β-Glucosidases and hydroxamic acid glucosides

-a proposed defense system in rye (*Secale cereale*)

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Abstract

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The thesis is an investigation of a proposed defense system in the Poaceae, consisting of glycosylated substrates being activated by β-glucosidase enzymes upon tissue rupture. The cyclic hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) are secondary metabolites that occur in high abundance as glucosides in the Poaceae, among them the cereals maize (*Zea mays*), wheat (*Triticum aestivum*) and rye (*Secale cereale*). Upon tissue disruption the hydroxamic acid glucoside is cleaved by β-glucosidase to release a toxic aglucone that is unstable and decomposes to the reactive benzoxazolinones. To protect the intact plant itself from the toxic products released by hydrolysis of the glucosides, the glucoside substrates and the enzyme need to be compartmentalized. In this study, the β-glucosidases in rye, wheat and maize seedlings are shown to be located in the epidermis and in cells associated with the vascular tissues. At the cellular level, β-glucosidase enzyme was located to plastids, cell walls and cytoplasm in all three species. To locate the hydroxamic acid glucoside substrates in rye, wheat and maize seedlings, tissue sections were stained with ferric chloride. In all three species staining was observed in the vascular bundles of shoots and coleoptiles, in the vascular cylinder in roots and in the outer cell layers of shoots, coleoptiles and roots.

In order to characterize the enzymatic part of the defense system a rye β-glucosidase cDNA was isolated. It was identified as a gene for a plastid located β-glucosidase (EC3.2.1.21) of glycoside hydrolase family 1. The sequence corresponding to the mature protein was expressed in *Escherichia coli*, purified and compared to purified intracellular β-glucosidase of rye. The recombinant protein was found to be homologous to the intracellular rye enzyme. In addition a cell wall enzyme from rye has been purified and identified as a glucan 1,3-β-glucosidase (EC3.2.1.58) of glycoside hydrolase family 3. Both the plastidic and the cell wall enzyme exhibited activity with DIBOA-glucoside and DIMBOA-glucoside as substrates and may therefore both be involved in the activation of the defense response.

Keywords: hydroxamic acids, rye, *Secale cereale*, β-glucosidase, secondary metabolism, plant defense, cyclic benzoxazinones

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Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals (I-V).

I. Nikus J, Jonsson LMV (1999) Tissue localization of β-glucosidase in rye, maize and wheat seedlings. Physiol Plant 107:373-378

II. Nikus J, Daniel G, Jonsson LMV (2001) Subcellular localization of β-glucosidase in rye, maize and wheat seedlings. Physiol Plant 111:466-472

III. Nikus J, Esen A, Jonsson LMV (2003) Cloning of a plastidic rye (*Secale cereale*) β-glucosidase cDNA and its expression in *E.coli*. (Physiol Plant, in print)

IV. Nikus J, Jonsson LMV. Cell wall glucan 1,3-β-glucosidase from rye (*Secale cereale*) active on hydroxamic acid glucosides. (Manuscript)

V. Nikus J, Jonsson LMV. Localization of hydroxamic acid glucosides in rye (*Secale cereale*) tissues. (Manuscript)

Papers I-III were printed with the permission of the publisher.

Abbreviations

BOA	2-benzoxazolinone
6-BNGlc	6-bromo-2-naphtyl-β-D-glucopyranoside
DIBOAGlc	2-*O*-β-D-glucopyranosyl-4-dihydroxy-1,4-benzoxazin-3-one
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one
DIMBOAGlc	2-*O*-β-D-glucopyranosyl-4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
MBOA	6-methoxy-2-benzoxazolinone
4-MUGlc	4-methylumbelliferyl-glucoside
HCN	hydrogen cyanide
Hx	hydroxamic acids
HxGlc	hydroxamic acid glucoside(s)
oNPGlc	*o*-nitrophenyl-β-D-glucopyranoside
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase-anti-peroxidase
pNPFuc	*p*-nitrophenyl-β-D-fucopyranoside
pNPGal	*p*-nitrophenyl-β-D-galactopyranoside
pNPGlc	*p*-nitrophenyl-β-D-glucopyranoside
pNPGlu	*p*-nitrophenyl-β-D-glucopyranosiduronic acid
pNPXyl	*p*-nitrophenyl-β-D-xylopyranoside
SDS	sodium dodecyl sulfate
TEM	transmission electron microscope

Introduction

Plants, like all organisms, are part of ecosystems and need to communicate with their environment. They need to attract pollinators, repel herbivores and to compete with neighboring plants for water, nutrients and light. Plants are also, like animals, constantly exposed to attacks by pathogens. Since plants are sessile and have no ability to produce sound they have instead developed well defined chemical communication and defense systems. Small compounds, not involved in the primary metabolism, used in plant communication and defense are called secondary metabolites or secondary compounds. Secondary compounds are involved in the chemical communication of plants by determining plant color, smell and taste and plant defense by acting as plant toxins.

Knowledge about plant secondary metabolism is important for many areas of applied botany, among them agriculture and horticulture. Since secondary metabolites often have a function in plant interaction with other organisms they may be important in practical contexts as resistance factors or weed growth inhibitors. Plant content of secondary metabolites may for instance be decisive in developing systems for crop rotation and companion planting. The main study object of this thesis, rye (*Secale cereale*) is a species that appears promising in these types of agriculture practices due to its ability to inhibit weed growth (Barnes and Putnam 1986). Planting rye in fall, allowing it to grow during fall and early spring and then kill it chemically or by plowing before conventional planting in the spring, results in inhibition of growth of several weed species (Barnes and Putnam 1983). Intercropping of maize (*Zea mays*) with the grass *Melinis minutiflora* decreases the levels of infestation of maize by stem-borers. Volatile compounds produced by *M. minutiflora* repel female stem-borers and increase larval parasitism of stem-borers by attracting foraging females of the parasitoid wasp *Cotesia sesamiae* (Khan et al. 1997). Plant secondary compounds may also be used as markers in plant breeding and they are obvious subjects for biotechnological modifications for specific traits.

Secondary compounds involved in plant defense are either toxic, like for example alkaloids and non-protein amino acids, or become toxic when chemically altered. Toxins are often feeding repellents of visual or olfactory nature to give the herbivore a warning of its presence before they start feeding on the plant, but many toxins provide feeding repellency through their bitter taste. Toxins may occur in surface waxes or be secreted by glandular hairs on the leaf, as for example the secretion of a toxic quinone from glandular hairs of *Primula obconica* (Harborne 1993).

Compounds that need to be chemically altered to become toxic are part of defense systems consisting of substrates and hydrolyzing enzymes located in different tissues or in different organelles of the plant. These compounds are often stored as glucosides in the central vacuole. When plant tissue is mechanically injured during pathogen attack or by feeding herbivores, a β-glucosidase (*O*-β-glucosidase, EC3.2.1.21; myrosinase, EC3.2.3.1), comes in contact with the glucosides that are hydrolyzed whereby an aglucone is formed. The aglucone decomposes spontaneously to a more toxic breakdown product.

In this thesis, a proposed defense system occurring in important cereals such as maize, wheat and rye has been studied and further characterized. The defense system consists of hydroxamic acid glucosides (HxGlc) and β-glucosidases. In the work, the localization of the glucoside substrates and the β-glucosidases has been studied and two β-glucosidases in rye have been isolated and characterized. As background information, the four plant defense systems known from the literature consisting of glucosides and glucosidases are described below.

Substrate – enzyme defense systems

Glucosinolates

Glucosinolates are thioglucosides that occur in crucifers and have been found to be toxic to animals. More than 120 different glucosinolates have been found, although any certain plant species has its own glucosinolate profile consisting of only a few. Upon tissue rupture, the glucosinolates are hydrolyzed by a thioglucosidase (myrosinase) and the aglucones that are formed disintegrate to reactive nitriles, cyanoepithioalkanes, thiocyanates, and isothiocyanates (mustard oils). In rapeseed (*Brassica napus*) and *Arabidopsis thaliana,* the enzyme and its substrate are compartmentalized in different cells (Rask et al. 2000). In seeds, leaves and roots of rapeseed, myrosinase has been shown to be located in protein bodies/vacuoles (myrosin grains) in specific myrosin cells scattered throughout the tissue (Höglund et al. 1992, Bones and Rossiter 1996). In leaves of *A. thaliana*, as in rapeseed, myrosinase is located to myrosin cells present only in the phloem parenchyma (Andréasson et al. 2001). In contrast to rapeseed, the seeds of *A. thaliana* do not seem to contain any myrosinase (Larsen Petersen et al. 2002). The glucosinolate substrates have been located to sulfur-rich cells between the phloem in flower stalks of *A. thaliana* (Koroleva et al. 2000). In seeds of *B. juncea* the glucosinolate sinigrin was located to protein bodies/vacuoles in non-myrosin, aleuron-like cells (Kelly et al. 1998). In the same species, myrosinase was co-localized with sinigrin to protein bodies/vacuoles

in aleurone-like cells, in addition to a localization of myrosinase to myrosin grains in specific myrosin cells. Mustard oils have an acrid smell and are probably emitted continuously in trace amounts as a feeding repellent. Toxic symptoms include severe gastroenteritis, salivation, diarrhea and irritation of the mouth.

Cyanogenic glucosides

Cyanogenic glucosides are *O*-β-glucosides from which hydrogen cyanide (HCN) is released upon tissue damage. The sugar moiety is enzymatically cleaved by an *O*-β-glucosidase and the corresponding aglucone is formed. The aglucone is then spontaneously, or enzymatically by a α-hydroxynitrile lyase, converted to carbonyl compounds and HCN (Poulton 1990). Compartmentalization of the substrate and enzyme prevent the release of HCN in high amounts in the intact plant although the localization of the substrate and its hydrolyzing enzyme differs between species (Poulton 1990). In sorghum seedlings the substrate, dhurrin, is located to epidermis and the enzyme, dhurrinase, is located to chloroplasts in the underlying mesophyll cells (Kojima et al. 1979, Thayer and Conn 1981). In cassava roots, however the compartmentalization seems to be at subcellular level since the enzyme, linamarase, as well as its substrate, linamarin, are present throughout the root tissue (Kojima et al. 1983). In cassava leaf tissue, linamarase has been located to cell walls (Mkpong et al. 1990). In cassava roots however it has been shown that the subcellular localization differs between cultivars of low and high cyanide content (Santana et al. 2002). In high cyanide content cultivars, linamarase was located to cell walls, whereas in low cyanide content cultivars, linamarase was located to both cell walls and vacuoles of laticifer (latex containing) cells. About 75 different cyanogenic glucosides have been identified in about 300 plant species, but the ability of a plant to release hydrogen cyanide has been recognized in over 3000 species, both monocotyledons and dicotyledons. HCN binds to cytochrome oxidase and blocks electron transfer through the mitochondrial electron transport chain and thereby inhibits respiration.

Avenacosides

Avenacosides are steroidal glycosides found in oat (*Avena sativa*) and were discovered due to their anti-fungal activities. The non-toxic form contains sugar residues at C3 and C26 (Nisius 1988). Damage of the oat cell by the infection of fungi leads to immediate hydrolysis of the glucose group at position C26 by an *O*-β-glucosidase, named avenacosidase after its substrate (Gus-Mayer et al. 1994b). Only the hydrolysis product, 26-desgluco-avenoside shows anti-fungal activity. Avenacosidase is compartmentalized in plastid

stromacenters in oat leaves (Nisius 1988, Gus-Mayer et al. 1994b, Kim et al. 2000).

Hydroxamic acid glucosides

HxGlc are mainly restricted to the Poaceae but have also been found in several dicotyledonous species (Baumeler el al. 2000, Sicker et al. 2000). They are found in high quantities (mM levels) in rye, wheat (*Triticum aestivum*) and maize. Hydroxamic acids (Hx) were, like the avenacosides, first discovered due to their anti-fungal properties (Wahlroos and Virtanen 1959) but have since then been implicated in various other biological functions such as defense against insects, fungi and bacteria, allelopathy and modification of auxin effects on elongation (Niemeyer 1988). In maize and wheat, DIMBOAGlc (2-O-β-D-glucopyranosyl-4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is the main HxGlc, whereas in rye it is DIBOAGlc (2-O-β-D-glucopyranosyl-4-dihydroxy-1,4-benzoxazin-3-one) (Hofman and Hofmanová 1969). In the intact plant, the substrates and their hydrolyzing enzymes are kept apart by compartmentalization. When the tissue is injured the substrates come in contact with *O*-β-glucosidase enzymes and the glucose moiety is cleaved off (Fig. 1). The resulting Hx aglucone decomposes spontaneously to benzoxazolinones, DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) to MBOA (6-methoxy-2-benzoxazolinone) and DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) to BOA (2-benzoxazolinone) with liberation of formic acid (Niemeyer 1988). The benzoxazolinones and the aglucones have been shown to be more toxic than the corresponding glucosides against insects, bacteria and fungi (Niemeyer 1988, Sicker et al. 2000). In maize seedlings, HxGlc hydrolyzing β-glucosidase has previously been located to plastids (Esen and Stetler 1993).

The contents of HxGlc have been determined in many studies either as the levels of HxGlc or as the levels of Hx aglucones after hydrolysis. Using the latter method it was shown for wheat that concentrations of Hx/HxGlc are highest in the young seedling and decreases with plant age (Zúñiga and Massardo 1991). This was later confirmed by Nakagawa and co-workers (1995), who showed the decrease of HxGlc as well as their corresponding aglucones by plant age. However, Copaja et al. (1999) suggested that this decrease was due to growth dilution effects. In maize, it was found that the decrease of DIMBOAGlc with plant age might be due to transformation into other derivatives (Cambier et al. 2000).

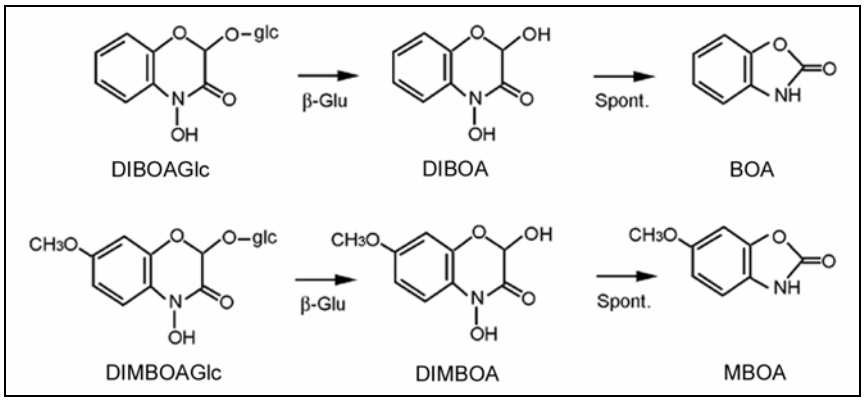

Figure 1. Hydrolysis of DIBOAGlc and DIMBOAGlc by β-glucosidase and the subsequent formation of benzoxazolinones (BOA and MBOA).

Biological activities of hydroxamic acids

Hx have been found to react with both proteins and nucleic acids (Niemeyer 1988). The reactivity of Hx towards sulfhydryl groups of proteins has been suggested to be the reason for their inhibitory effect on various enzymes. The protease papain, for instance, with a single free cysteine residue located at the active site was irreversibly inhibited by DIMBOA (Niemeyer and Pérez 1987). Friebe and co-workers (1997) showed an inhibitory effect of DIBOA and DIMBOA on plasma membrane H^+-ATPase from roots of *A. sativa* and *Avena fatua*. This inhibition may also be due to the reactivity of Hx towards sulfhydryl groups since at least one exposed cysteine residue at the active site is of importance for maintenance of enzyme conformation (Friebe et al. 1997). In addition, DIMBOA was shown to have an inhibitory effect on the electron transport and thus ATP production in isolated mitochondria and chloroplasts of maize (Massardo et al. 1994). Both DIBOA and DIMBOA have been shown to be mutagenic in a test with *Salmonella typhimurium* (Niemeyer 1988). The reactivity of Hx offers an explanation to the various biological effects observed. These are shortly described below. In the following, the term "resistance" is used meaning having a negative effect on the survival rate or reproduction of a pathogen or herbivore.

Resistance to insects

Hx have been shown to have a negative impact on the survival and reproduction of aphids (summarized by Niemeyer and Pérez 1987). Argandoña et al. (1980) found inverse correlations between Hx content in different varieties of rye and wheat and the growth rate of the aphid *Metopolophium dirhodum*. When the plants grew older and the Hx levels decreased, the growth rate of the aphid populations

increased. In the same paper it was also reported that aphids fed with artificial diets containing DIMBOA or MBOA had lower survival rate than aphids fed on artificial diets lacking DIMBOA or MBOA. Correlations have also been shown in maize between high Hx levels and resistance to the European corn borer *Ostrinia nubilalis* (Klun and Robinson 1969, Klun et al. 1970).

In addition to the reports based on correlations and work with insects on artificial diets, Hx have been shown to be induced by infestation with insects. Hx levels in the wounded tissue of maize stems and leaves increased upon infestation with larvae of the corn borer *Sesamia nonagrioides* (Gutierrez et al. 1988). In wheat, several cultivars including one *T. durum* cultivar showed increased levels of DIMBOA in leaf tissue upon infestation with the aphids *M. dirhodum* and *Rhopalosiphum padi* (Niemeyer et al. 1989, Gianoli and Niemeyer 1997a). Induced changes in Hx levels were however later shown in wild wheat (*Triticum uniaristatum*) to be due to translocation rather than enhanced local synthesis of Hx (Gianoli and Niemeyer 1997b).

Resistance to fungi

As early as 1959, Wahlroos and Virtanen reported about the anti-fungal effect of hydroxamic acids and their breakdown products on snow mold (*Fusarium nivale*). Fungi grown on medium containing DIMBOA, MBOA or BOA showed smaller colony diameter than fungi grown on an identical medium but lacking these compounds. Later reports indicated inverse correlations between infection ratings of Northern corn leaf blight-producing fungus (*Helminthosporium turcicum*) and plant DIMBOA levels, as well as inhibition of *H. turcicum* spore germination by DIMBOA (Couture et al. 1971, Long et al. 1975). These and other inhibitory effects of Hx on fungal growth are summarized by Niemeyer (1988). More recent studies of the fungal pathogen *Gaeumannomyces graminis* that causes the disease take-all in wheat and barley showed that DIBOA was a more potent fungal growth inhibitor than DIMBOA which is in correlation with the resistance of rye to take-all (Wilkes et al. 1999).

Resistance to bacteria

Bacterial stalk rot of maize is caused by a certain strain of *Erwinia chrysanthemi*. Maize is, however, resistant to rot caused by other isolates of *Erwinia chrysanthemi* and other soft rotting *Erwinia* species. It has been shown that DIMBOA inhibits the growth of several soft rotting *Erwinia* species at concentrations of 0.2-0.3 mM and that strains non-pathogenic to maize were more sensitive to DIMBOA than pathogenic strains. DIMBOA was therefore proposed

to be involved in the resistance towards *Erwinia* (Corcuera et al. 1978).

Allelopathy

Allelopathy, the chemical interaction of plants within the same species or between plants of different species, is important in the plant competition for water, nutrients and light. Hx have been ascribed a role in this interaction in many reports. DIBOA and BOA were shown to have an inhibitory effect on root growth of cress (*Lepidium sativum*) and barnyardgrass (*Echinochloa crusgalli*) (Barnes et al. 1987). DIMBOA and MBOA from *Triticum durum* were shown to have a growth inhibiting effect on roots of the weed *A. fatua* (Pérez 1990). In addition, MBOA was shown to inhibit seed germination on *A. fatua*. Rye root exudates containing DIBOA inhibited root growth of *A. fatua* whereas wheat root exudates without detectable amounts of Hx did not (Pérez and Ormeño-Núñez 1991).

Modification of auxin effect

A possible explanation to the growth inhibitory effect of Hx on other plant species might be their ability to modify auxin action. Venis and Watson (1978) reported that methylated benzoxazolinones are able to inhibit the binding of auxin to membrane receptors. MBOA was shown to have an inhibiting effect on auxin-induced bending (at concentrations of 0.6 μM or higher) and elongation (at concentrations of 0.6 mM) of oat coleoptiles (Hasegawa et al. 1992). In contrast, DIMBOA had a supporting effect on auxin-induced elongation of maize coleoptiles at a concentration of 20 μM (Park et al. 2001). The differences between the results of these experiments might be explained by different sensitivities of the plant species tested. Maize already contains DIMBOA and a UDP-glucose:Hx-glucosyltransferase (Bailey and Larson 1989, von Rad et al. 2001), to detoxify DIMBOA, that might interfere with the experiment. For instance, it has been shown that *A. thaliana* transformed with the genes for UDP-glucose:Hx-glucosyltransferases isolated from maize are less sensitive to the allelopathic substances DIBOA and DIMBOA in growth assays than wt *A. thaliana* that does not contain glucosyltransferases acting on these substances (von Rad et al. 2001). Another possible explanation is that in maize only MBOA and not DIMBOA is a potent inactivator of auxin-induced shoot elongation (Hoshi-Sakoda et al. 1994). Maize *bxbx* mutant, deficient in DIMBOA synthesis grow normally, although extremely susceptible to pathogen attack (Frey et al. 1997). It has been shown, though, that the *bxbx* mutant still contains trace amounts of DIMBOA, which might be enough to maintain a normal growth

(Melanson et al. 1997). However DIMBOA, when exuded from Hx/HxGlc containing plants, may be more important in plant competition with other plants for water, light and nutrients by inhibiting the growth of neighboring plants than in growth regulation of the plant itself.

Biosynthesis of hydroxamic acid glucosides

Five genes, *Bx1* to *Bx5*, are involved in the biosynthesis of DIBOA in maize (Frey et al. 1997). *Bx1* encodes an indole synthase, homologous to the alpha subunit of tryptophan synthase (TSA) that catalyzes the indole formation in tryptophan synthesis. Hx shares with tryptophan the synthesis pathway from indole, starting with the indole precursor indole-3-glycerol phosphate. The BX1 enzyme catalyzes a reaction in which free indole is released. In contrast bacterial and fungal tryptophan synthesis is dependent on TSA forming a complex with the beta subunit of tryptophan synthase (TSB), which catalyzes the formation of tryptophan from indole. Free indole is then usually not released (Frey et al. 1997). A similar TSA/TSB complex dependent tryptophan synthesis also exists in *A. thaliana* (Radwanski et al. 1995). *Bx2*-*Bx5* encode cytochrome P450 monooxygenases which catalyze the reactions from indole to DIBOA. Two genes are involved in the conversion of DIBOA to DIMBOA, *Bx6* and *Bx7* (Frey et al. 2003). *Bx6* encodes a 2-oxoglutarate-dependent dioxygenase that catalyzes the hydroxylation of DIBOA at position 7. The resulting product is finally methylated by the *O*-methyltransferase BX7 to generate DIMBOA. The BX7 enzyme, however, has not yet been identified. Two glucosyltransferase genes have been isolated from maize seedlings (von Rad et al. 2001), *Bx8* and *Bx9*. Their gene products are both able to catalyze the conversion of DIBOA and DIMBOA to DIBOAGlc and DIMBOAGlc, respectively.

Induction studies of *Bx4* and *Bx5* by the bacterial pathogens *Erwinia stuartii* and *Acidovorax avenae* showed that the expression of both genes were upregulated by both *E. stuartii* and *A. avenae* (Persans et al. 2001). These facts, together with the finding that a *bxbx* mutant maize, deficient in DIMBOA synthesis (Melanson et al. 1997), is extremely susceptible to pathogen attack (Frey et al. 1997) would support the idea that Hx are involved in plant defense.

Counterpart genes to *Bx1*-*Bx5* from maize are also found in wheat (Nomura et al. 2002). The presence of Hx, in addition to Poaceae, in several dicotyledonous species in the families Acanthaceae, Ranunculaceae and Scrophulariaceae suggests the development of this biosynthetic pathway relatively early in the evolution, even

before monocotyledons and dicotyledons diversed (Sicker et al. 2000, Gierl and Frey 2001).

β-Glucosidases

Enzyme classification is based on the type of reaction that the enzyme catalyzes and on its substrate specificity (EC classification). This classification does not necessarily reflect structural features of the enzyme. Since many enzymes, especially glycoside hydrolases show broad substrate specificity there is a need for a classification based on amino acid sequence similarities. Glycoside hydrolases that catalyzes the hydrolysis of *O*-glycosyl linkages between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety are classified into 90 different families based on their sequence similarities (Coutinho and Henrissat 1999). β-Glucosidases of EC3.2.1.21 and 3.2.1.1 belong to glycoside hydrolase family 1.

β-Glucosidase is one of the oldest enzymes known. As early as 1837 Wöhler and Liebig (1837) reported about β-glucosidase (emulsin) action in almond on the substrate amygdalin, a cyanogenic glucoside found in almond. β-Glucosidases occur widely among all organisms and most of them share some common features: they are exoglucolases with a subunit size of 55 to 65 kDa and acidic pH optima (pH 5-6) that require β-glucosides as substrates (Esen 1993). In plants, β-glucosidases have been implicated in numerous different plant functions, such as growth regulation by the release of active plant hormones (Campos et al. 1992, Brzobohatý et al. 1993, Falk and Rask 1995, Dietz et al. 2000), lignification (Hösel el al. 1982, Dharmawardhana et al. 1999) and plant defense by the release of plant toxins as reviewed in the previous section. In the genome of *A. thaliana* 45 putative β-glucosidase genes have been found that can be divided into 8 to 10 subfamilies (Joshi et al. 2002). Of these genes 41 encode *O*-β-glucosidases and 4 encode *S*-β-glucosidases (myrosinases). However, at least 4 *O*-β-glucosidase genes and 1 *S*-β-glucosidase gene seem to be pseudogenes (Joshi et al. 2002). In rice, maize and sorghum, only two β-glucosidase sequences have until today been reported (Table 1). In table 1, plant family 1 β-glucosidases with EC number 3.2.1.21 that have been sequenced and reported to database are summarized, with their proposed substrate as well as their subcellular localization as reported in the literature or as determined here from database programs. The table shows that the localization varies but in general is either plastidic or extracellular. The crystal structure has only been determined for maize Glu1 and for the cyanogenic β-glucosidase linamarase from white clover (Barrett et al. 1995, Czjzek et al. 2001). Both Glu1 and linamarase show an $(\alpha/\beta)_8$ barrel structure with all residues in one domain and the active site appearing as a slot-like flat pocket.

An unrooted phylogenetic tree of the family 1 glycosyl hydrolases of table 1 is shown in fig 2. This tree is based on an internal, conserved region of 336 amino acids. Although β-glucosidases from different plant species exhibit high degree of sequence homology, they do not necessarily share substrate specificity. For example, dhurrinase-1 from sorghum hydrolyses exclusively its natural substrate dhurrin, whereas maize Glu1, with 70% sequence identity with dhurrinase hydrolyzes a broad spectrum of substrates in addition to its natural substrate DIMBOAGlc (Cicek et al. 2000). Dhurrin binds to maize Glu1 and DIMBOAGlc binds to dhurrinase-1 with high affinity, but the hydrolysis of the β-glucosidic bond does not occur. This might be because the binding of the aglucone moiety does not position the glucosidic bond in correct angle and distance with respect to the catalytic residues of the active site (Cicek et al. 2000).

Enzymes that hydrolyse HxGlc have previously only been characterized as β-glucosidases (EC3.2.1.21) of family 1 glycoside hydrolases from soluble protein extracts of maize, wheat and rye (Cuevas et al. 1992, Babcock and Esen, 1994, Cicek and Esen 1999, Sue et al. 2000a, Sue et al. 2000b). Wahlroos and Virtanen (1959) first reported about enzymatic Hx aglucone formation when fresh rye or maize plants were crushed. A β-glucosidase, partly purified from maize was shown to exhibit activity on DIMBOAGlc as well as DIBOAGlc (Cuevas et al. 1992). The maize HxGlc β-glucosidase was later purified and characterized by Babcock and Esen (1994) and was shown to be a homodimer of 60 kDa monomers with a plastid localization. Later, HxGlc β-glucosidases were also purified and characterized from rye and wheat (Sue et al. 2000a, b). The wheat and maize enzymes show lower K_m and higher V_{max} with DIMBOAGlc than DIBOAGlc (Oikawa et al. 1999, Sue et al. 2000a). The rye enzyme however shows lower K_m with DIMBOAGlc than DIBOAGlc but higher V_{max} with DIBOAGlc than DIMBOAGlc (Sue et al. 2000b) The higher affinity for DIMBOAGlc than DIBOAGlc of the wheat and maize enzymes is in accordance with that DIMBOAGlc is the main HxGlc in these species.

Table 1. Summary of data found in GenBank of family 1 β-glucosidases (EC3.2.1.21) from different plant species. The subcellular localization is as reported or *has here been determined from the web-based programs pSORT on the pSORT www server (Nakai and Kanehisa 1992) or ChloroP v 1.1 from the Center for Biological Sequence Analysis on the Expasy server (Emanuelsson et al. 1999). **Activity on substrate not reported.

Species	Common name	GenBank	Enzyme	Localization	Putative substrate(s)	Reference
Arabidopsis thaliana	thale cress	AF183827	β-glucosidase	ER or exported outside*	unknown	unpublished
Arabidopsis thaliana	thale cress	AF386967	β-glucosidase	ER or exported outside*	unknown	unpublished
Arabidopsis thaliana	thale cress	U72155	β-glucosidase	exported outside*	unknown	Malboobi and Lefebvre 1997
Avena sativa	oat	X78433	avenacosidase (As-Glu1, As-P60)	plastidic*	avenacosides**	Gus-Mayer et al. 1994(a)
Avena sativa	oat	AF082991	As-Glu2	plastidic	unknown	Kim et al. 2000
Brassica napus	rapeseed	X82577	β-glucosidase	exported outside*	zeatin-*O*-glucoside	Falk and Rask 1995
Costus speciosus	crape ginger	D83177	furostanol glucoside 26-*O*-β-glucosidase	plastidic*	furostanol glucosides	Inoue et al. 1996
Dalbergia cochinchinensis	thai rosewood	AF163097	dalcochinase	exported outside*	dalcochinin-8-*O*-β-glucoside	Ketudat Cairns et al. 2000
Hordeum vulgare	barley	L41869	β-glucosidase	exported outside*	(1-2,1-3,1-4)-β-glucans	Leah et al. 1995
Manihot esculenta	cassava	S35175	linamarase	exported outside	lotaustralin, linamarin**	Hughes et al. 1992
Manihot esculenta	cassava	U95298	linamarase	ER or plasma membrane*	lotaustralin, linamarin**	unpublished
Manihot esculenta	cassava	X94986	linamarase	exported outside*	lotaustralin, linamarin**	Liddle et al. 1998
Oryza sativa	rice	U28047	β-glucosidase isozyme 1	exported outside*	gibberellin glucoside	unpublished
Oryza sativa	rice	AY056828	β-glucosidase isozyme 2	exported outside*	unknown	unpublished
Pinus contorta	lodgepole pine	AF072736	coniferin β-glucosidase	exported outside	coniferin, syringin	Dharmawardhana et al. 1999
Polygonum tinctorium	indigo plant	AB003089	β-glucosidase	Intracellular (unclear if plastidic)*	unknown	unpublished
Prunus avium	sweet cherry	U39228	β-glucosidase	exported outside or vacuolar*	unknown	Wiersma and Fils-Lycaon 1996
Prunus serotina	black cherry	U50201	cyanogenic β-glucosidase	protein bodies	(R)-prunasin	Zhou et al. 2002
Prunus serotina	black cherry	U26025	cyanogenic β-glucosidase	protein bodies	(R)-amygdalin	unpublished
Secale cereale	rye	AF293849	β-glucosidase	plastidic	DIBOAGlc, DIMBOAGlc	(III)
Sorghum bicolor	sorghum	U33817	dhurrinase-1	plastidic*	dhurrin	Cicek and Esen 1995
Sorghum bicolor	sorghum	AF253508	dhurrinase-2	plastidic*	dhurrin	unpublished
Trifolium repens	white clover	X56734	non-cyanogenic β-glucosidase	exported outside	unknown	Oxtoby et al. 1991
Trifolium repens	white clover	X56733	linamarase	exported outside*	lotaustralin, linamarin**	Oxtoby et al. 1991
Zea mays	maize	U25157	Glu1	plastidic	DIMBOAGlc	Esen 1992
Zea mays	maize	U44087	Glu2	plastidic*	DIMBOAGlc	Bandaranayake and Esen 1996

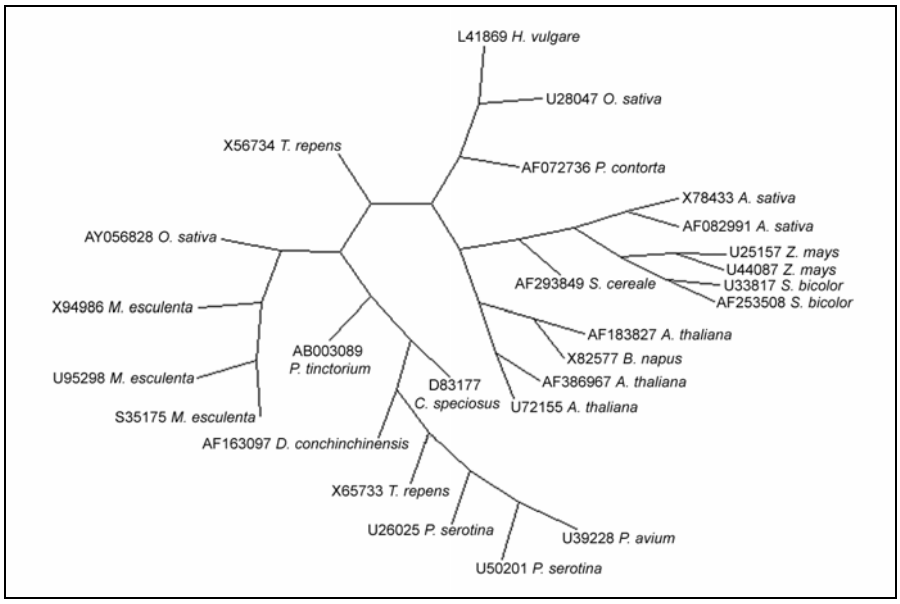

Figure 2. Unrooted phylogenetic tree of β-glucosidases listed in table 1 based on partial sequences corresponding to amino acid 125 to 460 of the rye enzyme (AF293849). Sequences were aligned using ClustalW (ClustalWWW server at the EBI). The unrooted tree was constructed using GeneBee (WWW-server at www.genebee.msu.su).

Rye

The present work focuses on rye. The main reason for studying rye is that rye contains DIBOAGlc as the main HxGlc in shoots and roots, whereas maize and wheat contain DIMBOAGlc as the main HxGlc in both shoots and roots. It was therefore expected that β-glucosidase of rye would exhibit different substrate specificity than the enzyme from maize and wheat. Furthermore, the HxGlc/β-glucosidase system in rye is particularly interesting because of the potential use of rye in intercropping systems due to its allelopathic properties. DIBOA, exuded from rye roots, and its breakdown product BOA were shown to have an inhibiting effect on growth of several weed species as reviewed above (Barnes et al. 1987, Pérez and Ormeño-Núñez 1991).

Rye is native to southwestern Asia and cultivated rye (*Secale cereale*) is believed to have originated from either *Secale montanum*, a wild species found in southern Europe and nearby parts of Asia, or from *Secale anatolicum*, a wild rye found in Syria, Armenia, Iran, Turkestan, and the Kirghis steppe. Rye was found as a widely distributed weed in wheat and barley fields in southern Asia and had coevolved with wheat and barley for over 2000 years before its value as a crop was recognized. The close relationship of rye with barley and wheat can be shown by phylogenetic mapping of several grass

species based on the amount of DNA in the pre-replication, diploid, mitotic nucleus (C-value) of the identified species (Freeling 2001). The English and Dutch that settled in the northeastern areas of what is now the United States brought rye to the Occident. Rye is cultivated in temperate regions in USA, Canada and Northern Europe and is mainly used as bread grain. Since many rye varieties are very cold hardy it is often cultivated where conditions are unfavorable for wheat. In addition, rye grows rapidly from seeds and will even grow on soils of limited fertility. To combine grain quality and productivity of wheat with the vigour and hardiness of rye, plant breeders developed a hybrid between wheat and rye called triticale. Rye is reported to tolerate aluminum, hydrogen fluoride, low pH, high pH, drought, poor soil, salt, sand, water logging, weeds and several diseases caused by nematodes, virus, bacteria and fungi (Duke, 1978). Still rye is susceptible to some diseases of which ergot (*Claviceps purpurea*) is the most serious disease. Breeding aims are higher seed yield, improved stability, resistance to powdery mildew and brown rust and better food and baking qualities. Fast growing, fine stemmed varieties with high leaf mass are particularly suited for use as green fodder. Rye literature is collected at Center for New Crops and Plant Products at Purdue University, URL: http://www.hort.purdue.edu/newcrop/ (accessed 15-mars-2003).

Aims of the project

The overall aim of this project was to gain more knowledge about the HxGlc/β-glucosidase system and its function in plants. Several questions were of interest, not only with regard to characteristics of a plant defense system but also for applications in agricultural practice and plant breeding. The studies of allelopathy had indicated that Hx aglucone was an active allelopathic compound and that DIBOA from rye was exuded, whereas DIMBOA from wheat was not (Pérez and Ormeño-Núñez 1991). This difference between wheat and rye, we postulated, might either be due to differences in the localization of substrate or enzyme or differences between the β-glucosidases from the different species in substrate specificity. This prompted an investigation of the properties of the rye β-glucosidase enzyme and the localization of enzyme and substrate in root tissue. The same questions were of interest for understanding how the defense system might function in the defense to pathogens and insects. As reviewed above, correlations have been found between levels of HxGlc and resistance to pathogens and insects. However, different types of pathogens or insects have quite different strategy for invasion. What are the actual levels of compounds that the attackers encounter? Do they encounter the glycosides or the aglucones? Where are the

glycosides in the intact tissue and will invasion by any type of pathogen or insect lead to the release of aglucones?

Against this background, the work in the thesis focused on two main questions: 1) The localization of the HxGlc and β-glucosidase and 2) the characterization of β-glucosidase acting on HxGlc from rye. The first question was approached by immunhistochemical and immunocytochemical methods using an antiserum against β-glucosidase purified from maize to study the localization of β-glucosidase. The localization of HxGlc was studied by ferric chloride staining of tissue sections. To characterize rye β-glucosidase, a β-glucosidase cDNA from coleoptiles of rye was isolated. The cDNA was analyzed and the sequence corresponding to the mature protein was expressed in *E.coli* and the recombinant protein was characterized and compared to purified native intracellular β-glucosidase. Since the localization work had showed the presence of β-glucosidase also in cell walls, a cell wall located β-glucosidase from rye was purified and characterized.

Results and discussion

Tissue localization of β-glucosidase

In order to determine the tissue localization of β-glucosidase an immunohistochemical approach was carried out with tissue sections from seedlings of maize, wheat and rye. An antiserum against SDS-denatured maize Glu1 was available (Esen 1992, Babcock and Esen 1994) and it was first examined whether the antibody would recognize β-glucosidases in rye and wheat as well. Enzyme extracts were prepared from shoots and roots of all three species in parallel. β-Glucosidases were partially purified by separations on an ion exchange column. In fractions eluting from the column only one peak of β-glucosidase activity was detected in all three species, measured with both pNPGlc and DIBOAGlc as substrates (I). The fractions exhibiting β-glucosidase activity were pooled and separated by SDS-PAGE and native PAGE and β-glucosidases were detected by immunoblotting. Results from native PAGE showed one immunoreactive band in each extract with the same migration as β-glucosidase activity stained with the chromogenic substrate 6-BNGlc in a parallel PAGE. Results from SDS-PAGE showed two closely migrating immunoreactive bands, one major and one further migrating minor band, in extracts from all three species. Double bands are sometimes shown also with purified proteins in SDS-PAGE and might be due to incomplete SDS-denaturation or in-gel renaturation. From the results of the above described experiments it was concluded that the maize Glu1 antiserum recognizes rye and

wheat β-glucosidases and thus was suitable for immunolocalization studies.

Using the maize Glu1 antiserum, β-glucosidase was located in tissues of rye, wheat and maize seedlings by immunohistochemistry (I). For detection, peroxidase-anti-peroxidase (PAP) staining was used and the results were observed in a light microscope. In rye tissue, β-glucosidase was located to epidermis of shoots and roots, to bundle sheath cells in shoots and to all cells except those of the vascular bundles in coleoptiles. In wheat, the enzyme was mainly located to epidermal cells in shoots and roots and to a minor extent to parenchymatic cells between the vascular bundles in shoots. In wheat coleoptiles, β-glucosidase was located to epidermis and the underlying cell layer. In maize, β-glucosidase was found in bundle sheath cells in shoot bases and coleoptile, in the epidermis in roots and in cells within the vascular tissue in shoot tips. The PAP-staining of maize tissue is in accordance with a previous localization of β-glucosidase in maize tissue by activity staining using an azo-coupling technique (Mace 1973). In contrast, Brzobohatý et al. (1993) found by immunocytochemical analysis the localization of the maize β-glucosidase to be restricted to zones of active cell division in the root tip. However, the difference between these and our results might be due to different expression pattern of the maize enzyme during plant development. In shoots of all three species staining was most intense in the basal parts and decreased towards the leaf tip. In roots, staining was more equally distributed along the root.

Subcellular β-glucosidase localization

In order to determine the subcellular localization of β-glucosidases of rye, wheat and maize, the same antiserum was used as primary antibody as in the immunohistochemical studies described above (II). For detection, gold-labeled secondary antibodies were used and the results were observed in a transmission electron microscope (TEM). In all three species, β-glucosidase was detected in cytoplasm, plastids and cell walls. The localization of β-glucosidase acting on HxGlc in plastids and cell walls have for rye been confirmed in this thesis by biochemical studies (III, IV). With regard to the cytoplasm localization it is proposed that it reflects cell wall β-glucosidase in the exportation pathway out of the cell.

The electron microscopy studies complemented and added to the results concerning tissue localization as follows. By immunohistochemistry we did not detect any β-glucosidase staining in rye root cortex, but the electron microscopy results showed gold staining in plastids of some cortex cells of rye roots. In wheat we

observed a weak gold staining in plastids of bundle sheath cells that was not observed before. In maize, β-glucosidase staining had not been observed in epidermis, but the immunocytochemical work revealed gold staining in cell walls of stomatal guard cells of shoots. A guard cell localization has also been shown for myrosinase in tissues of rapeseed (Höglund et al. 1991) and *A. thaliana* (Husebye et al. 2002). Localization in guard cells is in agreement with the proposed involvement of the glucosinolate/myrosinase system as well as the HxGlc/β-glucosidase system in plant defense, since this is a prime location for pathogen invasion. A negative response should not be taken as a proof that the enzyme is entirely absent from the tissue. For instance, in these experiments plastidic β-glucosidase could not be detected in rye coleoptiles, but the coleoptiles were later shown to contain plastidic β-glucosidase in addition to the immunoreactive cell wall glucan 1,3-β-glucosidase (III, IV and V).

Intracellular β-glucosidase

In order to characterize the rye HxGlc β-glucosidase activities, a β-glucosidase cDNA was isolated from rye coleoptiles (III). The cDNA was cloned and sequenced. In the same period, a β-glucosidase from rye was purified and the N-terminal sequence determined (Sue et al. 2000b). The amino acid sequence deduced from the isolated cDNA clone was analyzed with web-based chloroplast targeting prediction programs and found to predict an N-terminal chloroplast targeting peptide with a cleavage site between amino acid 49 and 50 that confirmed the N-terminal sequencing of the protein (Sue et al. 2000b). When aligning the deduced amino acid sequences corresponding to the mature β-glucosidases of rye with maize Glu1 and Glu2, the rye enzyme shows 65% sequence homology to the maize enzymes. Similar to other family 1 *O*-β-glucosidases of EC3.2.1.21, the rye enzyme contains the conserved peptide motifs TFNEP and ITENG, which are part of the active site and contain the catalytic glutamic acid residues E190 and E406.

Previous studies of a catalytically inactive mutant of maize Glu1 indicated that the four amino acids W378, F198, F205 and F466 of the maize Glu1 sequence are key amino acids in aglucone recognition and substrate specificity and that A467 interacts with the 7-methoxy group of DIMBOAGlc (Czjzek et al. 2000). Of these, the highly conserved tryptophan at position W378 and the phenylalanine at position F198 (position 197 of the rye sequence) are conserved in rye. However, the phenylalanines at position F205 (position 204 of the rye sequence) and F466 (position 463 of the rye sequence) are histidine and glycine, respectively, in rye and the amino acid corresponding to A467 is serine (position 464 of the rye sequence).

This confirms the functional importance of F198 and W378, and the alterations in F205, F466 and A467 are in accordance with the difference in substrate specificity of the intracellular rye β-glucosidase and maize Glu1 enzymes.

The sequence corresponding to the mature protein, based on N-terminal sequencing of the protein (Sue et al. 2000b), was cloned and expressed in *E. coli*. The recombinant protein was purified and compared to purified native rye intracellular β-glucosidase. The recombinant β-glucosidase showed similar substrate specificity and activity as the native enzyme. They both hydrolyzed DIBOAGlc, DIMBOAGlc, pNPGlc, oNPGlc, pNPFuc readily, pNPGal to a lesser extent but did not hydrolyze pPNGlu and substrates based on cell wall components. Kinetic studies of the recombinant β-glucosidase showed similar kinetic variables as the native enzyme on the substrates hydrolyzed. K_m varied between about 1 mM for pNPFuc and about 3 mM for DIBOAGlc and V_{max} varied between about 50 nkat mg^{-1} for pNPGal and about 2500 nkat mg^{-1} for DIBOAGlc. In contrast to the rye enzyme that shows lower K_m with DIMBOAGlc than DIBOAglc but higher V_{max} with DIBOAglc than DIMBOAGlc, the wheat and maize enzymes show lower Km and higher Vmax with DIMBOAglc than DIBOAGlc (Oikawa et al. 1999, Sue et al. 2000a). Both the wheat and the maize enzymes hydrolyze pNPGlc as well as pNPFuc readily and pNPGal to a lesser extent as the rye enzyme (Babcock and Esen 1994, Sue et al. 2000a).

Both the recombinant and the native enzyme showed immunoreactivity with the maize Glu1 antibody, migrated similarly in native PAGE and hydrolyzed the substrates used for gel staining, 6-BNGlc and 4-MUGlc. In SDS-PAGE, however the native enzyme migrated at 67 kDa whereas the recombinant β-glucosidase migrated at approximately 69 kDa due to 13 amino acids derived from the cloning vector used. From this it was concluded that the recombinant β-glucosidase is homologous to the intracellular enzyme and that the intracellular β-glucosidase has a plastidic localization.

The tissue distribution of intracellular β-glucosidase in 8-day-old rye seedlings was studied by immunoblotting, β-glucosidase staining and activity measurements of enzyme extracts from different parts of the plant. The enzyme was found throughout the rye plant with the highest activity levels in the basal, younger parts of the shoot. This is in accordance with the PAP staining of rye tissue sections that showed the most intense staining in the basal parts of the shoots and the coleoptiles (I).

Extracellular β -glucosidase

Since the localization studies had shown the presence of β-glucosidase in cell walls of rye in addition to a plastidic enzyme, it was important to find out whether this enzyme was able to hydrolyze DIMBOAGlc and DIBOAGlc. The β-glucosidase from cell walls was therefore purified and analyzed with regard to kinetic properties and amino acid sequence (IV). The cell wall β-glucosidase hydrolyzed the same substrates including DIBOAGlc and DIMBOAGlc, as the intracellular enzyme (III) but with some differences in the kinetic properties. As the intracellular β-glucosidase, the extracellular β-glucosidase did not hydrolyze pNPGlu, or the putative cell wall substrates coniferin and syringin. However, in contrast to the plastidic enzyme, the cell wall β-glucosidase did hydrolyze laminaribiose, gentiobiose and cellobiose (disaccharides). The K_m values for the accepted substrates varied between 0.5 mM for cellobiose to 2 mM for DIBOAGlc and V_{max} varied from 10 nkat mg^{-1} for pNPGal to 3000 nkat mg^{-1} for DIBOAGlc. In contrast to the plastidic β-glucosidase, the cell wall enzyme showed lower K_m for DIBOAGlc than for DIMBOAGlc but higher V_{max} for DIMBOAGlc than for DIBOAGlc. In SDS-PAGE the cell wall enzyme migrated at approximately 69 kDa under reducing conditions whereas the intracellular β-glucosidase migrated further in the gel at 67 kDa.

The cell wall enzyme was identified as a glucan 1,3-β-glucosidase (EC3.2.1.58) of family 3 glycoside hydrolases based on peptide sequencing, substrate specificity and kinetic properties. It shows high homology to barley ExoII (AAC49170.1) and wheat exohydrolase (AAM13694.1) and one of the peptide sequences contains the characteristic catalytic aspartic acid residue (D306 of barley ExoII) at the active site of family 3 glycoside hydrolases. In contrast to the intracellular β-glucosidase, for which the aglucone moiety of the substrate binds to the active site and thus determines substrate specificity based on its homology to the maize Glu1 (Cicek et al. 2000), the glucose moiety binds to the active site of glucan 1,3-β-glucosidases (Varghese et al. 1999). This is probably the explanation to the ability of the cell wall glucan 1,3-β-glucosidase to hydrolyze DIBOAGlc and DIMBOAGlc. In contrast to the three-dimensional $(\alpha/\beta)_8$ barrel structure of family 1 β-glucosidases with all residues in one domain (Barrett et al. 1995, Czjzek et al. 2001), the three-dimensional structure of the family 3 enzyme ExoI from barley shows a two-domain conformation (Varghese et al. 1999). The first domain (residues 1-357) forms an $(\alpha/\beta)_8$ barrel and the second domain (residues 374-559) forms an $(\alpha/\beta)_6$ sheath consisting of five parallel β strands and one anti-parallel β strand. The two domains are connected with a helix-like strand of 16 residues. The active site is located at the interface of the two domains.

Several exported β-glucosidases have been reported to be glycosylated, determined either experimentally or from their amino acid sequences (Oxtoby et al. 1991, Hughes et al. 1992, Falk and Rask 1995, Hrmova et al. 1996, Leah et al. 1995, Dharmawardhana et al. 1999, Varghese et al. 1999, Ketudat Cairns et al. 2000). Surprisingly, no protein glycosylation of the rye cell wall glucan 1,3-β-glucosidase could be detected (IV).

Previously only intracellular, soluble β-glucosidases (EC3.2.1.21) of glycoside hydrolase family 1 has been considered to be involved in the hydrolysis of HxGlc (Cuevas et al. 1992, Babcock and Esen, 1994, Cicek and Esen 1999, Sue et al. 2000a, Sue et al. 2000b). However, the characterization of the cell wall associated enzyme from rye indicates that glucan 1,3-β-glucosidases (EC3.2.158) also might be involved in the release of the toxic Hx aglucones and benzoxazolinones upon tissue rupture. It should be noted that the cell wall enzyme contributed to a small extent, only 5 % (not shown) to the total β-glucosidase activity as measured in enzyme extracts. The broad substrate specificity shown for glucan 1,3-β-glucosidases enables these enzymes to perform a variety of different functions during plant development (Hrmova et al. 2002).

Similar to the intracellular β-glucosidase, the cell wall glucan 1,3-β-glucosidase was present throughout the plant with the highest activity levels in the basal, younger parts of the shoot as measured in extracts from different parts of the plant with DIBOAGlc and by immunoblotting in 8-day-old rye seedlings.

Tissue localization of hydroxamic acid glucosides

Measurements of Hx/HxGlc levels in different plant parts (shoot top, shoot base, coleoptile, root above tip, root tip) of young rye seedlings showed that HxGlc are present throughout the plant with the highest levels in the shoot base (V). Both DIBOAGlc and DIMBOAGlc are present in all tissues although DIMBOAGlc only at low levels in the shoot. DIBOA aglucone was detected in extracts from rye tissue, but at low levels that probably reflect enzymatic hydrolysis of DIBOAGlc during preparation rather than the presence of DIBOA in the intact plant. In root exudates, the only Hx present was DIBOA. No DIMBOA aglucone was detected in rye tissues or in root exudates. The results are in accordance with previous reports. Hofman and Hofmanová (1969) showed that the main HxGlc of rye shoot is DIBOAGlc and that there are minor amounts of DIMBOAGlc, and Pérez and Ormeño-Núñez (1991) reported that DIBOA was the only Hx found in rye root exudates.

The localization of Hx/HxGlc in different tissues has previously been determined in maize leaves and roots. Argandoña and Corcuera (1985) separated maize vascular veins from the rest of the tissue and found that the concentration of DIMBOA (DIMBOAGlc) was higher in the vascular tissue than the total leaf or root. In later works, tissue was fractionated and it was found that DIMBOA (DIMBOAGlc) was present in both vascular bundles and parenchymatic cells in maize leaves (Massardo et al. 1994). DIMBOAGlc was also found to be present in phloem sap of wheat as analyzed in aphid stylet sap after cutting (Givovich et al 1994). High levels of Hx/HxGlc in the phloem might be an explanation to the observation that hyphae were never present in the phloem tissue in maize infected by the Northern corn leaf blight-producing fungus (*Helminthosporium turcicum*) (Hilu and Hooker 1964). Hyphae were also only rarely observed in the xylem of infected resistant plant leaves. Analysis of the expression pattern of the genes *Bx2* to *Bx5* involved in HxGlc synthesis in maize seedlings showed that expression of these genes were restricted to parenchymatic cells in both shoots and roots with the highest levels in the shoots (Frey et al. 1995).

A direct method to locate HxGlc in plant tissues is based on the forming of specific complexes with ferric chloride ($Fe(III)Cl_3$) which gives a bluish color (Hamilton 1964). In methanol, the complex with DIBOAGlc gives a pale purple color, DIBOA a dark violet color, DIMBOAGlc a grayish blue color and DIMBOA a bright blue color (Iwamura et al. 1996). Iwamura and co-workers used staining with ferric chloride of intact wheat tissues to locate Hx/HxGlc in wheat seedlings. Staining was found in cortical cells in the root, cells immediately underlying the epidermis in the coleoptile and to a lesser extent in the leaf folded inside the coleoptile (Iwamura et al 1996).

Here, staining with ferric chloride during very short incubation times (within 30 seconds) was used to locate Hx/HxGlc in tissues of 6-day-old rye seedlings (V). The results show staining in the vascular tissues and the cell layers underneath epidermis of roots, shoots and coleoptiles. Based on the Hx/HxGlc measurements in different plant parts of rye and the characteristics of the staining color and intensities of the different Hx and HxGlc, it is proposed that the pale purple color in the shoot is due to staining of DIBOAGlc and the darker staining in the coleoptiles and roots is due to staining of DIMBOAGlc. In shoots the cell layers beneath epidermis were stained on both upper and lower leaf sides. In coleoptiles the outermost cell layers were only stained on the outer side of the coleoptile and was often skewed to one side of the plant. This asymmetric staining was also observed for PAP-staining of β-glucosidase in the coleoptiles of rye. If this PAP-staining pattern could be ascribed to the intracellular or the extracellular β-

glucosidase could not be revealed since both the cell wall as well as the plastidic β-glucosidase were detected by gold staining in this tissue. The same ferric chloride staining pattern as shown for rye was also shown for wheat and maize. In view of the reported effects of Hx on auxin-induced elongation, it is tempting to speculate that the uneven distribution of HxGlc and β-glucosidas is related to cell elongation in these tissues.

Conclusions

-Rye contains at least two different HxGlc hydrolyzing enzymes of which one is a β-glucosidase (EC3.2.1.21) belonging to glycoside hydrolase family 1 and the other is a glucan 1,3-β-glucosidase (EC3.2.1.58) belonging to glycoside hydrolase family 3.

-The family 1 β-glucosidase is intracellular and located in plastids, whereas the family 3 glucan 1,3-β-glucosidase is extracellular and cell wall associated. Both enzymes hydrolyze DIBOAGlc and DIMBOAGlc with high efficiency as reflected in their K_m and V_{max} values.

-HxGlc and their hydrolyzing β-glucosidases are present throughout the plant. In shoots, the highest levels are in the basal parts of the shoot, whereas no clear distinction has been found in the different parts of the root system.

-In rye leaves, the HxGlc and the β-glucosidases are compartmentalized in different tissues, since the HxGlc are located in the cell layers beneath the epidermis and in vascular bundles, whereas the hydrolyzing β-glucosidases are located in the epidermis and in bundle sheath cells.

-In rye coleoptiles, the HxGlc were found beneath the epidermal cell layer and in vascular bundles whereas the β-glucosidase was found in all tissues except for the vascular bundles. Both the glucosides and the enzymes were often distributed on one side of the tissue. Thus, the compartmentalization may be on the subcellular level or there may be an asymmetric distribution of glucosides and β-glucosides on different sides of the coleoptile.

-In rye roots, the HxGlc and the β-glucosidases are compartmentalized either in different cells or subcellularly in different organelles. HxGlc are located in cortex cells underlying the epidermis and in the vascular cylinder. The hydrolyzing enzymes are located in epidermis as well as co-localized with the HxGlc in cortex cells. In the cortical cells, however, the enzyme is compartmentalized in plastids and the HxGlc (by suggestion) in the central vacuole.

-In maize roots and coleoptiles the compartmentalization of HxGlc and β-glucosidases is in different cells, since the HxGlc were located in the cell layers beneath the epidermis and in the vascular bundles, whereas the β-glucosidases were located to the bundle sheaths of coleoptiles and to the epidermis of roots. In maize shoots, however the HxGlc were located to the vascular bundles and the enzymes to

the bundle sheaths as well as in the vascular bundles similarly as the substrate. If the compartmentalization of substrate and enzymes in the vascular bundles is in different or the same cells remains unclear since the cellular localization of the HxGlc is unknown.

-In wheat shoots and roots, the compartmentalization of HxGlc and β-glucosidases is in different cells, since the HxGlc are located in the cell layers beneath the epidermis in both shoots and roots, in the vascular bundles of shoots and in the vascular cylinders of roots, whereas the β-glucosidases are located in epidermis of shoots and roots and in bundle sheaths of shoots. In coleoptiles, however the enzymes are, in addition to their localization in epidermis, co-localized with the substrates in cells beneath the epidermis. If the enzymes and substrates are subcellularly compartmentalized or if they are located at different sides of the coleoptile remains unknown.

The distribution of HxGlc and their hydrolyzing β-glucosidases, internally in association with the vascular tissues and externally in epidermis and cell layers beneath epidermis supports the proposed function in plant defense. Localization of a defense system around the vascular tissue will provide an ideal protection of the phloem from phloem-feeding aphids. An externally located defense system will give protection against fungal invasion trough the epidermis. The high levels of both substrate and enzyme in the actively growing, basal parts of the shoots will protect this soft and vulnerable tissue from invading pathogens. It may also be an indication of an involvement of the HxGlc/β-glucosidase system in plant growth and development.

Future perspectives

The work has shown the localization and probable involvement of two β-glucosidases of different glycoside hydrolase families in the hydrolysis of HxGlc in rye seedlings. For a more certain identification and characterization of the molecular properties, the extracellular glucan 1,3-β-glucosidase needs to be cloned and expressed in a heterologous system as was done for the plastidic enzyme.

HxGlc have been localized at the tissue level with a staining method. For a more precise localization of the glucosides both at tissue level and at the subcellular level, a possible approach would be to use antibodies raised against a conjugate of hydroxamic acid glucosides with bovine serum albumin in immunolocalization studies. During tissue sectioning, HxGlc must first be coupled to tissue proteins by prefixation to prevent diffusion of HxGlc in the tissue during fixation and dehydration and to prevent that HxGlc are washed off during rehydration (Sossountzov et al. 1988).

Both HxGlc and β-glucosidase were distributed on one side of the coleoptile. To investigate whether the glucosides and the β-glucosidase are distributed on the same side or different sides, the localization could be investigated in coleoptiles that have been induced to bend in one direction and carefully marked to indicate the different sides. Such experiments would also give indications whether the β-glucosidases and the HxGlc may be involved in cell elongation.

Rye exuded DIBOA but not DIMBOA and differences between rye cultivars in the ability to exude DIBOA have been reported. To understand this and to understand the differences between rye and wheat cultivars in the ability to exude Hx, the mechanism of root exudation is the main question. Root exudation of secondary compounds can occur by cells, budded of the root cap, bursting in the surroundings and thereby letting all the cell contents out or by secretion with the mucigel (polysaccharides, vitamins, organic acids, enzymes, amino acids) made by dictyosomes in the peripheral cells of the root cap (Juniper et al. 1977, Moore et al. 1998). The fact that rye and wheat varities differ in their capacity to exude Hx/HxGlc (Pérez and Ormeño-Núñes 1991, Wu et al. 2001) indicate that the process is specific and probably is carried out using specific efflux transporters. ATP-binding cassette (ABC)-type transporters have been suggested to be involved in transport of secondary metabolites into the vacuole (Martinoia et al. 2000), as well as in secretion of secondary metabolites out of the cell (Jasiński et al. 2001). The HxGlc might be transported out of the cell as glucosides and then

converted to the corresponding aglucones by the cell wall glycoside hydrolases. As a start, a screening of a cDNA library from root tips for ABC transporter genes may give possible candidates for investigation of affinity for HxGlc.

In order to study the function of HxGlc and β-glucosidase in the defense against insects and pathogens, the transformation of *A. thaliana* with the biosynthetic pathway of DIBOAGlc would be a suitable approach. *A. thaliana* has already been transformed with the glucosyltransferase genes *Bx8* and *Bx9* involved in DIBOAGlc and DIMBOAGlc synthesis and the transformed plants showed increased tolerance against DIBOA and DIMBOA as compared to wild-type plants (von Rad et al. 2001). By transferring the genes *Bx1-Bx5* and *Bx8* from the DIBOAGlc biosynthetic pathway to *A. thaliana* one would obtain a useful tool for functional analysis of Hx/HxGlc. Questions to be studied are for instance, possible effects on auxin induced cell elongation as well as the effects towards insects. This approach has previously been carried out for cyanogenic glucosides and demonstrated that dhurrin prevented insect herbivory (Tattersall et al. 2001).

The above approach may be combined with the transformation of genes for the β-glucosidase enzymes studied in this thesis to DIBOAGlc accumulating plants to study their involvement in HxGlc hydrolysis in the intact plant or upon tissue rupture and in the release of Hx in root exudates. For this the extracellular glucan 1,3-β-glucosidase needs to be cloned and sequenced. We investigated in enzyme extracts from *A. thaliana* the presence of HxGlc hydrolyzing activity and this was not found (results not shown).

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