

RESEARCH PAPER

Removing the mustard oil bomb from seeds: transgenic ablation of myrosin cells in oilseed rape (*Brassica napus*) produces *MINELESS* seeds

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Abstract

Many plant phytochemicals constitute binary enzyme–glucoside systems and function in plant defence. In brassicas, the enzyme myrosinase is confined to specific myrosin cells that separate the enzyme from its substrate; the glucosinolates. The myrosinase-catalysed release of toxic and bioactive compounds such as isothiocyanates, upon activation or tissue damage, has been termed ‘the mustard oil bomb’ and characterized as a ‘toxic mine’ in plant defence. The removal of myrosin cells and the enzyme that triggers the release of phytochemicals have been investigated by genetically modifying *Brassica napus* plants to remove myrosinase-storing idioblasts. A construct with the seed myrosin cell-specific *Myr1.Bn1* promoter was used to express a ribonuclease, barnase. Transgenic plants ectopically expressing barnase were embryo lethal. Co-expressing barnase under the control of the *Myr1.Bn1* promoter with the barnase inhibitor, barstar, under the control of the cauliflower mosaic virus 35S promoter enabled a selective and controlled death of myrosin cells without affecting plant viability. Ablation of myrosin cells was confirmed with light and electron microscopy, with immunohistological analysis and immunogold-electron microscopy analysis showing empty holes where myrosin cells normally are localized. Further evidence for a successful myrosin cell ablation comes from immunoblots showing absence of myrosinase and negligible myrosinase activity, and autolysis experiments showing negligible production of glucosinolate hydrolysis products. The plants where the myrosin defence cells have been ablated and named ‘*MINELESS* plants’. The epithiospecifier protein profile and glucosinolate levels were changed in *MINELESS* plants, pointing to localization of myrosinases and a 35 kDa epithiospecifier protein in myrosin cells and a reduced turnover of glucosinolates in *MINELESS* plants.

Key words: Anti-nutritional factors, *Brassica*, epithiospecifier protein, gene, glucosinolate–myrosinase system, GMO, metabolic engineering, myrosin cell, myrosinase, transgenic ablation.

Introduction

Glucosinolates (GSLs) are a group of allelochemicals that are present in the order Capparales, containing the Brassicaceae family (Sørensen, 1990; Bellostas *et al.*, 2007). GSLs are hydrolysed by myrosinase (MYR) upon plant tissue rupture and used as defence chemicals against

herbivores. GSLs are nitrogen- and sulphur-containing secondary metabolites that share a core consisting of a β -thioglucoside moiety and a sulphonated oxime, but differ by a variable side chain derived from one of several amino acids (Kliebenstein *et al.*, 2001; Mithen, 2001a; Grubb and

Abel, 2006; Tripathi and Mishra, 2007; Yan and Chen, 2007).

The enzyme MYR (EC 3.2.1.147) is a β -thioglucosidase that hydrolyses GSLs to a variety of products such as isothiocyanates, thiocyanates, nitriles, epithionitriles, and oxazolidine-thiones depending on the nature of the GSL, reaction conditions, and the presence of protein cofactors (Bones and Rossiter, 1996, 2006; Rask *et al.*, 2000). These protein cofactors include epithiospecifier protein (ESP), nitrile-specifier proteins (NSPs), and thiocyanate-forming protein (TFP) which in turn depend on ferrous/ferric ions (Bones and Rossiter, 1996; Foo *et al.*, 2000; Lambrix *et al.*, 2001; Burow *et al.*, 2007a; Kissen and Bones, 2009).

MYRs are present in specialized cells known as 'myrosin cells' (Bones and Iversen, 1985; Thangstad *et al.*, 2004; Kissen *et al.*, 2009) that are dispersed throughout plant tissues. MYR is present in palisade parenchyma, phloem idioblasts, guard cells, and ground tissue cells (Bones and Iversen, 1985; Bones *et al.*, 1991; Andreasson *et al.*, 2001). Immunocytochemical and *in situ* hybridization studies carried out on seeds of Brassicaceae have shown MYR to be exclusively present in myrosin cells of embryonic cotyledons and the radicle periphery (Thangstad *et al.*, 1990, 1991; Bones *et al.*, 1991; Höglund *et al.*, 1992; Geshi and Brandt, 1998; Kelly *et al.*, 1998; Husebye *et al.*, 2002; Kissen *et al.*, 2009).

The localization of GSLs is still unclear, although some studies have shown sinigrin to be localized in all cells except myrosin cells of *Brassica juncea* seeds (Kelly *et al.*, 1998), and radiolabelled GSLs appear to be transported to specific cells (Thangstad *et al.*, 2001). In *Arabidopsis thaliana* flower stalks, GSLs are thought to be present in S-cells (sulphur-rich cells) (Koroleva *et al.*, 2000) and are neighbours to the scattered idioblast cells containing MYR, which have been named myrosin phloem cells (Husebye *et al.*, 2002). The MYR genes in *Brassica napus* can be divided into three subfamilies, MA, MB, and MC (Xue *et al.*, 1992; Falk *et al.*, 1995). There are in total ~25 MYR-encoding genes in *B. napus*, two of which have been characterized and cloned, and which are members of MYR subfamilies MA (*Myr1.Bn1*) and MB (*Myr2.Bn1*), respectively (Lenman *et al.*, 1993; Thangstad *et al.*, 1993). *Myr1.Bn1* is a myrosin cell-specific gene which displays a highly specific expression in seed myrosin cells. The expression from its promoter has been shown to be restricted to this cell type (Thangstad *et al.*, 2004).

The ESP has been described as a MYR cofactor that drives the hydrolysis of alkenyl GSLs such as progoitrin, sinigrin, and gluconapin towards the production of epithionitriles (cyanoepithioalkanes) and alkyl GSLs towards nitriles instead of other possible hydrolysis products (MacLeod and Rossiter, 1985; Bernardi *et al.*, 2000; Foo *et al.*, 2000; Lambrix *et al.*, 2001; Kissen and Bones, 2009). Ferrous/ferric ions and MYR together with the ESP are essential for epithionitrile formation (MacLeod and Rossiter, 1987; Zabala *et al.*, 2005; Burow *et al.*, 2006). In *B. napus*, two isoforms of ESP polypeptides with molecular masses of 39 kDa and 35 kDa have been purified, partially sequenced,

and characterized (Bernardi *et al.*, 2000; Foo *et al.*, 2000). Two anti-ESP antibody-reactive bands of 37 kDa and 43 kDa have also been observed in broccoli and cabbage (Matusheski *et al.*, 2006).

Oilseed rape meal is a high protein feed for livestock. It contains the GSLs progoitrin or epiprogoitrin, gluconapin, and glucobrassicinapin, and has mostly been used in ruminants. Intact GSLs can serve as contact cues for feeding or oviposition stimulation (Halkier and Gershenzon, 2006). Moreover, MYR activity is also considered to be important for plant defence against specialist insects that have adapted themselves to intact GSLs, but less important for defence against generalists, which are susceptible to intact GSLs (Li *et al.*, 2000).

GSLs are themselves biologically inactive, but GSL hydrolytic products (thiocyanates, isothiocyanates, nitriles, and oxazolidine-2-thione) produced by the enzyme MYR during processing of oilseed rape meal are biologically active (Mawson *et al.*, 1993). The thiocyanates interfere with iodine availability, whereas 5-vinyl-1,3-oxazolidine-2-thione is responsible for the morphological and physiological changes of the thyroid and leads to goitre formation. Nitriles are known to affect liver and kidney functions (Elfving, 1980; Mithen, 2001b; Bellostas *et al.*, 2007; Tripathi and Mishra, 2007). Thus it is important to devise technologies that prevent the hydrolysis of GSLs in ruminants as an alternative to lowering the concentration of GSLs.

The MYR-catalysed release of toxic and bioactive compounds such as isothiocyanates by hydrolysing GSLs, upon activation or tissue damage, has been termed 'the mustard oil bomb' (Matile, 1980; Bones and Rossiter, 1996). The biological/ecological significance of the GSL–MYR system has been the subject of interest for several years, has been analysed to a large extent, and reviewed (Rask *et al.*, 2000; Kliebenstein *et al.*, 2005; Hopkins *et al.*, 2008; Müller, 2009; Textor and Gershenzon, 2009). An ecological role for 'the mustard oil bomb' from seeds has been studied by analysing the GSL–MYR defence system in *B. juncea* cotyledons during seedling development in defence against the generalist herbivore, *Spodoptera eridania* (Wallace and Eigenbrode, 2002), by testing the seed nutritional quality against the yellow meal worm/common beetle generalist (*Tenebrio molitor*) (Davis and Sosulski, 1974; Davis *et al.*, 1981, 1983; Eriksson *et al.*, 2002), and as an allelochemical in *Brassica nigra* (Lankau and Strauss, 2007).

The objective of this study was to produce transgenic *B. napus* plants with seeds that lack myrosin cells. Ablation of cells and tissue by the controlled expression of lethal genes has been performed previously, but its widespread success has often been limited by secondary effects on non-targeted tissue. Genetic ablation studies in plants have focused on engineering of male and female sterility, blocking anther dehiscence and sexual reproduction in, for example, tobacco, tomato, wheat, and populus trees, and genetic ablation of flowers in *A. thaliana* (Goldman *et al.*, 1994; Beals and Goldberg, 1997; De Block *et al.*, 1997; Nilsson *et al.*, 1998; Goetz *et al.*, 2001; Roque *et al.*, 2007; Wei

et al., 2007; Wang *et al.*, 2008). The objective of this study was to produce transgenic *B. napus* plants with seeds that lack myrosin cells using a genetic ablation strategy. The very first genetic cell ablation strategy induced male sterility in *B. napus* with the barnase gene regulated by the tapetum-specific TA 29 promoter (Mariani *et al.*, 1990). Barnase is a 110 amino acid extracellular potent RNase that is secreted by the bacterium *Bacillus amyloliquefaciens* and that is used as a digestive enzyme for nutritional purposes or/and as a defence toxin. Barstar is an 89 amino acid intracellular inhibitor of barnase that is produced constitutively by the bacterium. Barstar binds specifically to barnase, forming inactive barnase–barstar complexes (Hartley, 1989).

In the present study, the *Myr1.Bnl* gene promoter was used for this purpose, because *Myr1.Bnl* expression has been shown to be restricted to myrosin cells (Thangstad *et al.*, 2004). The expression of cytotoxic barnase driven by the *Myr1.Bnl* gene promoter resulted in controlled cell death of myrosin cell idioblasts. Not unexpectedly, the expression of barnase only (*pMyr1.Bnl:Barnase*) was embryo lethal. Due to the very toxic nature of barnase, barstar has often been co-expressed under the control of a constitutive promoter, to avoid plant lethality. It has also been shown that the co-expression of barstar under the control of a constitutive cauliflower mosaic virus (CaMV) promoter 35S leads to the ablation of myrosin cells without secondary effects in seeds. Controlled cell death (ablation) of myrosin cells produced *MINELESS* seeds—seeds with a dramatic reduction of MYR-containing toxic mines. The genetic ablation was successfully achieved using the promoter constructs *pMyr1:Barnase* in combination with *35S:Barstar*.

Materials and methods

Genetic constructs and molecular methods

The sequence of the *Myr1.Bnl* gene is given in GenBank (accession Z21977.3). The cloning procedure of the *Myr1.Bnl* promoter is as described by Thangstad *et al.* (2004). Standard molecular biology methods were employed (Sambrook *et al.*, 1989). *Escherichia coli* DH5 α (Bethesda Research Laboratories), JM109 (Promega, Madison, WI, USA), and MX1061 (Plant Genetic Systems, Ghent, Belgium) 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, La Jolla, CA, USA) and pGEM3, 5, and 11 (Promega) were used for subcloning. Briefly, the procedure for cloning is as follows. A *SalI*–*EcoRI* 1142 bp fragment containing the partial *Myr1.Bnl* promoter, the barnase-encoding gene (Mariani *et al.*, 1990), and the *Nos* terminator (Depicker *et al.*, 1982) was constructed in several cloning steps and inserted into pBI101.1, between the *SalI* and *EcoRI* restriction sites of the polylinker. The resulting plasmid was cut with *HindIII*–*SalI* and the remaining 2520 bp of the *Myr1.Bnl* promoter inserted utilizing the internal *SalI* site to obtain a binary plasmid carrying the full-length promoter, linked to the barnase gene and a *Nos* terminator, the *pMyr1.Bnl:Barnase* construct (Fig. 1A). To generate the *pMyr1.Bnl:Barnase:Barstar* plasmid construct (*Myr-BarnBar*=*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 *pMyr1.Bnl* promoter fragment inserted, giving rise to a plasmid containing the full-length *MYR Myr1.Bnl* promoter, barnase, *Nos* terminator, and CaMV35S:Barstar:3'g7 terminator (Fig. 1B). The constructs shown were verified by restriction digests and sequencing. The two constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404 (Clontech, Palo Alto, CA, USA) by electroporation and used to transform *B. napus*.

Production and selection of transgenic *Brassica napus* plants

Transformation of *B. napus* was performed essentially as described by Moloney *et al.* (1989). Seeds of *B. napus* cv. Westar were surface-sterilized in 1% sodium hypochlorite for 20 min, washed in sterile water three times, and planted in jars containing MS medium (pH 5.8) (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.8% agar gel (Sigma). Seeds were then germinated under controlled conditions at 22 °C in a 16 h light/8 h dark photoperiod and at a light intensity of 70–80 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The cotyledons with the cotyledonary petioles from 5-day-old seedlings were used as the explants for transformation using *A. tumefaciens* LBA4404 containing the binary plasmid constructs *pMyr1.Bnl:-Barnase* and *pMyr1.Bnl:Barnase:Barstar*. After co-cultivation, the selection of transformants was done on the basis of kanamycin resistance from the NPTII gene in the constructs. After regeneration of transgenic shoots, the verification of inserted constructs was conducted using PCR with genomic DNA as the template and specific primers for the inserted genes (barnase and barstar) essentially as described by Strittmatter *et al.* (1995). After repetitive transfers and hormone-induced rooting, plants (designated T₀) were transferred to soil and grown in controlled-environment rooms. Plants were allowed to self-pollinate and flowers were covered with paper bags. At maturity, the siliques were harvested and seeds (designated T₁) were collected from three transgenic lines of the *pMyr1.Bnl:Barnase:Barstar* construct and were verified by MYR activity and quantitative real-time PCR (qRT-PCR). Only one line from the *pMyr1.Bnl:Barnase* construct was obtained, and due to embryo lethality seeds could not be further characterized.

MYR activity and protein assays

The MYR activity was measured using the GOD-Perid assay (Bones and Slupphaug, 1989), by measuring the amount of glucose liberated from hydrolysis of the GSL sinigrin. The assay was performed using citrate buffer (50 mM, pH 5.5), sinigrin (15 mg ml⁻¹), and GOD-Perid reagent (Roche, Basel, Switzerland). In order to calculate specific MYR activity, the total protein content of samples was also measured using Bradford reagent (BioRad Laboratories, UK). The specific MYR activity is described as nmol glucose min⁻¹ mg⁻¹ protein.

RNA extraction and qRT-PCR analysis

QRT-PCR was performed to verify the expression of the transgenes in the three selected lines of *MINELESS* (*pMyr1.Bnl:Barnase:Barstar*). Frozen cotyledons from the wild type (Westar) and transgenic (*MINELESS*) lines were homogenized in pre-cooled (–80 °C) 2.0 ml tubes containing 5 mm stainless steel beads for 2 min at full speed using a TissueLyszer homogenization device (Qiagen, Valencia, CA, USA). Total RNA was extracted with a Spectrum Plant Total RNA Kit (Sigma-Aldrich), additionally treated with on-column DNase (RNase-free DNase Set, Qiagen), and RNasin[®] ribonuclease inhibitor (1 U μl^{-1}) (Promega Corporation, Madison, WI, USA) was added. The RNA was quantified using NanoDrop ND 1000 (Nanodrop Technologies, Wilmington, DE, USA) and analysed by formaldehyde–agarose gel electrophoresis. The cDNA was synthesized from 1 μg of total RNA using

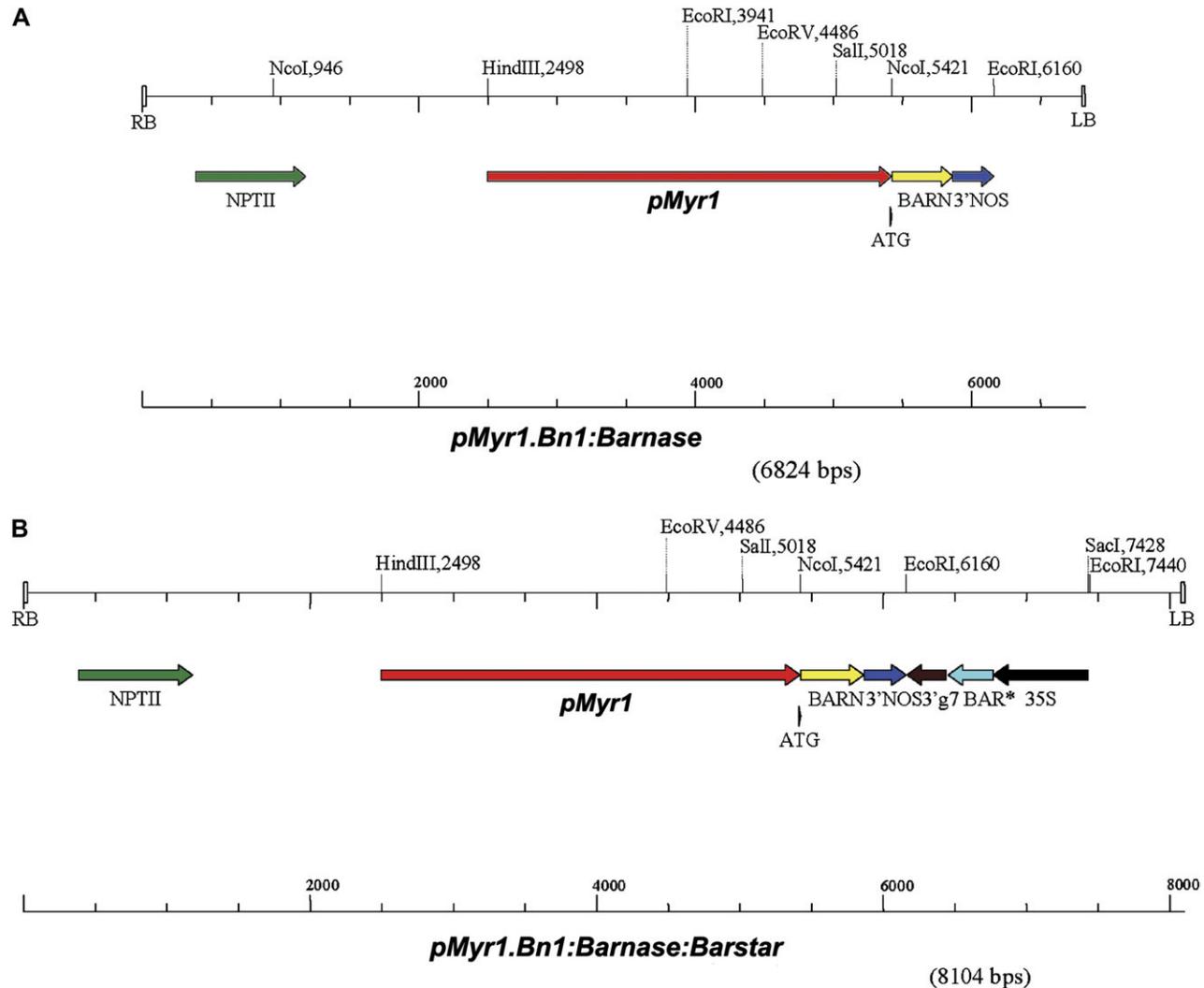


Fig. 1. Map of the myrosinase promoter (*pMyr1* as a *HindIII*–*NcoI* 2923 bp fragment) transformation constructs in pBI101. (A) *Myr1.Bn1* promoter: Barnase fusion (Barnase:3'NOS as a *NcoI*–*EcoRI* fragment). (B) *Myr1.Bn1* promoter: Barnase–35S: Barstar (35S: Barstar *EcoRI*–*EcoRI* as an orientation-selected insert) double fusion used to ablate myrosin cells in *Brassica napus* seeds. LB, left border; RB, right border; 3'NOS, nopaline synthase terminator; *NPTII*, kanamycin selection; 3'g7, g7 terminator; BARN, barnase gene; BAR*, barstar gene; 35S, CaMV promoter, restriction sites, and total size (bp) of the constructs. Arrows denote transcriptional orientation.

the QuantiTect® Reverse Transcription Kit (Qiagen). The sequences for gene-specific primers used in qRT-PCR are given in Supplementary Table S1 available at *JXB* online. The qRT-PCR was carried out using the LightCycler 480 SYBR Green I Master Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Each 20 µl reaction contained 0.5 µM of each forward and reverse primer, and a cDNA quantity corresponding to 0.05 µg of total RNA. PCR was performed in a LightCycler 480 (Roche, Basel, Switzerland) as follows: (i) 5 min at 95 °C; (ii) 45 amplification cycles; 95 °C for 10 s, 55 °C for 15 s (*Barstar* and *ESP*), or 53 °C for 15 s (*MYR*); 72 °C for 11 s; and (iii) 95 °C for 5 s, 65 °C for 1 min and 97 °C continuously for analysis of dissociation curves. Cp values and melting curves were calculated by the LightCycler 480 analysis programs using the second derivative maximum method. The PCR efficiency was determined employing LinReg PCR (Ramakers et al., 2003) and the relative expression ratios were calculated by using the Relative Expression Software Tool (REST) 2005 V.1.9.12 (Pfaffl et al., 2002). The RNA amount per sample was normalized using *Allene oxide synthase* as a reference gene.

MINELESS seed screening to select seeds for structural analysis of myrosin cells

To proceed with the structural analysis of myrosin cells through light, fluorescence, and transmission electron microscopy and GSL quantification, wild type and *MINELESS* seeds were plated on moistened filter paper-lined Petri dishes and were kept imbibed for 4 h at room temperature. To select *MINELESS* seeds with low or negligible MYR activity, a fast version of the GOD-Perid assay was performed as follows. After removal of seed coats from imbibed seeds, one cotyledon from a single seed was crushed with a solution of citrate buffer (50 mM, pH 5.5) and sinigrin (15 mg ml⁻¹), GOD-Perid (750 µl) was then added and after an incubation of 5 min the development of green colour indicated MYR activity. The other cotyledons from *MINELESS* single seeds (i.e. seeds that gave no green colour/very light green colour in the GOD-Perid assay) were further processed for structural analysis of myrosin cells as described below. In parallel, Westar wild type seeds were also run to see the colour differences between the wild type and *MINELESS* seeds.

Light, fluorescence, and transmission electron microscopy for structural analysis of myrosin cells

Cotyledons of imbibed seeds of *MINELESS* seeds and wild type seeds were fixed, dehydrated, and embedded as described by Thangstad *et al.* (1991) with 6 d of infiltration with LR-White. This material was further sectioned into 1 μm and 600–700 \AA thick sections to observe myrosin cells under a light microscope and a transmission electron microscope. For light microscopy, semi-thin sections were stained with toluidine blue. Slides were examined and photographed with a research microscope (Eclipse 800; Nikon, Tokyo, Japan), equipped with a digital camera (SPOT RT; Diagnostic Instruments, Burroughs, MI, USA). Semi-thin sections were used for the detection of MYR with the polyclonal antibody K089 (Thangstad *et al.*, 1991). Positive cells were visualized with fluorescein isothiocyanate (FITC)-conjugated streptavidin (DAKO, Glostrup, Denmark). The immunogold labelling of thin sections was carried out as described in Thangstad *et al.* (1991) with minor modifications. The goat anti-rabbit secondary antibody (Amersham, Buckinghamshire, UK) was conjugated with 15 nm colloidal gold, and the post-embedding on-grid osmium staining was performed with 2% osmium tetroxide. The sections were examined and micrographs were taken with a Jeol 1200 EX (Japan) electron microscope at 60 kV.

Extraction of MYR and MYR-binding proteins (MBPs)

Single seeds and 5-day-old cotyledons of wild type and transgenic (*MINELESS*) lines were extracted in 100 μl of imidazole-HCl buffer (10 mM, pH 6.0). Single seeds were defatted before extraction with *n*-hexane and the pellet was air dried. The samples were centrifuged at 4 $^{\circ}\text{C}$, and supernatants were transferred to dialysis membranes (12 000–14 000 Da MWCO) (Spectra/Por), dialysed overnight in imidazole-HCl buffer, centrifuged at 4 $^{\circ}\text{C}$, and used for MYR activity assay and protein assays.

For the analysis of expression of MBPs, another set of single seeds was ground in liquid N_2 , transferred to 100 μl of imidazole-HCl buffer (10 mM, pH 6.0) containing phenylmethylsulphonyl fluoride (PMSF; 1 mM), and incubated for 30 min at 4 $^{\circ}\text{C}$ before centrifugation and sampling of the supernatant.

The MYR and MBP extracts were further processed for SDS-PAGE and immunoblot analysis.

SDS-PAGE and immunoblot analysis

A 10 μg aliquot of protein samples was solubilized in standard SDS buffer prior to separation by SDS-PAGE using 7.5% and 12% polyacrylamide gels in a Hoefer miniVe vertical electrophoresis system. For immunoblotting, gels were blotted onto a 0.45 μm nitrocellulose membrane (Bio-Rad) in Hoefers blot module as described by the manufacturer. MYRs and MBPs were detected by the 3D7 and 34:14 monoclonal antibodies, respectively (Lenman *et al.*, 1990). The detection was visualized using a chemiluminescent detecting system (Super Signal West Pico, Pierce, Rockford, IL, USA).

ESP expression analysis and ESP activity assay through gas chromatography–mass spectrometry (GC-MS)

Wild type and *MINELESS* single seeds were extracted in 0.6 ml of imidazole-HCl buffer (100 mM, pH 6.8). A part of the extract from single seeds was used for determination of MYR activity by GOD-Perid assay, separation by SDS-PAGE, and immunoblot analysis. Immunoblot analysis was carried out as described above, and the ESP was detected by the anti-ESP polyclonal antibody characterized earlier (Foo *et al.*, 2000). The rest of the extract was used for ESP activity assay. To proceed with the ESP activity

assay, the extract was subdivided into two parts. One part was incubated in an assay mixture containing ferrous ions (0.6 mM ferrous ammonium sulphate), dithiothreitol (DTT; 1 mM), sinigrin (14 mM), and MYR (50 μl from an affinity-purified preparation from *Sinapis alba* seed with the ability to hydrolyse 865 μmol sinigrin min^{-1} as tested by GOD-Perid), while in the other part MYR was omitted. 1-Dodecanol was used as an internal standard. After 4 h incubation at 30 $^{\circ}\text{C}$, the mixtures were extracted twice with dichloromethane and the combined organic phases dried with anhydrous MgSO_4 and concentrated using a flow of nitrogen gas. ESP activity was determined by GC-MS on a Varian Star 3400 CX gas chromatograph coupled with a Varian Saturn 3 mass spectrometer. A polar column, Chrompack CP-Wax 52CB (30 $\text{m} \times 0.32 \text{ id} \times 0.25 \mu\text{m}$ film thickness), was used for all separations. The carrier gas flow (He) was held at 50 ml min^{-1} (injector) and 30 cm s^{-1} (column). The injector was kept at 200 $^{\circ}\text{C}$ (split injection), and the GC temperature program was held at 35 $^{\circ}\text{C}$ for 1 min, followed by 40–210 $^{\circ}\text{C}$ at a rate of 7 $^{\circ}\text{C min}^{-1}$. The MS detector was set at 200 $^{\circ}\text{C}$, and a mass range of m/z 40–300 was recorded (EI mode at 70 eV). GSL hydrolysis products were tentatively identified by using a mass spectra database search (NIST MS Database, 1998), and by comparing with mass spectra from the literature (Spencer and Daxenbichler, 1980).

GSL analysis by high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS)

Wild type and *MINELESS* seeds were cut into two parts, with one part used for GSL analysis and one part for MYR activity assay. After recording the dry weight, half of the seed was extracted with 3 \times 1 ml methanol (70%) at 70 $^{\circ}\text{C}$. The extracts were heated at 70 $^{\circ}\text{C}$ in a heated block (Techne, Dry-Block, DB-2P) for 10 min. After cooling, the samples were centrifuged at 15 000 g for 10 min at 4 $^{\circ}\text{C}$. The supernatants, stored on ice between the three extractions, were then combined and filtered through 0.45 μm filters (Millipore Corporation, Bedford, MA, USA). GSLs were desulphonated on a DEAE-A25 Sephadex ion exchange column (Amersham Biosciences, Uppsala, Sweden) supplemented with sulphatase (50 μl , Type H-1, from *Helix pomatia*, Sigma, St Louis, MO, USA) that was prepared as described by Graser *et al.* (2001). Benzyl GSL was used as an internal standard. The desulpho-GSLs were freeze-dried (Virtis Benchtop), reconstituted in water (100 μl), and analysed by HPLC (Agilent HP 1100 Series). Reverse-phase HPLC was performed on a Supelcosil LC 18 (250 $\text{mm} \times 2.1 \text{ mm}$, 5 μm spherical particles) Supelco column (Bellefonte PA, USA). The mobile phases were: (A) deionized water and (B) acetonitrile. The following gradient was used: 0–2 min, 3% B; 2–17 min, 3–40% B; 17–22 min, 40% B; 22–22.10 min, 40–100% B; 22.10–32 min, 100% B; 32–32.10 min, 100–3% B; 32.10–50 min, 3% B. The flow rate was 0.3 ml min^{-1} with UV detection at 229 nm. The injection volume was 50 μl . The LC-MS analysis was performed using a HPLC-DAD (Agilent HP 1100 Series) coupled to an Agilent 1100 Series LC/MSD trap mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with an APCI interface. The instrument was configured in positive ion chemical ionization. The column, solvents with gradient, flow rate, and injection volume were the same as described above for HPLC. The APCI settings were: nebulizer pressure, 60 psi; drying gas flow, 5 l min^{-1} ; drying gas temperature, 350 $^{\circ}\text{C}$; APCI vap temperature, 400 $^{\circ}\text{C}$; corona current, 4000 nA; capillary voltage, 3500 V; compound stability; 100%; trap drive level, 100%. The individual GSLs were identified by diode array UV spectra and mass spectra. The identity of the compounds was confirmed by comparison with retention times, UV spectra, and MS spectra of authentic standard desulpho-GSLs (mixture of isolated *B. napus* desulpho-GSLs, BIORAF foundation, Denmark). The correction factors at 229 nm from Brown *et al.* (2003) and Daun and McGregor (1991) were used to calculate the concentration of the GSLs.

Results

Selection of transgenic plants

Three out of four kanamycin-resistant T₀ lines of *pMyr1.Bn1:Barnase* (Fig. 1A) amplified the expected 160 bp PCR product from the inserted barnase gene (Strittmatter *et al.*, 1995), and were transferred to soil. Only one of these plants developed to maturity. Three out of six kanamycin-resistant T₀ lines of *pMyr1.Bn1:Barnase:Barstar* (Fig. 1B) were verified positive, amplified the expected 160 bp product from barnase and the 235 bp product from barstar (Strittmatter *et al.*, 1995; data not shown), and were transferred to soil.

The morphological appearance of *pMyr1.Bn1:Barnase* and *pMyr1.Bn1:Barnase:Barstar* transgenic (T₀) lines showed major differences. The single transgenic *pMyr1.Bn1:Barnase* line obtained after transfer to soil produced a viable plant, but no viable seeds. The majority of the embryos were terminated during silique development, and showed necrosis and altered morphology. Due to this embryo lethality, viable seeds could not be further characterized. Transgenic lines with the genetic construct *pMyr1.Bn1:Barnase:Barstar* had a close to normal morphological phenotype, where flowering was unaffected and viable seeds were produced.

Transgenic lines show barstar expression, low MYR activity, and down-regulation of GSL metabolism genes

To confirm the production of transgenic *B. napus* plants, 5-day-old cotyledons of three different transgenic T₁ lines of *pMyr1.Bn1:Barnase:Barstar* were analysed for barstar expression. All three transgenic lines showed barstar expression, while no barstar expression was detected in wild type Westar as verified by qRT-PCR (data not shown).

Seeds from three transgenic T₁ lines of *pMyr1.Bn1:Barnase:Barstar* were analysed for MYR activity by enzymatic glucose release using sinigrin as a substrate. All tested seeds ($n=7-8$) of transgenic lines TL1 and TL2, and five out of seven seeds of TL3, had lower MYR activity than the lowest MYR activity value of the wild type. Moreover, 5-day-old cotyledons of three transgenic lines and the wild type showed significantly low MYR activity (nmol glucose min⁻¹ mg⁻¹ protein) in all three transgenic lines as compared with wild type Westar (Supplementary Fig. S1B at *JXB* online).

The gene expression of GSL metabolism genes *Myr1* and *ESP* by qRT-PCR showed significant down-regulation in 5-day-old cotyledons of three transgenic lines as compared with the wild type (Supplementary Fig. S2 at *JXB* online). The *Myr1* and *ESP* genes showed down-regulation with log₂ ratios of -3.8, -5.3, and -2.4, and -3.4, -4.1, and -2.6, respectively, in three transgenic lines (TL1, TL2, and TL3) (P -values: *Myr1* <0.01, 0.00, 0.05; and *ESP* <0.05, 0.01, 0.00).

The transgenic line (TL1) that showed low MYR activity for both seeds (30.4 nmol glucose min⁻¹ mg⁻¹ protein) and cotyledons (7.9 nmol glucose min⁻¹ mg⁻¹ protein) was selected for further characterization. These seeds were

termed *MINELESS*, as the ‘toxic mines’ (myrosin cells) had been genetically ablated in the mature seeds.

Structural analysis of *MINELESS* seeds through light and transmission electron microscopy shows empty and degraded myrosin cells

The targeted myrosin cells from semi-thin sections of *MINELESS* seeds (radicles and cotyledons) stained with toluidine blue appeared empty when observed under light microscopy (Fig. 2A, B) while wild type tissues showed the expected distribution of myrosin cells (Bones *et al.*, 1991). A myrosin cell from a wild type section stained with toluidine blue is shown as an insert in Fig. 2B. Semi-thin sections (1 μm) were prepared for immunofluorescence microscopy. The anti-MYR antibody K089 (Thangstad *et al.*, 1991) followed by FITC-conjugated secondary antibody provided specific labelling for myrosin cells in wild type sections (Fig. 2C) while no specific labelling could be seen in MYR-negative *MINELESS* seed sections (Fig. 2E). *MINELESS* seeds with a weak/moderate MYR activity (described later) (Fig. 4) had both empty myrosin cells and myrosin cells with partially intact myrosin grains (Fig. 2D). It was also possible to observe different stages of cell degradation, with both unablated and semi-ablated myrosin cells.

The myrosin cell structures from wild type and *MINELESS* seeds were further analysed using transmission electron microscopy and immunogold-electron microscopy (EM). Myrosin cells of the wild type had a typical even granulated matrix in the myrosin grains (protein bodies/protein vacuoles) (Fig. 3A). In *MINELESS* plants, these

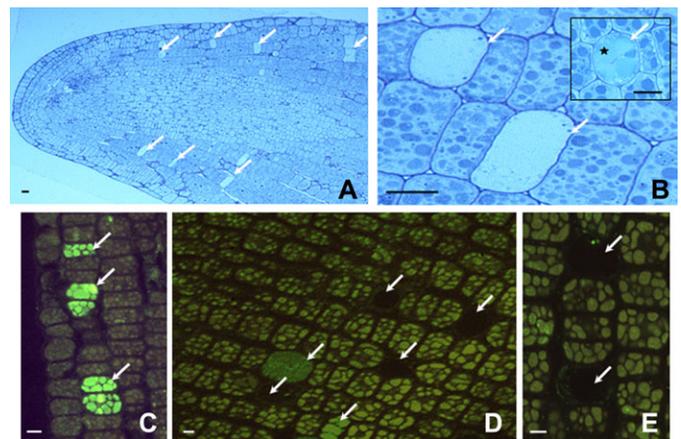


Fig. 2. Structural analysis of myrosin cells from semi-thin sections of wild type and *MINELESS* seeds by light microscopy. (A and B) Sections from *MINELESS* seeds stained with toluidine blue where the insert in (B) shows a normal myrosin cell from the wild type marked with a star. (C–E) Sections labelled with anti-myrosinase polyclonal antibody (K089) and FITC-conjugated secondary antibodies. (C) Wild type section; (D and E) *MINELESS* sections. White arrows indicate ablated or normal myrosin cells. Bars=10 μm. The exposure time in D and E was increased to visualize the ablated myrosin cells.

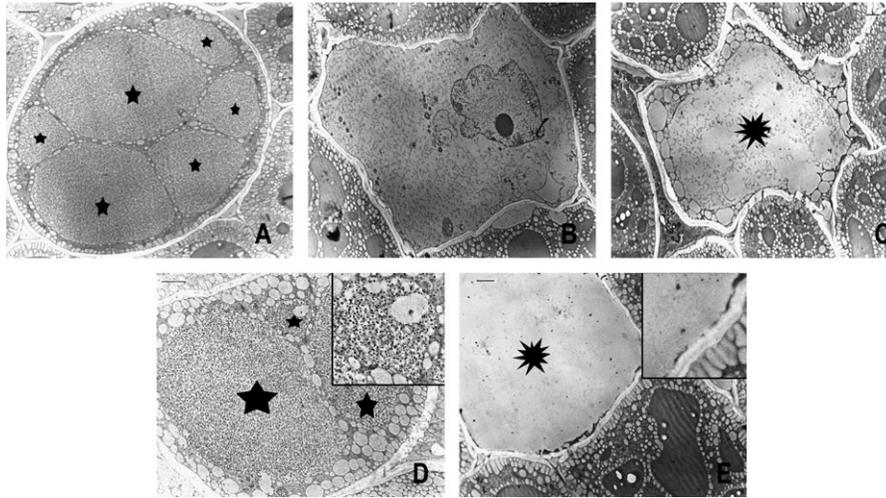


Fig. 3. Transmission electron microscopic structural analysis of myrosin cells from thin sections of wild type and *MINELESS* seeds. (A) Unlabelled section from wild type seeds showing a myrosin cell with the typical granulated matrix inside myrosin grains marked by stars. (B–E) Immunogold-EM sections labelled with anti-myrosinase (K089) and 15 nm colloidal gold. (B–C, E) *MINELESS* sections showing different stages of myrosin cell ablation. (D) Wild type section. Myrosin grains in the wild type (D) are marked by stars and ablated myrosin cells in *MINELESS* (C, E) are marked by starbursts. Inserts demonstrate specific detection of myrosinase inside a myrosin grain (D) and specific ablation inside a myrosin cell (E). Bars=2 μm .

cells appeared electron transparent and shrunken (Fig. 3B, C), proving that the cell content had been ablated. The surrounding aleurone-like cells appeared structurally normal, showing that the achieved ablation was highly cell specific. Further ultrastructural analysis using immunogold-EM of thin sections (600–700 Å) and the anti-MYR antibody K089 (Thangstad *et al.*, 1991) showed that the ablated cells, or other surrounding tissue, did not contain MYR (Fig. 3E). While protein bodies/vacuoles of myrosin cells from wild type tissues were densely labelled with immunogold particles localizing MYR (Fig. 3D and insert), no labelling was seen in *MINELESS* tissues (Fig. 3E and insert). It could also be observed that myrosin cells activated for ablation had characteristics of apoptosis such as nuclear membrane blebbing (Fig. 3B). A notable feature of the ablated cells is the apparent absence of any effect on neighbouring cells (Fig. 3E and insert).

MINELESS seeds show no or negligible MYR enzyme activity and are devoid of the MYR isoenzymes

In total 100 *MINELESS* and 50 Westar wild type single seeds were tested for MYR activity using sinigrin as a substrate. The distribution of specific MYR activity of wild type and transgenic single seeds is shown in Fig. 4. Sixty percent of wild type seeds possessed MYR activity in the region of 200–400 nmol glucose $\text{min}^{-1} \text{mg}^{-1}$ protein, while almost all (96%) transgenic seeds were found to have activity <200 nmol glucose $\text{min}^{-1} \text{mg}^{-1}$ protein. Seeds with no or negligible activities (i.e. <10 nmol glucose $\text{min}^{-1} \text{mg}^{-1}$ protein) were classified as *MINELESS* seeds. Of the transgenic seeds examined, 23% were found in this category (Fig. 4). Out of 50 wild type seeds that were tested for MYR activity, most seeds showed MYR activity in the range of

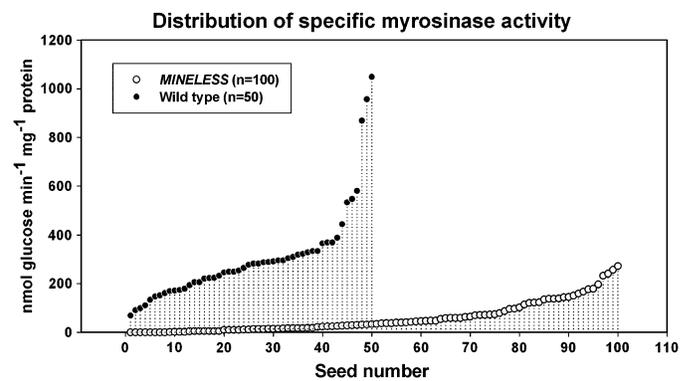


Fig. 4. Distribution of specific myrosinase activity in wild type and *MINELESS* single seeds.

201–400 nmol glucose $\text{min}^{-1} \text{mg}^{-1}$ protein and only a few seeds had MYR activity beyond this range (Fig. 4).

Immunoblot analysis using an anti-MYR monoclonal antibody (3D7 antibody) specifically labelled the three major bands for MYR polypeptide classes of *B. napus*, denoted as 75, 70, and 65 kDa in wild type protein extracts (Fig. 5A). All these three bands were absent in the protein extracts from *MINELESS* seeds.

Expression of MBPs is unaffected in MINELESS seeds

Through immunoblot analysis and by using the monoclonal anti-MBP antibody 34:14 (Lenman *et al.*, 1990) that recognizes different MBPs (Falk *et al.*, 1995; Geshi and Brandt, 1998; Eriksson *et al.*, 2002), no clear differences were observed between the wild type and *MINELESS* seeds (Fig. 5B). In some seeds, including both wild type and *MINELESS*, the 110 kDa MBP isoform was only faintly expressed, while in others it was strongly expressed. The

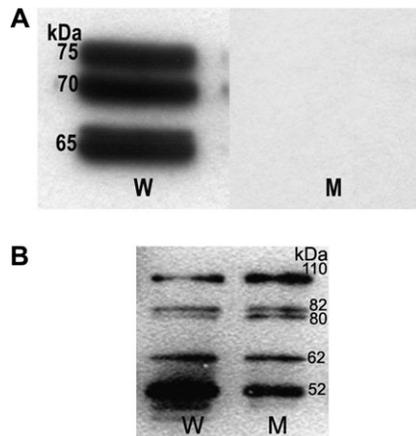


Fig. 5. Immunoblot analysis of wild type (W) and *MINELESS* (M) single seeds. (A) Expression of myrosinase proteins was detected with anti-myrosinase 3D7 antibodies. (B) Expression of myrosinase-binding proteins (MBPs) was detected with 34:14 antibodies. A 10 μ g aliquot of total protein was loaded in each lane.

52 kDa MBP band was visible in all *MINELESS* seeds examined, but the band intensity was low compared with that in wild type seeds.

MINELESS seeds show the absence of one isoform of ESP and inability to hydrolyse sinigrin

The effect of myrosin cell ablation on GSL hydrolysis and ESP activity was determined on single *MINELESS* and wild type seeds. GC-MS analysis was carried out on individual autolysed seeds using sinigrin as a substrate. From wild type seeds the expected allyl isothiocyanate (All ITC), 3,4-epithiobutane nitrile (3,4ETBut NIT), and allyl nitrile (All NIT) were produced upon hydrolysis of the GSL sinigrin due to the presence of endogenous MYR and ESP (Fig. 6A). *MINELESS* seeds showed a greatly reduced ability to hydrolyse the GSL sinigrin (Fig. 6B). In *MINELESS* seeds, the total hydrolytic product formation was <3.3 nmol hydrolysis product mg^{-1} seed as compared with 39.8 nmol hydrolysis product mg^{-1} seed for the wild type (Fig. 6C). These differences in the production of hydrolytic products between the wild type and *MINELESS* were significant ($P < 0.02$).

Addition of MYR purified from *S. alba* (Bones and Slupphaug, 1989) restored the GSL hydrolysis capacity in *MINELESS* extracts (Fig. 6C). Immunoblot analysis using a polyclonal anti-ESP antibody (Foo *et al.*, 2000) detected two bands of 35 kDa and 39 kDa in wild type seeds (Fig. 6D). In comparison, *MINELESS* seeds lacked the 35 kDa isoform (Fig. 6D). As expected from the low ESP levels detected in the immunoblot experiments, the production of allyl nitrile and 3,4-epithiobutane nitrile was much lower in *MINELESS* than in wild type extracts, which presumably are due to low MYR in *MINELESS* extracts (Fig. 6B). After addition of exogenous MYR, the only significant difference was the lower production of 3,4-epithiobutane nitrile in *MINELESS* as compared with the wild type ($P < 0.01$) (Fig. 6C).

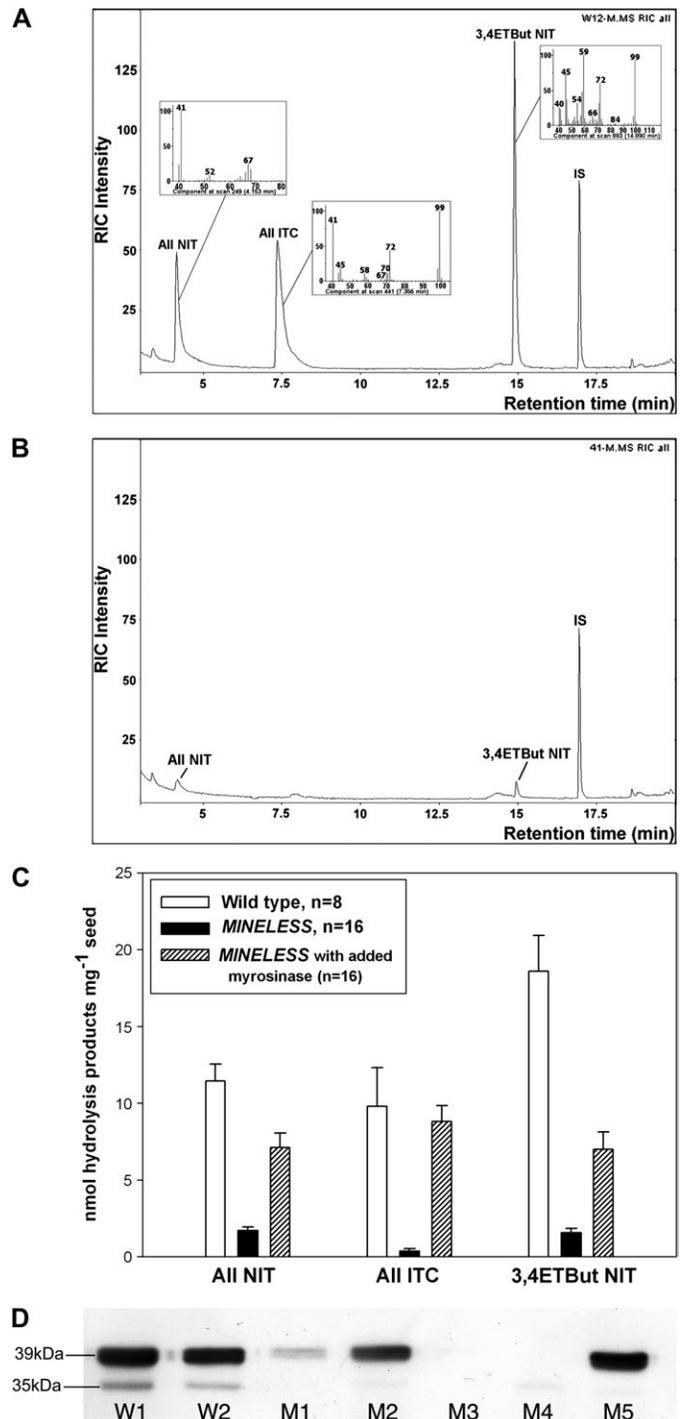


Fig. 6. Epithiospecifier protein (ESP) activity assay by assessing sinigrin hydrolysis product formation and immunoblot analysis of ESP expression from single wild type and *MINELESS* seeds. (A) GC chromatogram of a wild type single seed showing sinigrin hydrolysis products. (B) GC chromatogram of a *MINELESS* single seed showing sinigrin hydrolysis products. (C) Total hydrolysis products produced in a wild type single seed, a *MINELESS* single seed, and a single *MINELESS* seed supplemented with purified myrosinase. Error bars represent SE. (D) ESP expression in wild type (W1 and W2) and *MINELESS* (M1–M5) single seeds. A 10 μ g aliquot of total protein was loaded in each lane. The expression of ESP was detected with the anti-ESP antibody as described by Foo *et al.* (2000).

MINELESS seeds show alterations in GSL content and profile

The total GSL content and corresponding GSL profile was analysed from seeds split into two equal halves; the remainder was used for analysis of MYR activity. *MINELESS* seeds which showed MYR activity in the range of 0–10 nmol glucose min⁻¹ mg⁻¹ protein (Fig. 7A) were

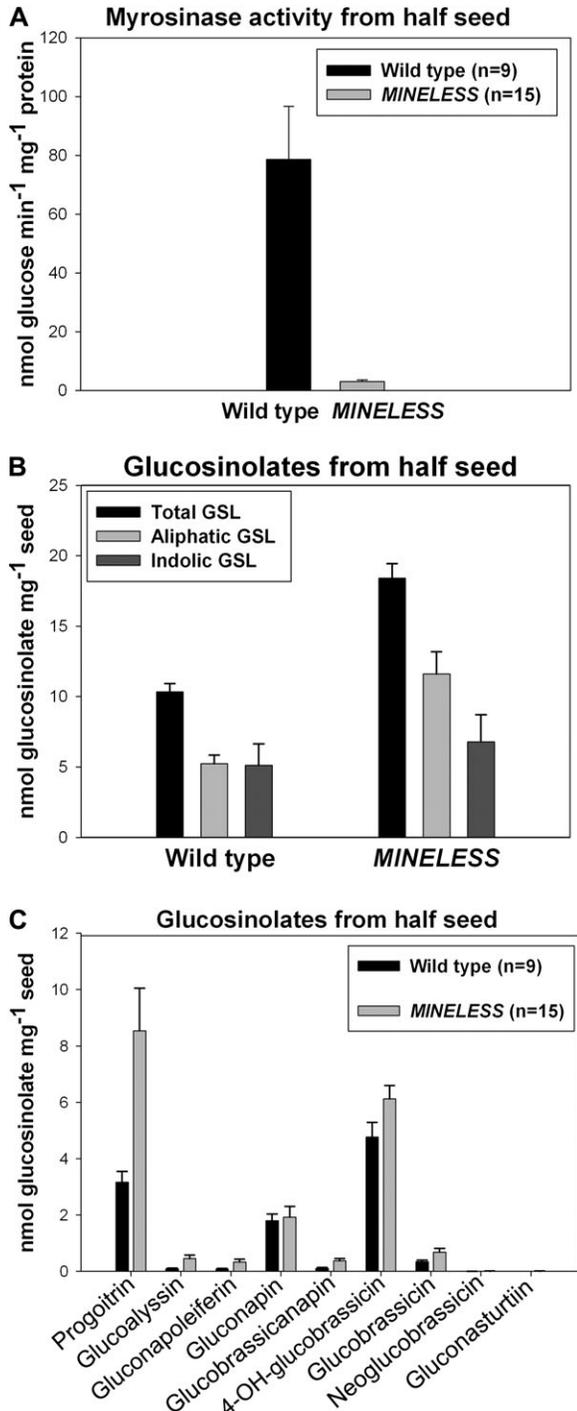


Fig. 7. Myrosinase activity and glucosinolates from half a seed of the wild type and *MINELESS*. (A) Myrosinase activity. (B) Aliphatic, indolic, and total glucosinolate content. (C) Glucosinolate profile and content of individual glucosinolates. Error bars represent the SE.

analysed for GSL. The total GSL concentration was increased in *MINELESS* seeds as compared with the wild type (Fig. 7B) ($P < 0.01$). The aliphatic GSLs in *MINELESS* seeds were >2-fold higher than in wild type seeds. 2(*R*)-2-hydroxy-3-butenyl (progoitrin), 5-methylsulphinyl-pentyl (glucoalyssin), 2-hydroxy-4-pentenyl (gluconapoleiferin), 4-pentenyl (glucobrassicinapin), and 4-hydroxy-3-indolylmethyl (4-hydroxyglucobrassicin) were increased in *MINELESS* seeds as compared with the wild type, with progoitrin being the highest (Fig. 7C). Aliphatic GSLs in *MINELESS* seeds were >2-fold higher than in the wild type seeds. All aliphatic GSLs except 3-butenyl (gluconapin), and the indolic GSL 3-indolylmethyl (glucobrassicin) showed a significant increase in *MINELESS* seeds ($P < 0.04$).

The distribution of MYR activity versus total GSL content from each examined *MINELESS* seed is visualized in a scatter plot (Fig. 8). The total GSL content ranged from 6 nmol GSL mg⁻¹ seed to 37 nmol GSL mg⁻¹ seed. The majority of *MINELESS* seeds (10 out of 15 seeds), however, contained 10–30 nmol GSL mg⁻¹ seed (Fig. 8). In contrast, most of the wild type seeds possessed a total GSL content in the range of 10–13 nmol GSL mg⁻¹ seed, and two wild type seeds were even below this range (Fig. 8).

Discussion

Confirmation of transgenic *MINELESS* *B. napus* plants

The detection of barstar only in transgenic lines and lack of its expression in the wild type confirmed the production of transgenic *MINELESS* plants. Moreover, the significantly reduced MYR activity in both seeds and cotyledons and strong down-regulation of glucosinolate metabolism genes (*Myr1* and *ESP*) in three transgenic lines as compared with the wild type by qRT-PCR analysis provided confirmation of the production of transgenic *MINELESS* *B. napus* plants (Supplementary Figs S1, S2 at *JXB* online). *Myr1* and *ESP* are the important genes in the GSL degradation pathway, and the strong down-regulation of these genes in transgenic lines confirms removal of myrosin cells. An almost similar level of down-regulation of the transcripts of *Myr1* and

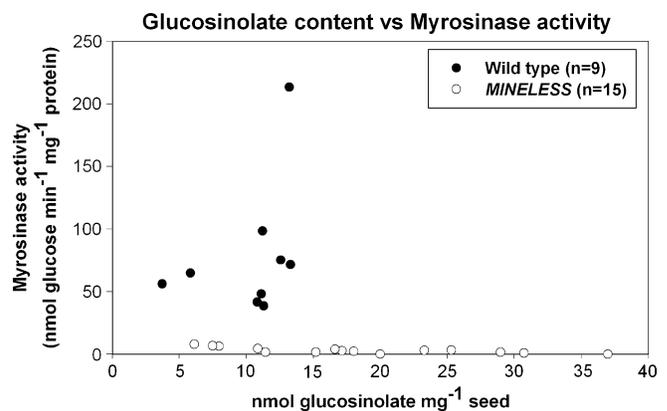


Fig. 8. Scatter plot showing glucosinolate content versus myrosinase activity from wild type and *MINELESS* single seeds.

ESP as measured with qRT-PCR provides another confirmation that ESP is associated with MYR. ESP has previously been described as a MYR cofactor and plays an important role during GSL degradation (MacLeod and Rossiter, 1985; Bones and Rossiter, 1996; Bernardi *et al.*, 2000; Foo *et al.*, 2000; Burow *et al.*, 2009; Kissen and Bones, 2009).

The genetic ablation of myrosin cells and production of MINELESS seeds

The use of T-DNA insertion knockouts in *A. thaliana* has made possible a large-scale elucidation of biochemical signalling pathways. However, this approach has limitations in the case of redundancy of members within gene families, as found in the MYR genes and its substrate pathways (Barth and Jander, 2006). An alternative cell-specific strategy to remove MYR from *B. napus* seed was therefore embarked on.

Since MYR is present in myrosin cells, transgenic seeds were expected to be free of MYR activity. This would prevent GSL hydrolysis and formation of anti-nutritional compounds in seed meal fed to ruminants. The term *MINELESS* seeds has been adopted to illustrate that the ‘the toxic mines’ (myrosin cells) have been removed. The present genetic cell ablation study has proven that the expression of a cell-specific cytotoxic construct encoded by the RNase barnase, driven by the stringent cell-specific *Myr1.Bn1* promoter, results in controlled cell death of myrosin cells in *MINELESS* seeds.

Previously, genetic ablation has been used as a tool to study male sterility. Tapetum-specific promoters were fused to cytotoxic genes and were shown to cause controlled cell death during the development phase (Nasrallah *et al.*, 1991; Block and Debrouwer, 1993; Roberts *et al.*, 1995; Zhan *et al.*, 1996; Beals and Goldberg, 1997; De Block *et al.*, 1997; Lee *et al.*, 2003; Roque *et al.*, 2007). The use of barnase has been limited by the ectopic expression and disruption of non-targeted tissue. The usefulness of barnase as a factor leading to cell ablation apparently depends on the specificity of the promoter used to control its expression. In the current study, a MYR promoter was used to direct the expression of cytotoxic barnase specifically to myrosin cells (*pMyr1:Barnase*). As a result, the barnase gene was strongly expressed in myrosin cells of maturing embryos/developing seeds of transgenic plants. However, due to the high toxicity of barnase, the viable seeds could be obtained only in the case of the co-expression of the barnase inhibitor barstar [*pMyr1.Bn1:Barnase:Barstar* (*MyrBarnBar=MINELESS*)]. Formation of inactive complexes (barnase–barstar) protects the embryo from widespread cell death, and restricts the ablation to single myrosin cells. The K_D of a barnase–barstar complex has been estimated to be $\sim 10^{-15}$ M. A K_D in this range indicates that once barnase–barstar complexes are formed, they are highly stable and rarely dissociate (Goldberg *et al.*, 1995). The fact that *MINELESS* seeds could germinate normally confirms the relative stability of the barnase–barstar complex.

Structural analysis of MINELESS seeds confirms myrosin cell ablation

The empty holes, visualizing the ablated myrosin cells, had the same distribution as myrosin cells in wild type Brassicaceae (Bones and Iversen, 1985; Bones *et al.*, 1991; Thangstad *et al.*, 1991; Höglund *et al.*, 1992). Structural analysis did not provide any indications suggesting that other cells were affected by the myrosin cell ablation. The use of transmission electron microscopy made it possible to examine the ultrastructure of the targeted myrosin cells in *MINELESS* in comparison with the wild type. Different levels of ablation were observed, from partial to completely empty remains surrounded by a cell wall (Fig. 3B, C, E). The identity of the ablated cells as myrosin cells was confirmed by immunogold-EM labelling of myrosin grains in wild type myrosin cells (Fig. 3D) compared with the absence of labelling in *MINELESS* seeds (Fig. 3E).

Transgenic seeds show reduced, but variable MYR activity

The MYR activity in transgenic seeds was reduced, but varied considerably among single seeds (Fig. 4). This was expected due to segregation of the transgenes and possibly also due to the cell- and time-specific variation of the *pMyr1Bn1* promoter. MYR activity and GSL levels in the wild type are controlled by several genes and can be regarded as a continuous trait in *Arabidopsis* (Pfalz *et al.*, 2007), and is even more complex in *B. napus*. The continuous trait variation is also apparent in *MINELESS*, as is visible in Fig. 4, indicating a segregation of the T₁ *MINELESS* phenotype. Despite the observed variability, most of the transgenic seeds showed MYR activity < 40 nmol glucose min^{-1} mg^{-1} protein. The major fraction of measured MYR activities from the wild type single seeds were in the range of 200–400 nmol glucose min^{-1} mg^{-1} protein, similar to levels reported in other cultivars of *B. napus* (Bones, 1990). Due to the complex situation of variability in MYR activity among single transgenic seeds, the MYR activity assay by the GOD-Perid method or the fast version of the GOD-Perid method was used as a tool for further characterizations. The *MINELESS* seeds which showed MYR activity in the range of 0–10 nmol glucose min^{-1} mg^{-1} protein were processed further.

MINELESS seeds show absence of MYR isoenzymes, but presence of MBPs

Immunoblot characterizations of protein extracts from *MINELESS* seeds using the 3D7 anti-MYR antibody showed the absence of all three bands of MYR isoenzymes found in *B. napus* (the 65, 70, and 75 kDa classes of MYRs), while all these bands, as expected, were present in wild type protein extracts (Fig. 5A, B). MYRs are glycosylated dimeric proteins with subunit molecular weights in the range of 62–75 kDa in plants (Bones and Rossiter, 1996). In wild type seed extracts, the presence of 65, 70, and 75 kDa MYR isoenzymes is consistent with the occurrence of three

different MYR isoenzymes in mature seeds of *B. napus*. These are encoded by the MB, MC, and MA gene family members, respectively (Lenman *et al.*, 1990; Falk *et al.*, 1995; Rask *et al.*, 2000). Immunolocalization studies of wild type plants indicate the presence of isoenzymes in myrosin cells (Thangstad *et al.*, 1990, 1991; Bones *et al.*, 1991; Höglund *et al.*, 1992; Kelly *et al.*, 1998). The absence of the 65, 70, and 75 kDa isoenzyme bands in *MINELESS* seed extracts provides direct evidence that the transgenic seeds are impaired in the synthesis and/or storage of MYR because of myrosin cell ablation. Therefore, it can be concluded that the *Myr1.Bn1* promoter activates the expression of barnase in myrosin cells, thereby leading to their ablation.

Furthermore, immunoblot analysis of MBPs from wild type and *MINELESS* seeds indicates that the MBPs are present in both wild type and *MINELESS* seeds. It should be noted that MBPs are highly variable in expression (Taipalensuu *et al.*, 1997). The presence of all MBPs in *MINELESS* seeds corresponds well with the results of a previous study by Eriksson *et al.* (2002), where immunohistochemical analysis showed that the MBPs are present in most cells, but not in myrosin cells. Further, Eriksson and co-workers have shown that the expression of MYR in seed myrosin cells of *B. napus* antisense plants was unaffected by the down-regulation of seed MBPs (Eriksson *et al.*, 2002).

ESP is affected in MINELESS seeds

Two isoforms of ESP that have been characterized in *B. napus* have molecular masses of 39 kDa and 35 kDa (Bernardi *et al.*, 2000; Foo *et al.*, 2000), while in broccoli and cabbage ESP has been shown to be 37 kDa and 43 kDa (Matusheski *et al.*, 2006). Consistent with these studies, two polypeptides with molecular masses of ~39 kDa and 35 kDa were detected in wild type *B. napus* seeds in the present study (Fig. 6D). Immunoblot analysis of *MINELESS* seeds using the same anti-ESP antibodies showed the absence of only the 35 kDa polypeptide or of both the 39 kDa and 35 kDa polypeptides. This indicates a possible co-localization of ESP with MYR in seed myrosin cells. The co-localization of MYR and ESP in myrosin cells of *Brassica* seeds and cotyledons should be further confirmed with the use of other experimental approaches. Other possible explanations are that myrosin cells contain a factor that affects either ESP transcription and *de novo* ESP synthesis, or the generation of the 35 kDa band from the 39 kDa band (e.g. by proteolytic cleavage/processing). In *A. thaliana*, ESP has been reported to be localized to the epidermis of all above-ground organs except the anthers and in S-cells of the stem below the inflorescence, but not in myrosin cells (Burrow *et al.*, 2007b). It should, however, be noted that MYRs also have a different localization in *A. thaliana* compared with *Brassica* species (Andreasson *et al.*, 2001; Husebye *et al.*, 2002; Thangstad *et al.*, 2004).

The presence of detectable levels of hydrolysed products in the GC-MS analysis (Fig. 6B) might indicate that the *MINELESS* seeds are not completely MYR free, but could have trace amounts (see also 'Structural analysis'). How-

ever, these small amounts of hydrolysed products in the tested *MINELESS* seeds could also be due to thermal or other enzymatic degradation of GSL (Bones and Rossiter, 2006). Another possibility is that peroxisome-localized PEN2 proteins recently reported to have thioglucosidase activity are responsible for a low GSL-hydrolysing activity (Bednarek *et al.*, 2009; Clay *et al.*, 2009).

External addition of MYR to *MINELESS* seed extracts restored the capacity of *MINELESS* seed extract to degrade sinigrin, confirming that MYR is the major limiting factor for GSL hydrolysis in *MINELESS*. Since the degradation products, both in the wild type and in *MINELESS*, come from added sinigrin, one would expect almost the same levels of hydrolysis products from *MINELESS* (with added MYR) as were observed in wild type seed extracts. However, this assumption holds for the allyl nitrile and allyl isothiocyanate levels, but not for the 3,4-epithiobutane nitrile levels. The level of the latter is significantly higher in wild type seed extracts as compared with *MINELESS* seed extracts with or without addition of MYR (Fig. 6C). The activity assay used to hydrolyse sinigrin is designed to favour the formation of epithionitriles. A close to complete restoration of 3,4-epithiobutane nitrile production in *MINELESS* is therefore expected if ESP levels are similar to wild type levels. 3,4-Epithiobutane nitrile is not a major hydrolysis product from *MINELESS* extract degradation of sinigrin even after addition of MYR. It is suggested that this is due to the absence of one or both ESP isoforms in *MINELESS* tissue that was observed by immunoblot.

The GSL profile is altered and total GSL content is increased in MINELESS seeds

The GSL profile of *MINELESS* seeds is somewhat altered as compared with the parent cultivar Westar (wild type) (Fig. 7C), and affects both aliphatic and, to a lesser extent, indolic GSLs. Overall, the profiles resembled GSL profiles from previous studies performed with *B. napus* seeds (Fenwick and Heaney, 1983; Fenwick *et al.*, 1983; Sang *et al.*, 1984; Kraling *et al.*, 1990; Daun and McGregor, 1991; Shahidi *et al.*, 1997; Matthaus and Luftmann, 2000). Evidence of a rapid turnover of GSLs even in unchallenged plants has been reported (James and Rossiter, 1991; Rosa *et al.*, 1994; Svanem *et al.*, 1997; Thangstad *et al.*, 2001; Petersen *et al.*, 2002). The average GSL content of *MINELESS* seeds was increased (Fig. 7B), but varied considerably among single seeds (Fig. 8). The GSL level of the wild type was similar to that of its parent cultivar 'Canola' at ~10 nmol GSL mg⁻¹ seed (Downey, 1990). An interesting observation is that the GSL content showed variation in *MINELESS* seeds with up to 37 nmol GSL mg⁻¹ seed, which was above the level of Canola.

The elevated levels of GSLs in the *MINELESS* seeds might resemble an ongoing biosynthesis but an absence of *in vivo* catabolism/hydrolysis. In seeds of the Brassicaceae family, e.g. *B. napus*, *S. alba*, and *Armoracia rusticana*, a comparable correlation between MYR and GSL levels can be observed (Bones and Iversen, 1985; Li and Kushad,

2004). However a study on *A. thaliana* (Barth and Jander, 2006) has indicated that the developmental decreases in GSL content during senescence and germination occur independently of MYRs (TGG1 and TGG2). Various stresses, such as mechanical wounding (Bodnaryk, 1992) and insect and pathogen attack (Brader *et al.*, 2001; Mewis *et al.*, 2005; Mewis *et al.*, 2006; Kim and Jander, 2007; Kusnierczyk *et al.*, 2007, 2008; Ahuja *et al.*, 2009; Bednarek *et al.*, 2009; Clay *et al.*, 2009) have been reported to induce alterations in the GSL profile and amount.

Conclusions

The targeted cell ablation strategy has been applied to genetically remove the MYR-containing myrosin cells from *B. napus* seeds. The production of *MINELESS* seeds is the first step on the way to reduce the anti-nutritional properties of rapeseed meal. In addition, the *MINELESS* plants, which lack the GSL–MYR defence, comprise an important model to study the plant defence system and other related functions. Further work will concentrate on the production and analysis of homozygous lines, and application of these seeds to study their nutritional quality. Studies of nutritional quality of the transgenic seeds, possible changes in secondary metabolic processes, and plant defence mechanisms are expected to yield new information, which will contribute to a better understanding of the GSL–MYR system.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Specific myrosinase activity in seeds and cotyledons of the wild type and three transgenic lines.

Figure S2. Quantitative real-time PCR (qRT-PCR) analysis of glucosinolate metabolism genes (*Myr1* and *ESP*) in 5-day-old cotyledons of the wild type and transgenic lines.

Table S1. Primer sequences used for the qRT-PCR in Fig. S2.

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