plants and their herbivores and pathogens. The glucosinolate/myrosinase system is a good example of the complex regulation of defense strategies mediated by ESP, ESM and TFP and possibly by MBP and other myrosinase associated proteins. The ability of some specialized insects to control the type of glucosinolate breakdown products formed via a nitrile specifier protein or to prevent glucosinolate hydrolysis by a desulfatase activity are intriguing examples of how biochemical adaptations to herbivory are met by counteradaptations from the herbivores. Similar complex regulatory systems are most likely to be found in the other two component defense systems in plants as the biochemical and molecular characterization of these systems advance.

The wound and jasmonate inducibility of MBP and MyAP as well as the change in plant glucosinolate profile observed in response to jasmonate treatment and insect attack (Mikkelsen et al., 2003; Mewis et al., 2005) demonstrate that although the glucosinolate-myrosinase system is a preformed defense system, several of the components are regulated in response to wounding and insect attack adding yet a dimension to the complexity of the glucosinolate/myrosinase system. Such regulation at several different levels might also be encountered in the less intensively studied remaining two-component systems described above.

Detailed knowledge on plant defense systems including the genes and enzymes of the biosynthetic and catabolic pathways, metabolon formation and metabolic cross talk will allow further exploitation and enhancement of the plants' own defense mechanisms by molecular breeding based on natural variation or even production of plant species with tailor made pest resistance introduced by transformation with entire pathways. Such approaches are likely to reduce the footprint of agricultural production on the environment and may limit the use of chemical pesticides. The tools of molecular breeding have enabled the transfer of the entire dhurrin biosynthetic pathway from sorghum to Arabidopsis with resistance to specific insects as a measurable new trait (Tattersall et al., 2001) and without significant inadvertant effects on the metabolome (Kristensen et al., 2005). This provides a unique example of a plant engineered to produce a new defense compound which confers insect resistance while at the same time adhering to the principle of substantial equivalence. In these

dhurrin producing Arabidopsis plants, dhurrin hydrolysis was very slow due to the lack of the second component, namely the corresponding specific β-glucosidase. This resulted in a slow HCN release rate and demonstrated the need for both components of the two-component defense system. *L. japonicus* BGD2, which efficiently hydrolyzes dhurrin, has been crossed into the dhurrin producing Arabidopsis plants to create a true cyanide bomb with immediate release of HCN upon tissue disruption (Morant et al., work in progress).

Plant defense systems including the two-component systems and secondary metabolites in general present a vast unexplored and hence unexploited potential of great agricultural, medicinal and industrial importance.

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