Thesis

Functional Analysis of Plant Idioblasts (Myrosin Cells) and their role in Defense, Development and Growth

by

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CONTENTS

1. ACKNOWLEDGEMENTS

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2.GENERAL SYMMARY

Glucosinolates are natural plant products known as flavor compounds, cancer-preventing agents, and biopesticides. There is a strong interest in controlling the level of glucosinolates to improve flavor and nutritional qualities of food crops and to study the physiological role of glucosinolates in plants. The role of the myrosinase-glucosinolate system in plant defense-related reactions of Cruciferae is accepted. As metabolism of glucosinolates in healthy intact plants has been reported, this indicates that products produced by hydrolysis of glucosinolates may also be important in the growth and development of the plant.

The main focus in this thesis has been to study the effect of preventing glucosinolate hydrolysis by removal of the hydrolyzing enzyme, myrosinase. By the use of the *Myr1.Bn1* promoter, we have made myrosinase free seeds of *Brassica napus* by controlled cell death (genetic ablation) of myrosin cells. The glucosinolate substrates were mainly unaffected by this ablation supporting their localization in non-myrosin cells and a cellular separation of enzyme and substrate. GC and GC-MS analysis confirmed that seeds with ablated myrosin cells had lost the ability to produce isothiocyanates. The effects of genetically ablating myrosin cells were tested for two other components in this complex enzyme system. The epithiospecifier protein (ESP) had lost the expression of one of its isoforms (39 kDa ESP) by the ablation indicating a cellular co-localization with myrosinase in myrosin cells for this specific isoform. The expression of myrosinase binding proteins (MBPs) was not severely affected supporting localization to non-myrosin cells in the *B. napus* seed. Plants grown from seeds with ablated myrosin cells (*MINELESS*) showed a bushy phenotype and morphological abnormalities in flower and silique. Seed maturation was delayed and seed production reduced. Parallel experiments performed in our lab with *Arabidopsis thaliana* produced similar results. The tissues affected were consistent with the expression pattern directed by the myrosinase gene promoter used.

Ablating myrosinase proteins affected the behavior of the specialist cabbage aphid *Brevicoryne brassicae* and the generalist peach potato aphid *Myzus persicae*. When seedlings of *MINELESS* and wild type were challenged by these aphid species, a difference in preference was observed. *B. brassicae* preferred wild type seedlings with high levels of myrosinase proteins, while *M. persicae* preferred *MINELESS* seedlings with a reduced level of myrosinase compared to wild type. Statistical analysis revealed that the *MINELESS* seedlings were more susceptible to attacks by aphids, regardless of aphid species. Furthermore, the number of aphids established on the seedling influenced the myrosinase activity and the height of the plant.

Thus, by ablating myrosinase proteins, new insights into the functions of the myrosinase-glucosinolate system have been elucidated. This complex system seems important not only in the defense against pests, but also show potential to influence the growth and development of the plant.

3. LIST OF PAPERS

Paper 1

Borgen Birgit Hafeld, Thangstad Ole Petter, Grønseth Liv, Seem Martin, Husebye Harald, Rossiter John Trevor and Bones Atle Magnar. (2002). "*MINELESS* Plants Reveal Multiple Roles for Myrosin Cell Idioblasts in Defense, Development and Growth". (submitted).

Paper 2

Borgen Birgit Hafeld, Honne Bjørn Ivar, Thangstad Ole Petter, Rossiter John Trevor, and Bones Atle Magnar. (2002). "Controlled cell death of myrosin cells increases the susceptibility for aphid attack in *MINELESS* seedlings". (manuscript).

4. GENERAL INTRODUCTION

The Brassicaceae is a large plant family with around 350 genera and 3000 species. The family contains many agriculturally important crops. Vegetables such as broccoli, all species of cabbage, cauliflower, brussel sprouts, turnip and rapeseed are cultivated mainly for their oil content and for food purposes. Consumption of these vegetables has been suggested to be beneficial for human health due to their presumed anti-carcinogenic effects, and there is therefore a great interest in these products with regard to food quality and safety. *Brassica* oilseed production has also increased over the past decades to become one of the worlds` most important sources of vegetable oil. Fatty acids beneficial to humans favor rapeseed oil due to its low content of saturated fatty acids and high content of the monounsaturated fatty, acid oleic acid. In addition, the polyunsaturated fatty acids linoleic acid and linolenic acid are present in appropriate amounts. The seed meal remaining after oil extraction has a high protein content with a well-balanced content of amino acids and is valuable as animal feeding.

A common feature of many plants belonging to the Brassicaceae family, is that they contain secondary metabolites called glucosinolates (Rodman 1991). Upon tissue damage, the glucosinolates become hydrolyzed by the myrosinase enzyme, the only known plant S-glycosidase. The hydrolysis produces unstable intermediates that spontaneously rearrange to one or more toxic products such as isothiocyanates, thiocyanates and nitriles. For review of the myrosinase-glucosinolate system see Bones & Rossiter (1996) and Rask et al. (2000). The products are highly reactive and participate in the interaction between plants and their consumers and thereby influence humans, animals, insects and fungi. The presence of the potentially toxic glucosinolates and also of erucic acid, both naturally present in *Brassica* species, has therefore limited the use of these plants. Erucic acid can cause negative effects to the metabolism of lipids. Development of new varieties has, however, increased the yield

of the *Brassica* crops and improved the quality of rapeseed oil and meal. Today, new "double-low" varieties containing low levels of both glucosinolates and erucic acid are available.

The myrosinase-glucosinolate system seems to play several important but not fully understood roles in the life of the Brassicaceae plants. The toxic products formed from the glucosinolates are considered as important compounds involved in plant protection. Defense against herbivores, pathogens and pests may be one of the main functions of this system (Bones & Rossiter 1996, Rask et al. 2000). Furthermore, glucosinolates may act as a potential storage form of nitrogen, carbon and especially sulfur (Underhill 1980). Apart from this, glucosinolates have also been suggested to be precursors of growth regulators in plant development (Bestwick et al. 1993, Bak et al. 2001). In order to coordinate these diverse events, the plant must have a complex regulatory network for this system.

4.1 GLUCOSINOLATES

Glucosinolates are naturally occurring anions characterized by having a thioglucose moiety, a sulfonated oxime, and a side chain derived from aliphatic, aromatic, and indole amino acids. Glucosinolates are found exclusively in the plant kingdom in the order Capparales, which includes the economically important oilseed rape and *Brassica* vegetables, and the model plant *Arabidopsis thaliana* L. Upon tissue damage, the glucosinolates are hydrolyzed by endogenous thioglucosidases (myrosinases) to produce a variety of degradation products. Typically isothiocyanates, thiocyanates and nitriles are formed, which have a wide range of biological effects (Fenwick et al. 1983, Chew 1988, Fahey et al. 2001). There is a rising interest in being able to control the level of specific glucosinolates in crops to improve the flavor and

nutritional qualities of food crop, and to study the physiological role of glucosinolates in plants.

4.1.1 Glucosinolate structure

More than 120 different glucosinolates have been identified to date (Fahey 2001). These occur predominantly, but not exclusively, in the family Brassicaceae (Fenwick and Heaney 1983). The glucosinolates all have a glucose moiety linked via a sulfur to an aglycone, and differ mainly in their R-group substituents, which can be either aliphatic, aromatic or indolyl (Fig. 1). The structure may also include functional groups such as hydroxyl, methylthio-groups, esters or ketones (Sørensen 1990). Each plant species contains only a limited number of compounds, which give rise to unique profiles of glucosinolates. In *Brassica napus* over 30 different glucosinolates have been detected (Sørensen 1990), whereas 23 different glucosinolates have been reported in *Arabidopsis thaliana* (Hogge et al. 1988). Common glucosinolates found in *B. napus* are listed in Table 1.

4.1.2 Glucosinolate distribution and regulation

Glucosinolates occur in all parts of the plant (Fenwick et al. 1983) but the distribution of the glucosinolates that have been examined varies among plant organs with both quantitative and qualitative differences affected by developmental stage (Sang et al. 1984, Clossais-Besnard & Larher 1991, Porter et al. 1991) and environmental factors such as light (Hasegawa et al. 2000), soil fertility (Booth & Walker 1992) or plant growth regulators (Bodnaryk & Yoshihara 1995). Glucosinolate content in plants is about 1% of dry weight in some tissues of the Brassica vegetables (Rosa et al. 1997) although the content is highly variable and can approach 10% in the seed. Different types of stress condition such as fungal infection (Li et al. 1999b, Butcher et al. 1974), insect damage (Koritsas et al. 1991, Ludwig-Müller et al. 1999), wounding (Bartlet et

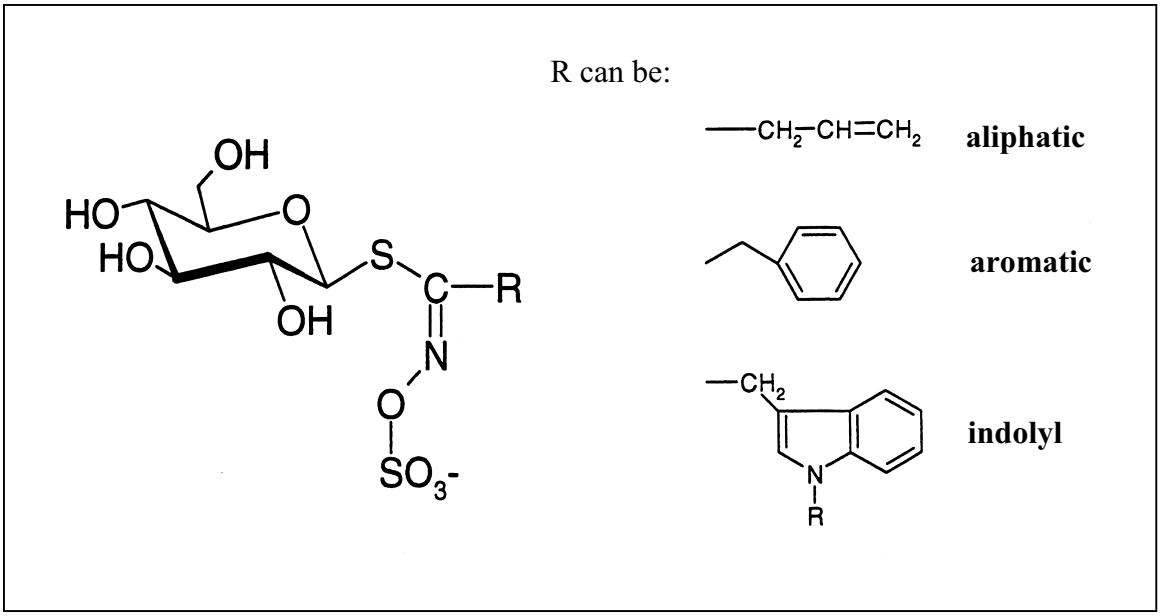

Figure 1. The basic structure of glucosinolates.

Table 1. Common glucosinolates found in *B. napus*.

***** The suffix "glucosinolate" is added to each systematic name.

Side chain structure (denoted R in Fig. 1.)		**Trivial name**	**Semisystematic name***
Aliphatic	R_1=CH_2	Sinigrin	2-propenyl (allyl)
	R_1=CH_2-CH_2	Gluconapin	3-butenyl
CH_2=CH—R_1—	R_1=CH(OH)-CH_2	Progoitrin	2-hydroxy-3-butenyl
	R_1=CH_2-CH_2-CH_2	Glucobrassicanapin	4-pentenyl
	R_1=CH_2-CH(OH)-CH_2	Gluconapolieferin	2-hydroxy-4-pentenyl
Indolyl	R_2=R_3=H	Glucobrassicin	3-indolylmethyl
R_3, CH_2—, N, R_2	R_2=OCH_3, R_3=H	Neoglucobrassicin	1-methoxy-3-indolylmetyl
	R_2=H, R_3=OH	4-hydroxyglucobrassicin	4-hydroxy-3-indolylmethyl
Aromatic	R_4=R_5=H, R_6=OH	Glucosinalbin	4-hydroxybenzyl
R_6—, CH—R_4—, R_5	R_4=CH_2, R5=R_6=H	Gluconasturtiin	2-phenylethyl

al. 1999, Koritsas et al. 1991, Bodnaryk 1992) and other forms of stress (Bartlet et al. 1999, Blake-Kalff et al. 1998) also have significant effects on levels of specific glucosinolates in the growing plants, and this may affect distribution among plant organs. Generally, biosynthetic activities are high in young leaves (Bennett et al. 1995, Bergman 1970, Lykkesfeldt & Møller 1993), shoots and silique walls (Lein 1972) and decrease as the tissue matures (Clossais-Besnard & Larher 1991, Porter et al 1991). When leaves mature the glucosinolate content decreases most noticeably when flowers appear and then again during seed development (Booth et al 1991). The dynamic changes of glucosinolate levels in a certain tissue depend on regulation of the novo biosynthesis, transport and degradation of glucosinolates (discussed further below).

In *Brassica napus* the aliphatic glucosinolates occur predominantly in the seed, whereas the indole glucosinolates are found predominantly in vegetative tissues (Clossais-Besnard & Larher 1991) and the aromatic phenethylglucosinolate constitutes the major glucosinolate in root (Sang et al. 1984). In young leaves of *B. napus* there is an approximate distribution between the aliphatic, aromatic and indolyl glucosinolates of 16, 23 and 61 percentage, respectively (Blake-Kalff et al. 1998). The aliphatic glucosinolates are degraded during leaf development at the same time *de novo* synthesis of indole glucosinolates occurs. Consequently old leaves contain only aromatic and indolyl glucosinolates (Blake-Kalff et al. 1998). The reason for this specific degradation is not known. Generally, however, defense compounds that are no longer needed are often degraded so that stored energy and nutrients can be recirculated into primary metabolic pathways. Mechanical wounding and cabbage stem flea beetle attacks in *B. napus* lead to an increase of the indolyl glucosinolate level while the level of aliphatic glucosinolates decreases (Bodnaryk 1992, Doughty et al. 1995b). *B. napus* leaves infested with the dark-leaf spot pathogen, *Alternaria brassicae*, gave a similar response (Doughty et al. 1991). In cotyledons of *Brassica juncea,* wounding, insect attack and treatment with methyl jasmonate, induce indole

glucosinolates. The levels of both the aliphatic and aromatic glucosinolates remain constant (Bodnaryk 1994). Cotyledons with low glucosinolate content showed increased susceptibility to nonspecialist herbivores, such as slugs (Glen et al. 1990). Several biologically active molecules are implicated in the biosynthesis of certain glucosinolates. Indole glucosinolates have been shown to accumulate systemically in plants treated with jasmonate, a signal molecule associated with reactions pertaining to wounding and herbivory, which argues for a role of indole glucosinolates in this context (Bartlett et al. 1999, Doughty et al. 1995b, Li et al 1999a, Möllers et al. 1999). Similarly, salicylic acid, a phenolic compound involved in pathogen related responses, seems to induce indole glucosinolates in roots (Ludwig-Müller et al. 1997) and also increases the concentration of 2-phenylethylglucosinolate (gluconasturtiin) in oilseed rape leaves (Kiddle et al. 1994). In contrast, treatment with abscisic acid results in low levels of indole glucosinolates in *in vitro* grown embryos (Möllers et al. 1999). Induction of glucosinolates also occurs after nitrogen starvation (Blake-Kalff et al. 1998). The large variation in glucosinolate profiles between individual tissues at different developmental stages, indicates a complex regulation of glucosinolate biosynthesis.

4.1.3 Glucosinolate biosynthesis

The glucosinolates are all synthesized from protein amino acids (e.g. alanine, methionine, valine, leucine or isoleucine for the alipathic glucosinolates, phenyl-alanine and tyrosine for the aromatic glucosinolates and tryptophan for the indole glucosinolates) and a number of chain-elongated homologues (e.g. many of the precursors of aliphatic glucosinolates such as homo-methionine, dihomo-methionine, trihomo-methionine). The dominating glucosinolates are synthesized from methionine (aliphatic), phenylalanine (aromatic) and tryptophan (indole). The biosynthetic pathway has recently been reviewed by Halkier (1999) and Mithen et al. (2000). The initial step in the biosynthesis of the glucosinolates proceeds, as in that of cyanogenic

glycosides, by N-hydroxylation of the precursor amino acid followed by de-carboxylation to form an aldoxime. The metabolism of the oxime to the parent glucosinolate is not well understood (Halkier 1999). It has been proposed that the aldoxime is being N-hydroxylated to an *aci*-nitro compound which subsequently reacts with a thiol donor (e.g. cysteine to produce a cysteine conjugate, S-alkyl-thiohydroxime) (Ettlinger & Kjær 1968). This reaction may be catalyzed by glutathione-S-transferase-like enzymes, which have not yet been characterized. The conjugate is then hydrolyzed by C-S lyase (Kiddle et al. 1999) to thiohydroximate, that is subsequently glucosylated by a soluble UDPG:thiohydroximate glucosyl-transferase (S-GT), thereby producing the desulphoglucosinolate. S-GTs are present in all glucosinolate producing crucifers, whereas they are absent in all non-glucosinolate plants examined, indicating a function of S-GTs in glucosinolate biosynthesis (Halkier 1999). The final step of glucosinolate biosynthesis is catalyzed by 3`-phospho-adenosine 5`-phosphosulphate:desulphoglucosinolate sulphotransferase (PAPS-ST) to convert desulphoglucosinolate to glucosinolate. A proposed model for the biosynthesis of glucosinolates is shown in Fig. 2.

Glucosinolates are related to cyanogenic glucosides as both groups of natural plant products are derived from amino acids and have oximes as intermediates. Cyanigenic glucosides are found throughout the plant kingdom and are considered to have arisen very early in evolution (Saupe 1981). On the contrary, glucosinolates are, with the exception of the genus *Drypetes*, found exclusively in the order Capparales, and thus considered a recent evolutionary event. This has led to the hypothesis that glucosinolate biosynthesis might have evolved based on a “cyanogenic pre-disposition” (Rodman et al. 1998), i.e. the pathway of cyanogenic glucoside biosynthesis has been adapted to produce glucosinolates. This hypothesis is supported by the demonstration of cytochrome P450 dependent oxime production in some glucosinolate producing plants (see below). Furthermore, the primary and tertiary structures of the degradative enzymes, myrosinases for glucosinolates and cyanogenic

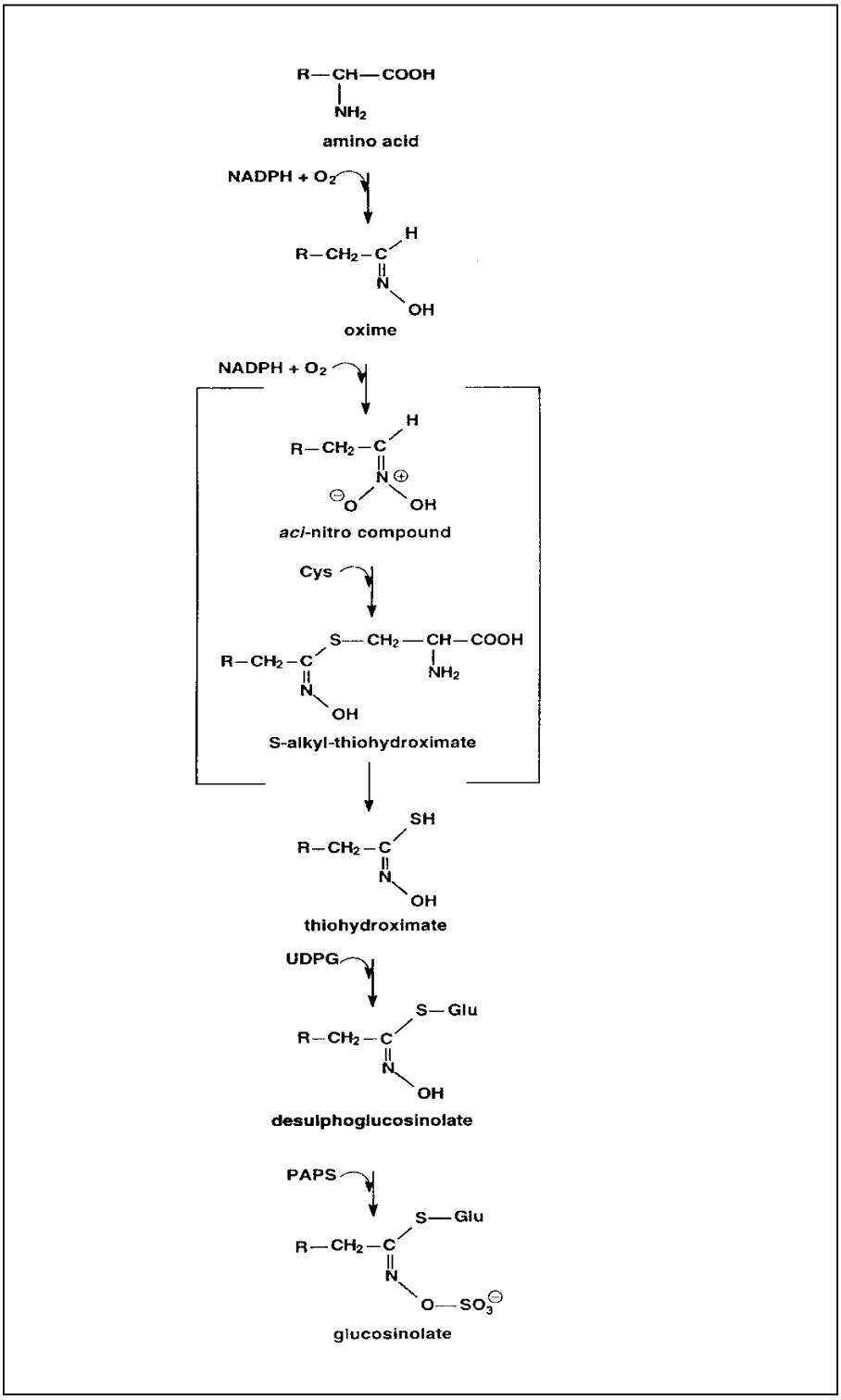

Figure 2. A model for the synthesis of the glucone moiety of glucosinolates. The boxed intermediates have so far limited biochemical evidence. R = variable side chain, PAPS = 3`-phosphoadenosine 5`-phosphosulpate. (From Halkier 1999).

glucosidases for cyanogenic glucosides, are very similar (Rask et al. 2000, Rodman et al. 1998). Cythochromes P450, belonging to the CYP79 family, catalyze the oxime production in cyanogenic glucoside biosynthesis (Andersen et al. 2000).

The biosynthetic pathway of the cyanogenic glucoside dhurrin in *Sorghum bicolor* has been characterised (Halkier et al. 1995). Cytochrome P450 CYP79A1 was shown to catalyze the conversion of tyrosine to p-hydroxyphenylacetaldoximes (Sibbesen et al. 1995). Introducing CYP79A1 from sorghum into *Arabidopsis*, which normally lacks p-hydroxybenzyl glucosinolate, leads to the production of high levels of this glucosinolate (Bak et al. 1999). CYP79A1 homologues have been identified in several glucosinolate producing species (Bak et al. 1998). In *Arabidopsis* cytochrome P450 CYP79A2, CYP79B2 and CYP79B3, and CYP79F1 have been shown to catalyze the conversion of phenylalanine (Wittstock & Halkier 2000), tryptophan (Hull et al. 2000, Mikkelsen et al. 2000), dihomo-methionine and trihomo-methionine (Hansen et al. 2001) to their corresponding oximes, respectively. The characterization of CYP79A1 homologues found in *A. thaliana* show that these enzymes are highly substrate specific and thought to determine the substrate specificity of glucosinolate biosynthesis. This indicates that after diverging away from cyanogenic glucosides the CYP79s involved in glucosinolate biosynthesis have developed new substrate specificity's, e.g towards tryptophan as no tryptophan-derived cyanogenic glucosides have been identified. There seems to be no feedback inhibition on the pre-oxime biosynthetic genes. Introduction of CYP79A1 into *A. thaliana* which produced the tyrosine-derived glucosinolate p-hydroxybenzylglucosinolate (p-OHBG) not found in the wild type, increased the total glucosinolate content 4-fold but the composition and level of major individual endogenous aliphatic and indole glucosinolates were not significantly different from control plants (Petersen et al. 2001). Accordingly, post-oxime enzymes in *A. thaliana* seem to have high biosynthetic capacity, and combined with the low substrate specificity of the post-oxime enzymes this provides a very flexible system for metabolic engineering of *A. thaliana* plants with altered

glucosinolate profiles (Grootwassink et al. 1990). This includes modification of the production of endogenous glucosinolates by alteration of the endogenous preoxime enzymes of which several have been identified in *A. thaliana* (Hull et al. 2000, Wittstock & Halkier 2000). This also includes metabolic engineering of novel glucosinolates derived from non-endogenous oximes introduced into the plant by, e.g. transformation with CYP79 homologues with substrate specificity's different from those of the endogenous CYP79s. As *Arabidopsis* is closely related to *Brassica* species it is thought that information concerning the genetic control of glucosinolate biosynthesis obtained in *Arabidopsis* will be transferable to *Brassica* (Halkier 1999). Glucosinolate research in the *Arabidopsis* era has recently been reviewed by Wittstock & Halkier (2002).

4.1.4 Glucosinolate localization and transport

Glucosinolates have been detected in all organs of Brassica plants but the capacity of *de novo* biosynthesis varies between organs at different stages (Bergman 1970, Porter et al. 1991). The cell-specific localization of glucosinlates is still not clear. Glucosinolates were first reported to be stored in vacuoles together with ascorbic acid (Groob & Matile 1979). Ascorbic acid is able to modulate myrosinase activity in a concentration dependent manner, i.e. inhibition at higher concentration and activation at lower concentration (Bones & Slupphaug 1989). The dual function of ascorbic acid sheds light on the possibility of co-localization of glucosinolates and myrosinases. Recent immuno-histocemical analysis has, however, shown that the glucosinolate sinigrin is localized to protein bodies in non-myrosinase cells in seeds (Kelley et al. 1998), assuming that this also is valid for other glucosinolates. This view is supported by results obtain in this thesis (Paper I), and indicates that the enzyme and its substrate are not compartmentalized in the same cell. The physical separation of the substrate and the enzyme suggests a role in the prevention of the formation of toxic products *in*

vivo, but poses a question as to how glucosinolates or myrosinases are transported to interact apart from tissue damage.

Several studies have suggested that glucosinolates are metabolized *in vivo* during development and not only upon tissue disruption (Cole 1978 and 1980, Svanem et al. 1997, Petersen et al. 2002). These findings indicate a role for the glucosinolates and myrosinases in the control of plant growth and development and not only in defense upon tissue damage. It has however been debated whether glucosinolates are turned over during the germination processes or merely diluted as a consequence of growth (McGregor 1988, Clossais-Besnard & Larher 1991). Recently, a group of cells (S-cells) has been reported, situated between the phloem of vascular bundle and the endodermis, containing extraordinary high levels of glucosinolates (> 100 mM) in *Arabidopsis* flower stalk (Koroleva et al. 2000). Husebye et al. (2002) reported the localization of myrosinase enzymes and glucosinolates in different but neighboring cells in the *A. thaliana* phloem bundle. Although the cells may have cytoplasmic connection, tonoplast transporters are needed because both glucosinolates and myrosinases are reported to be located in vacuoles (Thangstad et al. 1991, Höglund et al. 1991, Kelly et al. 1998). It has been shown that both intact glucosinolates and desulpho-glucosinolates possess the physio-chemical properties allowing phloem-mediated transport (Brudenell et al. 1999). In developing seedlings of *B. napus* findings have suggested that glucosinolates might be transported as desulpho-glucosinolates (Rossiter & James 1990, Rossiter et al. 1990, Thangstad et al. 2001). Recently, the presence of intact glucosinolates in the phloem has also been demonstrated in *Arabidopsis* (Chen et al. 2001). Analysis of glucosinolate profiles of seed and leaf tissue of *B. napus* F1 hybrids from reciprocal crosses between cv. Cobra and a synthetic line, showed that the profile of the aliphatic glucosinolates in the seed was identical to the profile in the leaves of the maternal parent (Magrath & Mithen 1993). Petersen et al. (2002) obtained a similar result for *Arabidopsis*. This suggests that glucosinolate biosynthesis and glucosinolate interconversions (i.e. hydroxylation)

do not take place within the embryo and that fully formed glucosinolates or desulpho-glucosinolates are transferred from maternal tissue into the developing seeds. In support of phloem transport, aphid feeding experiments on black mustard *B. nigra* have shown that there was more than 10 mM sinigrin in phloem sap of young leaves, whereas there was only about 1-2 mM glucosinolates in mature, presenescent and senescent leaves (Merritt 1996). From radio-labeled feeding of p-hydroxybenzyl-glucosinolate on a rosette leaf of *A. thaliana*, it was proposed that glucosinolate synthesis in mature leaves is readily loaded into phloem together with photo-assimilates in the osmotically driven translocation stream from source to sink (Chen & Andreasson 2001). In addition to possible nutritional function in case of environmental stress, the phloem transport of glucosinolates may help the plant to defend itself against phloem-feeding organisms and also gain the ability to coordinate the synthesis and use of protective resources between various organs.

4.1.5 Glucosinolate degradation

The glucosinolates are hydrolyzed by myrosinases to produce thioglucose, sulfate, and an unstable intermediate, which rearranges spontaneously to produce several de-gradation products, of which many have pronounced biological effects. Most frequently the unstable aglycone undergoes a proton-independent Lossen rearrangement with a concerted loss of sulfate to yield an isothiocyanate. The structural diversity of the hydrolysis products is dependent on; (1) the side chain of the glucosinolate; (2) the conditions of the environment (pH, ferrous ions); and (3) the presence of epithiospecifier protein (Chew 1988), see Fig. 3. At neutral conditions rearrangement of the aglycone will usually result in formation of an isothiocyanate, while at acidic conditions the nitrile derivative is the dominant product. In the presence of ferrous ions the relative amount of nitriles to isothiocyanates increases at all pHs (Uda et al. 1986 a ,b). In combination with ferrous ions, epithiospecifier protein produces an epithionitrile when the glucosinolate contains a terminal double

bond (Tookey 1973). Indole- and benzyl-glucosinolate derived aglycones are unstable and rearrange to form the corresponding alcohol and thiocyanate. Degradation of glucosinolates that contain hydroxylated side chains yields oxazolidine-2-thiones in a spontaneous cyclic reaction.

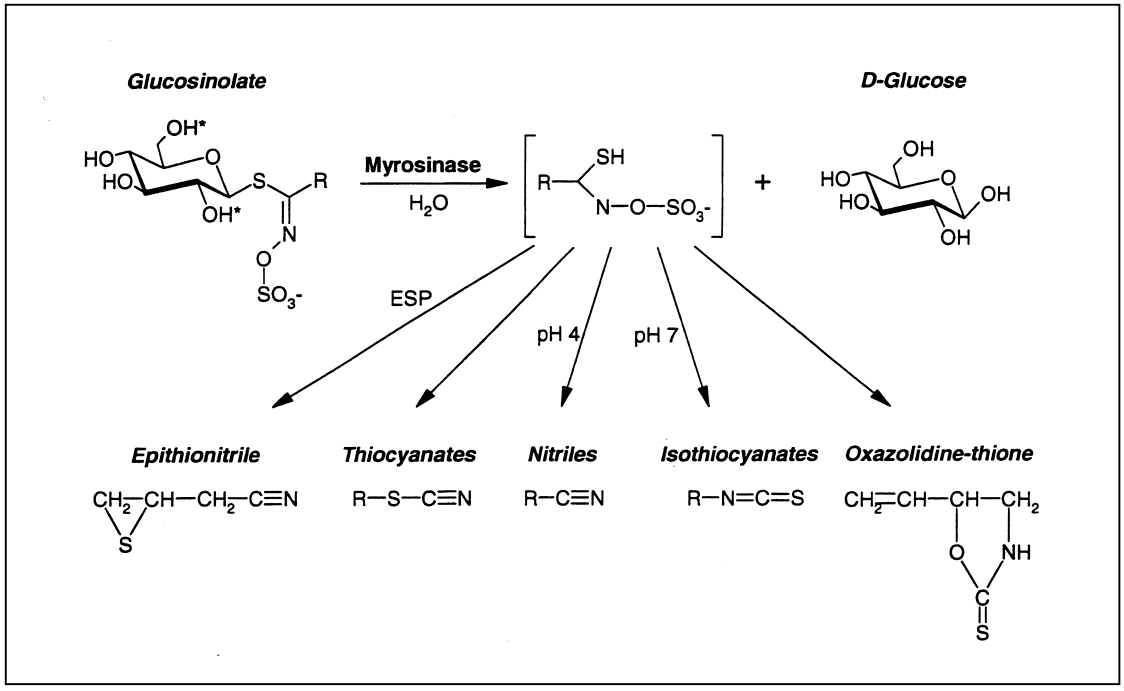

Figure 3. The myrosinase catalyzed hydrolysis of glucosinolates. Stars denote positions in the glucose moiety known to be acylated in certain glucosinolates. ESP = epithiospecifier protein. (From Rask et al. 2000).

4.2 MYROSINASES

The hydrolysis of glucosinolates is catalyzed by the enzyme myrosinase (EC 3.2.3.1) also known as thioglucoside glucosidase (TGG). The myrosinases are the only known plant S-glycosidases and they all seem to be glycosylated.

4.2.1 The myrosin cell

The term myrosin cell was first used by Guignard (1890) and describes this special type of cell discovered by Heinricher (1884) assumed to contain myrosinase. The importance of the myrosin cells as a taxonomic tool has been generally accepted, and Jørgensen (1981) used occurrence and distribution of myrosin cells as two of several criteria for the classification of the order Capparales. The appearance of myrosin cells is easily distinguished from surrounding cells in the electron microscopy (Fig. 4). The myrosin cells contain less lipid, have a high content of endoplasmic reticulum and harbor smooth-looking protein bodies referred to as myrosin grains (Bones & Iversen 1985). Myrosin cells occur as scattered cells in roots, stems, leaves, petioles, seeds and seedlings. Immunohistochemical studies of *B. napus* seeds showed that the myrosinase was found exclusively in the myrosin cells (Thangstad et al. 1990, Höglund et al. 1991). By *in situ* hybridization experiments it has been shown that myrosinase transcripts are present in these cells and that they presumably synthesize the enzyme (Falk et al. 1995a, Lenman et al. 1993, Xue et al. 1995). The signal peptides and presence of sites for N-linked glycosylation, suggest transport and glycosylation through the ER-Golgi complex (Thangstad et al. 1993, Höglund et al. 1992).

The myrosin cells comes in different shapes and sizes according to localization. In mesophyll tissue from cotyledons the shape may vary from isodiametric cells of normal size to elongated cells with an area 7-8 times larger than the surrounding

aleurone cells. Myrosin cells in longitudinal sections from cortex tissue in hypocotyls appear rectangular or cubed, while in roots they are elongated in the direction of the organ axis. The grain size varies from 2.5 to 10 µm, interspersed with oleosomes. Distribution, occurrence and morphology of myrosin cells reported in early studies have been reviewed by Bones & Iversen (1985). During differentiation of the myrosin cells the myrosin grains seem to fuse. A recent analysis of the subcellular organization of myrosinase in myrosin cells of *B. napus* embryos has shown, by the use of a monoclonal anti-myrosinase antibody and confocal microscopy, that the myrosin grains actually formed a continuous reticulum throughout the cell (Andreasson et al. 2001). The authors thereby suggested denoting the myrosinase-containing structure as a myrosin body rather than myrosin grains.

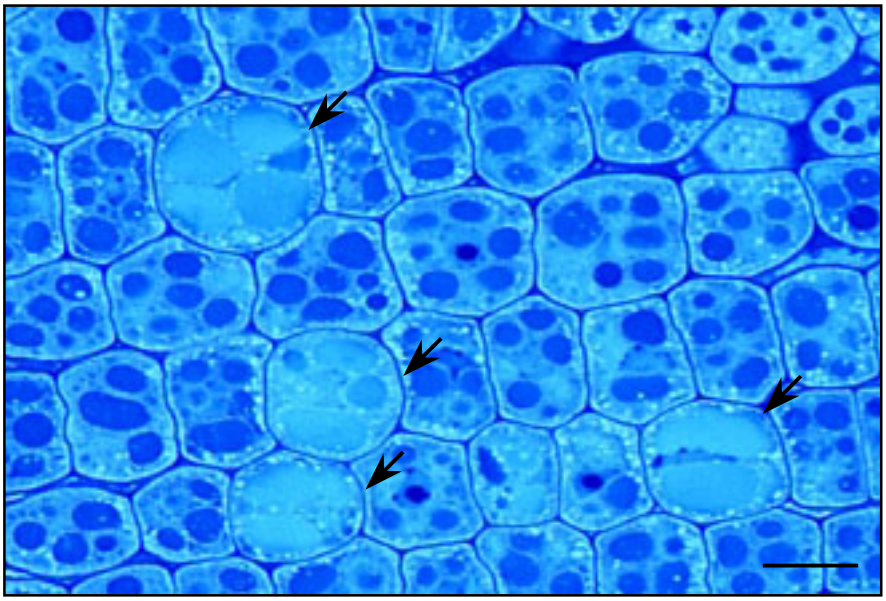

Figure 4. *Brassica napus* seed cotyledon. Light microscopy section of myrosin cells and aleurone cells stained with toludine blue. Arrows mark myrosin cells. Scale = 10 µm.

In seeds of *B. napus* the myrosin cells appear around 20 days after pollination and increase in number until they reach two to five per cent of the total amount of embryonic cells (Höglund et al. 1991). There seems to be no active formation of myrosin cells during seedling development and the myrosin cells found in seedlings are subsequently produced in the seed. As a consequence, there is a decrease during aging of the plant in the relative number of myrosin cells and myrosinase activity (Bones & Iversen 1985). In seedling cotyledons, leaves, old flower buds and siliques, myrosinase is present in the phloem parenchyma and in the ground tissue (Andreasson et al. 2001). In mature petals and stems myrosin cells were found in the phloem. This is in contrast to an earlier study, which reported a consistent staining of myrosin cells in the xylem of petals and stem (Höglund et al. 1991). In the root myrosinase containing idioblast cells are mainly located in the cortex region. In very young flower buds, no myrosinase containing cells have been found but they are present in the phloem of the pedicel (Andreasson et al. 2001). The localization of myrosinase to vascular tissue and the outermost cell layers of the organs support reports which suggest that the myrosinase-glucosinolate system has a role in the defense system against invading organisms (Rask et al. 2000).

The intracellular localization of myrosinase has been much debated throughout the years, especially in relation to the storage site of glucosinolates. By the use of antibodies against myrosinase (Thangstad et al. 1990, Höglund et al. 1991) and the glucosinolate sinigrin (Kelly et al. 1998), it was found that the enzymes were localized to the interior of the myrosin grains and the glucosinolate to protein bodies in non-myrosin cells in seeds.

Whereas myrosinase in *B. napus* is expressed in both the ground tissue and the phloem tissue, the Arabidopsis myrosinase is confined to cells of the phloem parenchyma (Husebye et al. 2002, Andreasson et al. 2001). Adjacent sulfur-rich cells

(S-cells) containing extraordinary high concentrations of glucosinolates have been found in Arabidopsis flower stalks (Koroleva et al., 2000).

4.2.2 Myrosinase genes

So far seven genes encoding myrosinase have been characterized by sequence determination, three from *B. napus* (Thangstad et al. 1993, Lenman et al. 1993), one from *B. campestris* (Machlin et al. 1993), and three from *A. thaliana* (Xue et al. 1995, Zhang et al. 2002). The exon/intron organization of all functional myrosinase genes characterized is conserved with 12 exons separated by 11 introns. One of the *B. napus* genes and one of the *A. thaliana* genes were non-functional pseudogenes (Thangstad et al. 1993, Zhang et al. 2002). Sequence comparison has showed that myrosinases belong to the family 1 of β-glucosidases (Thangstad et al. 1993). Plant O-β-glucosidases and the myrosinase genes seem to share a similar exon/intron organization, although certain exons are fused or split. This provides strong evidence for a shared evolutionary history for this type of genes since it is highly unlikely that identical exon/intron organization would arise by convergence (Rask et al. 2000).

In *B. napus* myrosinases can be divided into three different gene families; the MA, MB and MC family (Xue et al. 1992, Lenman et al. 1993, Thangstad et al. 1993, Falk et al. 1995a). Southern blot analysis has suggested the presence of approximate 5 MA genes, 10-15 MB and 5 MC myrosinase genes. The MA and MC genes have been reported to be exclusively expressed in the seed embryo (Lenman et al. 1993) but recent investigation has revealed that the MA promoter *Myr1.Bn1* is also expressed in developing siliques and the flower stalk (Paper I). The MB genes are expressed throughout plant development, in both embryonic and vegetative tissues (Falk et al. 1992, Lenman et al. 1993, Xue et al. 1993).

In *A. thaliana* three myrosinase genes, TGG1, TGG2 and TGG3 have so far been identified, none of them classified into the MA, MB or MC family. Just recently, three new myrosinase genes has been reported found in *A. thaliana* (Zhang, Andersson, Meijer, abstract to the Arabidopsis conference 2002). These genes denoted TGG4, TGG5 and TGG6 seem to belong to a new subfamily of myrosinase genes not earlier discovered.

4.2.3 Myrosinase isoenzymes

The existence of multiple forms of myrosinase has been shown in many plants. The isoenzymes are all glycosylated dimeric proteins with subunit molecular weights reported to be from 62 to 75 kDa (Bones & Rossiter 1996). The carbohydrate content varies between 9-23 per cent of the total molecular mass giving rise to myrosinase proteins with slightly different sizes. Distribution of the myrosinase isoenzymes is both organ- and species-specific and changes during development (James & Rossiter 1991, Xue et al. 1992, Lenman et al. 1993) and during stress exposure (Bones et al. 1994). The different isoenzymes show diversity in physio-chemical characteristics including differences in pH optima, temperature optima and kinetic properties (for review, see Bones & Rossiter 1996).

Different tissues contain different glucosinolates and a tempting thought has been that different myrosinase isoenzymes have different substrate specificity. However, based on the three-dimensional structure of a myrosinase from *S. alba* (Burmeister et al. 1997), this explanation could not be proven valid (see 4.2.5). But the reports of the substrate specificity of myrosinase isoenzymes are still limited. One report observed no substrate differences between isolated isoenzymes (Sørensen 1990). Even so, two different myrosinases from *B. napus* have been shown to degrade different glucosinolates at different rates, although both enzymes showed the highest preference for the aliphatic glucosinolates and least preference for the indolylic forms (James &

Rossiter 1991). The number of glucosinolates and isoenzymes present in the plant are not correlated, and myrosinases seem to accept a wide range of glucosinolate substrates. But when considering substrate specificity one should also be aware of the possibility that the specificity could be affected by associated factors like epithio-specifier protein (ESP), myrosinase binding proteins (MBPs) or other myrosinase associated proteins or components.

Western analysis of protein extracts from *B. napus* seeds shows the presence of three classes of myrosinase subunits with apparent molecular sizes of about 75, 70 and 65 kDa. MA genes, MC genes and MB genes are encoding these classes, respectively (Falk et al. 1995a, Lenman et al. 1993). The size differences of the myrosinases are probably almost entirely due to different extents of glucosylation, since the protein moieties of MA, MB and MC myrosinases have similar molecular masses. Myrosinases that belong to the MA family occur as free dimers (140 kDa), while the MB and MC myrosinase families are found in high molecular complexes (200-1000 kDa) with myrosinase binding proteins (MBP) and the myrosinase associated protein (MyAP) (for review, see Rask et al. 2000). Myrosinases found in these complexes are enzymatically active, although complex formation renders some of these myrosinases partially insoluble (Eriksson et al. 2002, Geshi & Brandt 1998, Falk et al. 1995b).

4.2.4 Myrosinase activity

The reported activities in different tissues varies, but all tested organs/tissues contain some myrosinase activity (Bones 1990). The hypocotyls contain the highest specific myrosinase activity of the seedling organs. The specific activity in hypocotyls was more than twice that in cotyledons and several times that in seedling roots. Except for the roots of fully grown plants where a high activity was observed, organs of mature plants normally contained low myrosinase activity. References to several other studies of myrosinase activity can be found in Bones (1990).

Ascorbic acid (vitamin C) is a water-soluble antioxidant present at millimolar concentration in plant cells (Wheeler et al. 1998), where it is probably stored in the vacuole (Grob & Matile 1979). Different myrosinase isoenzymes isolated from different species or within a species, have been reported to show varying response to ascorbic acid (Tsuro & Hata 1968, Henderson & McEwen 1972, Bones & Rossiter 1996). In general, high concentrations of ascorbic acid inhibit and low concentrations activate myrosinase activity (Bones & Slupphaug 1989). In *B. napus* the optimum concentration of ascorbic acid has been shown to be 0.3-0.5 mM (Bones & Slupphaug 1989). The total level of glucosinolates drops during early seedling growth, while the endogenous ascorbic acid concentration increases (Sukhija et al. 1985). This may explain the observed increase in the myrosinase activity during germination (James & Rossiter 1991). It has also been found that particularly isoenzymes with molecular weight around 65-58 kDa are activated by ascorbic acid, while isoenzymes with molecular weight around 75-77 are not activated (James & Rossiter 1991, Bones et al. 1994). Ascorbic acid at low concentrations (0.05-1 mM) has been shown to be an uncompetitive activator of myrosinase, decreasing V_{max} and K_m in parallel, while at higher concentrations (> 2,5 mM) it functions as a competitive inhibitor (Shikita et al. 1999, Ohtsuru & Hata 1979). The exact mechanism behind ascorbic acid activation of myrosinase is so far not fully understood.

4.2.5 Structure and catalytic mechanism of myrosinase

The three-dimensional structure of a myrosinase from *Sinapis alba* has been resolved by Burmeister et al. (1997), see Fig. 5. The crystallized myrosinase most probably belongs to the MA subfamily (Xue et al. 1992) and is a dimer of two identical subunits, each consisting of more than 499 amino acids. The myrosinase protein folds into a $(\beta/\alpha)_8$ barrel structure common to other O-glycosidases and is heavily glycosilated. The dimer is stabilized by a Zn^{2+} ion and each subunit contains three

disulphide bridges, which probably contribute to the remarkable stability of the myrosinase enzymes. The myrosinase structure also shows a substantial number of salt bridges and hydrogen bonds between charged and neutral atoms. This has been linked to thermostability and stability against denaturation under high salt conditions. Indeed, myrosinase is stable against denaturation (Pessina et al. 1990) and has its maximal activity between 55°-65°C (Hochkoeppler & Palmieri 1992). The large amount of carbohydrate (13 kDa per dimer) is most likely needed to maintain molecular stability and solubility in the dehydrated environment of the seed. The crystallization trials showed that myrosinase is very soluble and can only be precipitated using 66 % saturated ammonium sulphate (Burmeister et al. 1997). The hydrophobic substrate pocket formed by the $(\beta/\alpha)_8$ barrel structure fits the side chain of glucosinolates. Myrosinase has two conserved arginine residues in its active site important for correct binding and positioning of the substrate sulphate group. This is probably the site specifying the enzymes to be an S-glycosidase. Myrosinase belongs to family 1 of glycosyl hydrolases and shows both sequence and structural similarities with O-β-glucosidases found within this family. Especially the β-glucosidase linamarase from *Triticum repens* have similar properties, except for the O- and S-glycosidase mechanisms.

The catalysis by myrosinase occurs in two steps; a glycosylation step and a deglycosylation step. The conserved glutamate (Glu 409) functions as a catalytic nucleophile attacking the substrate sugar ring forming a glycosylenzyme-intermediate followed by a concomitant release of the aglycone. The glucose is together with the sulfate group involved in recognition. The significant enzyme-substrate interactions are established by all the hydroxyl groups of the sugar moiety. This can probably explain why acylated glucosinolates do not seem to be degraded by myrosinase (Sørensen 1990). The aglycone of a glucosinolate is an excellent leaving group, which explains why a glutamate otherwise conserved in glycosidases in myrosinase has been

replaced with a glutamine residue (Gln 187). The second deglycosylation step differs from other β-glycosidases due to this amino acid replacement (Burmeister et al. 1997).

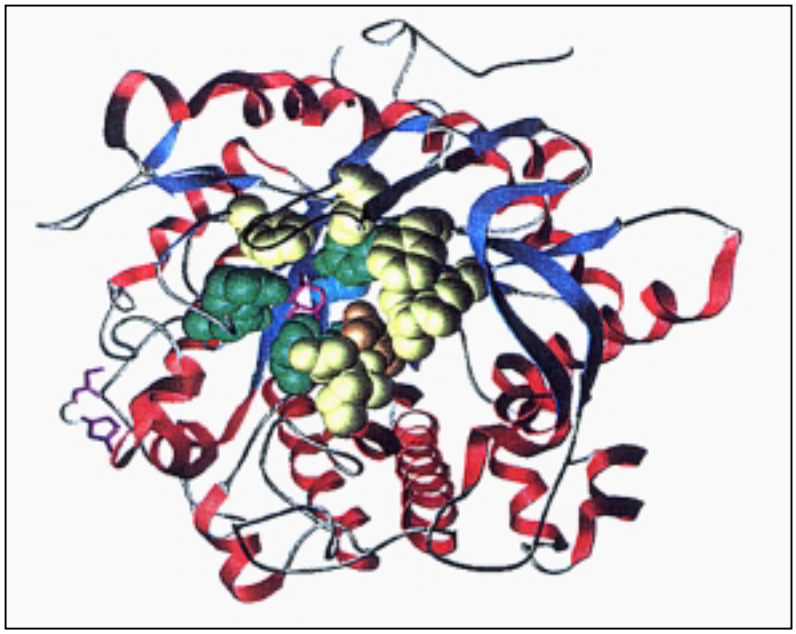

Figure 5. Three-dimensional structure of a myrosinase from *Sinapis alba* showing the (β/α)-structure without disulfide bridges and glycosilation. The secondary structure elements are shown in blue (β-sheets) and in red (α-helices). The two monomers are coordinated by a Zn ion shown in white and its ligands in purple. Residues involved in glucose-ring recognition are shown in green and residues recognizing the aglycone in yellow. The catalytic nucleophile is drawn in cyan and the glutamine residue corresponding to general acid/base in related O-glycosides in orange. The inhibitor 2-deoxy-2-fluoroglucotropaelin molecule in magenta covalently bound to the nucleophile indicates the position of a substrate in the active site. (From Rask et al. 2000 based on Burmeister et al. 1997).

Myrosinase seems able to transform glucosinolates with different side-chains at similar rates. Additionally, many O-glycosides can be hydrolyzed by myrosinase to some extent if they have good leaving groups (Botti et al. 1995). The lack of a good leaving group (sulfate) in desulphoglucosinolates could be a possible explanation as to why these compounds are not degraded by myrosinase (Hanley et al. 1990). Several hydrophobic residues surrounding the hydrophobic part of the aglycone is present close to the active site of myrosinase and they are conserved in the myrosinase sequences examined (Rask et al. 2000). Analysis of different forms of myrosinase is needed in order to study any potential differences evolved in substrate specificity and in turnover for different aliphatic, aromatic and indolyl glucosinolates.

4.3 PROTEINS INTERACTING WITH MYROSINASE

4.3.1 Epithiospecifier protein (ESP)

Allylic glucosinolates (alkenyl glycosinolates with terminally unsaturated carbon) such as progoitrin, sinigrin, gluconapin and glucobrassicanapin can give rise to highly toxic cyanoepithioalkanes (epithionitriles). The exact mechanism behind the formation of epithionitriles from aliphatic glucosinolates is not known but depends on the presence of a protein known as an epithiospecifier protein (ESP) (MacLeod & Rossiter 1985). The presence of ferrous ions and myrosinase together with ESP are essential for epithionitrile formation (MacLeod & Rossiter 1987; Zabala, Grant, Bones, Bennett Rossiter, unpublished). A generalized mechanism for the formation of cyanoepithionitriles and simple nitriles has been described (Foo et al. 2000). Crucial to the mechanism is the formation of an iron ligand with the thiohydroximate, which in the absence of ESP results in the formation of a nitrile but in the presence of ESP an epithionitrile.

On the basis of gel filtration, the molecular mass of ESP has been shown to be 30-40 kDa (Tookey 1973). Two classes of ESP polypeptides with molecular masses of 39 and 35 kDa have recently been purified and characterized in *B. napus* (Foo et al. 2000). ESP does not have myrosinase activity but seems to interact with myrosinase at a site different from the substrate-binding site, as suggested by kinetic studies where ESP inhibited myrosinase non-competitively (MacLeod & Rossiter 1985). The presence of ESP in a given plant seems to correlate with the presence of the appropriate substrates, i.e. allylic glucosinolates (Kaolla et al. 1980), but not the presence of myrosinase since some plants, e.g. *S. alba* and *Armoracia lapathifolia*, contain myrosinase but not ESP (Petroski & Tookey 1982). The ESP protein has thus been described as a myrosinase cofactor that drives the hydrolysis of some glucosinolates towards the production of cyanoepithioalkanes instead of the other possible hydrolysis products (Bernardi et al. 2000). ESP does not seem to require any specific myrosinase, as ESP is also active with myrosinase isolated from the aphid *Brevicoryne brassicae* (Jones et al. 2001).

The *Arabidopsis* ESP protein seems to be a more general "nitrile-specifying" protein. Lambrix et al. (2001) found that alkenyl glucosinolates were converted to epithionitriles and nonalkenyl glucosinolates were converted to simple nitriles, whereas in the absence of the ESP protein these substrates were converted to isothiocyanates.

The specificity of ESP to generate epithionitriles suggests a specific function of these compounds for the plant. Some studies have suggested that epithionitriles are particularly toxic to certain insects (Peterson et al. 2000, Galletti et al. 2001). Alternatively, some isothiocyanates are volatile and have been shown to act as hostfinding cues for specialist herbivores (Städler 1992). The presence of the ESP protein and alteration from isothiocyanate to nitrile formation may thereby allow the plant to escape detection by such insects. However, Lambrix et al. (2001) have

recently shown that in *Arabidopsis* the presence of functional ESP protein and the production of nitriles were associated with increased herbivory by the generalist *Trichoplusia ni* (*T.ni*). It should nevertheless be noted that although *T. ni* feeds readily on *Arabidopsis* in experimental situations, it has not been reported as a herbivore on *Arabidopsis* in the wild (Jander et al. 2001). In addition, the effect of nitrile formation on specialist herbivores and on predators was not investigated. Nitriles may be directed against other pests or might attract natural enemies of the herbivores. Nevertheless, this study indicates a key role for ESP in plant insect interaction by modification of plant glucosinolate hydrolysis profiles. ESP in correlation with herbivore feeding in *Arabidopsis* has recently been reviewed by Eckardt (2001). Loss of ESP function by loss of gene expression and loss of a large fragment of the coding region, as shown in ecotypes that do not form nitriles, indicates that plants might have been under selection pressure to abolish ESP activity (Lambrix et al. 2001).

4.3.2 Myrosinase binding proteins (MBPs)

Two types of myrosinases, MB and MC, present in *B. napus* seeds occur in complexes with non-myrosinase proteins. The proteins identified in these complexes are denoted myrosinase binding proteins (MBP) with molecular masses ranging from 30 to 110 kDa (Lenman et al. 1990, Falk et al. 1995b, Taipalensuu et al. 1996, Geshi & Brandt 1998). There are both seed specific and vegetatively expressed members of the MBPs (Taipalensuu et al. 1997a). The MBP50 and MBP52 are exclusively found in the seed, while the high molecular mass MBPs are found throughout the plant, which may indicate that they have different functions. The MBPs are most probably derived from a single large precursor, which is expressed predominantly in the seeds but also in other plant organs (Taipalensuu et al. 1997c). Alternative splicing or proteolytic cleavage of precursor protein probably generates MBP of different sizes (Taipalensuu et al. 1997c). MBPs are composed of several types of repeats from a few amino acid residues to approximately 160 residues (Taipalensuu et al. 1997b,c). The repeats are

jacalin-like and several proteins containing one or several of such repeats have been suggested to be involved in the defense against pathogens and pests (Peumans & van Damme 1995). Different MBPs have different solubility and some MBPs need the presence of a detergent in order to become soluble (Geshi et al. 1998, Eriksson et al. 2002). Myrosinase activity has been reported to redistribute from the soluble to the insoluble fraction of *B. napus* leaf extracts upon wounding (Taipalensuu et al. 1997b), and this might be due to binding to the concomitantly induced MBPs.

The exact function of MBPs has not been established but they may have a role in determining specificity or stability of the myrosinase complexes. The MBPs expressed vegetatively are induced in response to wounding, jasmonic acid and abscisic acid but the induction is antagonized by salicylic acid (Taipalensuu et al. 1997b,c, Geshi & Brandt 1998). This supports the possible regulatory role of these proteins. Seed MBP and MBP70 have been experimentally verified to have lectin activity (Taipalensuu et al. 1997b, Geshi & Brandt 1998) and might bind carbohydrates present in the insect gut or on the surface of fungi. Lectins, together with chitinases, glucanases and glycosidases, are the only known plant proteins that can recognize and bind foreign glyco-conjugates resulting in cytotoxic and growth inhibiting activities (Peumans van Damme 1995). Many plant lectins have been shown to be involved in the defense mechanisms of the plant (Chrispeels & Raikhel 1991), and the MBPs might have a synergistic effect that enhances the action of glucosinolate degradation products in the defense against pests and pathogens.

The MBPs are probably co-localized together with the glucosinolates in the *B. napus* seed (Kelly et al. 1998, Andreasson et al. 2001, Paper I). During germination MBP disappears outside myrosin cells and are co-localized together with myrosinases in the myrosin cells in 5 and 7 days old seedlings (Geshi et al. 1998, Andreasson et al. 2001). This is concomitant with a dramatic decrease of mainly alipathic glucosinolates and is in agreement with the observation by James and Rossiter (1991) that the

myrosinase activity increased during the same period. This might suggest a general storage protein-related function for MBP but also that MBP could be involved in the degradation of glucosinolates by being a part of the transport mechanism to the myrosin cells. Degradation of glucosinolates into nutritional components in intact myrosin cells could then be possible. The presence of MBP in myrosin cells later during development may support the latter idea. However, MBPs constitute a gene family with several members in which the different genes are structurally distinct although certain motifs are shared (Taipalensuu et al. 1997b), and different MBPs may have different function.

Just recently, the existence of novel MBP homologues specifically expressed in floral organs in *Arabidopsis* has been shown (Takechi et al. 1999, Capella et al. 2001). The function of these MBP genes in flowers of *Arabidopsis* is currently unknown. The *Arabidopsis* myrosinase TGG1 and the MBP genes, named MBP1 and MBP2, are expressed in the flowers in a developmentally similar way (Capella et al. 2001). However, TGG1 mRNA is present in tissues where MBP transcripts are absent, a strong indication that MBP1 and MBP2 are not required for TGG1 function (Capella et al. 2001). The expression of *Arabidopsis* MBPs restricted to floral organs is distinct from the expression profiles of *B. napus* MBPs described previously with both induced (vegetatively expressed) and not induced (seed-specific) MBPs. *Arabidopsis* seedlings treated with MeJA show a small induction of MBP1 and TGG1 but not MBP2 (Capella et al. 2001). It has been suggested that these MBPs have a role in male fertility since *coi*1 flowers, an *Aarabidopsis* mutant insensitive to jasmonic acid with production of male-sterile flowers, does not express them (Capellla et al. 2001). This could therefore indicate an alternative function of *Arabidopsis* MBPs not related to the myrosinase-glucosinolate system.

4.3.3 Myrosinase associated protein (MyAP)

The myrosinase associated protein is a 40 kDa monomeric glycoprotein that has also been identified in complexes together with MB and MC myrosinases as well as MBPs in *B. napus* (Taipalensuu et al. 1996). Based on amino acid sequence information and immuno-reactivity, the MyAP polypeptide is unrelated to MBP polypeptides (Taipalensuu et al. 1996). However, as for MBP, both seed-specific and vegetatively expressed MyAP isoforms exist. The latter forms are induced by wounding and by treatment with methyl jasmonate (Taipalensuu et al. 1996, 1997a). The cellular localization of MyAP is still unknown, but *in situ* hybridization of methyl jasmonate-treated *B. napus* leaves showed an increase of MyAP transcripts in all parenchymal cells (Andreasson et al. 1999). The exact function of MyAP has not been clarified, but the similarity of MyAP to lipases has prompted the suggestion that a possible role for MyAP is to release the acyl group from acylated glucosinolates thereby making the acylated glucosinolates available for hydrolysis (Taipalensuu et al. 1997a). This has, however, not been proved. In addition, acylated glucosinolates have not been found in *B. napus* although it is rich in MyAP. A major fraction of MyAP in *B. napus* seed is not bound in the myrosinase complexes (Taipalensuu et al. 1996), and this may further indicate a possible role of MyAP outside the myrosinase-glucosinolate system.

4.4 BIOLOGICAL SIGNIFICANCE OF THE MYROSINASE – GLUCOSINOLATE SYSTEM

4.4.1 Glucosinolate metabolism in defense

Plant secondary metabolites have been shown to serve as mediators in interactions between plants and insects and pathogens. The existence of both preformed and induced defense responses makes a complex network of possible defense strategies for the plant against intruding organisms. The preformed defense consists of many low

molecular compounds such as glycosides, alkaloids, phenols and calcium oxalate (Bennet & Wallsgrove 1994), while hormones and different elicitors initiating complex signal transduction pathways characterize the induced defense. The induced defense can further be divided into a hypersensitive response (HR) (Grant & Mansfield 1999) which is a quick local response due to the entry of pathogens, and a systemic response giving rise to a systemic acquired resistance (SAR). The SAR occurs in all parts of the plant and lasts for 2-3 weeks (Ryals et al. 1996). In the induced defense different signaling pathways seem to be involved depending on the nature of the attack. Mechanical damage by wounding or insect attack is known to elicit jasmonic acid (Peňa-Cortěs et al. 1993), while a pathogenic response triggers a salicylic acid-dependent pathway (Malamy et al. 1996). A cross-talk between the different pathways exists. Salicylic acid has been shown to inhibit synthesis of jasmonic acid and thereby inhibit wound-induced jasmonic acid responses (Peňa-Cortěs et al 1993). Furthermore, jasmonic acid or methyl-jasmonate applied exogenously onto the plant has been shown to induce the expression of several genes also induced by wounding or pathogenic attack. A type of pathogen induced response has also been shown to be independent of the salicylic acid pathway (Thomma et al. 1998), which contributes to the high complexity of the defense responses existing in plants.

The myrosinase-glucosinolate system has been shown to be modulated by wounding (Bodnaryk 1992), insects and pathogen attack (Koritsas et al. 1991, Doughty et al. 1991) and hormones involved in defense response signaling such as methyl-jasmonate (Bodnaryk 1994, Doughty 1995) and salicylic acid (Kiddle et al. 1994). Despite this, the system can to a large extent be considered as a preformed defense system, which is highly dynamic and is one of the first barriers to herbivores. Field and laboratory studies indicate that most species of crucifer feeders respond to glucosinolates or to their hydrolysis products (Rosa et al. 1997).

Effects on insects

Glucosinolates and the products of their degradation are considered to function as part of the plant`s defense mechanism against insect attack (Fenwick et al. 1983, Chew 1988) and to act as phagostimulants (Chew 1988). They have a major role in host plant localization and colonization by many phytophagous insects specifically adapted to brassicacious plants, and also in the location of these insects by parasites.

The effect of glucosinolates on insect feeding depends on the developmental stage of the host plant, their concentration and profile, and the insect species being considered. Several studies have shown a relationship between variation in the presence and/or concentration of aliphatic glucosinolates and the behavior of herbivores. Studies of individual glucosinolates and studies where herbivores were able to choose between glucosinolates, plants or populations are summarized by Raybould & Moyes (2001). Herbivores have been shown to respond differentially to glucosinolates with different side-chain structures (Huang & Renwick 1994). Generalist herbivores such as molluscs, tend to prefer to feed on plants with lower concentrations of glucosinolates. On the other hand, herbivores that specialize on crucifers tend to prefer to feed and lay eggs on plants with higher concentrations of glucosinolates. Giamoustaris & Mithen (1995) found that glucosinolate variation in *B. napus* affected amounts of herbivory caused by specialist herbivores such as *Psylloides chrysochephala* and *Pieris rapae*, and generalist herbivores such as pigeons and slugs. Compared with the low glucosinolate lines, the lines with high concentrations of glucosinolates were damaged more by the specialists and less by the generalists.

Among glucosinolates, the indoles have been demonstrated to be powerful stimulants for egg laying (Rosa et al. 1997). The strong effect of indole glucosinolates is attributed to their precursor, the amino acid tryptophan, which is nutritionally essential to insects (Koritsas et al. 1991). In host recognition an advantage of indole-3-ylmethyl glucosinolate (glucobrassicin) could be that indole glucosinolates do not yield volatile

aglycones following enzymatic hydrolysis by myrosinase that could attract natural enemies such as parasitoids or generalist predators that use such signals to locate their prey (Loon et al. 1992). Parasitoids of specialist herbivores have been shown to be attracted by higher glucosinolate concentrations (Raybould & Moyes 2001).

The concentration of indole glucosinolates is known to increase with the presence of jasmonic acid or by wounding due to mechanical damage or herbivory (Bartlet et al. 1999). Jasmonate acid or damage induced changes to the glucosinolate profile may benefit the plant by protecting it from fungal diseases (Doughty et al. 1995a) and generalist herbivores. This increase in total glucosinolates may not increase the susceptibility of the plant to some specialists because their stimulatory effect is offset by other elements of induced defense (Bartlet et al. 1999). This is because only indole glucosinolates, which do not produce volatile metabolites, are induced. Thus, the emission rates of compounds such as isothiocyanates, which are important attractants for crucifer specialists (Bartlet et al. 1997), would not be expected to increase after induction. The presence of ESP proteins in some plants may also allow the plant to change the hydrolysis profile from isothiocyanate to nitrile formation and thereby escape detection by specialist herbivores using isothiocyanate as host-finding cues (Lambrix et al. 2001), see also 4.3.3.

Despite the potential ecological importance of myrosinases, few studies have addressed the role of myrosinase activity in the defense against herbivores. Newman et al. (1992) showed the importance of myrosinase for defense in watercress, which deterred feeding to caddisflies, snails and amphipods. Deactivation of the myrosinase enzyme, and hence isothiocyanate production, by heating resulted in a shift in preference from leaves with a low glucosinolate (but also low nitrogen) content to leaves with high contents of glucosinolates and nitrogen. Addition of myrosinase to the testwater restored the deterrent. In a field experiment, feeding by *Phyllotreta* spp. flea beetles was 10.3% lower on *B. rapa* L. genotypes with high myrosinase activities

than those with low myrosinase activities (Mitchell-Olds et al. 1996). These two studies provide evidence that myrosinase variation influences herbivore feeding, and are in agreement with results obtained in this thesis (Paper II). A recent study concluded that myrosinase activity might be more important for plant defense against specialist insects that have adaptations to intact glucosinolates, but less important for defense against generalists, which are susceptible to the intact glucosinolates (Li et al. 2000). In a toxic experiment that used artificial diets, allyl isothiocyanates, but not allyl glucosinolates, were lethally toxic to the specialist (*Plutella xylostella*), whereas both the isothiocyanate and the glucosinolate were lethally toxic to the generalist (*Spodotera eridania*) (Li et al. 2000). A fundamental concept of chemical ecology assumes that antagonistic chemical interactions between plants and herbivorous insects co-evolve in a stepwise process; an advance in plant defenses exerts selective pressure on the insect's ability to overcome these defenses, and *vice versa* (Berenbaum & Zangerl 1998). Recent investigation has revealed that the crucifer specialist diamondback moth (*P. xylostella*) is capable of disarming the "mustard oil bomb" by its own glucosinolate sulfatase enzyme (Ratzka et al. 2002). The sulfatase prevents formation of toxic hydrolysis products arising from the myrosinae-glucosinolate system. This enzyme was shown to act on all major classes of glucosinolates, thus enabling diamondback moths to use a broad range of cruciferous host plants (Ratzka et al. 2002). Consequently, high concentrations of glucosinolates have no effect on herbivory by *P. xylostella*, while elevated myrosinase activity leads to reduced damage in *Brassica* (Li et al. 2000).

The specialist aphids *Brevicoryne brassicae* (cabbage aphid) and *Lipaphis erysmi* make their own myrosinase to hydrolyze plant-produced glucosinolates (Jones et al. 2001, Jones et al. 2002, Bridges et al. 2002). A potential role for the myrosinase-glucosinolate system in the cruciferous specialists is in generating isothiocyanates, which act as synergists for the alarm pheromone E-β-farnesene (Dawson et al. 1987). E-β-farnesene communicates a warning to other members of a developing colony.

Warning is probably most important in the early stages of plant colonization when a warning signal for predator attack is at its most useful. As with specialist crucifer-feeding insects of plants, these specialist pathogens and predators are probably adapted to the toxic glucosinolate breakdown products (Bartlet et al. 1999). For the polyphagous ladybird beetle, *Adalia bipunctata*, the specialist *B. brassicae*, was found to be more toxic than the generalist aphid *Myzuz persicae* when reared on plants with high glucosinolate concentrations (Francis et al. 2001).

Effects on microorganisms

Naturally occurring isothiocyanates possess a range of antifungal, antibacterial and antimicrobial activities and can thus repel microorganisms and molluscs (Fenwick et al. 1983). Nevertheless, a positive correlation between attacks of *Alternaria spp*. and glucosinolate presence has also been shown (Giamaustaris & Mithen 1997). This suggests that certain pathogens may become adapted, i.e. specialists which are analogous to herbivorous insects.

Allelopathic effects

There is an increasing interest in reducing or replacing pesticide application by use of natural phytotoxic plant residues. In this respect brassicacious plants may have a role to play. A number of cruciferous plants inhibit the growth of competing vegetation, and several glucosinolates and their hydrolysis products have been isolated and demonstrated to possess herbicidal or growth-inhibiting activity while others inhibit seed germination (Seigler 1998, Rosa et al. 1997).

4.4.2 Glucosinolate metabolism in growth regulation

Indole-3-acetic acid (IAA) is the primary plant auxin and regulates numerous biological processes, e.g. cell division, cell elongation, tropisms, cell differentiation,

fruit development, and senescence. IAA is synthesized both *de novo* and by hydrolysis of conjugates (Bartel 1997).

The biosynthetic routes resulting in IAA production and the mechanism securing an optimal IAA concentration at the cellular level are poorly understood, and several biosynthetic pathways have been proposed. Mutant studies have provided some knowledge of IAA and indole metabolism, and have led to a current picture of a metabolic grid consisting of several redundant pathways operating at different developmental stages (Normanly & Bartel 1999). Tryptophan (trp)-dependent as well as trp–independent pathways have been proposed to occur in *Arabidopsis* seedlings (Normanly et al. 1993). In most higher plants trp-dependent biosynthesis of IAA proceeds from two pathways (Bartel 1997). *The indole-3-acetonitrile (IAN) pathway* goes through trp, indole-3-acetaldoxime (IAOx) and IAN to IAA, and *the indole-3-pyruvic (IPA) pathway* goes through trp, IPA and indole-3-acetaldehyde to IAA. However, in species of Brassicaceae, indole glucosinolates can be converted by myrosinases into IAN and further hydrolyzed to IAA by nitrilases (Bartel & Fink 1994). It appears that IAN in all cases is the direct precursor of IAA, and thereby nitrilase is the key enzyme in IAA biosynthesis in Brassicaceae. In *A. thaliana*, increased levels of nitrilase have been reported in roots of sulfur-starving plants (Kutz et al. 2002). Induced nitrilase expression has also been implicated in virulent bacterial infection (Bartel & Fink 1994) and by methyl jasmonate exposure (Geshi, Brandt, unpublished). The involvement of the CYP79B2 and CYP79B3 genes in IAA and indole glucosinolate biosynthesis has been investigated (Hull et al. 2000, Mikkelsen et al. 2000). The presence of an IAOx producing CYP79 in the biosynthesis of indole glucosinolates was not necessarily expected as no tryptophan derived cyanogenic glucoside has been identified. Furthermore, indole glucosinolates are only present in four families in the Capparales order (Brassicaceae, Resedaceae, Tovariaceae, and Capparaceae) and are therefore considered a recent evolutionary event within glucosinolates (Ettlinger & Kjær 1968). The first committed step in the biosynthesis

of indole glucosinolates is the conversion of indole-3-acetaldoxime into an indole-3-S-alkyl-thiohydroximate. The initial step in this conversion is in *Arabidopsis* catalyzed by CYP83B1 (Fig.6) (Bak et al 2001). The knockout mutant of the CYP83B1 gene (*rnt1-1*) shows a strong auxin excess phenotype (Bak et al. 2001). The indole-3-acetaldoxime in excess seems to be channeled into IAA biosynthesis which leads to elevated IAA levels, and thereby increased apical dominance and reduced indole glucosinolate levels. Conversely, overexpression of CYP83B1 in *Arabidopsis* leads to a reduced IAA phenotype and loss of apical dominance and elevated indole glucosinolate levels (Bak et al. 2001). This indicates that increased N-hydroxylation of indole-3-acetaldoxime results in a net loss of IAA. Indole-3-acetaldoxime thus seems to constitute a metabolic branch point in IAA and indole glucosinolate biosynthesis (Fig.6), and the level of IAA may be regulated by the flux of indole-3-acetaldoxime through CYP83B1. The role of IAN in IAA biosynthesis appears to be restricted to glucosinolate producing species because they contain myrosinases that hydrolyze indole glucosinolates to IAA as well as nitrilases that can convert IAN to IAA (Thimann & Mahadevan 1964). Apart from their function as bioactive natural products, indole glucosinolates may thus have a role in IAA biosynthesis as a sink for indole-3-acetaldoximes as well as a source of the IAA precursor IAN by turnover of the indole glucosinolate pool. This indicates that there is a cross-talk between the biosynthetic pathways of indole glucosinolates and IAA at the IAOX branch point.

It is striking that no cyanogenic glucoside is known to be derived from trpytophan. An explanation may be that for a natural product biosynthetic pathway to share an intermediate in the biosynthesis of an essential hormone, a tight and controlled regulation is mandatory. CYP83B1 seems to fulfill these requirements as the closest relative. CYP83A1 with 63 % amino acid sequence identity was shown to not have indole-3-acetaldoximes as its physiological substrate (Bak & Feyereisen 2001). Instead, CYP83A1 seems to catalyze the initial conversion of aldoximes to thiohydroximates in the synthesis of glucosinolates not derived from tryptophan. The

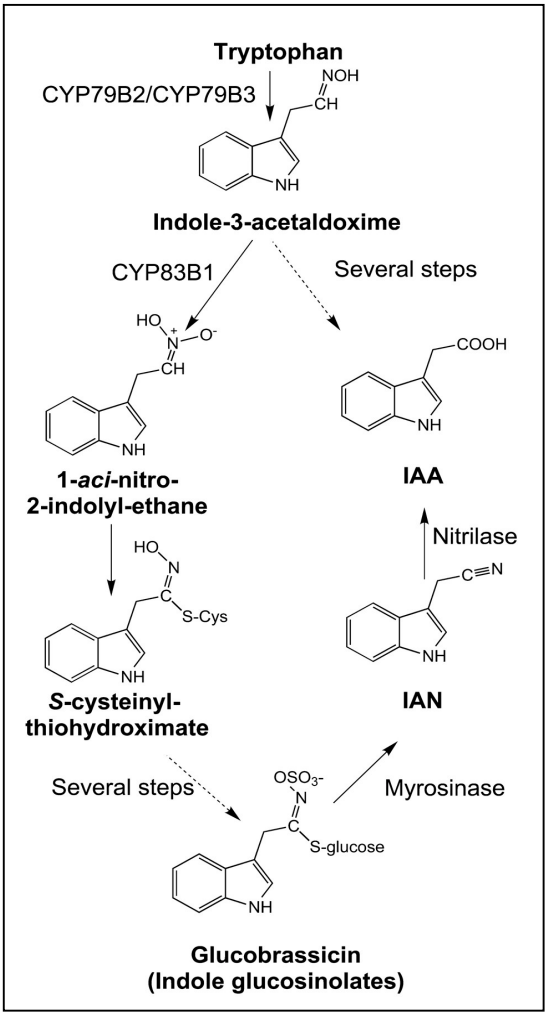

Figure 6. Indole-3-Acetaldoxime is the metabolic branch point between indole glucosinolates and IAA biosynthesis. (From Bak et al. 2001).

use of a separate CYP83 for indole glucosinolate biosynthesis ensures tight control of the flux of the shared intermediate, indole-3-acetaldoxime, for indole glucosinolate and IAA biosynthesis. The presence of putative auxin responsive elements (AuxREs) in the CYP83B1 but not in the CYP83A1 promoter also suggest that they have different functions (Bak & Feyereisen 2001).

4.4.3 Glucosinolates as storage compounds for nutrients

The glucosinolates contain sulfur and nitrogen and can thus be considered as sinks for these nutrients. Modulation of the myrosinase activity by sulfate and micronutrients has been reported (Visvalingam et al. 1998). *B. napus* has an extraordinarily high sulfur demand due to the high amounts of sulfur-containing amino acids and for the biosynthesis of glucosinolates, especially for synthesis of aliphatic glucosinolates originating from the sulfur-containing amino acid methionine (Chew 1988). As a consequence, *B. napus* takes up three times more sulfur than any high-yielding cereal crop (Schnug & Evans 1992). In field experiments Zhao et al. (1994) showed a clear influence of both nitrogen and sulfur supply on individual glucosinolates in *B. napus* seeds. An increase in nitrogen availability favored the hydroxylation step converting gluconapin to progoitrin. Sulfur starvation of young *B. napus* plants caused only a slight reduction in dry weight but resulted in the loss of the normally existing indolyl glucosinolates in cotyledons (Bodnaryk & Palanswamy 1990). *B. napus* cultivars with reduced content of aliphatic glucosinolates in the seed were found to be more sensitive to sulfur deficiency (Schnug 1989). This suggests a role for glucosinolates in survival strategy. In *B. napus* and in *Sinapis alba* decreasing sulfur supply results in a decrease in free sulfate and glucosinolate concentrations and also an increase in myrosinase activity (Schnug 1990, Bones et al. 1994). This latter may induce remobilization of sulfur from glucosinolates and their subsequent decrease. Aliphatic glucosinolates are degraded during germination and the glucosinolate-bound sulfur is released (James & Rossiter 1991). Sulfate seems to regulate the expression of myrosinase in an age-dependent manner but the expression of myrosinases was generally higher in plants with a normal sulfur supply (Bones et al. 1994). Sulfate has also been shown to indirectly regulate myrosinase activity by affecting the concentration of ascorbic acid since the myrosinase isoenzymes show different sensitivity to ascorbic acid (Björkman 1976, James & Rossiter 1991). In addition, sulfate generated from the degradation of the glucosinolate sinigrin was shown to be a competitive inhibitor of myrosinase activity with respect to both sinigrin and ascorbic acid (Botti et al. 1995).

In *B. napus* cultivars a maximum of only 8 % of the total amount of sulfur is bound in glucosinolates (Fieldsend & Milford 1994). This means that glucosinolates can not be the major sulfur long-term storage form during the life cycle. However, the glucosinolates may represent one-half of the sulfur content of the seeds (Josefsson 1970). After germination the expression of myrosinases rises, and this suggests a storage-compound function during development. Furthermore, glucosinolate levels have been found to vary according to photoperiod (Rosa et al. 1994) and environment (Bouchereau et al. 1996) without any difference in pest pressure. Induction of glucosinolates also seems to occur after nitrogen starvation (Blake-Kalff et al. 1998). Taken together, these results may indicate the existence of a highly regulated short-term nutritional storage/balance function involving different myrosinase isoenzymes. The system may thus be helping the plant during nutritional stress conditions.

4.4.4 Glucosinolates as anticarcinogenic compounds

There is now considerably evidence that human consumption of *Brassica* vegetables might reduce the risk of developing cancer due to their relatively high glucosinolate content (for review see Verhoeven et al. 1997, Talalay & Fahey 2001). Both isothiocyanate (the largest group of hydrolysis products) and thiocyanates can modify carcinogenesis through the induction of phase II detoxification enzymes such as NADPH-quinone reductase, glutathione-S-transferase or epoxide hydrolases. These enzymes metabolize potentially carcinogenic substances to more polar forms and thereby reduce their deleterious activities. In extracts from broccoli, sulforaphane in particular, the isothiocyanate of 4-ethylsulfinylbutyl-glucosinolate, has been shown to be a very potent inducer of detoxification enzymes (Zhang et al. 1992, Fahey et al. 1997). As potential protectors influencing human health, the World Health Organization (WHO) recommended 200 g of fruit and vegetables a day, including at least one Brassicaceae product (Plumb et al. 1996). In contrast to their presumed protective action some isothiocyanates show mutagenic potential in mammalian cells

and bacteria (Verhoeven et al. 1997). In addition, the thiocyanate ion shares the same binding site as iodine in the thyroid gland. High concentration of thiocyanate may therefore compete with the iodine binding, and a combination of low iodine intake and an excessive consumption of *Brassica* vegetables may contribute to the development of goiter (Mawson et al. 1994). Nevertheless, Mithen (2001) concluded that there is no evidence for any goitrogenic effect of *Brassica* consumption in humans. This was based on a study of adult volunteers who had included 150 g brussel sprouts in the diet without any effect on thyroid hormones (McMillan et al. 1986). Based on the demonstrated anti-carcinogenic properties of some of the hydrolysis products, these compounds are regarded as good candidates for producing "functional foods" designed for preventing cancer (Halkier 1999).

4.4.5 Glucosinolates in animals diet

Brassica vegetables can contribute indirectly to the human food chain by their extensive use as an animal feed. The seed meal remaining after oil extraction is used for its nutritional value, and is regarded as a rich protein source due to its high content of several important amino acids. Despite this, the presence of glucosinolates in the meal limits its use as an animal feed due to a number of anti-nutritional and negative physiological effects, such as liver, kidney and thyroid damage (Tookey et al. 1980, Fenwick et al. 1983). The extent to which thyroid function is impaired by glucosinolates is clearly related to species, intake, duration of feeding, and the nature of the compound (Rosa et al. 1997). In general, rapeseed glucosinolates do not affect thyroid function in ruminants due possibly to microbial breakdown of potential goitrogens in the gut. Today the use of "double low" varieties of oilseed rape that contain no erucic acid (has a negative effect on lipid metabolism) and low levels of aliphatic glucosinolates has, at least partly, diminished the problem.

4.4.6 Glucosinolates as condiment

Sulfur is associated with odor, and sulfur compounds contribute to the agreeable as well as the disagreeable flavors of many foods. Included among such compounds are the odoriferous breakdown products (isothiocyanates, thiocyanates and nitriles) of glucosinolates (flavorless sulfur precursor). The distinctive flavor of members of the Brassicaceae such as *Brassica nigra* (black mustard), *Armoracia lapathifolia* (horseradish) and *Brassica oleracea* (cauliflower, broccoli, cabbage, Brussels sprouts), is dependent on the hydrolysis of glucosinolates and on the nature and amounts of the products thus released (Fenwick et al. 1983). Isothiocyantes are of considerable flavor importance (MacLeod 1976) and descriptions such as pungent, lachrymatory, acrid, garlic-like, horseradish-like, and bitter have been associated with these compounds (Fenwick et al. 1983). The identification of glucosinolate-derived flavor is however quite complex because of the number of glucosinolates involved and the additional volatile compounds that does not arise from glucosinolate catabolism (Rosa et al. 1997).

5. AIM OF THE STUDY

The role of the myrosinase-glucosinolate system in defense-related reactions in plants of the Cruciferae is widely accepted (Bones & Rossiter 1996, Rask et al. 2000). A continuous turnover of glucosinolates during development of healthy intact plants has however also been reported (Cole 1978, 1980, Svanem et al. 1997, Petersen at al. 2002), suggesting that the products produced by hydrolysis of the glucosinolates may be important in normal growth and development of the plant. A goal in this study has been to examine the effect of removing myrosinase activity and thereby prevention of the hydrolysis of glucosinolates. Investigation of biochemical alterations in the seeds where myrosin cells had been ablated, and of morphological and biological alterations in developing plants grown from such seeds was desirable.

6. SUMMARY OF RESULTS AND DISCUSSION

The myrosinase-glucosinolate system is a complex system found primarily in species of the order Capparales. The main components of the system, myrosinase and glucosinolate have been known for more than a century (Bussy 1840). This system and its components have been pre-eminent subjects of chemical investigation of species within the Capparales. Degradation of glucosinolates produces products that affect the value of glucosinolate-containing plants when used as food for humans or for feeding animals (Chew 1988). Throughout the years of scientific investigation additional factors influencing the system have been revealed and the complexity increased. This indicates an important role of the myrosinase-glucosinolate system in the life of the cruciferous plants. The system shows potential for diverse functions. The obvious function is defense due to the toxic nature of the product formed by hydrolysis. These products have been shown to affect insects and microorganisms (Rosa et al. 1997, Rask et al. 2000). The glucosinolates, containing nitrogen and sulfur, may also be a sink for these nutrients, possibly under stress conditions. In addition, recent research has revealed indole glucosinolates to have a possible role in the plant's IAA regulation (Bak et al. 2001). The large number of different glucosinolates and isoenzymes reported further argues that specific hydrolytic products are needed for certain situations or developmental stages.

6.1 CONTROLLED CELL DEATH (ABLATION) OF MYROSIN CELLS AFFECT GROWTH AND DEVELOPMENT OF THE MINELESS PLANT (Paper I)

Genetic ablation of myrosin cells and myrosinase proteins in the seed was achieved by expression of a cell specific, cytotoxic construct encoded by the RNase barnase from *Bacillus amyloliquefaciens*. For development of viable seeds, coexpression of the barnase inhibitor barstar driven by the 35S promoter was necessary. *MINELESS* seeds were negative in myrosinase assays, showed no expression of myrosinase bands in western analysis, and did not produce isothiocyanates as confirmed by GC and GC-MS analysis. Standard light and electron microscopy as well as immunocytochemical LM-analysis with monoclonal antibodies and immunogold-EM analysis using polyclonal antibodies all showed that myrosin cells were targeted and ablated. No evidence for ablation of other cells than myrosin cells was observed.

The phenotype of grown *MINELESS* plants was altered (Paper I, Fig. 2). Major differences from wild type plants were a bushy appearance, elongated carpels, abnormal siliques with outgrowth, and a reduced and delayed seed production. The morphological phenotype of *MINELESS* plants indicated that other parts than the seed was affected by the barnase expression. By the use of transgenic plants carrying constructs containing the *Myr1.Bn1* promoter fused to the reporter gene GUS, we were able to show a positive GUS expression in the flower stalk and in developing siliques. The MyrGus expression was observed in the typical myrosin cells, phloem parenchyma cells, and in a fraction of guard cells (Paper I, Fig. 8). Stained cells were also frequently observed at branch points. The observed expression pattern of Myr1.Bn1:GUS and the observed phenotypic abnormalities in the *MINELESS* plants strongly suggest a link between the organs affected and the expression pattern of myrosinase.

It has been suggested that rapid metabolism of glucosinolates is needed to sustain plant growth (Clossais-Besnard 1991). Also, during the reproductive growth period and especially during flowering, accumulated glucosinolates from the vegetative growth period seem to be catabolized (Clossais-Besnard 1991). Considering the large number of different glucosinolates and isoenzymes reported, it may be that *Myr1.Bn1* has a role in the control of growth and correct development of the plant. The expression is high in seeds and at the start of germination. There is now also indication for expression at the stage of flowering and seed maturation. Important and critical stages in the life of the plant are thereby associated with *Myr1.Bn1* expression.

Reports over the last years have also started to link genetic modifications of endogen glucosinolate profiles to growth and to morphological alterations of plants (Mikkelsen at al. 2000, Reintanz et al. 2001, Bak et al. 2001, Kutz et al. 2002). We did not find any changes in the glucosinolate profiles of *MINELESS* seeds. But as it is the hydrolysis products and not the glucosinolates themselves that are biologically active, a termination of glucosinolate hydrolysis by a lack of myrosinase expression should be expected to influence the plant's morphology. We did not investigate the glucosinolate profile of grown *MINELESS* plants in this study. An induced biosynthesis of indole glucosinolates has been associated with different kind of stress (Doughty et al. 1991, Bodnaryk 1992, Bodnaryk 1994, Doughty et al. 1995, Bartlet et al. 1999, Li et al. 1999a, Møllers et al. 1999, Hull et al. 2000), and this might also be expected in grown *MINELESS* plants. The ablation of specific cells in the maturing embryo will most probably induce several stress signals in the plant.

The expression of the MA family has been regarded as seed specific (Lenman et al. 1993), but our findings of MA expression in other parts of the plant show that re-evaluation is required. The morphological alterations found in the *MINELESS* plants are in agreement with the expression pattern directed by the *Myr1.Bn1* promoter. This may be consistent with a role for Myr1.Bn1 in the development of new organs and/or

in regulation of growth. We cannot completely rule out that unknown processes affected by the genetic modification of the *B. napus* plant cause the observed effects. However, the fact that we obtain identical phenotypes in *B. napus* and *A. thaliana* make it more likely that the effects observed are due to the ablation of myrosin idioblast cells.

6.2 ABLATION OF MYROSIN CELLS AND ITS EFFECT ON PROTEINS INTERACTING WITH MYROSINASE (Paper I)

6.2.1 Myrosinase binding proteins (MBPs)

The *MINELESS* seeds did not show any significant variation in the expression of the MBPs compared to wild type seeds. The variation among single seeds was however considerable, with the greatest variation in the expression of the 110 kDa and 52 kDa MBP. This variation could not be correlated to differences in the myrosinase activity. The 52 kDa MBP was seen in all seeds examined, while the 110 kDa MBP was only faintly expressed in some seeds, both wild type and *MINELESS*. So far, there is no evidence for the existence of myrosinase-MBP complexes in intact cells of the seed embryo (Eriksson et al. 2002). This is in accordance with the results obtained here.

6.2.1 Epithiospecifier protein (ESP)

So far two classes of ESP polypeptides with molecular masses of 39 and 35 kDa have been purified and characterized in *B. napus* (Fo et al. 2000, Bernardi et al. 2000). The expression of the 39 kDa was affected by the MyrBarnBar construct. In seeds with no or very low myrosinase activity, the 39 kDa band was missing on western blots while the 35 kDa band was still present. This indicates a co-localization in the seed for

myrosinase and the 39 kDa ESP. The 35 kDa ESP must be localized in other cells than myrosin cells and this may further indicate different roles in the myrosinase-glucosinolate system for the two isoforms of ESP. The amount of hydrolysis products formed in *MINELESS* seeds was lower than in wild type even with added myrosinase, probably due to a lower total ESP expression (see 6.3 and Paper I).

6.3 GLUCOSINOLATE PROFILE AND HYDROLYSIS PROFILE IN MINELESS SEEDS (Paper I)

Ablation of myrosin cells did not affect the glucosinolate profile in *MINELESS* seeds severely (Figure 6, Paper I). This observation supports the recent indications that myrosin cells are free of glucosinolates (Kelly et al. 1998) and that myrosinases and glucosinolates are compartmentalized in different cells (Koroleva et al. 2000, Husebye et al. 2002).

As expected, no isothiocyanates were formed in extracts from *MINELESS* seeds with no myrosinase activity (Figure 7A, Paper I). Even so, trace amounts of epithionitriles and simple nitriles were detected, indicating that the seeds were not absolutely myrosinase-free. The assay used favors epithionitrile formation. Adding myrosinase to extracts of *MINELESS* seeds restored the glucosinolate hydrolysis activity and produced identical hydrolytic products as the wild type, but the distribution of the products was altered (Fig. 7B, Paper I). The reduction in the expression of total ESP in *MINELESS* seed may have led to reduced ESP affection on the unstable thiohydroximate-*O*-sulphonate intermediate and thereby a reduction in epithionitrile formation supplemented with myrosinase. In spite of that, a higher increase in nitrile formation should be expected in extracts of *MINELESS* seeds supplemented with myrosinase. However, the relatively small proportion of epithionitrile production in

MINELESS seeds with added myrosinase could be due to excessive quantities of myrosinase, and further experiments are needed to determine the optimum amount of myrosinase for the ESP/myrosinase assay. As *Crambe abyssinica* is known to only have the 35 kDa ESP (Foo et al., 2000), one might expect that normal expression of the 35 kDa ESP isoform would have been enough to compensate for production of a normal amount of epithionitriles and simple nitriles with added myrosinase.

The nature of the degradation products of the alkenyl glucosinolates is most likely regulated by ESP. ESP might regulate the bioactive profile of the Brassicaceae plant, and is unique inasmuch as it has no activity towards the initial substrate but only against the unstable intermediate. Lambrix et al. (2001) have reported the *Arabidopsis* ESP to be a more general nitrile-specifying protein giving rise to both epithionitriles and simple nitriles (see 4.3.1). Although highly speculative, one possibility could be that the 39 kDa ESP is involved in epithionitrile formation (from alkenyl glucosinolates) while the 35 kDa ESP is involved in nitrile formation (from non-alkenyl glucosinolates). Lambrix concluded that epithionitrile formation was not dependent on the presence of ferrous ions. But recent investigation has confirmed the role of Fe^{2+} in both epithionitrile and simple nitrile formation (Zabala, Grant, Bones, Bennett, Rossiter, unpublished). They have demonstrated that the formation of the thiirane ring had an absolute requirement for Fe^{2+}, ESP and myrosinase *in vitro*. *In vitro* nitrile formation required also Fe^{2+} and myrosinase but not ESP. In spite of that, a higher increase in nitrile formation should be expected in extract of *MINELESS* seeds with added myrosinase. Clearly, more information on the role of ESP in glucosinolate hydrolysis is required.

6.4 MINELESS SEEDLINGS ARE MORE SUSCEPTIBLE TO APHID ATTACK (Paper II)

Modulating the possibility for glucosinolate hydrolysis in the plant affects the emission of volatile compounds released, and reduced emission of compounds such as isothiocyanates will most probably affect specialist and generalist insects differently. Seedlings of wild type and *MINELESS* were infested with the aphid species *Brevicoryne brassica* (specialist) and *Myzus persicae* (generalist). For the Brassica specialist only 31 % of the aphids were established on *MINELESS* seedlings after six days, while 66 % of the generalist aphids were found on *MINELESS* seedlings. Considering that the glucosinolate profiles were the same for Westar and *MINELESS* (6.3), the major difference between these seedling types is the amount of formed hydrolysis products by the difference in the activity of the myrosinase enzymes. This supports the theory that variation in myrosinase activity is important in the defense against herbivores (Mitchell-Olds et al. 1996, Newman et al. 1992). Using seedlings with increased differences in the myrosinase activity between wild type and *MINELESS* (design 2) further lowered the preference of the specialist for *MINELESS* seedlings. This indicates that volatiles like isothiocyanates are important for the specialist aphid in stimulating feeding. The generalist aphid did not seem affected by this increase in difference. The *MINELESS* seedlings were further found to be more susceptible for aphid infestation in general, compared to the wild type, regardless of aphid species. This is consistent with the defense-related role of the myrosinase-glucosinolate system. Plants with a weakened defense system, incapable of releasing toxic compounds, will most probably be more susceptible to attack.

Seedlings infested by aphids increased their myrosinase activity. For the generalist many aphids on a seedling increased the activity more than few aphids. For the specialist, few aphids increased the activity in the seedling while many aphids maintained the same activity as in control seedlings (Table 2, Paper II). How this

arises is an open question but it might be a strategy used by the specialist aphid, as isothiocyantaes have also been demonstrated to be toxic to specialist insects (Li et al. 2000). This is further discussed in paper II.

The height of the seedlings at the end of the experiments was found to be affected by how many aphids they had been infested with (Fig. 6, Paper II). The height was also slightly more affected by the generalist. *M. persicae* has been reported to spend more time in xylem ingestion probably as a kind of detoxification mechanism (Cole, 1997). This may have affected the water transport in the seedlings more severely than feeding by *B. brassicae*. Cell expansion rates seam mainly to be associated with how much water is absorbed regardless of the type of environmental stress condition (Ikeda et al. 1999).

6.5 THE MYROSINASE-GLUCOSINOLATE SYSTEM IS COMPLEX AND MOST LIKELY HAS SEVERAL FUNCTIONS IN THE PLANT

Plants produce a vast array of natural products, often referred to as secondary metabolites, to accommodate their biotic and abiotic environments. These natural products are produced at a high expense of energy, with natural selection as a driving force. The myrosinase-glucosinolate system represents an intricate example of secondary metabolism in plants. The system consists of more than 120 different substrates and several enzyme isoforms, and a complex network for regulating the expression of the different components must exist. The system has so far mainly been implicated in plant defense. Constitutive defenses, like the myrosinase-glucosinolate system, are considered as more expensive than induced defenses where the cost of synthesis, storage and maintenance only occurs when the plant is under attack (Karban & Baldwin 1997). As several of its components are changed in response to stress

(Bones & Rossiter 1996, Rosa et al. 1997, Rask et al. 2000), the system can also be considered as partly inducible. Recently, costs of resistance have been demonstrated for plants containing the myrosinase-glucosinolate system (Mauricio & Rausher 1997, Siemens & Mitchell-Olds 1998). Plant genotypes with high constitutive levels of myrosinase were associated with significant decreases in seed production when compared to genotypes with low levels of myrosinase, which showed no induced responses that could repel herbivores (Siemens & Mitchell-Olds 1998). In field populations there was selection for higher levels of glucosinolate concentration in populations of *A. thaliana* exposed to herbivory than in populations protected from herbivory, and there was also a negative correlation between seed production and glucosinolate level (Mauricio & Rausher 1997, Mauricio 1998). But due to the possible additional functions such as nitrogen and sulfur storage and attraction of pollinators and specialized insects predators (Gershenzon 1994), it is difficult to measure the exact cost of producing glucosinolates. Most probably the net cost is lowered because of additional functions of the system. Evidence now continues to accumulate to support the role of the myrosinase-glucosinolate system in multiple functions. Double-low oilseed rape plants seem to be more sensitive to sulfur deficiency than single-low plants (Schnug 1989). Reports over the last years have also started to link genetic modifications of endogen glucosinolate profiles to growth and morphological alterations of plants (Mikkelsen et al. 2000, Reintanz et al. 2001, Bak et al. 2001, Kutz et al. 2002). The results reported in this thesis further support a role for the myrosinase-glucosinolate system in multiple functions.

An overview of some of the functions and biological implications of the myrosinase-glucosinolate system is outlined in fig 7. The expression and metabolism of the components of the system is highly affected by different stimuli such as herbivory, nutrient supply, expression of plant hormones and photobiology.

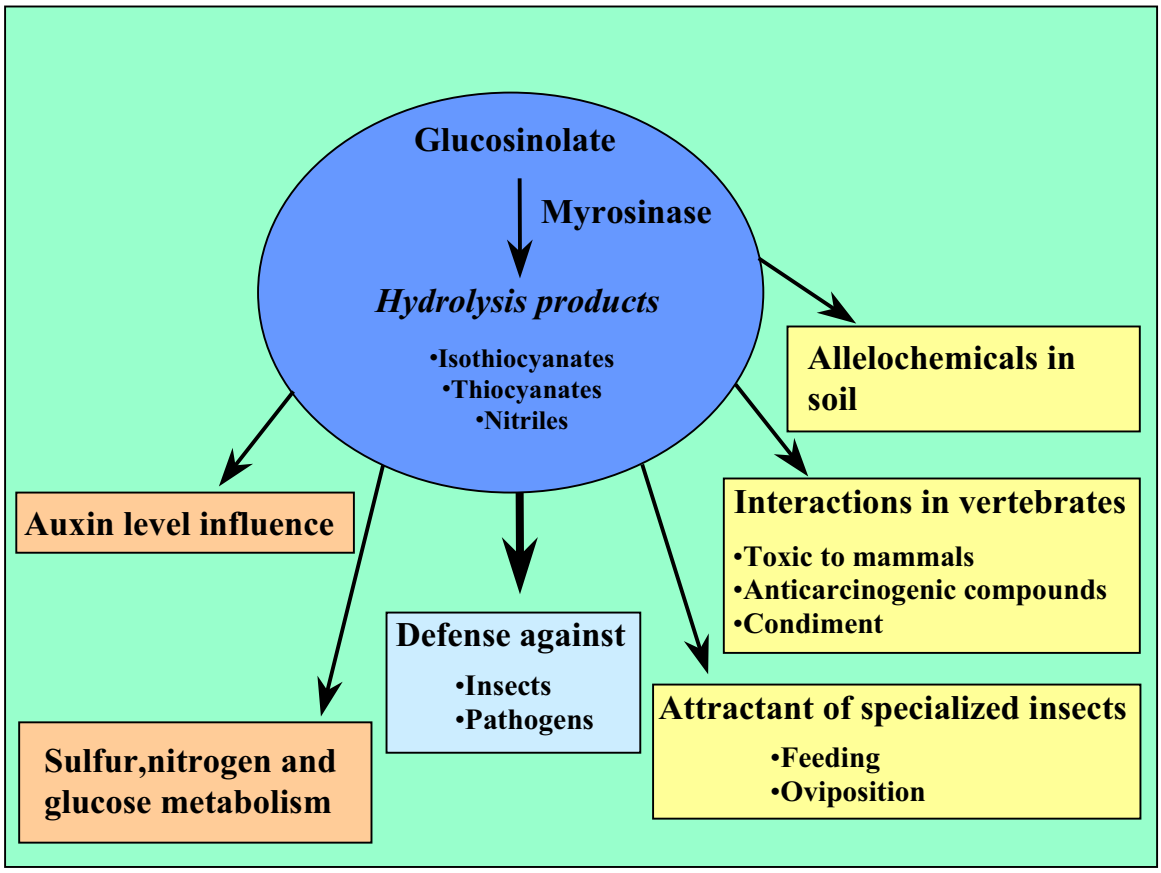

Figure 7. Functional and biological implications of the myrosinase-glucosinolate system.

7. FUTURE PERSPECTIVES

The GUS expression found in flower stems and developing siliques as directed by the *Myr1.Bn1* promoter, reveals new action for the MA family outside the seed. A more thorough study of the *Myr1.Bn1* expression in different tissues and at different developmental stages should be completed. The existence of ablated myrosin cells in flower stems and siliques should also be proven for the *MINELESS* plants.

One of the ESP isoforms was missing in *MINELESS* seeds with ablated myrosin cells. Both cellular and subcellular localization of different ESP isoforms should be examined. ESP might play a key role in modifying the glucosinolate hydrolysis profile. A systematic study of ESP activity in different organs at different developmental stages and with different kind of stress factors would be advantageous.

The *MINELESS* construct seems to work well in *A. thaliana* giving the same phenotypic changes as in *B. napus*. Regarding the much shorter life cycle and its known genome sequence, further studies of growth and development in correlation with myrosinase expression might be more fruitful in *A. thaliana*.

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Paper I

MINELESS Plants Reveal Multiple Roles for Myrosin Cell Idioblasts in Defense, Development and Growth.

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ABSTRACT

Myrosinases are hydrolytic enzymes involved in degradation of glucosinolates thereby producing an array of degradation products with toxic effects. Myrosinases of *Brassica napus* seeds are localized in myrosin cell idioblasts. We have used the *Myr1.Bn1* promoter to express the cytotoxic RNase barnase in myrosin cells thereby introducing controlled cell death of myrosin cell idioblasts. Controlled cell death (ablation) of myrosin cells produce *MINELESS* seeds – seeds without myrosin cell idioblasts. *MINELESS* seeds produced plants characterized by increased branching (bushy phenotype), early emerging and elongated carpels and outgrowth of secondary siliques. Seed maturation was delayed and seed production reduced. The tissues affected co-incidence with the expression pattern directed by the myrosinase gene promoter used. Parallel experiments with *Arabidopsis thaliana* produced similar results. Myrosinase is one of several enzymes in a complex enzyme system that also includes myrosinase-binding proteins, myrosinase-associated proteins and epithiospecifier proteins (ESP). One of the isoforms of ESP (39 kDa) is lost by the ablation of myrosin cells indicating a cellular co-localization with myrosinase in myrosin cells. Myrosinase binding proteins are not reduced by myrosin cell ablation supporting localization to other cells in the seed. The glucosinolate substrates are mainly unaffected by myrosin cell ablation supporting localization in non-myrosin cells and a cellular separation of enzyme and substrate. GC and GC-MS analysis of glucosinolate hydrolysis products clearly show that ablation of myrosin cells are associated with a loss of ability to produce isothiocyanates. This activity can be restored by external addition of myrosinase, supporting that the majority of glucosinolates are present in other cells than myrosin cells. Taken together, these results and the phenotype associated with the *MINELESS* plants strongly indicate that the myrosinase-glucosinolate system has a role not only in defense but also in regulation of growth and development.

INTRODUCTION

The complex myrosinase-glucosinolate system of Brassicacaea plants consists of more than 25 myrosinase encoding genes, a large number of myrosinase binding (MBPs) and myrosinase associated proteins (MyAPs) and co-factors like epithiospecifier proteins (ESPs) and ascorbic acid and a total of more than 100 different glucosinolate substrates. Most myrosinases occurs in protein complexes were the other constituents (MBPs, MyAPs, ESPs, Fe) some of which appear to control the products formed during hydrolysis. The role of the myrosinase-glucosinolate system in defense-related reactions in plants of the Brassicaceae is widely accepted (Bones and Rossiter, 1996; Rask et al., 2000). The toxic nature of the hydrolysis products together with "the mustard oil bomb" model from Lüthy and Matile (1984) and "the toxic mine" model from Bones and Rossiter (1996), has pointed out plant defense as a major biological function for this system. In both models, disruption of cell membranes or active transport is necessary to activate the system. Glucosinolate breakdown products have been proposed to act as allelochemicals and to play a role in plant defense (Bones and Rossiter, 1996; Rask et al., 2000). Naturally occurring isothiocyanates have been shown to possess a range of antifungal, antibacterial and antimicrobial activities, and thereby be capable of repelling microorganisms, insects and mollusks (Fenwick et al., 1983; Sarwar et al., 1998). Glucosinolates and their breakdown products are also clearly involved in host-plant recognition by specialized pests. Some isothiocyanates attract pests of Brassicaceae, which use these volatile compounds as feeding and oviposition stimuli (Fenwick et al., 1983; Rask et al., 2000). The aphids *Brevicoryne brassicae* and *Lipaphis erysmi* make their own myrosinase to hydrolyze plant produced glucosinolates (Jones et al., 2001; Bridges et al., 2002).

Turnover of glucosinolates during development of healthy intact plants as reported by Cole (1978, 1980), Svanem et al. (1997) Thangstad et al. (2001) and Petersen et al. (2002) can,

however, not be explained by existing models. Glucosinolates and the hydrolyzing enzyme, myrosinase, seem to be localized in different but neighboring cells. Kelly et al. (1998) reported the glucosinolate, sinigrin, to be localized in all cells of *Brassica juncea* seeds except cells with myrosinase. Koroleva et al. (2000) showed that distinct phloem cells of Arabidopsis flower stalks, denoted S-cells (sulfur-rich cells), contained extraordinary high concentrations of glucosinolates (> 100 mM). These S-cells have been shown to be neighbors to scattered idioblast cells containing myrosinase and named myrosin phloem cells (Husebye et al., 2002). Localization of glucosinolates and myrosinase in different cells and metabolism of glucosinolates in intact plants require a transport system for enzymes and/or substrates. Brudenell et al., (1999) have shown that both intact glucosinolates and desulphoglucosinolates possess the physio-chemical properties allowing phloem-mediated transport. Strong evidence for a glucosinolate/desulphoglucosinolate specific transport system has recently been provided (Thangstad et al., 2001). Using cryo-sectioning, microautoradiography and pulse-chase feeding of tritium-labeled desulphoglucosinolate to *B. napus* siliques, radiolabel was localized to specific cells of seeds resembling the pattern of myrosin cells (Thangstad et al., 2001).

The hydrolyzing enzyme myrosinases (β-thioglucoside glucohydrolase, EC 3.2.3.1) are glycosylated dimeric proteins with subunits of molecular masses in the range of 62 to 75 kDa (Bones and Rossiter 1996). The structure of *Sinapis alba* myrosinase has been determined by X-ray crystallography and the enzymatic mechanism of hydrolysis elucidated (Burmeister et al., 1997). Isoenzymes have been purified and characterized and shown to have varying degree of glycosylation, varying degree of ascorbic acid activation, and varying hydrolysis rates on different glucosinolates (Bones and Rossiter, 1996). Immunocytochemical localization (Thangstad et al., 1990; Bones et al., 1991; Höglund et al., 1992; Geshi and Brandt, 1998; Kelly et al., 1998; Husebye et al., 2002), and *in-situ* hybridization (Xue et al., 1993; Lenmann et al., 1993; Xue et al., 1995; Thangstad, Gilde, Chadchawan, Seem, Husebye, Bradley, and

Bones (submitted)) have localized the myrosinase enzymes and their transcripts to specific cells denoted myrosin cells (Guignard, 1890). Myrosin cells are dispersed throughout the tissues and appear as parenchymatic cells, phloem idioblasts, guard cells and ground tissue cells (Bones and Iversen 1985; Andreasson et al., 2001). A large family of myrosinase genes are present in *B. napus*, consisting of at least 25 different genes (Xue et al., 1992; Falk et al., 1992; Thangstad et al., 1993; Beisvåg, Winge and Bones, unpublished). These can be divided into three subfamilies; *MA, MB* and *MC* (Xue et al., 1992; Falk et al., 1995). Thangstad et al. (1993) following the recommendations of The International Society for Plant Molecular Biology (1993) named the two characterized functional nuclear myrosinase genes of *B. napus Myr1.Bn1* and *Myr2.Bn1*. *Myr1* and *Myr2* equals the MA and MB subfamilies, respectively.

Myrosinases of the subfamilies MB and MC are found in complexes together with myrosinase binding proteins (MBPs). MBPs belong to a large family of proteins ranging from 30 to 110 kDa (Falk et al. 1995; Taipalensuu et al., 1997b; Geshi and Brandt, 1998). The function of the MBPs has not been identified, but due to their lectin activity MBPs have been suggested to bind to carbohydrates present in the insect gut or on the surface of fungi (Rask et al., 2000). A second group of proteins shown to interact with the myrosinase-glucosinolate system is the epithiospecifier protein (ESP). In the presence of ESP and myrosinase epithionitriles are produced. A mechanism for generation of epithionitriles with mechanistic similarities to cytochrome P450 mediated iron dependent oxidation has been suggested (Foo et al., 2000). Two isoforms of ESP polypeptides with molecular masses of 39 and 35 kDa have so far been purified and characterized in *B. napus* (Foo et al., 2000; Bernardi et al., 2000).

Controlled cell death ("genetic ablation") can be achieved by directing expression of a toxic gene product from a tissue-specific promoter. As the Myr1.Bn1 promoter specifically directs expression to myrosin cells, the myrosinase-glucosinolate system is well suited for ablation studies. An advantage using the cytotoxic RNase barnase from *Bacillus*

amyloiquefaciens is the possibility to modulate barnase activity by co-expression of the barnase inhibitor, barstar. Barstar inhibits barnase activity by forming a very stable one-to-one complex (Hartley, 1989).

Indole-glucosinolates in species of Brassicaceae can be converted by myrosinase into indole-3-acetonitrile (IAN), and then hydrolyzed to indole-3-acetic acid (IAA) by nitrilase (Bartel et al., 1994). Further studies have shown that CYP79B2 and CYP79B3 gene products are involved both in the biosynthesis of indole glucosinolates and IAA in *A. thaliana* (Hull et al., 2000; Mikkelsen et al., 2000). Indole-3-acetaldoxime seems to be a metabolic branchpoint between indole glucosinolates and IAA biosynthesis. The reported turnover of the indole glucosinolate pool to IAA trough IAN indicate that there is cross-talk between the biosynthetic pathways of indole glucosinolates and IAA (Bak et al., 2001). The reports of the nutritionally modulation of myrosinase expression (Bones et al., 1994; Visvalingam et al., 1998; Blake-Kalff et al., 1998), downregulation of myrosinase expression by salicylic acid treatment (Taipalensuu et al., 1997a), and induction of myrosinase activity by blue light (Hasegawa et al., 2000) and methyl-jasmonate (Taipalensuu et al., 1997a), further support roles of the myrosinase-glucosinolate system in other mechanisms than defense.

The present study is part of an analysis of idioblasts and their function in plants. We show that the *Myr1.Bn1* promoter can be used for myrosin cell idioblast specific expression. Using the *Myr1.Bn1* promoter to express the cytotoxic RNase barnase we were able to specifically control cell death of myrosin cells. The activity of myrosinase in myrosin cell ablated seeds was completely lost. Ablation of myrosin cells also provide a tool for analysis of the localization of myrosinase associated proteins like epithiospecifier protein and myrosinase binding proteins. The glucosinolate profile and concentration in seeds with ablated myrosin cells addresses whether substrate and enzyme is physically separated into different cells or present in different compartments of the same cell. Observations of transgenic plants grown

from the *MINELESS* seeds indicated that the myrosin cell ablation affected branching of the plant and induce development of secondary siliques. We therefore set out to analyze the expression pattern of the Myr1.Bn1 in detail. Transgenic plants expressing GUS under control of the Myr1.Bn1 promoter show that the Myr1.Bn1 promoter directs expression to myrosin cell idioblasts in seed, stems, flower stalk and siliques. We propose a model were myrosin cell idioblasts not only are toxic mines distributed in the tissues, but also are involved in control of development and growth of the plant.

RESULTS

Controlled cell death of myrosin cells

An expected outcome of expressing barnase under control of the myrosinase promoter is the controlled cell death of myrosin cells in the developing seed. This was obtained using the promoter constructs Myr1:Barnase (*MyrBarn*) and Myr1:Barnase in combination with 35S:Barstar *(MyrBarnBar=MINELESS*). The *MyrBarn* transgenic lines did not produce viable seeds. Two clones were further evaluated, *MyrBarn1* had a strongly altered phenotype with a much-reduced size of the plant. Flowering started when the plant was about the size of an Arabidopsis, but no seed was produced and embryos were terminated in the silique at an early stage. *MyrBarn2* had no altered phenotype and it flowered normally, but very few seeds were produced. The vast majority of the embryos were terminated in the silique at an early stage. Embryos that were dissected out and examined had necrosis and altered morphology (data not shown). Necrosis could be seen as localized spots likely identifying ablated myrosin cells and surrounding cells. Three clones with the construct *MyrBarnBar* had close to normal phenotype, flowering was normal and viable seeds were produced. The clone *MyrBarnBar1* was chosen for further characterization because of the higher amount of seeds showing no myrosinase activity. Models of the gene constructs used in this study are presented in Figure 1. We named these plants expressing the combined barnase and barstar construct *MINELESS* because the toxic mines (myrosin cells) was ablated.

Altered phenotype for the F2 generation of *MINELESS* plants

Twenty-five seedlings with one cotyledon showing myrosinase activity in the range of 0-10 nmol·min^{-1}·mg^{-1}were chosen for propagation and analysis. Flowering started at the same time or a few days earlier than wild type plants (*Brassica napus* cv. Westar). Although smaller, *MINELESS* plants needed more time than the wild type for seed development, taking six to eight months from sowing to harvesting of technical mature seeds. For Westar plants, four months from seed to seed was normal. Many carpels were already visible at a stage at which floral buds were still closed or just beginning to open (Figure 2E). Developing siliques were variable in size and seed content and about half of the developed siliques did not contain normal seeds. Some of these siliques were completely empty. In others maturing embryos had terminated during seed development and was dry and shrunken. And in some apparently mature seeds had started to grow within the silique. Some plants (36%) had a small percentage of abnormal siliques with three chambers instead of the normal two (Figure 2H). During maturation secondary new siliques were growing out of these abnormal siliques but no seeds were formed (Figure 2C). Reduced internodal length was frequently observed and these plants also had higher level of branching making them more bushy than normal. Characteristic features of the transgenic plants of the F2 generation are shown in Figure 2.

As a validation, transgenic plants of *A. thaliana* expressing the same Myr1:Barnase and 35S:Barstar construct (*AtMyrBarnBar=AtMINELESS*), was produced. A comparison of carpels of wild type (Figure 2F) and transgenic (Figure 2G) *A. thaliana* show similar phenotypic changes as in *B. napus* with an extended early approaching carpel. As for transgenic *B. napus* plants a bushy appearance with reduced internodal length, abnormal siliques and a reduced seed production was observed

Controlled cell death of myrosin cells, structural analysis

Due to the variable penetrance of different phenotypes in transgenic plants, myrosinase content of the transgenic seeds varied considerably and selection of seeds showing no myrosinase activity was necessary. Eight percent of the transgenic seeds had no detectable myrosinase activity. For practical reasons, seeds showing no myrosinase activity in the fast God-Perid method, were selected as appropriate seeds for examination and analysis in light (LM) and transmission electron microscopy (TEM). Targeted cells clearly appear empty on light microscopic sections stained with toluidine blue (Figure 3A and B). In seed tissues with no detectable myrosinase activity no normal myrosin cell could be observed, while tissues with normal myrosinase activity contained normal amounts of myrosin cells (see e.g, Bones et al., 1991). The empty, ablated cells seem to have the same distribution as normal myrosin cells in *Brassicaceae* (Bones and Iversen, 1985; Bones et al., 1991; Höglund et al., 1991). The structure of myrosin cells in wild type *B. napus* and the ablated/empty cell structures of *MINELESS* were further analyzed by TEM. Myrosin cells of *B. napus* appear with the typical granulated matrix in the protein bodies/protein vacuoles (Figure 3C). The targeted "myrosin cells" of *MINELESS* plants appear electron transparent (Figure 3D), indicating that the cell content has been degraded.

Immuno-gold EM analysis of thin sections (600-700 nm) using the anti myrosinase antibody K089 (Thangstad et al., 1991) show that the ablated cells do not contain myrosinase proteins (Figure 3D). Myrosin cells from wild type plants are strongly labeled (Figure 3C). When semi-thin sections (1 μm) were labeled with the same antibody against myrosinase (K089) and followed by FITC-conjugated secondary antibodies, no specific labeling could be seen in myrosinase negative transgenic sections (Figure 3E). From tissues with low myrosinase activity both empty cells and myrosin cells with intact myrosin grains could be seen (Figure

3F). In these sections all stages of degraded cells could be observed including myrosin cells with apparently normal structures. Immunocytochemical labeling of sections from wild type seeds (Figure 3G) produced a strong labeling. The tissue used for immuno-localization was embedded in LR-white and this is known to produce some non-specific background labeling. Notice that in sections with low or no specific labeling (Figure 3E and F), exposure time was increased to better visualize the distribution of the empty cells.

Variation in myrosinase content and expression of myrosinase isoforms

The specific myrosinase activity of wild type and transgenic seeds are shown in Figure 4A. Out of 100 transgenic seeds measured, 57 show specific myrosinase activity below the lowest detected activities in the wild type seeds. Only 4 of the transgenic seeds have activity in the wild types major range (201-400 $nmol \cdot min^{-1} \cdot mg^{-1}$). No transgenic seeds had activities in the wild type's upper range. Seeds with activities in the range of 0-10 $nmol \cdot min^{-1} \cdot mg^{-1}$ are classified as *MINELESS* seeds, while activities between 11-20 $nmol \cdot min^{-1} \cdot mg^{-1}$ are classified as *MINELESS* seeds with low activities. Of the transgenic seed examined, 38% are found in these two categories. *MINELESS* seeds were selected for further investigations (Figure 4B).

Immunoblot analysis (Figure 5A) confirmed the results obtained with the God-Perid assay. The wild type protein extracts produce bands consistent with the three major myrosinase polypeptide classes in *B. napus*, denoted as 75, 70 and 65 kD. Transgenic seeds with no or very low myrosinase activities show no protein bands for myrosinase isoenzymes. *MINELESS* seeds with a moderate myrosinase activity produced faint bands on immunoblot analysis.

Expression of myrosinase-binding proteins are not affected by the myrosin cell ablation

Immunoblot analysis of *MINELESS* seeds using the monoclonal anti-MBP antibody 34:14 (Lenman et al., 1990) did not show any significant differences between wild type and *MINELESS* seed (Figure 5B). This study was based on analysis of 44 wild type and *MINELESS* single seeds. Of the isoforms 110 kDa and 52 kDa MBP varied most. In some seeds, including both wild type and *MINELESS*, the 110 kDa MBP was only faintly expressed while in others it was strongly expressed. The 52 kDa MBP was seen in all seeds examined. The differences between single seeds could not be correlated to differences in myrosinase activity (data not shown).

One isoform of epithiospecifier protein is lost by myrosin cell ablation

Immunoblot analysis using a polyclonal anti-ESP antibody, Wye 5 (Foo et al., 2000) indicate that the 35 kDa isoform is unaffected while the 39 kDa isoform is lost from *MINELESS* seeds (Figure 5C). Transgenic seeds with a low but detectable myrosinase activity and expression do express the 39 kDa isoform of ESP (data not shown).

Glucosinolate profile and content are largely unaffected in *MINELESS* seeds

The profile of detected glucosinolates and the amount of total glucosinolates were mainly unaffected by the myrosin cell ablation. A small increase in the progoitrin content and a small decrease in the content of gluconapolieferin and gluconapin were observed. The difference was,

however, not significant. The relation between total aliphatic and total indolyl glucosinolates differed with only 0.1 % (w/w) in wild type and *MINELESS* seeds (Figure 6).

Analysis of glucosinolate degradation products proves that myrosinase is lost in *MINELESS* seeds.

To investigate the effect of myrosin cell ablation on hydrolysis of glucosinolates, products of glucosinolate hydrolysis were analyzed by GC and GC-MS. No isothiocyanates was formed in extracts from *MINELESS* seeds with no myrosinase activity (Figure7A). In the same extracts trace amounts of epithioalkanes and of nitriles were detected and identified by GC and GC-MS. The assay used favors epithionitrile formation. GC-analysis of samples from wild type seeds showed normal levels of isothiocyanates, epithioalkanes and nitriles (Figure 7A).

Adding myrosinase to the samples of *MINELESS* seeds restored glucosinolate hydrolysis but did not produce a similar distribution of products. The formation of isothiocyanates increased to normal levels, while supplementing myrosinase only had a very small effect on the epithionitrile formation indicating a reduced ESP activity for this seed. The levels of simple nitriles also increased markedly but less than the increase of isothiocyanates formed. A representative comparison of product formation in wild type and *MINELESS* single seed with and without added myrosinase is shown in Figure 7B.

The *Myr1.Bn1* is not exclusively expressed in the developing seed

Initial experiments indicated that the Myr1.Bn1 promoter only was active in developing seeds (Thangstad, Gilde, Chadchawan, Seem, Husebye, Bradley, and Bones (submitted)). It was, however, evident from the morphological phenotype of *MINELESS* that other parts than seeds were affected. We therefore set out to analyze the expression directed by the Myr1.Bn1 promoter more in detail. Transgenic plants carrying constructs containing the promoter of *Myr1.Bn1* fused to the GUS gene were used. Using an improved method for the histochemical detection of GUS expression (Husebye et al., 2002), we were able to show that the promoter of *Myr1.Bn1* also is active in the flower stalk and developing siliques (Figure 8). The Myr1.Bn1:GUS expression are observed in the typical myrosin cells (Fig. 8D), phloem parenchyma cells (Figure 8C) and in a fraction of guard cells (Figure 8B). Stained cells are frequently observed at branch points. When larger pieces of the stalk were used such as shown in Figure 8E, penetration of the staining substrate becomes limiting, resulting in poor staining in myrosin cells deep in the tissue.

DISCUSSION

Controlled death of myrosin cells and proof of myrosinase negative seeds

We have demonstrated the ability to produce myrosinase free and myrosin cell ablated seeds of *B. napus* using a myrosin cell specific promoter to direct expression of the cytotoxic RNase barnase. The *Myr1.Bn1* promoter is a strong promoter and co-expression of its inhibitor barstar inhibitor was necessary to obtain viable seeds. To our knowledge this is the first report of successful cell specific ablation in plants. We denote these plants as *MINELESS* because "the toxic mines" (myrosin cells) have been ablated.

Successful tissue specific ablation has been previously demonstrated in *B. napus* using the tapetum specific promoter pTA29 linked to the barnase gene, where male sterile plants were obtained (De Block and Debrouwer, 1993). The transgenic plants and their progenies were vegetatively normal.

The *Myr1.Bn1* is strongly expressed in maturing embryos and the necessity of co-expression of the barstar gene confirms this. Over-expression of the barnase gene was lethal to the embryo in MyrBarn plants. Selecting for seeds with no or very low myrosinase activity, i.e. a strong expression of the barnase gene, enhanced the number of empty siliques and terminated embryos in the siliques of the *MINELESS* F2 generation.

Three lines of evidence show that *MINELESS* seeds are myrosinase negative. The seeds are negative in myrosinase assays, show no signal in immunoblot analysis, and no isothiocyanates are produced as confirmed by GC and GC-MS analysis. This is consistent with localization of myrosinase to myrosin cells as reported earlier. Some reports indicate that there is expression of myrosinase also in other cells than myrosin cells (Kelly et al., 1998), a view not supported by our data.

Methods for immunolocalization of myrosinase and glucosinolates has been reported earlier (Bones et al., 1991; Thangstad et al., 1991; Kelly et al., 1998). Here we provide direct evidence for the expression of the *Myr1.Bn1* promoter in myrosin cells. Standard light and electron-microscopy as well as immunocytochemical LM-analysis with monoclonal antibodies and immunogold-EM analysis using polyclonal antibodies all show that myrosin cells are targeted and ablated. Furthermore, no evidence of ablation of other cells than myrosin cells was observed. It might be that a low expression of myrosinase in non-myrosin cells is hidden by counteraction of the expressed barstar. However, considering that the few arrested developing seeds of the MyrBarn plants showed necrotic spots resembling myrosin cell distribution, evidence point towards a myrosin cell specific expression. This is further supported by the total absence of myrosinase activity in seeds where all myrosin cells had been ablated. This is also consistent with the Myr1.Bn1:GUS expression pattern.

Glucosinolate content and profile of *MINELESS* seeds

Analysis of *MINELESS* seeds proves that the majority of seed glucosinolates are not localized in myrosin cells. This is consistent with the results we reported earlier after immunolocalization of the glucosinolate sinigrin in *Brassica juncea* seeds (Kelly et al. 1998), although several reports indicate a distribution of glucosinolates in all cells of the seed (Grob and Matile, 1979; Wei et al., 1981; Yiu et al., 1984). Thangstad et al. (2001) investigated the uptake, metabolism and localization of radiolabelled desulpho but-3-enylglucosinolate and the corresponding glucosinolates in developing seeds of *B. napus*. Using micro-autoradiography, the label was detected in distinct cells resembling the pattern of distribution of myrosin cells. The latter result indicating that either the desulphoglucosinolate/glucosinolate or some degradation products

after hydrolysis of the labeled glucosinolate are accumulated in specific cells. We did not find any significant change in the glucosinolate profile of *MINELESS* and wild type seeds, and consider it evident that glucosinolates are not accumulated in myrosin cells. Korolova et al. (2000) reported that glucosinolates of flower stalks of Arabidopsis are localized to sulfur rich cells (S-cells). More recently, Husebye et al. (2002) reported myrosinase to be localized to the neighboring phloem cell idioblast (M-cells) of the S-cells. Although we believe glucosinolates are present in most non-myrosin cells of seeds, more work is needed to sort out where different glucosinolates are localized. The fact that addition of myrosinase to extracts of *MINELESS* seeds restore production of glucosinolate degradation products suggests that all components needed for glucosinolate degradation except myrosinase is present in the seed. However, this does not exclude the possibility that the *in vivo* situation may be different.

MINELESS **seeds do not hydrolyze glucosinolates**

Aspects of the *in vivo* metabolism of glucosinolates are still open but recent results provide evidence for a rapid turnover of glucosinolates even in unchallenged plants (James and Rossiter, 1991; Rosa et al., 1994; Svanem et al., 1997; Thangstad et al., 2001; Petersen et al., 2002). Most reports conclude that glucosinolates are present in all cells of the seed, although some recent results indicate that myrosin cells are free of glucosinolates (Kelly et al., 1998) and that myrosinase and glucosinolate are compartmentalized in different but neighboring cells (Korolova et al., 2000; Husebye et al., 2002). Our results support the hypothesis that glucosinolates are localized in non-myrosin cells. This is based upon the fact that myrosin cell ablation do not affect the glucosinolate profiles of seeds. Supplementing myrosinase to extracts of *MINELESS* seeds completely restores the glucosinolate hydrolysis activity and produces

identical hydrolytic products as the wild type. This is consistent with myrosinase being the only missing component for glucosinolate hydrolysis in *MINELESS* seeds. The assay used to investigate the hydrolysis products has a longer incubation time than the myrosinase activity assay and is most likely the explanation for the small amount of epithionitriles and nitriles formed in seeds with no myrosinase activity.

Isoforms of epithiospecifier protein is most likely localized in different cells

Epithiospecifier protein (ESP) is a Fe^{2+} dependent protein that catalyses formation of epithionitriles during hydrolysis of glucosinolates with a terminal double-bond in the side-chain (Tookey, 1973). Immunoblot analysis of myrosinase negative *MINELESS* seeds using antibodies against epithiospecifier protein show that the 35 kDa isoform is unaffected while the 39 kDa isoform is lost. This indicates a co-localization of the 39 kDa ESP with myrosinase in myrosin cells of seeds. Since the 35 kDa ESP is not affected by controlled cell death of myrosin cells, this might further indicate different roles in the myrosinase-glucosinolate system for the two polypeptides in seeds. However, nothing is at present known about differential roles of 35 kDa and 39 kDa ESP and localization in different cells do not necessarily indicate functional diversification.

As expected, *MINELESS* seeds did not produce isothiocyanates. The observation that the product profile in *MINELESS* seeds with added myrosinase differed from Westar seeds was puzzling. Although the assay is designed to favor the formation of epithionitriles it did not increase noticeably. The amount of simple nitriles increased markedly but not as much as the formation of isothiocyanates. Recent investigation has confirmed the role of Fe^{2+} in both epithionitrile and simple nitrile formation (Zabala, Grant, Bones, Bennett, and Rossiter,

unpublished). The reduction in the expression of total ESP in *MINELESS* seed may have led to reduced ESP affection on the unstable thiohydroximate-*O*-sulphonate intermediate and thereby a reduction in epithionitrile formation supplemented with myrosinase. In spite of that, a higher increase in nitrile formation should be expected in extracts of *MINELESS* seeds supplemented with myrosinase. However, the relatively small proportion of epithionitrile production in *MINELESS* with added myrosinase could be due to excessive quantities of myrosinase. Further experiments are needed to determine the optimum amount of myrosinase for the ESP/myrosinase assay. One might expect that normal expression of the 35 kDa ESP isoform would have been enough to compensate for normal amount of epithionitriles and simple nitriles, as *Crambe abyssinica* is known to only have the 35 kDa ESP (Foo et al., 2000). Clearly more information on the role of ESP in glucosinolate hydrolysis is required.

MBP expression is unaffected in *MINELESS* seeds

Immunoblot analysis of MBPs in wild type and *MINELESS* seeds indicate that the quantities of MBPs are highly variable between single seeds. There is no evidence in the literature for the existence of myrosinase-MBP complexes in intact cells of the seed embryo (Eriksson et al., 2002). This is supported by our results showing no reduction of MBPs in *MINELESS* seeds. It is reasonable from recent results that glucosinolates are metabolized in intact plants (Cole 1978, 1980; James and Rossiter, 1991; Rosa et al., 1994; Svanem et al., 1997; Thangstad et al., 2001; Petersen et al., 2002). Glucosinolates are most likely transported into myrosin cells for endogenous hydrolysis (Thangstad et al., 2001), but the mechanism awaits elucidation. The MBPs have been suggested as members of this unknown glucosinolate transport mechanism (Andreasson et al., 2001). One could speculate that the plant attempts to compensate for the

absence of glucosinolate degradation products by increasing the expression of proteins involved in transport or hydrolysis of glucosinolates. However, our results do not support this, as there is no increase in MBPs.

Phenotype of *MINELESS* and expression of the Myr1.Bn1

In addition to the expected expression in myrosin cells of developing and mature seeds, the Myr1.Bn1 promoter also directs expression to some (but not all) guard cells, myrosin cells and phloem idioblasts of stem and flower stalk and to idioblast like cells of maturing silique wall. The observed expression pattern of Myr1.Bn1:GUS and the observed phenotypic abnormalities in the *MINELESS* plants strongly suggest a link between the organs affected and the expression pattern of myrosinase. The morphological changes are consistent with the expression of Myr1.Bn1 as verified by Myr1.Bn1:GUS plants. We cannot completely rule out that the observed effects are caused by unknown processes affected by the genetic modification of the *Brassica napus* plant. However, the fact that we obtain identical phenotypes in *B. napus* and *A. thaliana* make it more than likely that the effects observed is due to the ablation of myrosin cell idioblasts. We therefore propose that the phenotypic modifications observed is linked to the absences of one or more glucosinolate degradation product that have regulatory roles. It is further tempting to speculate that these products have an inhibitory role since the effects observed are overdeveloped carpels, production of secondary siliques and higher level of branching. The expression of the MA family (including the Myr1.Bn1 gene) has been regarded as seed specific (Lenman et al., 1993). Our results show that this needs to be re-evaluated. The expression pattern observed may be consistent with a role for Myr1.Bn1 in the development of new organs and/or in regulation of growth. The phenotypic alterations found in the *MINELESS*

plants are at locations where the *Myr1.Bn1*:*Barnase* construct is expressed. The reason why only a fraction of the guard cells were positive for GUS staining is an open question and await further investigations.

The myrosinase-glucosinolate system is likely to have multiple functions

Evidence continues to accumulate in support of roles of the myrosinase-glucosinolate system in multiple functions. The cell compartmentalization of myrosinase to specific idioblasts of epidermis (as guard cells), phloem, cortex and mesophyll (Bones and Iversen, 1985; Bones et al., 1991; Høglund et al., 1991; Husebye et al., 2002) form a network of cells in the tissues earlier described as toxic mines (Bones and Rossiter 1996). Reports over the last years have started to link genetic modifications of endogenous glucosinolate profiles to growth and morphological alterations of plants. A CYP79F1 knock-out mutant was reported by Reintanz et al. (2001) to have a modified glucosinolate profile (the biosynthesis of aliphatic glucosinolates was blocked) that was followed by a bushy phenotype. This is consistent with the bushy phenotype of the *MINELESS* plant. Likewise, over-expression of CYP79B2 and CYP83B1 (increased biosynthesis of indolglucosinolates) have been reported to result in plants with reduced growth and a low seed setting (Mikkelsen at al., 2000) and reduced apical dominance respectively (Bak et al., 2001). We did not find any changes in the glucosinolate profiles of *MINELESS* seeds. However, it is the hydrolysis products and not the glucosinolates that are biological active. We have shown that extracts of *MINELESS* seeds have to be supplemented with myrosinase to degrade glucosinolates. A termination of glucosinolate hydrolysis by a lack of myrosinase expression would in theory influence any biological process that was dependent on glucosinolate hydrolysis products. The myrosinase-glucosinolate system has been related to

homeostasis of the growth hormone IAA (Bak et al., 2001; Bak and Feyereisen, 2001). Hydrolysis of indolyl glucosinolates produces the growth hormone precursor IAN converted to IAA by nitrilase (Bartel, 1997). In *A. thaliana*, increased levels of both indolyl glucosinolates and nitrilase (NIT3) has been demonstrated in roots of sulfur-starving plants (Kutz et al., 2002). Thus, a regulatory loop apparently seems to exist in the turnover of the indolyl glucosinolate pool. The four *Arabidopsis* genes encoding nitrilase have been shown to have different expression pattern during development (Bartel and Fink, 1994). Induced nitrilase expression has also been implicated in virulent bacterial infection (Bartel and Fink, 1994) and by methyl jasmonate exposure (Geshi and Brandt, unpublished).

Work in our laboratory have shown that ablation of myrosin cells significantly changes the interaction between the aphids *Brevicoryne brassicae* (a *Brassica* specialist) and *Myzus persicae* (generalist) and wild type and *MINELESS* plants (Borgen, Honne, Thangstad, Rossiter, and Bones, unpublished). As expected the generalist preferred the *MINELESS* plants and the specialist the wild type plants in free choice experiments. In-depth characterization of plant-insect interactions is in progress and will provide supplementing information about the role of the myrosinase-glucosinolate system in the defense against pests.

By ablation of myrosinase and myrosin cells we have been able to produce *MINELESS* seeds and gain a fascinating insight into the way in which the myrosinase-glucosinolate system functions in plants. This complex system is important not only in defense against pests, but also seems to be involved in a strictly tissue/organ specific regulation of development and growth by enzymatic control of the turnover of the glucosinolate pool. We propose that this system is capable of making an array of products of which some alone or in combination provide activities that can influence cellular responses or activities. This makes the Brassicaceae plant highly capable to meet a changing environment.

METHODS

Plants

Brassica napus cv. Westar was generously provided by Dr. Wilfred Keller (Plant Biotechnology Institute, National Research Council, Saskatoon, Canada).

Bacterial strains, plasmids and molecular methods

Standard molecular methods were employed (Sambrook et al., 1989). *Escherichia coli* DH5α (Bethesda Research Laboratories), JM109 (Promega Corporation) and MX1061 (Plant Genetic Systems) were used for plasmid manipulations. Because of the toxicity of barnase, all plasmids containing this gene were propagated in the *E. coli* MX1061 strain, which has a chromosomal expression of the barnase-inhibitor gene barstar. Plasmids pBluescript II KS (Stratagene), pGEM3, 5 and 11 (Promega Corp.) was used for gene sub-cloning.

Myrosinase seed promoter constructs

The sequence of the Myr1.Bn1. myrosinase gene is given in Genbank (accession #AF323020). A Sal*I*-Eco*RI* 1142 bp fragment containing the partial myrosinase gene promoter, the Barnase encoding gene (Mariani et al., 1990) and nos-terminator (Depicker et al., 1982) was inserted into pBI101.1, between the Sal*I* and Eco*RI* restriction sites of the polylinker. The resulting plasmid was cut with Hind*III* - Sal*I* and the remaining 2520 bp of the promoter inserted utilizing the internal Sal*I* site to obtain a binary plasmid carrying the full-length promoter. To generate the Myr1.Bn1:Barnase:Barstar plasmid construct (MyrBarnBar = *MINELESS*) a cassette consisting of the Barstar gene (Mariani et al., 1992) under the CaMV35S promoter

with a 3`g7 terminator (Velten and Schell, 1985) was inserted at the EcoRI restriction site of pBI101.1 containing the 1142 bp fragment described above and the 2520 bp myrosinase promoter fragment inserted giving rise to a plasmid containing the full-length myrosinase Myr1.Bn1 promoter, Barnase, *Nos*-terminator and CaMV35S: Barstar: 3'g7. Details of the cloning are available upon request. The constructs were transformed into *Agrobacterium tumefaciens* using electroporation (BioRad). The strain LBA4404GV3101 (pMP90RK) (DSMZ, Germany) was used to transform *B. napus* and *A. thaliana*. The Myr1.Bn1:GUS construct (MyrGus) is described in Thangstad, Gilde, Chadchawan, Seem, Husebye, Bradley, and Bones (submitted).

Production and analysis of transgenic plants

Brassica napus

Transformation of *B. napus* was performed basically as described by Moloney et al. (1989). Cotyledons of *B. napus* cv Westar were transformed with *A. tumefaciens* LBA 4404 containing the binary plasmid constructs MyrBarn, MyrBarnBar and MyrGus. Selection of transformants was done on the basis of kanamycin resistance from the NPTII gene in the constructs. After regeneration of transgenic shots, verification of constructs in the transgenic clones were done using PCR with genomic DNA as template and specific primers for the inserted genes essentially as described by Strittmatter et al. (1995) for the barnase and barstar genes and similarly using primers for the β-glucoronidase gene. After repetitive transfers and hormone induced rooting, plants were transferred to soil and grown in controlled environment rooms. Plants were allowed to self-pollinate and flowers were covered with paper bags. After maturity, seeds were collected and analyzed.

Arabidopsis thaliana

The *MINELESS* construct was transferred into *Arabidopsis* ecotype Colombia by *Agrobacterium tumefaciens*-mediated vacuum infiltration according to Bechtold er al. (1993). Plants were selected by kanamycin resistance and grown on soil under a 16 hour light (25 ºC) and 8 hour dark (20 ºC) regime in controlled environment rooms.

Extraction of proteins

Single seeds of *Brassica napus* L. cv. Westar and from transgenic Westar (*MINELESS*) were defatted with n-hexan, the pellet air dried and resolved in 100 µl imidazole-HCL buffer (10 mM, pH 6.0). The supernatant was used for myrosinase activity determination and for the SDS-polyacrylamide gel electroforesis. For the analysis of MBP expression, single seeds were ground in liquid N_2, transferred to 100 µl imidazole-HCL buffer (10 mM, pH 6.0) containing phenylmethylsulfonyl fluoride, PMFS, (1 mM) and incubated for 30 min at 4ºC before centrifugation, the supernatant was used further.

Myrosinase and protein assays

Myrosinase activity was measured as the liberation of glucose with GOD-Perid assay as described by Bones and Slupphaug (1989). To select appropriate transgenic seeds with no or very low myrosinase activity for LM and EM analysis, a fast version of the GOD-Perid assay was performed as followed. One cotyledon from the seed was crushed with citrate buffer (50 mM, pH 5.5) and sinigrin (15 mg/ml), GOD-Perid was then added and after a short incubation

the development of a green color indicated myrosinase activity. The protein concentration was determined (Bradford, 1978) with bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Samples was solubilized in standard SDS buffer prior to separation by SDS-PAGE using 7,5% and 12% polyacrylamide gels in Hoefer miniVe vertical electrophoresis system. Gels were blotted to 0.45 μm nitrocellulose membrane (Bio-Rad) in Hoefers blot module as described by the manufacturer. Myrosinases and myrosinase binding proteins (MBP) were detected by the 3D7 and 34:14 monoclonal antibodies, respectively (Lenman et al., 1990). The epithiospecifier proteins (ESP) were detected by the anti-ESP polyclonal antibody Wye5 (Foo et al., 2000).

Immunohistochemical staining

Mature seeds from control and transgenic *B. napus* cv. Westar without the seed coat were fixed, dehydrated, embedded and sectioned (1 μm and 600-700 Å) as described by Thangstad et al. (1991) with six days of infiltration with LR-White. One cotyledon from every seed was tested for myrosinase activity by the fast GOD-Perid test described above. Semi-thin sections were used for the detection of myrosinase with the polyclonal antibody K089 (Thangstad et al. 1991). Positive cells were visualized with fluorescein-isothiocyanate (FITC) -conjugated streptavidin (DAKO, Denmark). Slides were examined and photographed with a research microscope (Eclipse 800; Nikon) equipped with a cooled digital camera (SPOT RT; Diagnostic Instruments, Burroughs, MI). The immuno-gold labeling of thin sections was carried out as described in Thangstad et al., (1991) with minor modifications. The goat anti-rabbit secondary antibody (Amersham, U.K) was conjugated with 15 nm colloidal gold and the post-embedding on-grid osmium staining were performed with 2 % osmium tetroxide. The sections were

examined and micrographs were taken with a Jeol 1200 EX (Japan) electron microscope at 60 kV.

Analysis of glucosinolates by HPLC

Glucosinolates from single seeds were extracted in boiling methanol (70 %). To remove oils and proteins n-hexan and a barium (0.5 M)/lead acetate (0,5M) solution was used. Desulpho-glucosinolates were made on a DEAE-A25 Sephadex ion exchange column with sulphatase (75 µl, Type H-1, Sigma). The desulphoglucosinolates were freeze dried (Virtis Benchtop), reconstituted in water (100 µl) and analyzed by HPLC. Benzyl glucosinolate were used as internal standard. Reverse phase HPLC was performed on a Sperisorb C-18 column (250 mm x 4.6 mm, 5 µm spherical particle). The mobile phases were: (A) deonized water, (B) 20 % acetonitrile/deionised water. The flow rate was 1.5 ml/min with UV detection at 230 nm. A mixture of *B. napus* desulphoglucosinolates was used as an identification standard (Bioraf Denmark).

ESP assay

Single seeds were extracted in 0.6 ml imidazole-HCL buffer (100 mM, pH 6.8). One half of the extract was incubated in an assay mixture containing ferrous ions (0.6 mM ferrous ammonium sulfate), DTT (1 mM), 2-propenylglucosinolate (sinigrin, 14 mM) and myrosinase (50 µl from an affinity purified preparation from *Sinapis alba* seed with high myrosinase activity as tested by GOD-Perid). In the other half myrosinase was omitted. After a 4 h incubation at 30ºC, the mixtures were extracted twice with dichloromethane and the combined organic phases dried

with anhydrous $MgSO_4$, filtered and concentrated using a flow of argon gas. ESP activity was determined by capillary GC (Varian Saturn 3400cx). Products was confirmed by their mass fragmentation patterns (Varian Saturn 3 GC-MS). Parts of the extract were used for determination of myrosinase activity by GOD-Perid assay and separation by SDS-PAGE.

Histochemical GUS detection

The histochemical detection of GUS was performed as described by Jefferson et al. (1987) with modifications as described by Husebye et al. (2002). GUS staining was performed using 0.4 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic-acid and sodium salt (DUCHEFA, Haarlem, The Netherlands). After staining, the samples were examined and photographed using a stereo microscope (SMZ1500; Nikon, Tokyo) equipped with a digital camera (Coolpix 990; Nikon) or a research microscope (Eclipse 800; Nikon) equipped with a cooled digital camera (SPOT RT; Diagnostic Instruments, Burroughs, MI).

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in non-commercial research purposes.

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Figure legends:

Figure 1. Schematic presentation of the *Myr1.Bn1* promoter Barnase, Barnase:Barstar and GUS fusion gene constructs. The figures are not drawn to scale.

Figure 2. Morphology of wild type and transgenic *B. napus* (**A-E** and **H)** and *A. thaliana* (**F-G)**. **A.** Wild type plant. **B.** *MINELESS* plant. **C.** *MINELESS* plant showing an abnormal silique with outgrowth of new siliques instead of seed development. **D.** Wild type flower and flower buds. **E.** *MINELESS* flower and flower buds. Visible carpels are appearing at a stage were floral buds are still closed. **F.** Arabidopsis wild type flower. **G.** *AtMINELESS* flower. **H.** *MINELESS* silique with three silique-chambers instead of the normal two.

Figure 3. Semi-thin and ultra thin sections from *B. napus* radicles (**A-B, D-G**) and cotyledon (**C**) embedded in LR-White. **A-B;** sections stained with toluidine blue, **C-D;** immunogold-EM sections labeled with anti-myrosinase (K089) and 15 nm colloidal gold, **E-G;** semi-thin sections labeled with anti-myrosinase polyclonal antibody (K089) and FITC conjugated secondary antibodies. Scale =10 μm (**A, B, E, F, G**) and 2 μm (**C, D**). White arrows indicates some of the myrosin cells, normal or ablated. Myrosin grains in LM and TEM sections are marked by an asterisk. Notice that the exposure time in E and F was increased to visualize the ablated myrosin cells. **A.** Ablated myrosin cells in *MINELESS*. **B.** Ablated myrosin cells at higher magnification. Inserted; Normal myrosin cell with myrosin grains. **C.** Myrosin cell from wild type containing 3 myrosin grains with immunogold labeled myrosinase proteins. **D.** Ablated myrosin cell from *MINELESS* with no immunogold labeling. **E.** LM-section from myrosinase negative *MINELESS* showing no immunolabeling in ablated cells. **F.** LM-section

from *MINELESS* with moderate myrosinase activity showing myrosin cells in different stages of degradation. **G.** LM-section from wild type showing labeled myrosin cells.

Figure 4. Myrosinase activity in wild type and *MINELESS* seeds. **A.** Distribution of specific myrosinase activity in individual wild type and *MINELESS* seeds. Wild type seeds with specific myrosinase activity above 200 $nmol \cdot min^{-1} \cdot mg^{-1}$, and *MINELESS* seeds with specific myrosinase activity below 20 $nmol \cdot min^{-1} \cdot mg^{-1}$were selected for further investigations. **B.** Myrosinase activity in selected wild type and *MINELESS* seeds.

Figure 5. Western analysis of wild type (L1), and *MINELESS* (L2) seeds. 10 µg of total protein was applied in each lane. **A.** Expression of myrosinase proteins detected with 3D7 antibodies **B.** Expression of myrosinase-binding proteins detected with 34:14 antibodies. **C.** Expression of epithiospecifier proteins detected with the Wye5 antibodies.

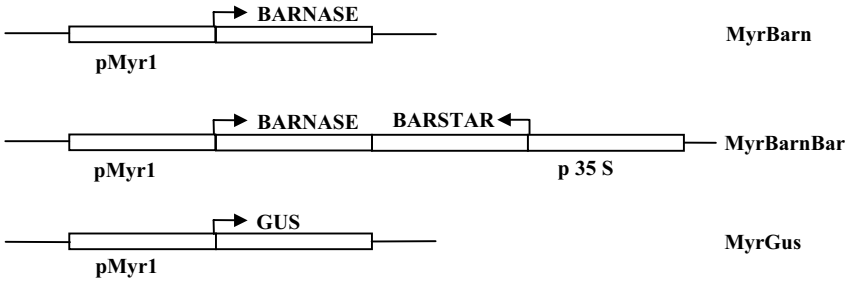
Figure 6. Major glucosinolates in wild type and *MINELESS* seeds. Error bars represent SE.

Figure 7. Analyses of ESP activity in single seeds and product formation from glucosinolate hydrolysis. **A.** GC chromatogram of wild type and *MINELESS* seed showing products formed by hydrolysis of sinigrin. **B.** Hydrolysis products produced in seeds from wild type, *MINELESS* and *MINELESS* supplemented with purified myrosinase.

Figure 8. Cells expressing the *Myr1.Bn1* promoter-directed GUS expression in flowering *B. napus*. Scale = 50 µm. Arrows; cells expressing GUS. Diamond arrows; guard cells not expressing GUS. **A.** GUS positive cells of silique. **B.** GUS positive and negative guard cells in silique. **C.** Flower stalk showing GUS positive cells resembling phloem myrosin idioblasts. **D.**

Myrosin cells from silique. The same type of positive cells could be seen in flower stalks. **E.** Flower stalk with branch point. Using large pieces of the stalk such as in this picture result in limited penetration of the staining substrate and thereby poorer staining of the deeper layer myrosin cells. There are more positive cells deeper in the stalk that do not appear in this picture.

Figure 1. Borgen et al.



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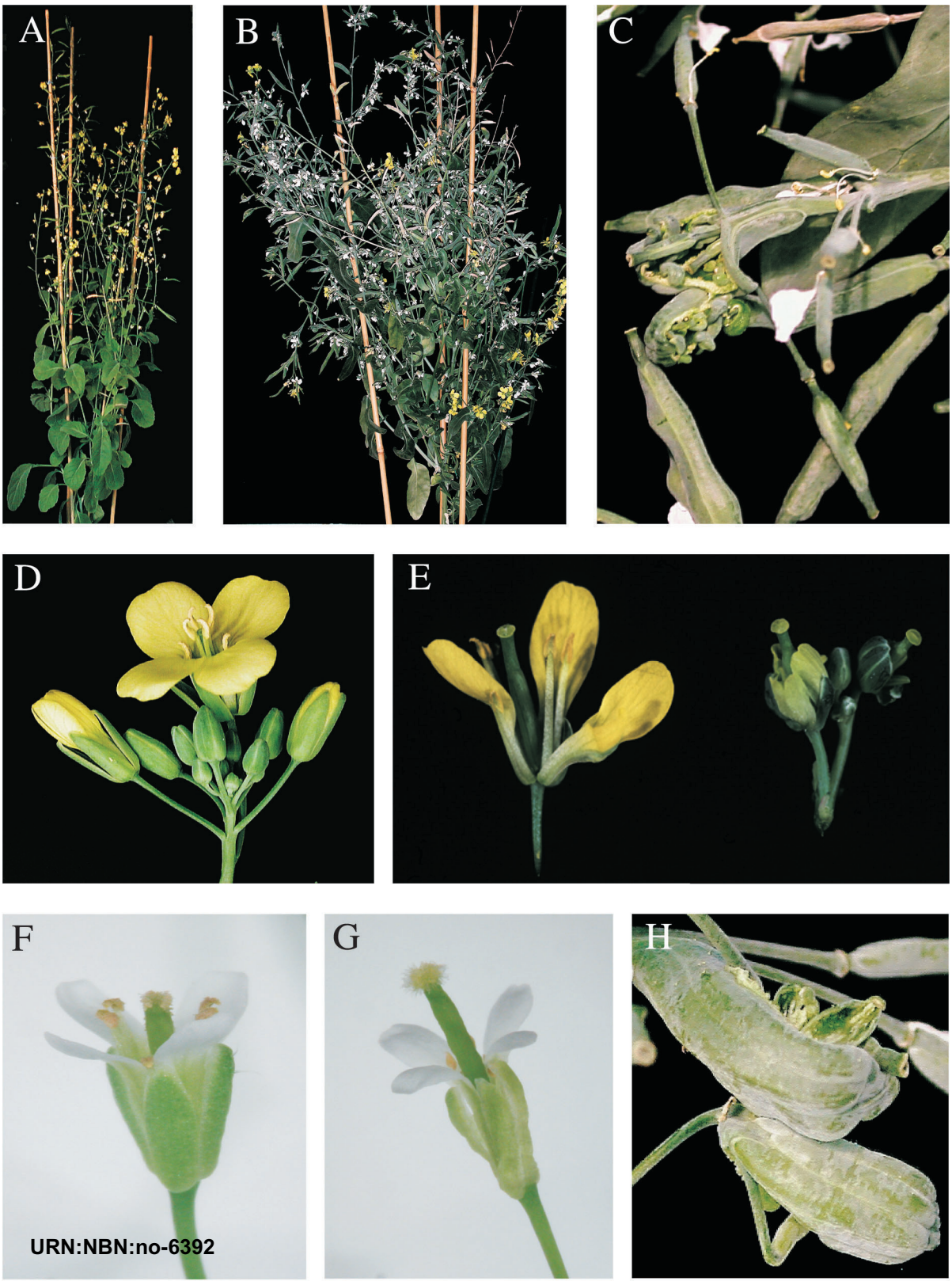
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Figure 3. Borgen et al.

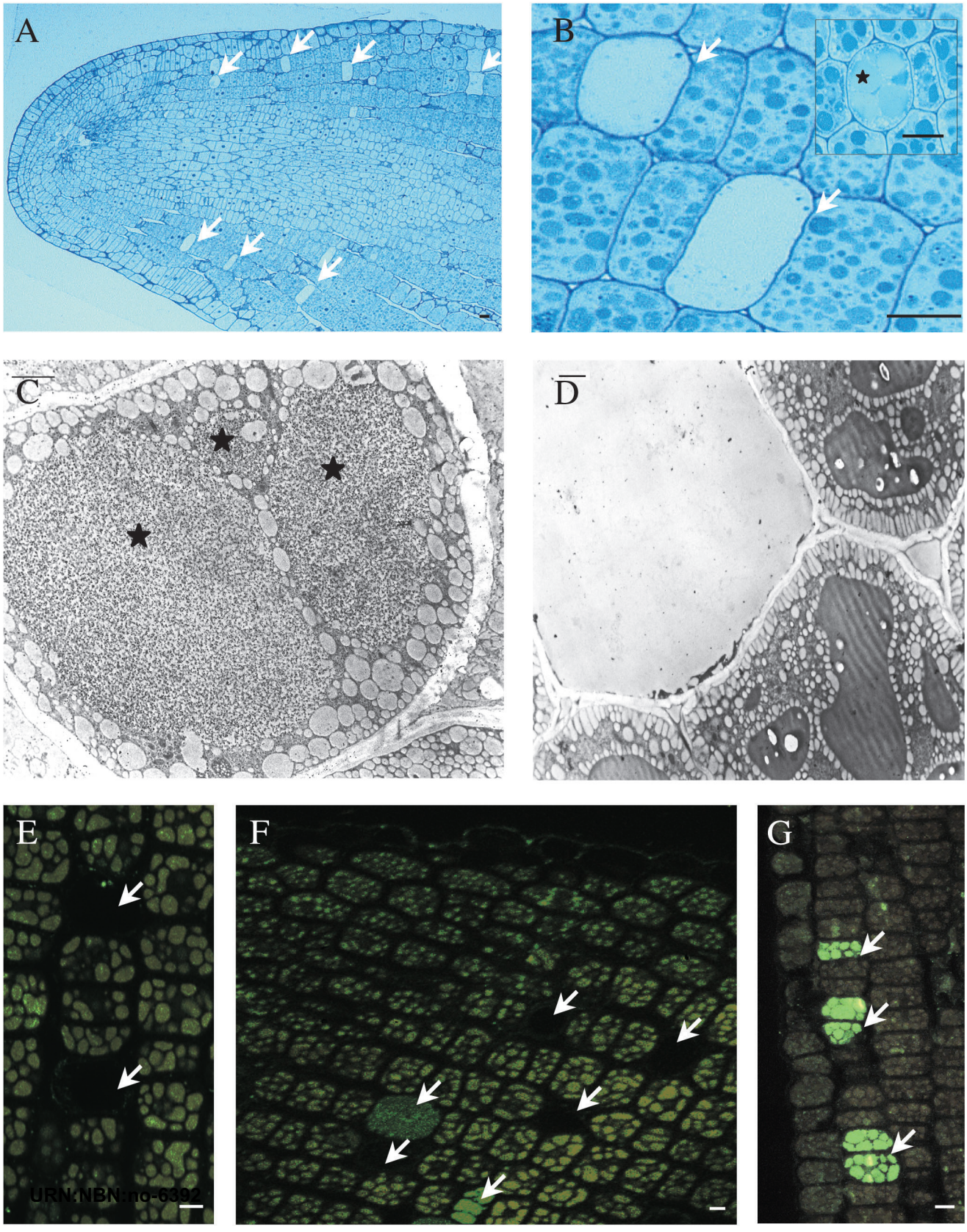

Figure 4. Borgen et al.

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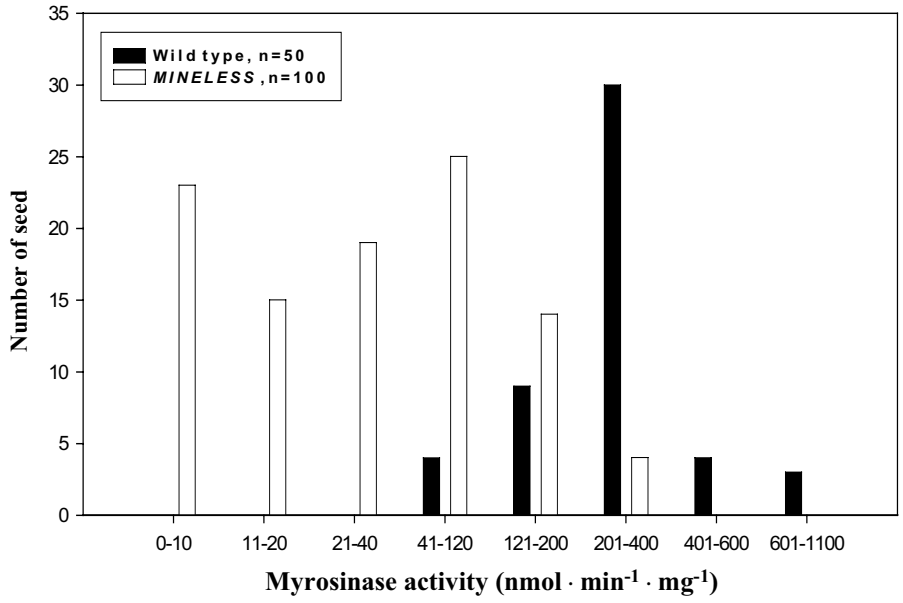

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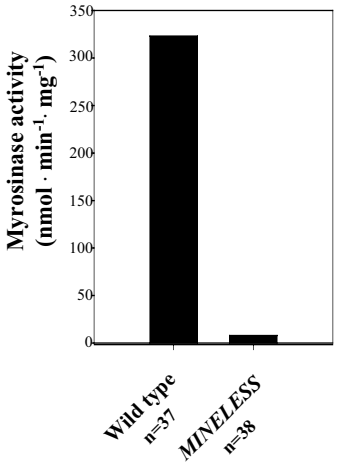

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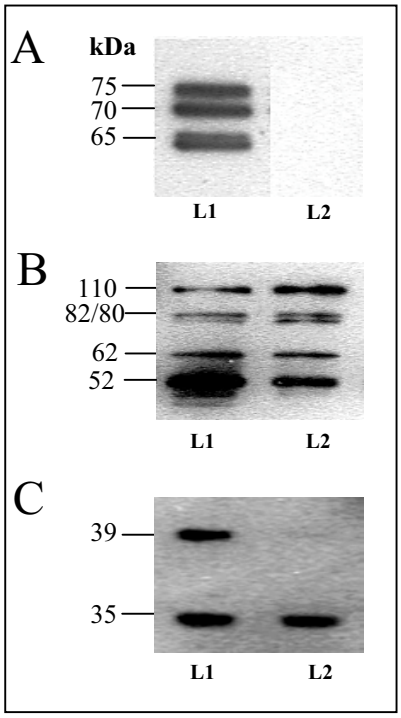

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Figure 6. Borgen et al.

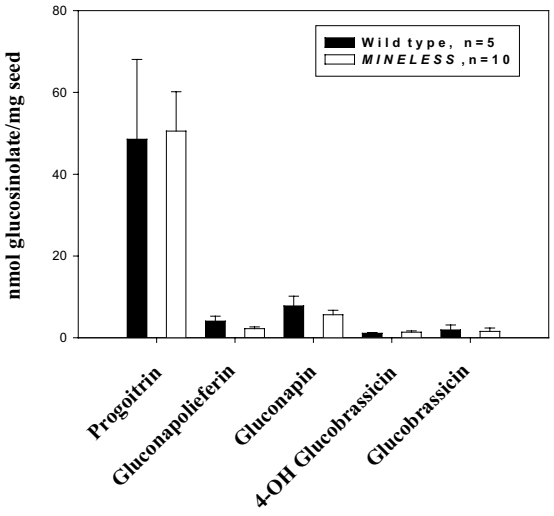

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Figure 7. Borgen et al.

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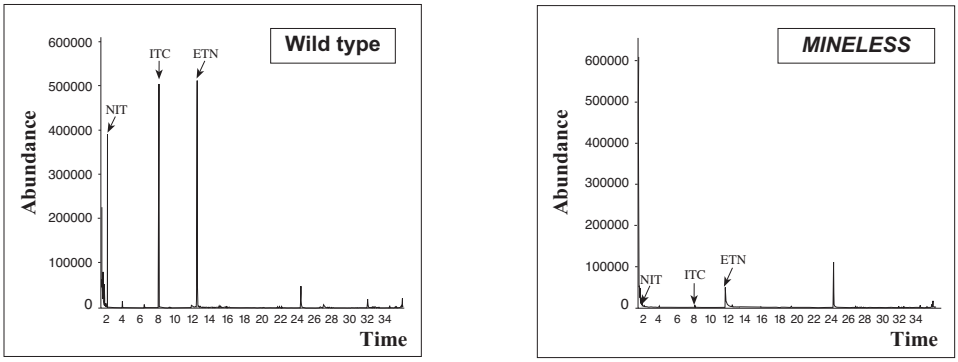

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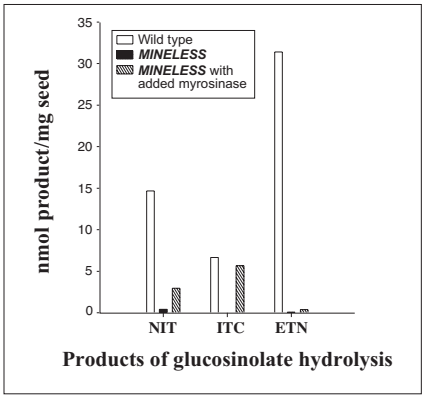
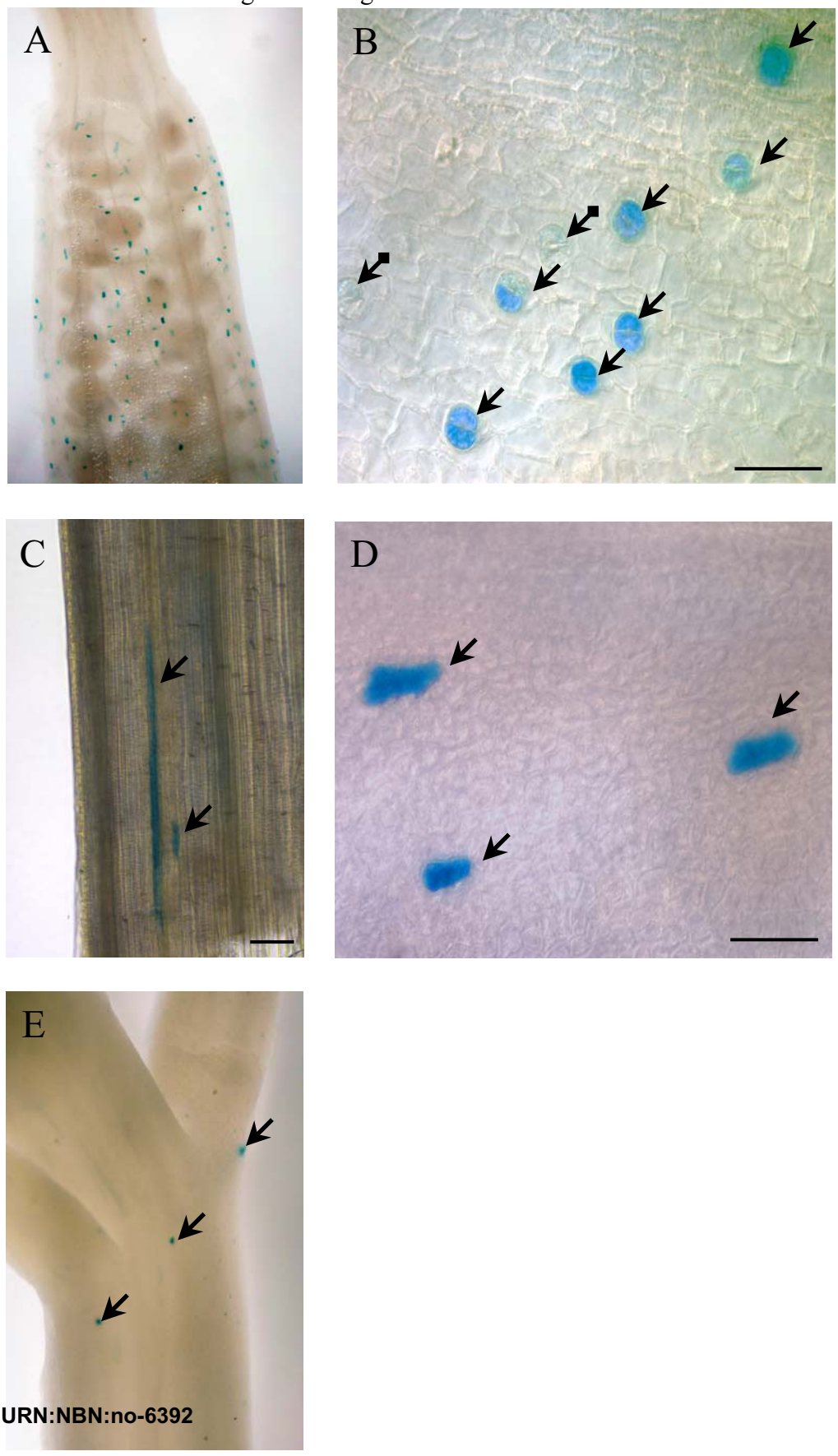


Figure 8. Borgen et al.



Paper II

Controlled cell death of myrosin cells increases the susceptibility for aphid attack in *MINELESS* seedlings.

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Key words: *Brassica napus*; *Brevicoryne brassicae*; *Myzus persicae*; myrosinase; glucosinolate

Abstract

We have used a cell specific promoter and genetic engineering to produce *Brassica napus* plants where selectively the myrosin cell idioblasts have been induced to undergo controlled cell death. The resulting seeds and plants thereof are named *MINELESS* and the seeds do not have myrosin cells and myrosinase making them unable to degrade glucosinolates to isothiocyanates and other biological active compounds. The effect of this myrosin cell ablation on interactions between the specialist *B. brassicae* and the generalist *M. persicae* and *MINELESS* and wild type plants have been studied in a free choice experiments where the baits were seedlings of *Brassica napus* cv. Westar or the *MINELESS* counterpart. The generalist *M. persicae* showed a strong and significant preference for the *MINELESS* plants. The specialist *B. brassicae* showed a significant preference for the wild type plants indicating that it uses one or more of the products of glucosinolate degradation in host plant selection. Based on the results with *MINELESS* seeds we propose that targeting and modification of the content of specific cells like myrosin cell idioblasts can be used to control plant-insect interactions.

Introduction

The β-thioglucoside glucohydrolase (EC 3.2.3.1) also named myrosinase, can upon contact with glucosinolates, a class of secondary metabolites characteristic of crusiferous plants, hydrolyze the substrates to diverse biological active products (Figure 1). The reaction products formed depend on pH and other factors such as the presence of ferrous ions, epithiospecifier protein and the nature of the glucosinolate side chain (for review see Bones & Rossiter, 1996; Rask et al., 2000). Ascorbic acid has been shown to modulate the activity of myrosinase enzymes inhibiting activity at high concentrations and activating activity at low concentrations (Bones & Slupphaug, 1989). Hydrolysis can likely occur as part of a natural physiological process or by tissue disruption caused by a pest or pathogen (Cole 1978, 1980; Bones & Rossiter, 1996; Svanem et al. 1997; Rask et al., 2000; Petersen et al. 2002). The enzyme and substrate are spatially separated in the plant. The myrosinase has been localized to specialized cells known as myrosin cells (Thangstad et al., 1990; 1991), while the glucosinolate sinigrin has been localized to aleurone-like cells in the seed (Kelly et al., 1998). Recently, glucosinolate-specific cells have been identified in the flower stalk of *Arabidopsis* (Koroleva et al., 2000), and these cells have been shown to be neighbors to scattered cells containing myrosinase (Husebye et al., 2002).

Aphids represent one of the serious insect pests of agricultural crops in most temperate regions, and plants have evolved different defense systems in order to escape herbivory. Volatile isothiocyanates, a possible glucosinolate breakdown product, have been shown to possess a range of antifungal, antibacterial and antimicrobial activities and thereby makes the plant capable of repelling microorganisms, insects and mollusks (Fenwick et al., 1983, Sarwar et al., 1998). Nevertheless, a number of insects have become adapted and are able to circumvent the detrimental effects of secondary metabolites contained in plants. These

specialized pests may even use these compounds in their own defense against natural enemies (Bartlet et al., 1999). The specialist cabbage aphid *Brevicoryne brassicae* is restricted to feed on glucosinolate containing crops and is able to make its own myrosinase to hydrolyze the plant produced glucosinolates to its own benefit (Jones et al., 2001; Jones et al., 2002, Bridges et al., 2002). For generalist insects, the nutrient status of the crop represented by free amino acid concentrations may be important to stimulate feeding (van Emden, 1990). The peach potato aphid *Myzus persicae* is a polyphagous aphid whose diet can include crucifers.

Reducing the glucosinolate hydrolysis by removal of myrosinase proteins affects the emission of volatile compounds released from the plant (Borgen, Thangstad, Grønseth, Seem, Husebye, Rossiter, and Bones, unpublished). Reduced emission of volatiles such as isothiocyanates will most likely affect specialist and generalist insects differently. In this study we have set out to study the effect of myrosin cell ablation in plant-insect interaction.

Materials and methods

Plants and aphids

Brassica napus cv. Westar was generously provided by Dr. Wilfred Keller (Plant Biotechnology Institute, National Research Council, Saskatoon, Canada). The production and analysis of the transgenic plants, *MINELESS*, were performed as described earlier (Borgen, Thangstad, Grønseth, Seem, Husebye, Rossiter, and Bones, unpublished). Briefly we used the *Myr1. Bn1* promoter to direct controlled cell death of myrosin cells by expression of the cytotoxic RNase barnase. *Brevicoryne brassicae* and *Myzus prsicae* were mass reared from individual aphids on the *B. napus* cv. Westar in a controlled environment chamber maintained at 18°C ± 2°C day and 15°C ± 2°C night respectively, with a photoperiod L16:D8.

Experimental design

The two aphid species were introduced to wild type and *MINELESS* seedlings in separate chambers within the same plant growth cabinet. Approximately 200-300 aphids were used in each experiment. Two experimental designs were used with two trials for each design. Selected germinating seeds were placed in 4-cm pots with soil and placed in plexiglas chambers with nylon nets before introducing the aphids. The soil was watered with dH_2O regularly. Lights were placed above the test boxes. The number of aphids on each seedling was counted at the same time every day under the six day long period of each experiment.

Experimental design 1; Wild type and *MINELESS* seeds were sown on filter paper watered with ascorbic acid (10 mM, pH 6.0) and placed in the dark. *MINELESS* and wild type seeds

were germinated until the appearance of 2-4 mm root tip. For *MINELESS*, myrosinase free seeds were selected. For wild type seeds, no special selection was made. The germinating seeds were arranged every other wild type and *MINELESS* in a square. Aphids were introduced by placing leaves from the rearing chambers with aphids in the middle of the floor in the test chambers.

Experimental design 2; *MINELESS* seeds were sown on filter paper watered with ascorbic acid (10 mM, pH 6.0) and placed in the dark. Wild type seeds were sown on filter paper watered with dH_2O and placed in the dark. *MINELESS* and wild type seeds were germinated until the appearance of 2-4 mm root tip. The selection of germinated seeds was performed as for design 1. The germinating seeds were arranged every other wild type and *MINELESS* in a circle. To reduce the distance between the seedlings and the introduced aphids a double floor was used in the chambers making the top of the pots at the same level as the floor of the chamber, see figure 3 for illustration. Aphids were introduced by placing leaves with aphids in the middle of the circle of pots with seedlings.

Control seedlings were treated as in design 1 but without introducing them to aphids.

Extraction of myrosinase proteins

Single seedlings of wild type and *MINELESS* were counted for number of aphids and measured for height. The aphids were removed before the seedling where crushed with immidazole-HCl buffer (150 µl 10 mM, pH 6,0) and centrifuged for 20 min at 13 000 g. After centrifugation the supernatant was dialyzed against the same buffer before a new centrifugation. The supernatant was used as the protein extract for myrosinase activity determination and for the SDS-polyacrylamide gel electroforesis.

Myrosinase and protein assays

Myrosinase activity was measured as the liberation of glucose with GOD-Perid assay as described by Bones and Slupphaug (1989). Protein was measured by the Bio-Rad protein reagent as described by the manufacturer using bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis and Western analysis

Samples were solubilized in standard SDS buffer prior to separation by SDS-PAGE using 7,5% polyacrylamide gels in Hoefer miniVe vertical electro-phoresis system. Biotinylated protein molecular weight marker (SP-1400, Vector) was used as standard. Gels were blotted to 0.45 μm nitrocellulose membrane (Bio-Rad) in Hoefers blot module as described by the manufacturer. Myrosinases were detected by the specific mouse anti-myrosinase 3D7 monoclonal antibody (Lenman et al., 1990) followed by rabbit anti-mouse Ig (P161; Dako, Glostrup, Denmark) conjugated with biotin and enhancement by streptavidin conjugated with horseradish peroxidase (Vector, SA-5004). The detection was visualized by a chemiluminescent detecting system (SuperSignal West Pico, Pierce).

Statistical treatment

The data has been analyzed applying general linear models (GLM) to ANOVA for nested or split plot designs. For number of aphids and for plant height, multivariate ANOVA with repeated measurement analyses have been applied. Effects of design, aphid species and plant genotype are all considered fixed, while trial within design and individual plants within plant genotype within cage are considered random effects. Where several mean squares are tested

against a common error, Bonferroni probabilities are applied (with n multiple comparisons/test, and probability p for the individual comparison/test, then the Bonferroni adjusted probability is n*p.).

Results

Selection of MINELESS seeds using ascorbic acid

Due to a limited number of *MINELESS B. napus* seeds available and the variable penetrance of no-myrosinase phenotypes in transgenic plants selection of seeds were needed. *MINELESS* seeds were germinated on filter paper watered with a high concentration of ascorbic acid (10 mM) in the dark. The *MINELESS* seeds showed different germination rate, and a correlation between germination order and myrosinase activity and expression was observed (Figure 2). The last germinating seeds showed no expression of myrosinase proteins in western analysis. Wild type seeds sown on ascorbic acid germinated more uniformly and the difference in myrosinase expression could not be correlated to order of germination. This way of selecting *MINELESS* seeds with reduced myrosinase activity was not completely reliable and a few seeds were clearly miss-selected. This was also evident in the western analysis of seedlings where these miss-selected seeds showed strong myrosinase expression. In series 2 the experiment was designed both to give aphids easier access to the plants and to optimize the difference in myrosinase activity. We therefore redesigned the chambers and selected *MINELESS* seeds by germination on ascorbic acid. By this ascorbic acid treatment we were able to obtained seedlings with low or no myrosinase activity.

Establishment of aphids

The specialist aphid *B. brassicae* showed strongest preference towards wild type seedlings in all trials. After six days only 31 % of the *B. brassicae* in average had settled on *MINELESS* seedlings. On the other hand, 66 % of the polyphagous aphid *M. persicae* had settled on

MINELESS seedlings. The structure of the experiment was changed in design 2 and this increased the difference between seedling preference for the specialist. *B. brassicae* had a 19 % decrease of infestation of *MINELESS* seedlings for design 2 compared to design 1. A slight but not significant decrease in preference towards *MINELESS* seedlings was also observed for *M. persicae* by the change in design**.** The difference in preference for seedling type by aphid species was significant ($P<0.001$). Percentage establishment of the two aphid species on different seedling type is shown in figure 3 for the two experimental designs used. In all trials *MINELESS* seedlings were more susceptible for aphid infestation compared to wild type seedlings regardless of aphid species ($P<0.01$).

The number of aphids that successfully infested developing seedlings varied greatly for the two aphid species used (Table 1). *M. persicae* had a lower mortality than *B. brassicae* when changing environment from the rearing chambers to the experimental chambers and seemed to adapt better to alterations in the environment. *B. brevicoryne* generally needed more time (24-36 h) than *M. persicae* (12 h) to find its host plants. Attempts to decrease this time by starving the aphids for 24 or 48 hours before introduction into the experimental chambers was tried without significant improvement (data not shown).

Myrosinase activity and myrosinase expression in wild type and MINELESS seedlings

Seedlings infested with aphids showed an increased myrosinase activity compared to control seedlings (Figure 4). The increase in activity was slightly higher with the generalist aphid in design 2. This was, however, not significant considering the two designs together, and this was reflected in the western analysis. The wild type control seedlings showed myrosinase activities below 200 $nmol \cdot min^{-1} \cdot mg^{-1}$. Infestation with aphids increased the activity up to 500 $nmol \cdot min^{-1} \cdot mg^{-1}$. The number of aphids that successfully infested single seedlings affected

the myrosinase activity (Table 2). Increasing numbers of the generalist aphid on single seedlings was correlated with increasing myrosinase activities. Seedlings infested with few specialist aphids also showed an increased myrosinase activity compared to control seedlings. But seedlings infested with more than 5 specialist aphids showed no significant increase in the activity compared to control seedlings. Thus, the specialist aphid seamed able to control the activation of the defense system, but this control was dependent on the establishment of many aphids on a single seedling.

The highest induction of myrosinase proteins after aphid infestation was seen in the *MINELESS* seedlings (Figure 5). Comparison of protein extracts from the two seedlings with the most and the least aphids in each trial, confirmed that the specialist and the generalist showed different preference for seedlings with high levels of myrosinase proteins. Most *B. brassicae* had settled on wild type seedlings that expressed high levels of myrosinase proteins, while *M. persicae* preferred *MINELESS* seedlings with a reduced level of myrosinase compared to the wild type (Figure 5).

Seedling height is affected by aphid infestation

Wild type seedlings were in average higher than *MINELESS* seedlings at the end of the experiments. In addition, both wild type and *MINELESS* seedlings infested with aphids showed a significant ($P<0.01$) reduced height compared to control seedlings (Figure 6). The final height was more affected by infestation with *M. persicae* ($P<0.01$) and there was a correlation between number of aphids and the reduction in plant height ($P<0.01$). As expected the significance was strongest at the end of the experiments.

Discussion

The idea of selection on ascorbic acid and in darkness was following from earlier observations. High concentration of ascorbic acid is known to reduce the myrosinase activity (Bones & Slupphaug, 1989). Likewise, seedlings grown in the dark also have a reduced myrosinase expression compared to light grown seedlings (Hasegawa et al., 2000). Analysis of the *MINELESS* seeds selected by delayed germination on ascorbic acid showed that the selection worked although it was not completely accurate. The few incorrect selected plants could easily be detected by an unexpectedly higher or lower number of aphids infesting them. The miss-selection could be proved by analysis of myrosinase expression and activity.

Our results provide direct support for the importance of myrosin cells and myrosinase activity in the defense against herbivores (Newman et al., 1992; Mitchell-Olds et al., 1996). The specialist and the generalist aphid responded differently to myrosinase variation in wild type and *MINELESS* seedlings. The glucosinolate profile of wild type and *MINELESS* seeds are identical (Borgen, Thangstad, Grønseth, Seem, Husebye, Rossiter and Bones, unpublished). The change in host plant selection is therefore likely due to the difference in myrosinase activity and the corresponding difference in capacity to produce glucosinolate hydrolytic products. The glucosinolate degradation products, mainly the isothiocyanates, are cues for plant localization and feeding stimulants for *Brassica* specialist herbivores (Bartlet et al., 1994; Rosa et al., 1997). As expected, the specialist preferred the wild type seedlings that had the highest natural emission of isothiocyanates, while the generalist preferred the *MINELESS* seedlings. The greater susceptibility for aphid infestation in *MINELESS* compared to wild type was also to be expected. The myrosinase-glucosinolate system is generally accepted to be a part of the defense system in Brassicaceae (Bones & Rossiter, 1996; Rask et al., 2000). Removal of the capacity to produce biological active degradation

products including both toxic compounds and volatiles with a potential role in signaling was expected to affect the interaction between plants and insects.

The difference in the myrosinase activity for wild type and *MINELESS* seedlings was more evident in design 2. Probably because of stronger emission of stimulating isothiocyanates, that increased the preference for wild type seedlings by the specialist. The generalist did not seem to be more repelled by wild type seedlings compared to design 1. Myrosinase activity has been reported to be of less importance to generalist (Li et al., 2000), and the differences in myrosinase activity may not have been large enough to influence *M. persicae* markedly. Nevertheless, western analysis showed that in trials most *M. persicae* settled on *MINELESS* seedlings that had a low level of myrosinase compared to wild type.

It should be noted that in experiments of longer duration than six days the observed difference in preference of host plant decreased (data not shown). This is due to developmental stage organ dependent activation of expression of other myrosinases of the MB class (Myr2 class) (Bones et al. 1994, Eriksson et al. 2001) The *MINELESS* plants have been design to selectively ablate myrosin cells of the seed, and most of the myrosinase enzyme system in other parts of the plant is intact.

Both MA and MB myrosinase proteins were induced in *MINELESS* upon aphid infestation. The cytotoxic construct with barnase in *MINELESS* seedlings affects myrosin cells with expression of the *Myr1.Bn1* promoter (MA promoter). These results might indicate that MA myrosinase proteins are expressed in more than one myrosin cell type. Downregulation of the Myr1.Bn1 promoter and thereby the barnase expression could be another possibility. If the MA is only in the same cell type this is not likely, as we would expect the MA itself also to be downregulated.

The relative induction of myrosinase activity and expression was much higher in *MINELESS* compared to wild type seedlings and indicates that there might be a threshold

level of myrosinase expression in *B. napus*. The levels of glucosinolates and myrosinase found in different *Brassica* plants have been correlated with costs for resistance (Mitchell-Olds et al., 1996).

As phloem-feeders, aphids use their stylets to penetrate leaves and stems until a suitable nutritional site (i.e. a sieve tube) is found. Results obtained in our lab has shown that while *M. persicae* apparently avoid penetrating myrosin cells while feeding, *B. brassicae* specifically targets myrosin cells (Husebye, Evjen, Rossiter, Bones, unpublished). In light of that, we expected to find a higher increase in the myrosinase activity in seedlings infested with the specialist. We did not find such a correlation but could observe a correlation between the number of aphids established on each seedling and the increase in myrosinase activity. This correlation was highly interesting for the specialist. When several specialist aphids were infested on a single seedling, they seemed able to prevent influencing the myrosinase activity for that particular seedling. This could be a strategy used by the specialist aphid, as high concentrations of isothiocyanates also have been demonstrated toxic against specialist insects, despite the attractiveness for feeding (Li et al. 2000). But how this could be achieved by specifically targeting myrosin cells is an open question. Glucosinolates have been shown to be sequestered by *B. brassicae* (Bridges et al., 2002), and a potential role for the myrosinase-glucosinolate system in the *B. brassicae* is suggested to be in generating isothiocyanates. *B. brassicae* has been shown to carry an endogenous myrosinase (Jones et al., 2001, 2002) that show activity towards the glucosinolates sinigrin and glucotropaeolin (Pontoppidan et al., 2001). Isothiocyanates can act as synergists for the alarm pheromone E-β-farnesene, a pheromone communicating warning to other members of the aphid colony (Dawson et al., 1987). Glucosinolates and myrosin cells are localized to different but neighboring cells in the plant (Kelly et al., 1998; Koroleva et al., 2000; Husebye et al., 2002). The spatial organization of glucosinolates and myrosinase in the aphid resemble that of the

plant, as the aphid myrosinase is compartmentalized into crystalline like microbodies (Bridges et al., 2002). The precise mechanism behind activation of the myrosinase-glucosinolate system in *B. brassicae* is still an open question. One way of avoiding the toxicity of sequestered secondary metabolites is seen in the neotropical butterfly *Heliconius sara*. *H. sara* can avoid the harmful effects of the cyanogenic leaves of *Passiflora auriculata* by metabolize the nitrile group of the cyclopentene ring to a thiol (Engler et al., 2000). Although speculative, *B. brassicae* may use an opposite strategy and inhibit the activity of the myrosinase enzymes in the plant. Targeting myrosin cells and reducing the enzyme activity may make the aphid able to feed in the phloem and to sequester glucosinolates without experience a high level of toxic compounds. Many aphids feeding on the same plant will over time probably affect the myrosinase activity in the whole plant. For the *B. brassicae* colony, triggering of the defense system by the first aphids to find a new feeding source may be advantageous. This will transmit positive signals to the specialist colony and warning signals to competing generalist insects. Once the colony is established, a lowering in the plant's myrosinase activity is favorable to avoid toxic levels and to avoid specialist predators that also are attracted by volatiles released by the myrosinase-glucosinolate system (Bartlet et al., 1999).

Small seedlings infested with a large colony of aphids may by time be severely weakened. The number of aphids on a single seedling influenced the seedling's heights, and infestation with *M. persicae* gave the shortest seedlings. *M. persicae* have been shown to spend more time in xylem ingestion than *B. brassica* and this may represent a mechanism for diluting toxic secondary plant compounds from the system of the generalist aphid (Cole, 1997). This would most probably influence the water transport and thereby the growth. Elongating of plant cells have be correlated with water supply. Cell expansion rates are mainly associated with how much water can be absorbed by elongating cells regardless of the

kinds of environmental stress condition applied (Ikeda et al., 1999). Aphids penetrating cells in both phloem and xylem may thus weaken the seedling more/or faster than aphids mainly feeding in the phloem.

In conclusion our results prove that ablation of myrosin cells of *Brassica napus* affect the behavior of insect pests. It seems evident that removing the myrosinase activity and thereby the production of glucosinolate degradation products increase the susceptibility of the plants. We therefore conclude that the myrosinase-glucosinolate system is a part of the defense system against insect pests. Based on the observation of a differential response in preference for the *MINELESS* and the wild type plants between the generalist and the specialist we propose that the glucosinolate degradation products are a major factor used for selection of host plant. The observations presented in this paper support the importance of the myrosinase-glucosinolate system in defense. *MINELESS* seedlings with a reduced myrosinase expression and thereby reduced production of toxic defense products were more susceptible for aphid attack in general and especially attractive for the generalist aphid. The interesting indications for the ability of *B. brevicoryne* to suppress the plant's defense response should be investigated further.

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Figure legends:

Figure 1. The hydrolysis of glucosinolates by the enzyme myrosinase.

Figure 2. Selection of *MINELESS* seeds using ascorbic acid. (A) Specific myrosinase activity for seeds correlated with germination order. (B) Western analysis of the same seed extracts as in A. 5 µg of total protein was applied in each lane. Myrosinase proteins were detected by the monoclonal anti-myrosinase antibody 3D7.

Figure 3. Percentage establishment of aphids on wild type and *MINELESS* seedlings after six days with infestation by aphids. (A) The mean of two trials with experiment design 1, n = 14. (B) The mean of two trials with experiment design 2, n = 10.

Figure 4. Specific myrosinase activity in wild type and *MINELESS* seedlings after six days with infestation by aphids. Error bars are SE of the activity between single plants in each design. n = 14 for design 1 and 10 for design 2 and control. (A) Seedlings infested by *B. brassicae*. (B) Seedlings infested by *M. persicae*.

Figure 5. Western analysis of wild type (Wt) and *MINELESS* (*M*) seedlings. Control is seedlings not introduced to aphids. The lane representing the plant with most aphids in a trial is marked by an asterisk. Lanes without asterisk represent the plant with least aphids in a trial. Myrosinase proteins were detected by the monoclonal anti-myrosinase antibody 3D7. 5 µg of total protein was applied in each lane

Figure 6. The mean final height of seedlings from all trials. Error bars are SE of the height between individual seedlings. n = 24 for seedlings infested by aphids, n = 10 for seedlings not introduced to aphids.

Figure 1.

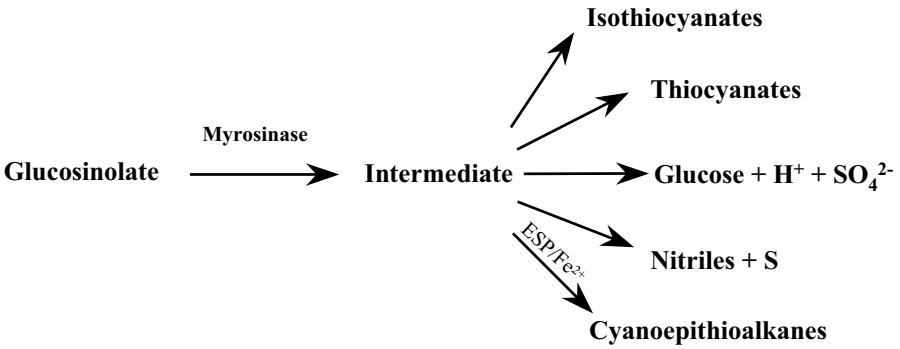

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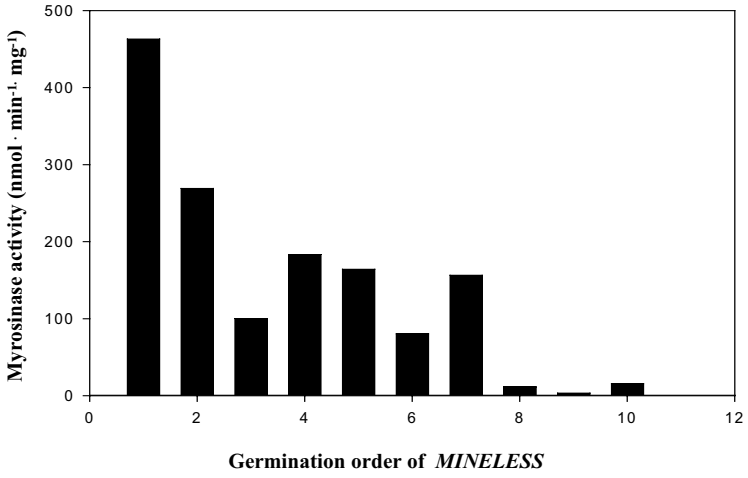
Figure 2.

A



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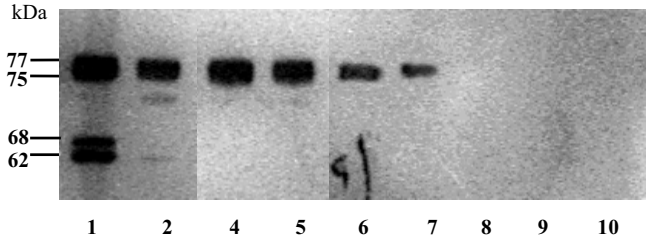

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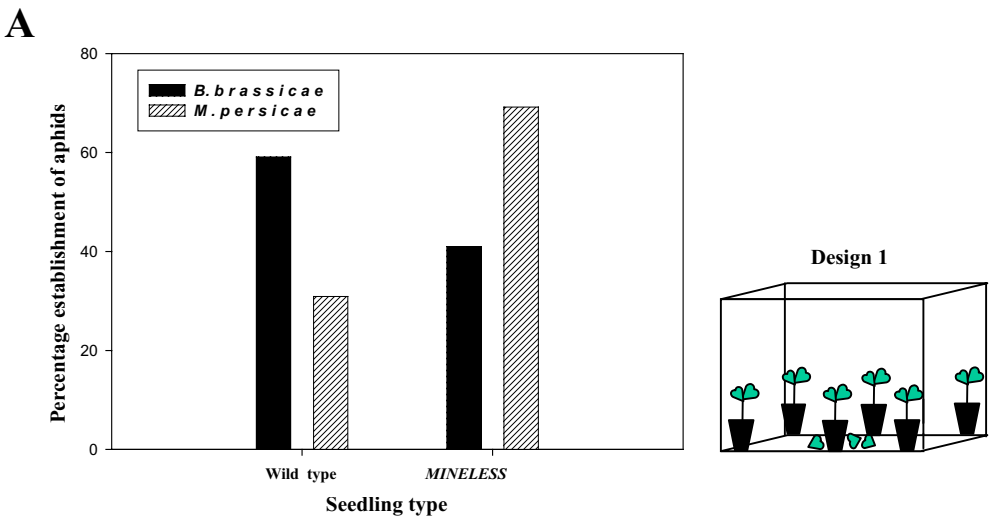
Figure 3.



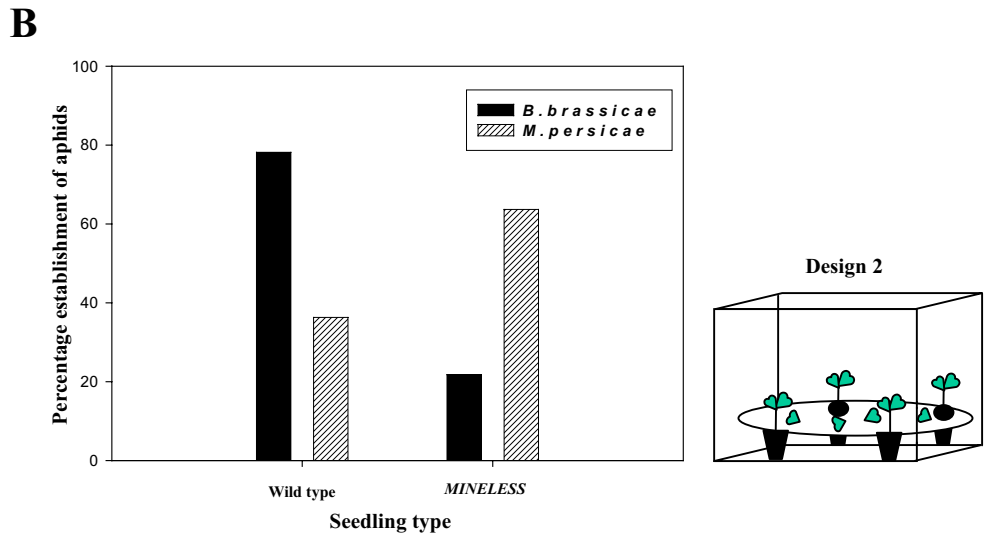


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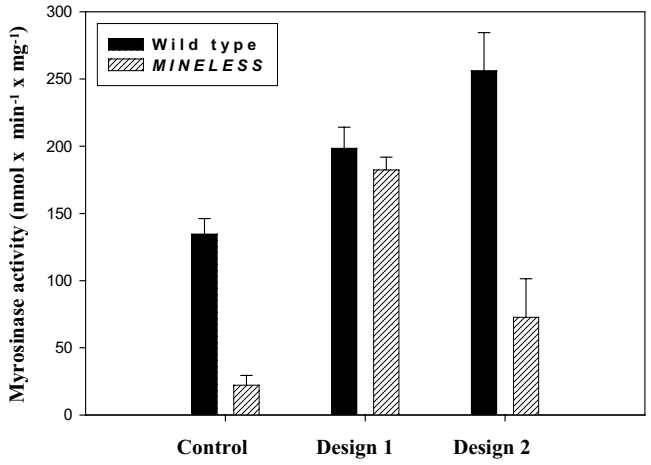
Figure 4.

A



B

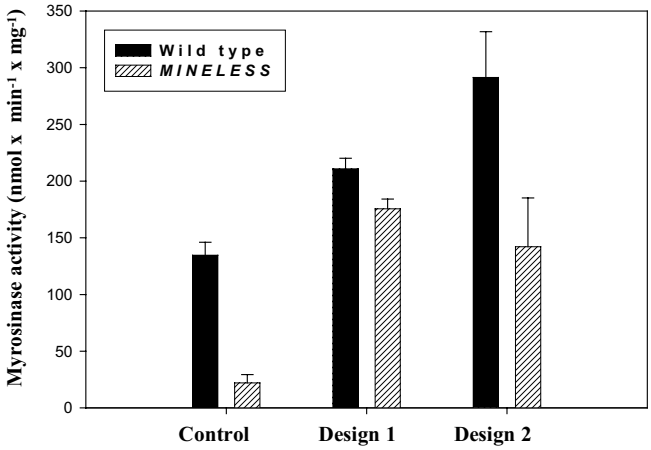


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Figure 5.

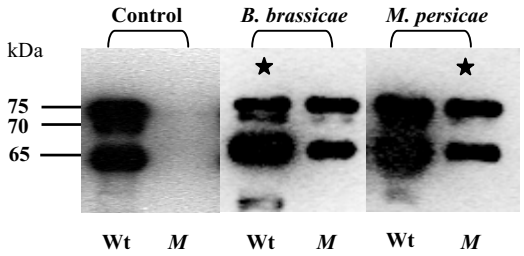

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Figure 6.

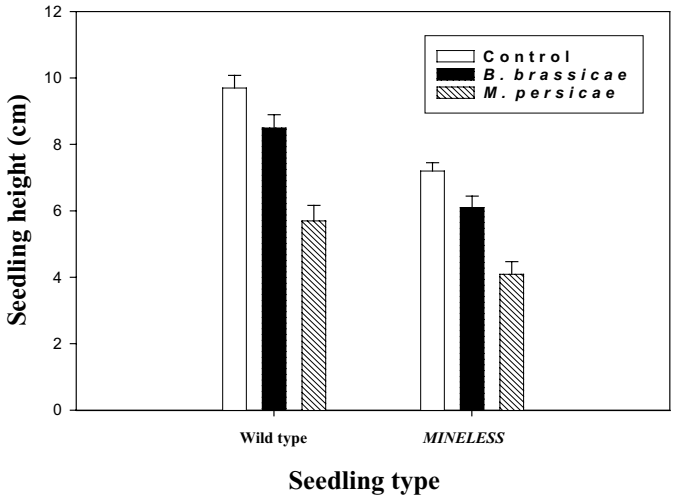

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Table 1. Mean number of aphids established on seedlings.

	B. brassicae	*M. persicae*
Trial 1	152	273
Trial 2	29	50
Trial 3	19	243
Trial 4	21	183

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Table 2. Specific myrosinase activity related to number of aphids on single seedlings

	Control No aphids	***M. persicae*** ≤ 30 aphids	***B. brassicae*** 1-5 aphids	***B. brassicae*** 6-15 aphids
($nmol \cdot min^{-1} \cdot mg^{-1}$)	< 200	≤ 500	> 200	≤ 200

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