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Sinapis phylogeny and evolution of glucosinolates and specific nitrile degrading enzymes

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ABSTRACT

Levels of sinalbin (4-hydroxybenzylglucosinolate) and 28 other glucosinolates were determined in leaves and roots of 20 species that were either phylogenetically close to *Sinapis alba, Sinapis arvensis*, or *Sinapis pubescens* (tribe Brassiceae, Brassicaceae), or were expected to contain arylalkyl nitrilase activity. Comparison with a molecular phylogenetic tree based on ITS DNA sequences identified two separate occurrences of sinalbin. The first in a group of species related to *S. alba* (including members of the genera *Coincya* and *Kremeriella*); and the second in *S. arvensis*, nested among sinalbin deficient species. Significant 4-hydroxyphenylacetonitrile degrading enzyme activity was found in both *S. alba* and *S. arvensis*, but in *S. alba* the major product was the corresponding carboxylic acid, while in *S. arvensis* the major product was the amide. Both investigated enzyme activities, nitrilase and nitrile hydratase, were specific, accepting only certain arylacetonitriles such as 4-hydroxy and 4-methoxyphenylacetonitrile. Only the *S. alba* enzyme required an oxygen in *para* position of the substrate, as found in sinalbin. Indole-3-acetonitrile, arylcyanides, and arylpropionitriles were poor substrates. The nitrilase activity of *S. alba* was quantitatively comparable to that reported in the monocot *Sorghum bicolor* (believed to be involved in cyanogenic glycoside metabolism). Glucosinolates derived from methionine were found in all *Sinapis* clades. Glucosinolate patterns suggested a complex evolution of glucosinolates in the investigated species, with several apparent examples of abrupt changes in glucosinolate profiles including chain length variation and appearance of glucosinolates derived from branched-chain amino acids. NMR data for desulfated homosinalbin, 9-methylsulphonylnonylglucosinolate, 3-methylpentylglucosinolate and related glucosinolates are reported, and a facultative connection between sinalbin and specific nitrilases is suggested.

1. Introduction

1.1. Glucosinolates and chemotaxonomy

Glucosinolates (Fig. 1) are amino acid derived secondary metabolites restricted to 16 plant families in the order Brassicales (formerly known as Capparales), including the Brassicaceae and Tropaeolaceae (Angiosperm Phylogeny Group, 2003; Halkier and Gershenzon, 2006). More than 120 individual structures are known, mainly differing in the amino acid derived side chain (Fig. 1; Fahey et al., 2001). While presence of glucosinolates were of considerable chemotaxonomic significance in initially defining the order (Kjær, 1974; Dahlgren, 1980; Rodman et al., 1998), the presence of individual glucosinolates or types of glucosinolates are more difficult to use as taxonomic markers. Although examples of natural genera with distinct glucosinolate profiles are known, glucosinolates can not be assumed to be neutral taxonomic markers due to their ecological effects, and several examples of evolutionary hotspots and intrageneric or intraspecific variation have been reported (Raybould and Moyes, 2001; Agerbirk et al., 2003; Kliebenstein et al., 2005; Windsor et al., 2005; van Leur et al., 2006).

The taxonomic delineation of the genus *Sinapis* is controversial (Warwick and Black, 1991). DNA sequence comparisons suggest that the genus is artificial (Warwick and Sauder, 2005), and species have been variously ascribed to *Sinapis* and *Brassica,* e.g. *Brassica aucheri* (Boiss.) O.E. Schulz (syn. *S. aucheri* Boiss.) and *S. alba* L. (syn. *B. hirta* Moench., *B. alba* (L.) Rabenh.) (Al-Shehbaz and Warwick, 1997). The glucosinolate sinalbin (4-hydroxybenzylglucosinolate) (**2**), originally named for its presence in *S. alba*, is also present in *S. arvensis* (Bennett et al., 2004). The natural distribution of sinalbin is poorly known, partially because data (compiled by Fahey et al., 2001) from earlier analyses (Daxenbichler et al., 1991) could not distinguish sinalbin and indole glucosinolates (**7**-**11**, Agerbirk et al., 2008) (Bold numbers indicate glucosinolates as illustrated in Figs. 1 and 5). As sinalbin appeared to be a rare glucosinolate (Bennett et al., 2004; Windsor et al., 2005), its

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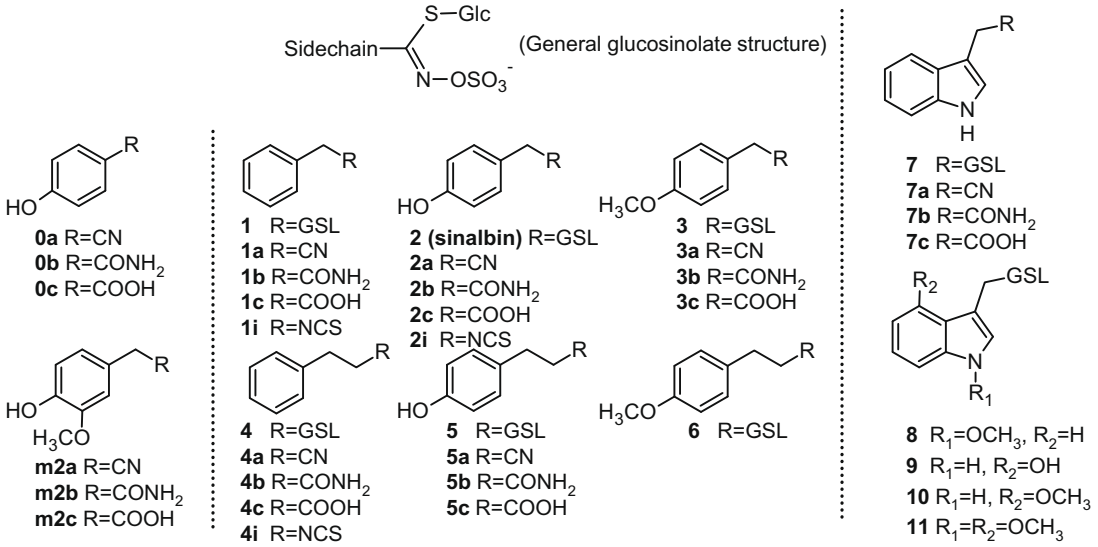

Fig. 1. Structures of some aromatic glucosinolates and derived compounds. GSL: The glucosinolate radical C(SGlc)NOSO_3^-. **1**, benzylGLS; **2**, 4-hydroxy**1** (sinalbin); **3**, 4-methoxy**1**; **4**, phenethylGLS; **5**, 4-hydroxy**4** (homosinalbin); **6**, 4-methoxy**4**; **7**, indol-3-ylmethylGLS (glucobrassicin); **8**, 1-methoxy**7** (neoglucobrassicin); **9**, 4-hydroxy**7**; **10**, 4-methoxy**7**; **11**, 1,4-dimethoxy**7**.

presence was suggested to be of chemotaxonomic value and possibly supporting an extension of the genus *Sinapis* (Bennett et al., 2004). The first purpose of this paper addresses the evolution and chemotaxonomic value of sinalbin and other glucosinolates (Section 1.5).

1.2. Glucosinolate biosynthesis

Sinalbin biosynthesis is still not certain. Sinalbin may in principle be derived directly from Tyr, or be an oxidized derivative of **1** (derived from Phe, Wittstock and Halkier, 2000). Classical investigations of sinalbin biosynthesis were inconclusive (Ettlinger and Kjær, 1968), whereas later investigations report that radiolabelled sinalbin was formed from radiolabelled Tyr in *S. alba* (Du et al., 1995). When *Arabidopsis thaliana* was genetically modified to express Tyr-specific CYP79A1, sinalbin accumulated, demonstrating that sinalbin in this case was derived from Tyr (Bak et al., 1999), but Tyr or Phe-specific CYPs have not yet been identified from sinalbin containing plants (Naur et al., 2003).

The majority of natural glucosinolates are derived from chain-elongated homologues of standard amino acids, such as **4-6** that are derived from homoPhe and the large number of homoMet and n-homoMet derived glucosinolates (see Section 2.5). Chain elongated phenolic glucosinolates (such as **5**) were unknown until recently (Agerbirk et al., 2001; Bennett et al., 2004), but it appeared possible that such glucosinolates could be formed in plants with the combined ability to biosynthesize homoPhe derived glucosinolates such as **4** and phenolic glucosinolates such as sinalbin, as is the case for several *Sinapis* species. The third purpose of this paper addresses the flexibility of aromatic glucosinolate biosynthesis (Section 1.5).

1.3. Glucosinolate metabolism via nitriles

Upon tissue disruption, glucosinolates are hydrolysed by enzymes (called myrosinases) into unstable aglucones that ultimately yield either isothiocyanates, nitriles or other kinds of products (Fig. 2). Some nitriles may also be formed from intermediates in the biosynthesis (Pedras et al., 2007). Some nitrile degrading enzymes in glucosinolate containing plants have been suggested to be involved in glucosinolate turn over in intact tissue, including a nitrilase in oilseed rape, *B. napus* (Bestwick et al., 1993) and three nitrilase isoenzymes in *Arabidopsis thaliana*. In *A. thaliana*, three similar genes named *NIT1*, *NIT2* and *NIT3* were originally believed to be involved in biosynthesis of the plant hormone indole-3-acetic acid (**7c**) (Bartel and Fink, 1994), but later it was shown that the corresponding nitrile **7a** was a very poor substrate of NIT1-3. (In the following, compound **xa** is the nitrile derived from glucosinolate **x**, **xb** is the amide derived from **xa**, **xc** is the carboxylic acid derived from **xa** and **xb**, and **xi** is the isothiocyanate

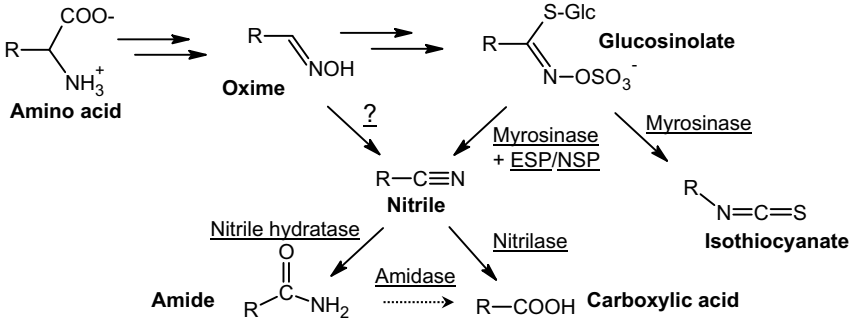

Fig. 2. Nitrile metabolism and possible connections to glucosinolate biosynthesis and degradation. ESP, epithiospecifier protein occurring in some plants. NSP, nitrile specifier protein occurring in some caterpillars.

derived from **x**, see Fig. 1). All three enzymes accepted a rather broad range of substrates, especially **4a** and allylcyanide, and it was proposed that the function of the NIT1-3 enzymes is to hydrolyse glucosinolate-derived nitriles (Vorwerk et al., 2001). However, *A. thaliana* contains a surprising variety of glucosinolate structures (Reichelt et al., 2002; Kliebenstein et al., 2005) that may give rise to a similar variety of nitrile structures. A useful test of the proposed involvement of nitrilases in glucosinolate metabolism would be to search for correspondence of nitrilase specificity and glucosinolate structures in other crucifers. A fourth nitrilase, NIT4, present in *A. thaliana* as well as a wide variety of higher plants, is responsible for metabolism of β-cyanoAla in the cyanide detoxification pathway which is related to ethylene biosynthesis, not glucosinolate metabolism (Piotrowski and Volmer, 2006).

All nitrilases are bi-functional enzymes that simultaneously catalyse two different reactions of a single substrate (Fig. 2): in addition to hydrolysis of nitriles to corresponding carboxylic acids and ammonia, they also catalyse the addition of water forming stable amide end products. Although plant nitrilases are both nitrilases (E.C. 3.5.5.1) and nitrile hydratases (E.C. 4.2.1.84), on the basis of the reactions catalysed (Fig. 2), the name nitrilase is preferred because sequence comparisons show similarity to bacterial nitrilases rather than nitrile hydratases (Piotrowski et al., 2001). The relative importance of the two reactions is variable, depending on the substrate (Osswald et al., 2002) and the enzyme (Piotrowski and Volmer, 2006).

1.4. Ecological effects of nitrile degrading enzymes

Larvae of the cabbage white butterflies (*Pieris* sp.) and related genera, which feed exclusively on plants containing the glucosinolate-myrosinase system, contain the gut protein NSP (nitrile specifier protein) which causes the glucosinolate-myrosinase reaction to yield nitriles instead of isothiocyanates (Fig. 2, Wittstock et al., 2004; Burow et al., 2006; Agerbirk et al., 2006; Wheat et al., 2007). The impetus of the present investigation of plant nitrile degrading enzymes was the discovery of host plant dependent metabolism of sinalbin in *P. rapae* caterpillars (Agerbirk et al., 2007). The metabolism involved a plant nitrile hydratase acting on the corresponding nitrile **2a** formed in the insect gut from sinalbin (**2**) ingested with Sinapis *arvensis* and *S. alba*. During our investigations, involvement of a nitrilase in metabolism of **1** ingested with *Tropaeolum majus* was also suggested (Vergara et al., 2006). The pattern suggested from these reports appeared to be that cruciferous plants with glucosinolate **1** or sinalbin (**2**) would contain nitrile degrading enzymes with specificity matching the corresponding nitriles **1a** and **2a**, in agreement with the presence of **4a** degrading enzymes in *A. thaliana* and *B. napus* containing glucosinolate **4** (Vorwerk et al., 2001; Bestwick et al., 1993). In order to test this hypothesis, we combined the taxonomic investigations of sinalbin and related glucosinolates with a screen for nitrile degrading enzymes with specificity for **2a.**

1.5. Purpose

The purposes of this study are: i) to deduce the evolutionary history of glucosinolate profile variation in the genus *Sinapis* and related species within a phylogenetic framework, ii) to investigate the taxonomic distribution of the aromatic glucosinolate sinalbin (**2**) and its correlation with substrate specificity of nitrile degrading enzymes with specificity for 4-hydroxyphenylacetonitrile (**2a**), and iii) to investigate structural flexibility of sinalbin biosynthesis in plants containing both sinalbin and phenethylglucosinolate (**4**).

2. Results and discussion

2.1. Phylogenetic tree of three groups of Sinapis species

The genus *Sinapis* is polyphyletic, as it forms three separate clades in the phylogenetic tree (Fig. 3), each of which show a closer relationship to *Brassica* species and/or other members of the tribe Brassiceae than to each other. The three *Sinapis* clades/lineages will be designated herein as "Arvensis", "Alba", and "Pubescens" clades. The polyphyletic origins of *Sinapis* had been found in earlier DNA studies based on restriction fragment length polymorphisms (Warwick and Black, 1991, 1993, 1997). The former *Sinapis* species *B. aucheri* was placed in *Brassica* based on its distinct fruit morphology, unique chromosome number ($n = 7$ chromosomes) and endemism to Iraq/Iran as compared with the Mediterranean distribution of the rest of *Sinapis* (Al-Shehbaz and Warwick, 1997). The three *Sinapis* clades correspond to the three morphologically-based sections of the genus recognized by Schulz (1936) and Baillargeon (1986). In the Arvensis clade, *Sinapis arvensis* ($n = 9$) was sister to *Brassica nigra* ($n = 8$), a relationship supported by

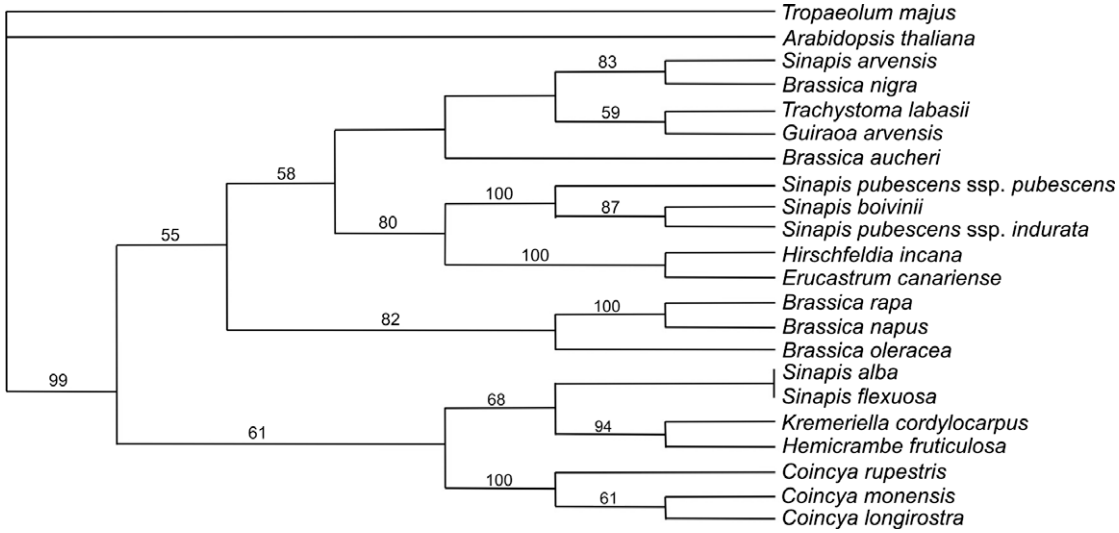

Fig. 3. ITS-based phylogenetic tree, based on maximum parsimony analysis of 131 parsimony informative characters, tree length = 326. Bootstrap values with over 50% support appear above branches.

numerous molecular and isozyme data sets (reviewed in Warwick and Black, 1991; Warwick and Sauder, 2005). *Sinapis pubescens* ($n = 9$) and *S. boivinii* ($n = 18$) formed a well supported clade, the Pubescens clade, with *Hirschfeldia incana* ($n = 7$) and *Erucastrum canariense* ($n = 9$). The Alba clade included *S. alba* and *S. flexuosa*, both with $n = 12$ chromosomes, and another $n = 12$ species, *Kremeriella cordylocarpus*, and *Hemicrambe fruticulosa* ($n = 9$) and *Coincya* spp. (all $n = 12$). No differences were detected in the ITS sequences nor in the chloroplast DNA genomes of *S. alba* and *S. flexuosa* (Warwick and Black, 1991), suggesting a recent divergence of these two species.

It has been argued that genera such as *Sinapis* and *Hirschfeldia* need be abandoned due to their lack of consistency with phylogenetic data (Al-Shehbaz et al., 2006). Indeed generic revision in the tribe Brassiceae would require the abandonment of at least ten of the most commonly known genera, including *Brassica* itself – not a trivial matter as many genera are traditionally recognized and include economically important or weedy taxa. As phylogenetic assessment of the family as a whole nears completion (e.g. Beilstein et al., 2006; Bailey et al., 2006), the tribal and generic taxonomy of the family should be altered to reflect phylogenetic data, such nomenclatural changes associated with revised generic delimitations are planned for the tribe Brassiceae (Al-Shehbaz, Warwick, and Hall, pers. comm.).

2.2. Taxonomic distribution of sinalbin and other aromatic glucosinolates

Sinalbin was found in the Arvensis and Alba clades, but not detected in the Pubescens clade (Table 2). Most species of the Alba clade contained sinalbin, suggesting that the ability to biosynthesize this glucosinolate originates in a common ancestor of the clade. Its absence in *H. fruticulosa* suggests a secondary loss of this feature, and very low levels in some *Coincya* species might indicate a secondary loss of the tendency to accumulate high levels of sinalbin. Intraspecific variability is expected in the genus *Coincya*, as apparent polymorphisms for high sinalbin levels in seeds have previously been reported (Vioque et al., 1994).

Sinalbin presence in *S. arvensis* appears to have evolved after the divergence of *S. arvensis* and *B. nigra*, as sinalbin was absent from the remaining investigated species in the Arvensis clade. The distantly related *Tropaeolum* spp. also contained low levels of sinalbin (and sinalbin was dominant in *T. peregrinum* seeds, results not shown), confirming that parallel evolution of sinalbin occurrence is possible in distinct lineages, as has also been demonstrated for morphological characters such as branched trichomes or particular fruit types (Beilstein et al., 2006).

In several species of the Alba clade, sinalbin co-occurred with **4** in roots (Table 2), prompting us to search for the oxidized derivatives **5** and **6**. However, neither the hydroxylated **5** nor the corresponding methoxylated **6** were detected in species of the Alba clade or in any other species investigated. [Authentic references of **5** and **6**, first reported by Bennett et al., 2004 based on LC-MS evidence, were obtained and identified (Table 7 and Table 8)]. Other known derivatives of **4** and **5** (Agerbirk et al., 2001; Bennett et al., 2004) were likewise not observed. This result suggests that a hypothetic enzyme converting **1** to **2** is too specific to include **4** as substrate, or that **1** in the Alba clade is not a biosynthetic intermediate of sinalbin, but rather a product of a parallel glucosinolate biosynthesis from Phe. In contrast, as both **1** and **3** were detected in *Tropaeolum* along with low levels of sinalbin, it seems possible that sinalbin in *Tropaeolum* is a biosynthetic intermediate in the formation of **3** from **1**. Unfortunately, chain elongated glucosinolates were not detected in *Tropaeolum*, so the hypothesis of structural flexibility in the possible oxidation of **1** could not be tested. Similarly, the absence of **4** in *S. arvensis* prevented the test of structural flexibility in the independently evolved sinalbin biosynthesis of this species. One or more of the indole glucosinolates **7**-**10** were detected in all tested species except *Tropaeolum* sp., mainly as methoxylated derivatives (**8**, **10**) in the roots. But the rare indole glucosinolate **11**, which combines the substitution pattern of **8** and **10** (Agerbirk et al., 2001), was only detected in a single species (the polyploid *S. boivinii*). Thus, structural flexibility in the biosynthesis of aromatic glucosinolates was generally absent.

Glucosinolates are known to be in part constitutive and in part inducible defences, and the possibility of changes in the glucosinolate profile due to induction should therefore be considered.

Table 1
Plant accessions and ITS sequences investigated

Plant species[a]	Source, accession number[b] (ITS sequence[c])
Arabidopsis thaliana (L.) Heynh. ('Col-0')	B. Halkier, Copenhagen Univ. (U43224)
Brassica aucheri Boiss. (=*Sinapis aucheri* (Boiss.) O.E. Schulz)	UPM 3735[d] (AY722413)
Brassica napus L. 'Excalibur'	Monsanto SAS, Toury, France (DQ003672)
Brassica nigra (L.) W.D.J. Koch (1)	Palermo Bot. Garden, Italy[d] (AY722422)
Brassica nigra (2)	Coll. B. Traw at Ithaca, New York
Brassica oleracea L.	BCN 3489[d] (AY722423)
Brassica rapa L.	BCN 2984[d] (AF531563)
Coincya longirostra (Boiss.) Greuter & Burdet	UPM 1175 (AY722430)
Coincya monensis (L.) Greuter & Burdet	UPM 4429 (AY722431)
Coincya rupestris ssp. *leptocarpa* (González-Albo) Leadlay	UPM 8862 (AY722432)
Erucastrum canariense Webb & Berthel.	UPM 5305 (AY722462)
Guiraoa arvensis Coss.	UPM 1550[d] (AY722468)
Hemicrambe fruticulosa Webb	UPM 2232 (AF039984)
Hirschfeldia incana (L.) Lagr.-Foss	Djebel Thaya, Algeria (BCN 2700)[d] (AY722470)
Kremeriella cordylocarpus Coss.& Durieu (1)	Copenhagen Bot. Garden 1103[d] (AY722471)
Kremeriella cordylocarpus (2)	UPM 1142
Sinapis alba L. 'SALVO' (1)	Advanta seeds, Capelle, The Netherlands
Sinapis alba. (2)	GAT SIN20/81[d] (AY722486)
Sinapis alba (3)	UPM 0560
Sinapis alba ssp. *mairei* (Lindb.) Maire	UPM 7619
Sinapis arvensis L. (1)	Coll. J.K. Nielsen at Kværkeby, Denmark (AY722487)
Sinapis arvensis (2)	NGB 6922
Sinapis arvensis (3)	Coll. A. Bornø Clausen at Slangerup, DK
Sinapis arvensis (4)	Coll. Th. Steinger at Basel, Switzerland
Sinapis arvensis (5)	Coll. Th. Steinger at Tblisi, Georgia
Sinapis boivinii Baillargeon	UPM 7630 (AY722489)
Sinapis flexuosa Poir.	UPM 0867 (Warwick unpubl. data)
Sinapis pubescens L. ssp. *pubescens*	UPM 3823 (AY722488)
Sinapis pubescens ssp. *indurata* (Coss.) Batt.	UPM 4689 (AY722490)
Sorghum bicolor (L.) Moench	AgriPro Seeds, Shawnee Mission, Kansas[e]
Trachystoma labasii Maire	UPM 3014[d] (AY722493)
Tropaeolum majus L. 'Jewel Mix' (1)	Weibull, Hammenhög, Sweden (AF254020)
Tropaeolum majus 'Empress of India' (2)	Weibull, Hammenhög, Sweden
Tropaeolum peregrinum L.	Chiltern Seeds, Ulverston, England

[a] Subspecies, cultivar or ecotype indicated when relevant. For taxa replicated in this work, the indicated numbering system is used, e.g. *S. alba* (1) is *S. alba* 'SALVO'.
[b] UPM: Crucifer seed bank at Dept. Biología Vegetal, Universidad Politécnica de Madrid, Spain, www.etsia.upm.es/DEPARTAMENTOS/biologia. Coll.: collected by. GAT: Genetic & Breeding Inst., Gatersleben, Germany. Tblisi: "NE of Tblisi 450 m asl". NGB: Nordic Gene Bank, www.nordgen.org.
[c] Sequence number of ITS sequence used for calculation of phylogenetic tree in Fig. 4. Sequences were obtained from GenBank, not from the listed plant accessions.
[d] Same material as investigated by Warwick and Sauder (2005).
[e] "Hybrid Sorghum Sudangrass Seed. Lot 4617", from K. Jørgensen, Univ. of Copenhagen.

Table 2
Levels (μmol/g dry wt.) of glucosinolates **1–4** and **7–10** in leaves (Le) and roots (Ro) of species of the genus *Sinapis* and selected other plant species, either related to *Sinapis* sp. or previously suggested to contain nitrilase

Glucosinolate	**1**		**2** (Sinalbin)		**3**		**4**		**7–10**[a]		*N*
Precursor	Phe		Phe/Tyr		Phe/Tyr		homoPhe		Trp		
Plant part:	Le	Ro	Le	Ro	Le	Ro	Le	Ro	Le	Ro	
Clade/species											
Arvensis clade											
S. arvensis (1)	n.d.	n.d.	**9**	**36**	n.d.	n.d.	n.d.	n.d.	**0.1**	**1.5**[10]	3
S. arvensis (2)	n.d.	n.d.	**50**	**20**	n.d.	n.d.	n.d.	n.d.	**0.4**	**2**[10,9]	2
S. arvensis (3)	n.d.	n.d.	**23**	**17**	n.d.	n.d.	n.d.	n.d.	**0.2**	**1.7**[10,9]	2
S. arvensis (4)	n.d.	n.d.	**23**	**25**	n.d.	n.d.	n.d.	n.d.	**tr.**	**4**[10,9]	2
S. arvensis (5)	n.d.	n.d.	**16**	**17**	n.d.	n.d.	n.d.	n.d.	**tr.**	**2**[10,9]	2
B. nigra (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.5**	**13**	**0.2**	**1.3**[8,10]	2
B. nigra (2)	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	**tr.**	**1.5**	**0.1**	**0.1**	2
Tra. labasii	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.1**	**11**[8]	2
G. arvensis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**tr.**	**5**[8,10]	2
B. aucheri	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**tr.**	n.d.	**tr.**	**6**[8,9]	2
Pubescens clade											
S. pubescens	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**20**	**0.2**	**0.8**[9]	2
S. boivinii	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**6**	**tr.**	**1.1**[7,10b]	2
S. pub. ssp. *indurata*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**58**	**0.2**	**0.4**[9]	2
Hir. incana	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.6**	**15**	n.d.	**2**[8,10]	2
E. canariense	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1.5**[7]	**6.9**[8]	2
Alba clade											
S. alba (1)	**9**	**0.7**	**40**	**11**	n.d.	n.d.	n.d.	**4**	**0.1**	**1.9**[10,8]	4
S. alba (2)	**0.4**	**tr.**	**32**	**5**	n.d.	n.d.	n.d.	**4**	**tr.**	**1**[8,10]	2
S. alba (3)	**4**	**0.2**	**29**	**7**	n.d.	n.d.	n.d.	**6**	**tr.**	**0.7**[10,8]	2
S. alba ssp. *mairei*	**2**	**0.3**	**67**	**12**	n.d.	n.d.	**tr.**	**5**	**tr.**	**0.4**[10,7]	3
S. flexuosa	n.d.	n.d.	**39**	**12**	n.d.	n.d.	n.d.	n.d.	**tr.**	**0.6**[10]	2
K. cordylocarpus (1)	**0.1**	n.d.	**76**	**11**	n.d.	n.d.	n.d.	**0.2**	**tr.**	**2**[8,10]	2
K. cordylocarpus (2)	**tr.**	–	**48**	–	n.d.	–	n.d.	–	**tr.**	–	2
Hem. fruticulosa	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2**	**28**	**0.4**	**1.3**[10,9]	2
C. rupestris	**tr.**	**tr.**	**tr.**	**tr.**	n.d.	n.d.	**tr.**	**67**	**0.3**	**6**[8,10]	2
C. monensis	n.d.	n.d.	**17**	**10**	n.d.	n.d.	n.d.	**19**	**0.2**	**5**[10,9]	2
C. longirostra	n.d.	n.d.	**0.2**	**0.2**	n.d.	n.d.	**0.1**	**28**	**0.2**	**10**[10,7]	2
With known or suggested nitrilase											
A. thaliana	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d[c]	n.d.	**4**[7]	**4**[8]	1
B. napus	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**tr.**	**0.5**	**3**[7,8]	**4**[8,10]	2
Tro. majus (1)	**24**	**9**	**tr.**	**tr.**	**0.1**	**1.4**	n.d.	n.d.	n.d.	n.d[d]	2
Tro. peregrinum	n.d.	**5**	**0.7**	**0.5**	**tr.**	**20**	n.d.	n.d.	n.d.	n.d[d]	2

Glucosinolates **5** and **6** were not detected in any of the species. tr: less than 0.1 μmol/g dw.
[a] Main individual indole glucosinolates as superscript.
[b] Trace of **11** in roots of this species.
[c] 0.02 μmol/g dry wt. reported by Brown et al. (2003).
[d] **7** is inducible in *Tropaeolum*, see text.

Absence of some glucosinolates in Table 2 and Table 6 may be due to lack of induction, improper choice of plant part or growth stage, or simply to lack of sensitivity, but this is a general problem in taxonomic screens. The problem was significantly reduced by inclusion of roots in the screen. Induction of **7** in *Tropaeolum* species has been reported (Ludwig-Müller et al., 2002) but usually, induction of glucosinolates involves those already present (Travers-Martin and Müller, 2007; Traw and Dawson, 2002).

2.3. Taxonomic distribution of 4-hydroxyphenylacetonitrile-degrading enzymes

As indicated earlier, **2a** nitrile hydratase was already known for *S. alba* and *S. arvensis*. In this study, the known high nitrile hydratase activity in *S. arvensis* (converting **2a** to **2b**) was found to be accompanied by a low nitrilase activity (converting **2a** to **2c**). In contrast, the previously reported low nitrile hydratase activity in *S. alba* was accompanied by a high nitrilase activity (Table 3). In both cases, the identities of the products **2b** and **2c** were confirmed by isolation and NMR spectroscopy (Table 8).

The ratio of nitrile hydratase to nitrilase, expressed as nitrilase activity relative to the sum of the two activities, was consistent among the 4-5 accessions of each species tested (Table 3), and it was assumed that the relatively constant ratio of the two products was due to presence of one nitrilase enzyme with two products (**2b** and **2c**).

The activity of such **2a** nitrilase was significantly higher in *S. arvensis* and in *S. alba* than in all other species (Table 3). Mean total nitrilase activity (including nitrile hydratase activity) in *S. arvensis* and *S. alba* was 1.0-2.4 and 1.3-8.8 nkat/g fresh wt., respectively, significantly higher that the activity in *S. flexuosa* (0.17 nkat/g fresh wt.) despite its recent divergence from *S.alba* (section 2.1). Activities in the remaining species, both sinalbin containing and sinalbin deficient, were generally even lower than in *S. flexuosa*, with the highest in sinalbin deficient *B. napus* (0.15 nkat/g fresh wt.). Our estimate of the activity in the latter species was comparable to previous estimates of nitrilase activity in *B. napus* using other substrates, e.g. 0.24 nkat/g fresh wt. in seedlings (Bestwick et al., 1993), and 0.13-0.24 nkat/g fresh wt. in leaves (Rausch et al., 1981). As **2a** nitrilase in seedlings of the monocot *Sorghum bicolor* was reported during the writing of this paper (Jenrich et al., 2008), we included mature leaves of this species. The activity, 2.4 nkat/g fresh wt., was comparable to the activity in *S. alba* at our assay conditions (Table 3).

Assays of nitrile degrading enzymes can be affected by amidase activity and the presence of specific or general enzyme inhibitors,

Table 3
Nitrilase and nitrile hydratase activities (nkat/g fresh wt., mean (S.D)) in sinalbin containing (name in bold) and related sinalbin deficient plant species (name not in bold) as well as other species known or suspected to contain nitrilase activities

Species (accession)	*N*	Nitrilase	Nitrile hydratase	Nitrilase (%)[a]	Amidase[b]
S. arvensis (1)	2	0.21 (0.25)	1.84 (2.11)	9 (1)	0.03
S. arvensis (2)	2	0.14 (0.02)	0.83 (0.13)	17 (0)	0.02
S. arvensis (3)	5	0.22 (0.13)	1.13 (0.56)	17 (3)	0.02
S. arvensis (4)	2	0.16 (0.02)	1.27 (0.50)	12 (5)	0.03
S. arvensis (5)	2	0.41 (0.47)	2.01 (2.13)	15 (3)	0.03
B. nigra (1)	2	0.02 (0.02)	tr.	83 (2)	0.03
B. nigra (2)	2	0.09 (0.03)	tr.	83 (0.3)	0.06
Tra. labasii	2	tr.	n.d.	–	0.04
S. pubescens	2	0.06 (0.05)	tr.	80 (1)	0.03
S. boivinii	2	tr.	tr.	81 (1)	0.03
Hir. incana	2	0.04 (0.02)	tr.	83 (0.7)	0.02
S. alba (1)	5	2.06 (1.08)	0.13 (0.07)	94 (1)	0.02
S. alba (2)	2	1.16 (1.33)	0.11 (1.3)	90 (4)	0.03
S. alba (3)	4	5.49 (3.44)	0.32 (0.23)	94 (0.7)	tr.
S. alba* ssp. *mairei	2	8.43 (2.29)	0.47 (0.15)	94 (0.2)	0.01
S. flexuosa	3	0.17 (0.05)	tr.	100 (0)	0.02
K. cordylocarpus (1)	2	tr.	tr.	88 (9)	tr.
K. cordylocarpus (2)	2	tr.	tr.	95 (7)	tr.
C. rupestris	3	n.d.	n.d.	–	0.02
C. monensis	2	tr.	tr.	92 (12)	0.02
B. napus	2	0.13 (0.07)	0.02 (0.02)	85 (2)	0.01
A. thaliana	2	n.d.	n.d.	–	0.01
Tro. majus (2)	2	n.d.	n.d.	–	0.11
Tro. peregrinum	2	0.02 (0.03)	tr.	62 (–)	0.03
Sorghum bicolor[c]	2	2.32 (1.31)	0.03 (0.04)	98 (0)	n.d.

The limit of detection (LOD) for quantitative determinations (60 min incubation) was approximately 0.02 nkat/g. "tr." signifies that the activity was below the LOD but that a trace of activity was detected after incubation over night. Otherwise, the activity was concluded to be "not detected" (n.d.).

[a] Nitrilase activity (%) relative to the sum of nitrilase and nitrile hydratase.

[b] $N = 1$ for amidase assay (nkat/g fresh wt.).

[c] Neither sinalbin nor any other glucosinolate detected in *S. bicolor* (results not shown).

but these do not appear to be factors in this study. Amidase (converting **2b** to **2c**) activity was very low (Table 3) and would not interfere with the measurements of **2b** produced by nitrile hydratase activity. Indeed, **2b** and **2c** (at 1 mM) were essentially stable for 24 hours in all extracts. There was also no significant inhibition of the nitrile degrading activities of *S. arvensis* or *S. alba* when mixed in variable proportions with *K. cordylocarpus* and *C. rupestris* extracts. From preliminary variation of the conditions, we found the extraction conditions (pH 7, extract not desalted, no chelating or reducing agents) suitable for *S. arvensis* and *S. alba*. Thus, there were no signs of instability in those species in which we did detect significant activities, and mixing with selected extracts devoid of apparent activity did not reduce short term stability. However, the possibility of false negative results due to a general extraction method not optimized for each tested species could not be ruled out completely.

2.4. Substrate specificity of nitrilase and nitrile hydratase activities in *S. alba* and *S. arvensis*

The specificities of the **2a** (4-hydroxyphenylacetonitrile) degrading enzyme activities in both *S. alba* and *S. arvensis* extracts were relatively high, but the methyl ether **3a** (4-methoxyphenylacetonitrile) was also a good substrate for the activities in both species, again mainly with amide formation in *S. arvensis* and carboxylic acid formation in *S. alba* (Table 4). Compound **1a,** lacking the phenol group of **2a**, was also a good substrate for the activity in the *S. arvensis* extract, but not in the *S. alba* extract. Other positions on the aromatic ring were important in both species, as even the relatively small additional 3-methoxy substituent of **m2a** (Fig. 1) reduced the activity significantly. The importance of the precise aromatic ring structure was also shown by the lack of activity with 3-indolylacetonitrile (**7a**). The distance between the aromatic ring and the nitrile functional group was critical, as neither the lower homologue **0a** nor the higher homologue **5a** were accepted by any of the enzyme extracts. The specificities distinguish the enzyme activities in both *Sinapis* species from the previously known nitrilases in *A. thaliana*, *B. napus*, *B. campestris*, and *Sorghum bicolor* (Rausch et al., 1981; Bestwick et al., 1993; Vorwerk et al., 2001; Jenrich et al., 2008), and suggest that the investigated enzyme activities are specifically involved in sinalbin metabolism, not in metabolism of other aromatic glucosinolates in the investigated plants, such as **1** or **4** (in *S. alba*) or **7** (in both species).

Table 4
Specificity of the nitrile degrading enzyme activities in crude extracts of *S. alba* (1) and *S. arvensis* (3)

Substrate	*S. arvensis* extract nitrile hydratase activity (mean (S.D.))	Nitrilase (%)[a]	*N*	*S. alba* extract nitrilase activity (mean (S.D.))	Nitrilase (%)[a]	*N*
0a	0.5* (0.6)	–	2	0.4* (1.2)	–	2
1a	96.5ns (17.7)	20 (6)	4	3.7* (1.6)	–	4
2a	100.0	20 (4)	4	100.0	94 (0.3)	4
m2a	15.4* (1.2)	19 (1)	2	1.3* (1.8)	–	2
3a	123.6ns (9.6)	19 (3)	3	81.1* (5.7)	95 (0.3)	3
4a	0.0* (0.0)	–	3	0.6 (–)	–	1
5a	0.7* (0.9)	–	2	0.2* (0.3)	–	2
7a	2.8* (0.1)	–	2	0.0* (0.0)	–	2

Relative activities statistically different from 100 ($P < 0.05$, concluded from 95% confidence intervals) are indicated with *, lack of stat. sign. with ns.

Activities of nitrile hydratase (converting **xa** to **xb**) and nitrilase (converting **xa** to **xc**) were measured for each substrate. The activity with **2a** (nkat/ml extract) was defined as 100, and remaining activities obtained with the same extract were expressed relative to this.

[a] Nitrilase activity (%) relative to the sum of nitrilase and nitrile hydratase determined from concentrations of **xb** and **xc** after incubation for 22 h. When one or both peaks were negligible or absent, the value was not calculated (–).

2.5. Phylogenetic distribution of aliphatic glucosinolates

Significant variation of several aliphatic glucosinolates were observed within *S. arvensis*, as well as among Sinapis *alba* and closely (*S. flexuosa*) or more distantly related species (*C. rupestris*, *E. canariensis*) (Fig. 4, Table 5, Table 6). Glucosinolates **15**, **17** and **18*R*** were previously known from *Coincya* (Vioque et al., 1994) and **15** from *S. alba* (Cole, 1976). Two long chain glucosinolates (**21** and **23)** had previously been identified in *S. arvensis* (Griffiths et al., 2001), while **25** (and the minor levels of **22** + **24**) had apparently not been observed before in the species (Fahey et al., 2001). Although incomplete, unassigned NMR data of desulfoderivatives of **21**, **23** and **25** had been reported (Yamane et al., 1992), it was considered relevant to report the complete, assigned spectral data (Table 5). Branched-chain glucosinolates (**27**, **28**) had not been reported previously from *Coincya*, *Erucastrum*, or *Hirschfeldia* (Fahey et al., 2001). The structure of **28** could not be identified from one-dimensional ^{1}H NMR alone, and dept, ^{13}C NMR, cosy, hsqc, hmbc and jres spectra were additionally recorded. Dept confirmed the methylpentyl side chain, and a combination of cosy, hsqc, and dept (data not shown) allowed us to conclude that the methyl group was in the 3-position (Fig. 4).

Most species in all three *Sinapis* clades accumulated Met derived glucosinolates, while only a few species accumulated other

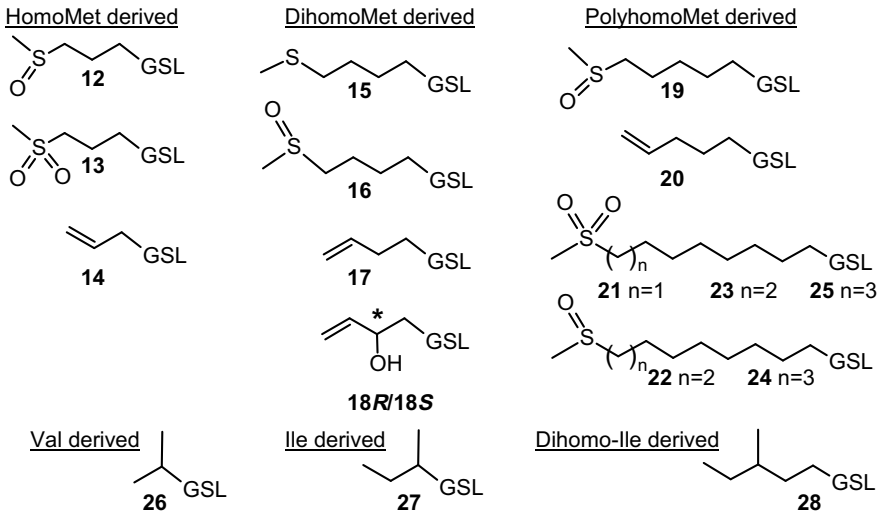

Fig. 4. Aliphatic glucosinolates identified in *Sinapis* sp. and related species. **12**, 3-methylsulfinylpropylGSL; **13**, 3-methylsulfonylpropylGSL; **14**, allylGSL (sinigrin); **15**, 4-methylthiobutylGSL; **16**, 4-methylsulfinylbutylGSL (glucoraphanin); **17**, 3-butenylGSL (gluconapin); **18*R***, *R*-2-hydroxy-3-butenylGSL (progoitrin); **18*S***, *S*-2-hydroxy-3-butenylGSL (*epi*progoitrin); **19**, 5-methylsulfinylpentylGSL; **20**, 5-pentenylGSL; **21**, 8-methylsulfonyloctylGSL; **22**, 9-methylsulfinylnonylGSL; **23**, 9-methylsulfonylnonylGSL; **24**, 10-methylsulfinyldecylGSL; **25**, 10-methylsulfonyldecylGSL; **26**, *iso*-propylGSL; **27**, *sec*-butylGSL; **28**, 3-methylpentylGSL.

Table 5
Identification of desulfo-derivatives (**dx**) of aliphatic glucosinolates (**x**), obtained by sulfatase treatment of native glucosinolates in extracts, by ^{1}H NMR (obtained in D_2O), molecular ion (*m*/*z* of $[M+Na]^+$ and HPLC retention time

Side chain[a]	1	2	3	4	5	Me
d15[b]	2.57 (*t*)	1.6–1.8 (*m*)	do.	2.62 (*t*)	–	2.09 (*s*)
d17[c]	2.70 (*t*)	2.41 (*qua*[d])	5.9 (*m*)	5.06 (*d*[d],10)+5.12(*d*[d],17)-	–	–
d18*R*[e]	2.81 (*m*[f])	4.55 (*m*)	5.92 (*m*)	5.21 (*d*[d],11)+5.30(*d*[d],17)-	–	–
d26[g]	2.90 (*m*)	1.22 (*d*)	–	–	–	1.23 (*d*)
d27[c,g]	2.71 (*m*)	1.54 + 1.69 (*m*)	0.91(*t*)	–	–	1.19 (*d*)
d28[h]	2.57 + 2.62 (*m*)	1.47 + 1.65 (*m*)	1.42 (*m*)	1.18 + 1.37 (*m*)	0.86 (*t*)	0.90 (*d*)

	1	2	3 to *n* − 3	*n* − 2	*n* − 1	*n*	Me
d23[i]	2.59 (*t*)	1.64 (*m*)	1.33 (*m*)	1.45 (*m*)	1.80[j](*qui*)	3.24[j] (*dd*)	3.06 (*s*)

Thioglucoside moeity, HPLC-DAD retention time (Rt) and *m*/*z* of $[M+Na]^+$

	d15	**d17**	**d18R**	**d21**[g]	**d23**	**d25**[g]	**d26**	**d27**	**d28**
1′	5.01[k]	4.99[k]	5.05[k]	4.97[k]	4.97[k]	4.97[k]	5.00[k]	4.98[k]	4.96[k]
Rt (min)	22.1	14.2	6.2	29.8	35.6	41.2	10.1	17.3	33.0
m/*z*	364	316	332	452	466	480	304	318	346

[1] H NMR data are reported as δ*H*/ppm (multiplicity, J/Hz).
[a] For all resolved doublets, triplets, quartets and quintets, J was 7–8 Hz unless otherwise indicated.
[b] Isolated from *S. alba* (1) roots.
[c] Isolated from *C. rupestris* leaves.
[d] With additional fine structure.
[e] Isolated from *C. rupestris* roots.
[f] ABX system.
[g] Isolated from *Sisymbrium* sp. leaves.
[h] Isolated from *E. canariense* leaves. Due to peak overlap, ^{1}H chemical shifts were extracted from 2D spectra.
[i] Isolated from *S. arvensis* (1) leaves. Chemical shifts for **d21** and **d25** were identical to those of **d23** (within 0.01 ppm).
[j] Assignment verified by COSY.
[k] Doublet, *J* = 10 Hz. Other thioglucoside signals were (±0.01 ppm and 1 Hz): 2′–5′: 3.40–3.58 (*m*), 6′a: 3.88 (*dd*, 12;2), 6′*b*: 3.70 (*dd*, 13;6).

Table 6
Aliphatic glucosinolates in *S. pubescens*, *S. arvensis*, *S. alba*, and their close relatives, with the non-Brassicaceae *T. peregrinum* for comparison

Species (taxon)	Plant part	**Glucosinolate** (*Side chain class*)													
		12 C3	**13** C3	**14** C3	**15** C4	**16** C4	**17** C4	**18**[a] C4	**19** C5	**20** C5	**21** C8	**23**[22] C9	**25**[24] C10	**27**[26] BCAA	**28** BCAA[b]
Trop. peregrinum	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.6**[2]	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.[tr.]	n.d.
S. arvensis (1)	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1.4**	**10**[c]	**3**[d]	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**tr.**	**0.6**[c]	**tr.**[d]	n.d.	n.d.
S. arvensis (2)	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	**tr.**	**0.5**[c]	**0.2**[d]	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	**tr.**	**tr.**[c]	**tr.**[d]	n.d.	n.d.
S. arvensis (3)	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	**tr.**	**1.0**[c]	**0.6**[d]	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	n.d.	**tr.**[c]	**tr.**[d]	n.d.	n.d.
S. arvensis (4)	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	n.d.	**0.1**[c]	**tr.**[d]	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	n.d.	n.d.	n.d.	n.d.	n.d.
S. arvensis (5)	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1.0**	**6**[c]	**0.7**[d]	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**tr.**	**0.2**[c]	n.d.	n.d.	n.d.
B. nigra (1,2)	Leaf	n.d.	n.d.	**47**	n.d.	n.d.	**0.3**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	n.d.	n.d.	**0.6**	n.d.	n.d.	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trac. labasi	Leaf	**tr.**	n.d.	**70**	n.d.	**tr.**	**0.1**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	**tr.**	n.d.	**54**	n.d.	**tr.**	**0.1**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G. arvensis	Leaf	**tr.**	n.d.	**61**	n.d.	n.d.	**0.1**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	**tr.**	n.d.	**150**	n.d.	n.d.	**0.1**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Brassica aucheri	Leaf	**0.1**	**6**	n.d.	n.d.	n.d.	n.d.	**tr.**^{R}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	**0.3**	**34**	n.d.	**tr.**	n.d.	n.d.	**0.2**^{R}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S. pubescens	Leaf	n.d.	n.d.	n.d.	n.d.	**tr.**	**41**	**tr.**^{S}	**tr.**	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	**0.1**	**31**	**tr.**^{S}	**tr.**	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.
S. boivinii	Leaf	**tr.**	n.d.	**8**	n.d.	**tr.**	**35**	n.d.	**tr.**	**tr.**	n.d.	n.d.[e]	n.d.[e]	n.d.	n.d.
	Root	n.d.	n.d.	**4**	n.d.	**0.1**	**27**	n.d.	**–**	**–**	n.d.	n.d.	n.d.	n.d.	n.d.
S. pub. ssp. *indurata*	Leaf	n.d.	n.d.	**tr.**	n.d.	**tr.**	**34**	n.d.	**tr.**	**tr.**	n.d.	n.d.[e]	n.d.[e]	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	**tr.**	**27**	n.d.	**tr.**	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.
H. incana	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	**2**	**0.2**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	n.d.	**3**	**8.3**^{R}	**tr.**	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.
E. canariense	Leaf	**tr.**	n.d.	**14**	**tr.**	n.d.	**0.2**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**3.0**
	Root	**tr.**	n.d.	**34**	n.d.	n.d.	**0.3**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2.1**
S. alba (1,2,3)	Leaf	n.d.	n.d.	n.d.	n.d.	**tr.**	**tr.**	**0.2**^{S}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S. alba (1)	Root	n.d.	n.d.	n.d.	**3**	**0.6**	**tr.**	**1.1**^{S}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S. alba (2)	Root	n.d.	n.d.	n.d.	**0.4**	**0.8**	**tr.**	**0.2**^{S}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S. alba (3)	Root	n.d.	n.d.	n.d.	**6**	**0.7**	n.d.	**0.5**^{RS}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S. alba* ssp. *mairei	Leaf	n.d.	n.d.	n.d.	n.d	**tr.**	**tr.**	**0.7**^{S}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	**1.8**	**0.4**	**tr.**	**2.0**^{S}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S. flexuosa	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
K. cordylocarpus (1)	Leaf	n.d.	n.d.	n.d.	n.d.	n.d.	**0.6**	**1.0**^{S}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	n.d	**tr.**	**tr.**	**0.6**^{S}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
K. cordylocarpus (2)	Leaf	n.d.	n.d.	n.d.	n.d.	**0.1**	**0.4**	**tr.**^{S}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
H. fruticulosa	Leaf	**tr.**	n.d.	**tr.**	n.d.	**0.1**	**59**	**0.9**^{S}	**tr.**	**tr.**	n.d.	n.d.	n.d.	n.d.	**1.3**
	Root	n.d.	n.d.	**tr.**	n.d.	**tr.**	**7**	**8.1**^{S}	**tr.**	**tr.**	n.d.	n.d.	n.d.	n.d.	**0.7**
C. rupestris	Leaf	**tr.**	n.d.	**tr.**	n.d.	**0.3**	**38**	**0.5**^{R}	**tr.**	**tr.**	n.d.	n.d.	n.d.	**1.6**	n.d.
	Root	n.d.	n.d.	**tr.**	**2**	**1.6**	**5**	**26**^{R}	**tr.**	n.d.	n.d.	n.d.	n.d.	**0.4**	n.d.
C. monensis	Leaf	n.d.	n.d.	**tr.**	n.d.	n.d.	**3**	**1.4**^{S}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	**0.2**	**0.9**	**0.3**	**1.5**^{R}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C. longirostra	Leaf	n.d.	n.d.	**tr.**	n.d.	**0.1**	**34**	**0.1**^{R}	**tr.**	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.
	Root	n.d.	n.d.	n.d.	**8**	**1.6**	**2**	**10**^{R}	**tr.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., Not detected; tr., less than 0.1 μmol/g dry wt.; –, not determined (LC–MS not carried out).
Species with high levels of sinalbin in bold.
[a] Predominantly the *R* or *S* stereoisomer as indicated.
[b] BCAA: Branched-chain amino acids. **26** and **27** from standard amino acids, **28** from chain elongated amino acid.
[c] **22** Also detected at ca. 10% of **23**.
[d] **24** Also detected at ca. 10% of **25**.
[e] Not detected according to LC–MS, in LC-DAD there was overlap with unidentified aromatic compounds, apparently not corresponding to any major peaks in LC–MS.

aliphatic glucosinolates (Table 6). There was a tendency for an inverse relationship between the content of sinalbin and aliphatic glucosinolates (**12**–**28**); species or accessions high in sinalbin were usually low in aliphatic glucosinolates (compare e.g. *S. alba* and *C. monensis* to *C. rupestris* and *C. longirostra*, and the *S. arvensis* accessions 1 and 5 to 2, 3 and 4). This pattern would suggest that evolutionary loss of high levels of sinalbin in *Coincya* sp. was accompanied with increased levels of aliphatic glucosinolates (or *vice-versa*).

With respect to chain length of Met derived glucosinolates (Table 6), most investigated species variously accumulated "C3" glucosinolates (**12**–**14**) with a trace of "C4" glucosinolates (**15**–**18**), or C4 glucosinolates with traces of C3 and "C5" glucosinolates (**19**–**20**), as would be expected from expression of homologues of either *MAM*2 (controlling chain elongation of Met to mainly homoMet) or *MAM*1 (controlling chain elongation of Met to mainly dihomoMet) (Kliebenstein et al., 2005). The two chain length phenotypes were mostly distinct (with dominance of either C3 or C4 glucosinolates, not both), except in the polyploid *S. boivinii*, which contained significant amounts of both C3 and C4 glucosinolates.

In the Alba clade, all investigated species (except *S. flexuosa* which was devoid of aliphatic glucosinolates) contained C4 aliphatic glucosinolates, suggesting that *MAM*2 like genes are

frequent and *MAM*1 like genes are rare or absent in the clade. In the Pubescens clade, both chain length types were observed, while in the Arvensis clade, four of five investigated species were dominated by C3 glucosinolates, while one species, *S. arvensis*, contained highly variable levels of long chain Met derived glucosinolates with 8–10 methylene groups in the side chains ("C8, C9 and C10 glucosinolates", **21**-**25**). The long chain glucosinolates could be due to an enzyme similar to MAM3 in *A. thaliana* (Textor et al., 2007), but of an even more processive nature. *S. arvensis* may be even more polymorphic for aliphatic glucosinolates than illustrated by the five accessions analysed here, as *S. arvensis* seeds from former Yogoslavia were dominated by the C9 glucosinolate **23** while seeds of the same species from Tunisia were dominated by the C3 glucosinolate **12** (Daxenbichler et al., 1991). There was no correlation between geographic origin and level of **21**–**25** in our data set (Table 1, compare origin of *S. arvensis* 1 and 5).

Variation in side chain modification of n-homoMet-derived glucosinolates (Halkier and Gershenzon, 2006) was limited within the investigated species, and can be interpreted in genetic terms (Giamoustaris and Mithen, 1996) with the Alba clade as example. In that clade, genes and enzymes responsible for both oxidation of side chain sulphur (*GSL-OXID* homologues converting, e.g., **15** to **16** or 3-methylthiopropylglucosinolate to **12**), removal of CH_3S- and desaturation (GSL-ALK homologues converting, e.g., **15** to **17**), and oxidation of the alkenyl glucosinolate **17** to the 2-hydroxyalkenyl glucosinolate **18** (GSL-OH homologues), all seemed to be present (perhaps except in *S. flexuosa*), resulting in a mixture of side chain types. Species lacking sinalbin were dominated by **17** in leaves and **18** in roots, while the parent C4 glucosinolate **15** was the dominating aliphatic glucosinolate in *S. alba*. The particular stereoisomer of **18** accumulated was variable even among closely related species, in agreement with a previous report of intraspecific variation of this character in *Coincya* (Vioque et al., 1994).

In three species of the Arvensis clade, non-hydroxylated alkenyl glucosinolates (**14**, **17**) dominated, while in two species (*B. aucheri*, *S. arvensis*), alkenyl glucosinolates were absent or almost absent. In the latter two species methylsulphonylalkyl glucosinolates (either **13** or **21**, **23**, **25**) were prominent, along with minor amounts of less oxidized methylsulphinyl glucosinolates (**12**, **22**, **24**) that are likely biosynthetic intermediates. Considering glucosinolate profiles only, as observed in this study and particularly the similarity of *B. aucheri* and a previously described Turkish accession of *S. arvensis* (Daxenbichler et al., 1991), the former placement of *B. aucheri* in the genus *Sinapis* appears as meaningful as the present placement in *Brassica*, but both genera are obviously not natural groups as defined currently.

The side chain modifying biosynthetic characters of the investigated species of the Pubescens clade appeared to be of the same types as deduced for the Alba clade, but the extent of 2-hydroxylation was typically lower, and the parent C4 glucosinolate **15** was typically absent.

Branched-chain glucosinolates (**26**, **27**, **28**) were detected in a few species only, and with a peculiar, scattered distribution (Table 6). Two of the occurrences (**27**, **28**) were in the Alba clade, in both cases in species that had apparently undergone a complete (*H. fruticulosa*) or partial (*C. rupestris*) secondary loss of sinalbin biosynthesis. Low levels of **27** and absence of **17** have also been reported in *S. alba* leaves (Cole, 1976; Nielsen et al., 1979), but we detected **17** but not **27** in the *S. alba* accessions by LC-MS. This data suggest that biosynthesis of detectable levels of branched-chain glucosinolates evolved as a consequence of the loss of sinalbin biosynthesis, in addition to the stimulation of the biosynthesis of existing aliphatic glucosinolates. The third occurrence of this glucosinolate type (in *E. canariense*) was likewise in a species that could be expected to have undergone a recent mutation in glucosinolate biosynthesis (from a profile dominated by C4 glucosinolates, as in the rest of the Pubescens clade, to a profile dominated by C3 glucosinolates). It was also peculiar to observe the similarity of the "new" glucosinolates, being in all three cases derived from the same amino acid, Ile (with or without chain elongation to dihomoIle). As the Ile derived glucosinolate **27** is common in *Sisymbrium* (Daxenbichler et al., 1991; Bennett et al., 2004), which is a sister group to the Brassiceae including *Sinapis* (Koch et al., 2001), it is possible that the ability to channel Ile into glucosinolate biosynthesis is a latent (as defined by Wink and Witte, 1983; Wink, 2003) character in the Brassiceae. The biosynthesis of **28** in *E. canariense* and *H. fruticulosa* would then be a combination of an ancient, re-activated trait (glucosinolates derived from Ile), combined with a typical Brassiceae trait, the biosynthesis of glucosinolates derived from chain elongated amino acids. In turn, some genes involved in biosynthesis of chain elongated Met derived glucosinolates may derive from genes involved in Leu biosynthesis (Binder et al., 2007).

Alternatively, it may be that the expression levels of the relevant enzymes in other species were simply too low for the glucosinolate to be detected, as was demonstrated for CYP79A2 from *A. thaliana* causing detectable accumulation of **1** only after homologous overexpression (Wittstock and Halkier, 2000). Previous reports of **27** in *S. alba* would support this interpretation (Cole, 1976; Nielsen et al., 1979).

2.6. Evolution and ecological effects of sinalbin (**2**) accumulation

The apparent evolution of accumulation of sinalbin at the expense of very bioactive glucosinolates in both the Alba and Arvensis clades is of ecological interest, given the purported roles of specific glucosinolates in nematode (Buskov et al., 2002; Lazzeri et al., 2004), disease (Brader et al., 2006), and general resistance (Traw and Feeny, 2008), and perhaps in specialist insect resistance (Bodnaryk, 1991; but see Hopkins et al., 1998; Nielsen et al., 2001). Low *in vitro* activity of sinalbin degradation products towards pests (Buskov et al., 2002; Lazzeri et al., 2004) has been reported and attributed to substitution reactions of **2i** (Fig. 5B). However, the inverse quantitative relationship of sinalbin and aliphatic glucosinolates (section 2.5) suggests that sinalbin may substitute for ecological effects of aliphatic glucosinolates *in vivo*. An alternative explanation, that biosynthesis of sinalbin would lower biosynthesis of other glucosinolates due to competition for enzymes or substrates, is less likely because endogenous leaf glucosinolates were not reduced in *A. thaliana* plants metabolically engineered to accumulate sinalbin (Petersen et al., 2001).

The apparently repeated gain and loss of high sinalbin phenotypes in the evolution of the Alba clade suggests that particular environments may variously select for and against accumulation of sinalbin. The fact that *S. alba* accumulated much more sinalbin than **1** (Table 2) suggests that accumulation of sinalbin is at least as advantageous as accumulation of **1** in environments were *S. alba* has evolved, despite the labile nature of **2i** (Fig. 5B). Possible advantages of accumulation of sinalbin rather than **1** or **4** in leaves could be its ability to liberate thiocyanate ion, which has been demonstrated to be an intermediate in biosynthesis of methylthiocyanate (Attieh et al., 2000; Attieh et al., 2002, Fig. 5C) as well as phytotoxic in its own right (Borek and Morra, 2005). The non-volatile nature of isothiocyanate **2i** produced from sinalbin, in contrast to **1i** and **4i**, could also reduce long distance attraction of crucifer specialist insects (Renwick et al., 2006). A similar situation of glucosinolates with non-volatile products in leaves and **4** in roots has been reported from *Barbarea vulgaris* (Agerbirk et al., 2003), and a rare chemotype accumulating **4** in leaves has recently been described (van Leur et al., 2006). The present paper provides additional plant species for experimental

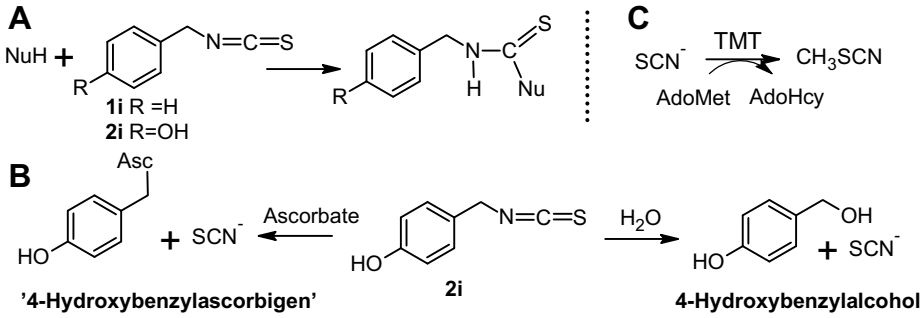

Fig. 5. (A) General reactivity of glucosinolate-derived isothiocyanates such as **1i** and **2i** with nucleophiles (Nu). (B) Competing nucleophilic substitution reactions of **2i** but not **1**. (C) Methylation of thiocyanate ion by thiol methyltransferase (TMT). AdoMet: *S*-adenosyl-L-methionine, AdoHcy: *S*-adenosyl-L-homocystein.

studies of ecological effects (van Leur et al., 2008) of variable glucosinolate profiles in closely related species or genotypes of the same species.

2.7. Evolution and function of 4-hydroxyphenylacetonitrile (*2a*) degrading enzymes

The infrequent occurrence of significant **2a** degrading enzyme activity in general, and its presence and specificity in some but not all of the sinalbin containing species investigated, may suggest a facultative function of **2a** degrading enzymes in plants containing sinalbin. Alternatively, the enzyme may be universally required in sinalbin metabolism, but in some cases confined to other organs or growth stages than investigated here.

Three observations suggest that the nitrilase/nitrile hydratase detected in *S. arvensis* has evolved independently of the nitrilase in *S. alba*: The product profile, dominated by amides, the somewhat different specificity, accepting **1a** without a *para*-positioned oxygen, and the isolated occurrence in a species where also the glucosinolate content appears to have evolved drastically after its divergence from the closely related *B. nigra*.

Nitrilases of the NIT4 family, widespread in higher plants, are involved in detoxification of cyanide produced in ethylene biosynthesis (Piotrowski and Volmer, 2006). But recently, a nitrilase of the NIT4 sequence family with ability to also hydrolyse **2a** was reported from *Sorghum bicolor*, a glucosinolate-deficient monocotyledonous plant (Jenrich et al., 2008). It was demonstrated that the cyanogenic glucoside dhurrin can degrade to **2a**, in addition to the usual cyanogenic degradation, and the **2a** nitrilase was suggested to be involved in detoxification of **2a** from this new pathway. It may be that higher plants have an inherent potential for the evolution of NIT4 with such specificity. Such potential could have been realised in *S. alba* and *S. arvensis*, representatives of two groups of sinalbin containing plants. Alternatively, the detected **2a** degrading enzymes could have evolved from NIT1-3 that were suggested to be involved in glucosinolate metabolism in *A. thaliana*.

If the epithiospecifier protein ESP, which controls hydrolysis of glucosinolates to nitriles rather than isothiocyanates (Fig. 2, Bones and Rossiter, 2006)), were present in *S. alba* and *S. arvensis*, but not in other sinalbin containing species such as *K. cordylocarpus* and *C. monensis*, the sporadic occurrency of **2a** degrading enzymes would be explained, but we are not aware of any reports of ESP in the two *Sinapis* species (Cole, 1976). However, both *S. alba* and *S. arvensis* are accepted by *Pieris* caterpillars, and in the gut of these, hydrolysis of sinalbin is directed towards **2a** by NSP (Fig. 2). Further conversion of **2a** to **2b** by the plant enzyme has been demonstrated in chewed *S. arvensis* tissue in caterpillar guts (Agerbirk et al., 2007), and it is possible that there is an ecological function of **2a** degrading plant enzymes in herbivore guts. Indeed, the first ecological function of an NSP product (**7a**) has been reported (De Vos et al., 2008). Another possible NSP product, **1a**, is a signal involved in *P. brassicae* mating behavior (Andersson et al., 2003).

The identification of phylogenetically related sinalbin containing plant species with widely different activities of **2a** degrading enzymes will facilitate future investigations of the involvement of such plant enzymes in plant or caterpillar glucosinolate metabolism (Vergara et al., 2006; Agerbirk et al., 2007), the structures of the metabolites formed, and their possible functions.

3. Experimental

3.1. General experimental procedures

All substrates and authentic references of products were from Sigma-Aldrich (Steinheim, Germany), except 3-(4-hydroxyphenyl)propionitrile (**5a**) (Alfa Aesar, Karlsruhe, Germany) and a number of amides (section 3.6). Equipment for HPLC-DAD and NMR spectroscopy was as previously described (Agerbirk et al., 2007).

3.2. Plants

Plants were grown from seeds in a greenhouse using enriched peat based growth medium (Pindstrup substrate No. 2). Suppl. light and heating were employed to ensure long day conditions (□16 h of light) and temp. above ca. 16 °C. Voucher specimens of plants previously investigated by SW have been deposited as reported elsewhere (Warwick and Sauder, 2005). Voucher specimens of additional species have been dep. at the herbarium of Dept. of Botany, Faculty of Life Science, KU, seeds of *S. arvensis* (3) were deposited at UPM (Table 1), and seeds of *S. arv.* (1) have recently been deposited after harvest in 2008.

Plants for purification of reference compounds were: *Barbarea verna* (Agerbirk et al., 2003), *Arabis hirsuta* (grown from seeds provided by Bennet, originally obtained from Botanischer Garten der J.W. Goethe Universität, Frankfurt, Germany), *Arabis* sp. [tentatively identified as *Arabis allionii* DC by Prof. N. Jacobsen, this species was originally obtained from the Bot. Garden of Giessen University (Germany) under the name "*A. aubrietioides*" by R.N. Bennett, who reported seed glucosinolate contents (Bennett et al., 2004) and contributed seeds to this study], and *Sisymbrium* sp. foliage (collected in Massachusetts by Chew).

3.3. Phylogenetic analysis

A phylogenetic tree was obtained from sequence data from the ITS region (internal transcribed spacers ITS-1 and ITS-2 of nuclear DNA, and the 5.8 rRNA gene) of 20 species of *Sinapis*, *Brassica* and related cruciferous species. Sequences for the 20 Brassiceae

members (Warwick and Sauder, 2005) were obtained from GenBank, along with those for the two outgroup taxa, *A. thaliana* and *T. majus*; GenBank accession numbers are given in Table 1. Maximum parsimony and bootstrap analyses of the 22 aligned ITS sequences were conducted using the computer program PAUP*, version 4.0b10 (Swofford, 2002), using procedures outlined in Warwick and Sauder (2005). The most parsimonious trees were generated using the heuristic search algorithm, with tree bisection-reconnection (TBR) branch-swapping, equal-weighted characters, 1000 random additions of the sampled taxa, and 1000 trees saved per replicate. A total of 1000 bootstrap replicates were generated in order to test the stability of particular nodes in the parsimony analysis using a full heuristic search, with options ACCTRAN, MULTREES = MULPARS, and TBR branch-swapping, and each replicate generated with simple addition sequence of taxa, with 1000 trees saved per replicate.

3.4. Determination and identification of glucosinolates

Roots and leaves of at least two individual plants of each accession were analysed separately. Roots were rinsed briefly in tap water followed by light drying with tissue paper and immediate lyophilization. Extensive rinsing of roots was avoided in order to reduce the risk of leaching and tissue damage. Some adhering peat was therefore accepted, meaning that reported levels are minimum estimates. Glucosinolates (GSLs) were extracted in boiling MeOH–H_2O (7:3) from lyophilised roots and leaves, adsorbed to anion exchange columns, and enzymatically converted to desulfoGSLs; which were identified and quantified by HPLC-DAD, all as prev. descr. (Agerbirk et al., 2007) except that an external std. of sinigrin (response factor 1.00) was used instead of an internal std., and an elution vol. of 5 ml was used from anion exchange columns. Generally accepted resp. factors were used (Wathelet et al., 2004), and **3**, **5** and **6** were assumed to have the same resp. factor as **2** (0.5). GSL identities were confirmed by comp. with auth. references (Agerbirk et al., 2003, **d1** and **d2** from *S. alba* roots, **d3** from *T. peregrinum* roots, **d4** from *B. verna* seeds, **d5** from *A. hirsuta* leaves and **d6** from *A. aubretioides* leaves (Table 7, Table 8)), and this procedure sufficed for the detection and quantification of all aromatic GSLs. In order to identify aliphatic GSLs, and to confirm detected presence of **1** and **2**, LC-MS was used on at least one root sample and one leaf sample of each plant species, supplemented with isolation of selected desulfoGSLs (Table 5), and comp. with **d14** from comm. avail. **14**, and **d16** from desulfated *A. thaliana* GSLs. Analytical LC–MS was carried out on an Agilent 1100 Series LC (Agilent Technologies) coupled to a HCT*plus* ion trap mass spectrometer (Bruker Daltonics). The column was a Synergy Fusion-RP column (Phenomenex; 2.5 μm, 100 Å, 2 × 100 mm), and the flow rate was 0.2 ml/min. The mobile phases were: A, 0.1% (v/v) HCOOH, 50 μM NaCl in water; B, 0.1% (v/v) HCOOH in MeCN. The grad. program was: 0-20 min, linear grad. 5-100% (v/v) B; 20-23 min, 100% B; 24-30 min, 5% B. The mass spectrometer was run in electrospray mode with detection of positive ions. Data handling was done with the native DataAnalysis software, and individual desulfoGSLs were identified based on the Na^+ adducts, $[M + Na]^+$. Determination of the dominant stereoisomer of **18R** and **18S** was done by visual comparison of the relevant *m*/*z* 332 $[M+Na]^+$ peaks (at 2.5 and 2.8 min), the early peak was identified as the *R* isomer. Retention times (min) in HLPC-DAD, of compounds not specified in tables, were: d**12**: 4.9; d**13**: 5.9; d**18R**: 6.2; d**18S**: 7.6; d**14**: 7.8; d**16**: 8.2; d**19**: 13.7; d**9**:19.7; d**7**: 26.6; d**10**:31.7; d**22**: 34.8; d**8**: 35.8; d**11**: 40; d**24**: 40.4.

Table 7
^{1}H NMR spectroscopic data (obtained in D_2O) for desulfated glucosinolates **d1–d6**. The number of side chain methylene groups affected selected ^{1}H and ^{13}C NMR signals in thioglucose moieties

	d1	d2	d3	d4	d5	d6
Side chain						
1	4.03 (*s*)	3.94 (*s*)	3.98 (*s*)	2.9–3.0 (*m*)	2.8–3.0 (*m*)	2.8–3.0 (*m*)
2	–	–	–	do.	do.	do.
2′ + 6′	7.4–7.5 (*m*)	7.21 (*d* ,8)	7.28 (*d*, 8)	7.3–7.4 (*m*)	7.17 (*d*, 8)	7.24 (*d*, 8)
3′ + 5′	do.	6.89 (*d*, 8)	7.01 (*d*, 8)	do.	6.85 (*d*, 8)	6.96 (*d*, 8)
4′	do.	–	–	do.	–	–
OMe	–	–	3.83 (*s*)	–	–	3.81 (*s*)
Thioglucoside moeity						
1	n.d.	4.7[a]	n.d.	4.90 (*d*, 9)	4.85 (*d*, 10)	4.85 (*d*, 11)
2–5	3.2–3.42 (*m*)	3.2–3.42 (*m*)[b]	3.2–3.43 (*m*)	3.4–3.5 (*m*)	3.4–3.6 (*m*)	3.4–3.6 (*m*)
6a	3.6–3.7 (*m*)	3.6–3.7 (*m*)	3.6–3.7 (–)	3.67 (*dd*, 12;5)	3.68 (*dd*, 13;5)	3.67 (*dd*, 13;5)
6b	do.	do.	do.	3.85 (*dd*, 13;2)	3.84 (*d*, 13)	ca.3.82[c]

[1] H NMR data are reported as δ/ppm (multiplicity, J/Hz). n.d., not detected due to peak overlap with DOH at 4.7–4.8 ppm.
[a] Revealed by hsqc.
[b] Hsqc revealed the following shifts (ppm): 2+3: 3.3, 4: 3.4, 5: 3.2.
[c] Peak overlap.

Table 8
^{13}C NMR spectroscopic data of desulfated glucosinolates **d1–d6** (spectra obtained in D_2O) and enzyme products **2b** and **2c** (spectra obtained in CD_3OD) (δ in ppm) as well as HPLC-DAD retention times (Rt) and UV absorption maxima obtained at the HPLC conditions described for desulfoglucosinolates and **2b** + **2c**, respectively

	d1	d2	d3	d4	d5	d6	2b	2c
Aglucone								
0[a]	155.3	155.7[c]	155.5	156.2	156.3	156.2	–	–
1	38.8	38.1	38.0	34.4	34.6	34.5	177.7	176.2
2	–	–	–	33.8	33.1	33.1	42.7	41.1
1′	136.9	128.5	129.4	141.4	133.3	134.0	127.6	126.8
2′ + 6′	128.9[b]	130.2	130.1	129.5	130.8	130.7	131.2	131.4
3′ + 5′	129.9[b]	116.7	115.3	129.5	116.3	115.0	116.4	116.3
4′	128.1	155.7[c]	158.8	127.3	154.8	158.2	157.5	157.5
OMe	–	–	56.2	–	–	56.2	–	–
Thioglucoside moeity								
1	81.9	81.9	81.9	82.3	82.3	82.3	–	–
2	72.9	72.9	72.8	73.0	73.0	73.0	–	–
3	77.9	77.9	77.9	77.9	77.9	78.0	–	–
4	69.7	69.7	69.7	70.0	70.0	70.0	–	–
5	80.6	80.6	80.6	80.9	80.9	80.8	–	–
6	61.2	61.1	61.2	61.4	61.4	61.4	–	–
Rt/min	21.4	16.3	25.5	28.2	23.0	32.4	16.1	25.6
UV(λ_{max}/nm)	–	225	226	–	222	222	222	223
	–	276	276	–	275	274	274	274

[a] By convention, the oxime carbon is numbered 0 in glucosinolates.
[b] Assignments may be exchanged.
[c] Same chemical shift of C0 and C4′ confirmed by hmbc.

3.5. Assay for nitrilase, nitrile hydratase, and amidase

Fresh foliage (1-1.5 g, younger leaves) was extracted in a 1:1 mixt. of 0.1 M aq. Na_2HPO_4 and NaH_2PO_4 as prev. rep. (Agerbirk et al., 2007). From each accession, at least two plants were extracted. Each extract was assayed for nitrilase and nitrile hydratase activity: To each of 3 aliquots (600 μl) of aq. 20 mM **2a** (for amidase assay: **2b**) in 'boil proof' Eppendorf tubes was added 200 μl crude extract, and the mixtures were incubated at 30 °C for either 0 min, 60 min or 22 h, imm. foll. by immersion for 2 min in boiling water, which served to stop the reaction. Each mixt. was de-proteinized by freezing, thawing, and centrifugation, and analysed by HPLC on a Luna phenylhexyl (Phenomenex) 250 ×

4.6 mm (5 μm) column, A=0.1% aq. CF_3COOH, B=MeOH, column temp. 40 °C, 1 ml/min, inj. vol. 10 μl, UV det. at 223 nm, 0% B for 2 min, then linear grad. 0-40% B over 28 min, followed by a brief wash (B) and eq. in A.

Stability of the products **2b** and **2c** was assayed for each accession by substituting the substrate with 600 μl 1.3 mM of **2b** or **2c** in assays. Assays without added substrate were also carried out in order to test whether **2a** (a known prod. of **2**) was present in the crude extract and whether product formation could occur independent of the nitrile substrate, this was not the case for any species. A minor peak of 4-hydroxybenzylalcohol (presum. formed from **2** via **2i** during extraction, Fig. 5) from the crude extract was observed in assays with extracts of *S. alba*, *S. flexuosa* and *K. cordylocarpus*. The ret. time (16.9 min) was close to that of **2b**, but base line sep. was accomplished. The rate of reaction was found to be constant for 60 min, so nitrilase, nitrile hydratase, and amidase activities were calc. from the diff. in product conc. at 60 min and 0 min. The nitrilase to nitrile hydratase ratio was expressed as nitrilase relative (%) to the sum of the activities calc. directly from the amounts of **2b** and **2c** observed after 22 h. incubation of **2a**, as [**2c**]/([**2b**]+[**2c**]). Identities of reaction products were confirmed by isolation and NMR (^{1}H NMR of **2b** and **2c** from both *S. alba* and *S. arvensis* enzymes, ^{13}C NMR of **2c** from *S. alba* enzyme and **2b** and **2c** from *S. arvensis* enzyme (Table 8).

3.6. Synthesis and identification of amides

The amides **0b**, **1b**, **m2b**, **3b**, **4b**, and **5b** were synth. by solid-phase synthesis. The corresponding carboxylic acid (5 equiv.), diisopropylcarbodiimide (5 equiv.) and 1-hydroxybenzotiazole (5 equiv.) were coupled overnight to a Tentagel SRAM resin (500 mg, 0.24 meq/g) in *N*-methylpyrrolidon (2 ml). The amides were cleaved from the resin using CF_3COOH/H_2O (19:1, 1 ml), evap. to dryness, lyophilized from H_2O/CH_3COOH (9:1), re-diss. in H_2O, isol. by prep. HPLC and their identities confirmed by accurate mass measurement of Na^+ adducts (on a micrOTOF-Q, Bruker Daltonics) in electrospray mode with detection of positive ions. Compounds were introduced in 80% aq. MeCN by direct loop injection. Expd. for [**0b**+Na]$^+$: 160.0369, found: 160.0370. Expd. for [**1b**+Na]$^+$: 158.0576, found: 158.0574. Expd. for [**m2b**+Na]$^+$: 204.0631, found: 204.0639. Expd. for [**3b**+Na]$^+$: 188.0682, found: 188.0688. Expd. for [**4b**+Na]$^+$:172.0733, found: 172.0734. Expd. for [**5b**+Na]$^+$: 188.0682, found: 188.0688.

3.7. Substrate specificities of nitrilase and nitrile hydratase

The assay was carried out with **0a**–**5a**, **7a** and **m2a** in parallel (with aliquots of a single enzyme extract). Due to the lipophilic nature of some substrates, EtOH (final conc. 10%) was included in all assays and [substrate] was 7.5 mM in all assays. The experiment was carried out in duplicate, and for substrates of particular interest in triplicate. The HPLC gradient was extended (0-60% B over 48 min). UV detection was at 223 nm for assays with **0a**, **2a**, **3a**, **5a**, and **7a**, at 252 nm for assays with **m2a** and at 212 nm for assays with **1a** and **4a**. Relevant product peaks were identified from comp. of ret. times and UV spectra with auth. standards (comp. no., Rt/min): 0 b, 13.0; **0c**, 22.8; **1b**, 27.0; **1c**, 30.0; **2b**, 15.2; **2c**, 25.3; **m2b**, 19.6; **m2c**, 29.7; **3b**, 31.6; **3c**, 42.1; **5b**, 21.0; **5c**, 32.4; **7b**, 32.2; **7c**, 40.9. In the case of assays with **4a**, a Supelcosil LC-ABZ (Supelco) 250 x 4.6 mm (5 μm) column was used (at 22 °C) because **4a** and **4c** coeluted (48 min) on the phenylhexyl column: **4a**, 25.0 min; **4b**, 23.0 min; **4c**, 29.1 min. Products of **2a**, **3a** and **5a** were quant. rel. to a std. curve of **2c**, while products of the remaining substrates were in each case quant. rel. to a std. curve of the carboxylic acid because of nearly identical UV spectra of corresponding acids and amides.

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