NITRILE-SPECIFIER PROTEINS INVOLVED IN GLUCOSINOLATE HYDROLYSIS IN ARABIDOPSIS THALIANA*
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SUMMARY
Glucosinolates are plant secondary metabolites present in Brassicaceae plants such as the model plant Arabidopsis thaliana. Intact glucosinolates are believed to be biologically inactive, while degradation products after hydrolysis have multiple roles in growth regulation and defense. The degradation of glucosinolates is catalyzed by thioglucosidases called myrosinases and leads by default to the formation of isothiocyanates. The interaction of a protein called epithiospecifier protein (ESP) with myrosinase diverts the reaction towards the production of epithionitriles or nitriles depending on the glucosinolate structure. Here we report the identification of a new group of nitrile-specifier proteins (AtNSPs) in A. thaliana able to generate nitriles in conjunction with myrosinase, and a more detailed characterization of one member (AtNSP2). Recombinant AtNSP2 expressed in E. coli was used to test its impact on the outcome of glucosinolate hydrolysis using a GC-MS approach. AtNSP proteins share 30-45% sequence homology with A. thaliana ESP. Although AtESP and AtNSP proteins can switch myrosinase-catalyzed degradation of 2-propenylglucosinolate from isothiocyanate to nitrile, only AtESP generates the corresponding epithionitrite. Using the aromatic benzylglucosinolate, recombinant AtNSP2 is also able to direct product formation to the nitrile. Analysis of glucosinolate hydrolysis profiles of transgenic Arabidopsis thaliana plants overexpressing AtNSP2 confirms its nitrile-specifier activity in planta. In silico expression analysis reveals distinctive expression patterns of AtNSPs, which supports a biological role for these proteins. In conclusion we show that AtNSPs belonging to a new family of A. thaliana proteins structurally related to AtESP divert product formation from myrosinase-catalyzed glucosinolate hydrolysis and thereby likely affect the biological consequences of glucosinolate degradation. We discuss similarities and properties of AtNSPs and related proteins and the biological implications.

INTRODUCTION
Brassicaceae plants such as oilseed rape (Brassica napus), turnip (Brassica rapa), white mustard (Sinapis alba), as well as the model plant Arabidopsis thaliana contain a group of secondary metabolites known as glucosinolates (GSLs)1 (1, 2). These are β-thioglucoside N-hydroxysulfates with a sulphur-linked β-D-glucopyranose moiety and a variable side chain that is derived from one of eight amino acids or their methylene group-elongated derivatives. Aliphatic GSLs are derived from alanine, leucine, isoleucine, valine or predominantly methionine. Tyrosine or phenylalanine give aromatic GSLs and tryptophan-derived GSLs are called indolic GSLs (for review: 3). Although over 120 different GSLs have been identified in total so far, individual plant species usually contain only a few GSLs (2). Quantitative and qualitative differences of GSL profiles are also observed within a species, such as for example for different A. thaliana ecotypes (4-6). In addition, GSL composition varies among organs and during the life cycle of plants (7, 8) and is affected by external factors (9). Intact GSLs are mostly considered to be biologically inactive. Most GSL degradation products have toxic effects on insect, fungal and bacterial pests, serve as attractants for specialist insects or may have beneficial health effects for humans (10-15). The enzymatic degradation of GSLs (Fig. 1A), which occurs massively upon tissue damage, is catalyzed by plant thioglucosidases called myrosinases (EC 3.2.1.147; glycoside hydrolase family 1). Depending on several factors (e.g. GSL structure, proteins, cofactors, pH) myrosinase catalyzed
hydrolysis of GSLs can lead to a variety of products (Fig. 1B; for review: 16, 17). Of these, isothiocyanates are the most common as their formation only requires myrosinase activity. Thiocyanates on the other hand are only produced from a very limited number of GSLs and their formation necessitates the presence of a thiocyanate-forming factor in addition to myrosinase (18). A thiocyanate-forming protein (TFP) has recently been identified in *Lepidium sativum* (19). Alkenyl GSLs, a subgroup of aliphatic GSLs containing a terminal unsaturation in their side chain, can lead to the production of epithionitriles through the cooperative action of myrosinase and a protein called epithiospecifier protein (ESP; 20) in a ferrous ion-dependent way (21-23). Both TFP and ESP contain a series of Kelch repeats (19). Kelch repeats are involved in protein-protein interactions and Kelch repeat containing proteins are involved in a number of diverse biological processes (24). In addition to isothiocyanates, nitriles are the major group of GSL hydrolysis products. Although ESP and TFP activities can generate nitriles (19, 21, 25, 26), indications for an ESP independent nitrile-specifier activity exist. The GSL hydrolysis profile of *A. thaliana* roots, an organ that does not show ESP expression or activity (27), reveals predominantly the presence of nitriles (28). In addition, leaf tissue of *A. thaliana* ecotypes supposedly devoid of ESP activity produces a certain amount of nitriles upon autolysis (21). Under acidic buffer conditions, a non-enzymatic production of nitriles from GSLs is observed (29 and references therein). Increasing Fe²⁺ concentrations have also been shown to favour nitrile formation over isothiocyanate formation from a number of GSLs in the presence of myrosinase and absence of ESP (21, 22). Therefore a non-enzymatic origin of this nitrile production cannot be excluded, although the presence of a nitrile-specifier protein is a tempting alternative. Although ESP is able to generate nitriles, it has also been shown that the conversion rates of GSLs to nitriles are lower than those of GSLs to epithionitriles for ESP (21, 22).

A nitrile-specifier protein (NSP) that is able to redirect the hydrolysis of GSLs towards nitriles has been cloned from the larvae of the butterfly *Pieris rapae* (30). This protein does however not exhibit sequence similarity to plant ESP and a corresponding plant nitrile-specifier protein has not yet been identified. We report here the identification of a group of six *A. thaliana* genes with some sequence similarity to *A. thaliana* ESP, providing evidence for a new family of nitrile-specifier proteins, and a more detailed characterization of one member that possesses nitrile-specifier activity in vitro, when applied exogenously to plant tissue and after ectopic expression in the two *A. thaliana* ecotypes Col-0 and C24. Despite its sequence homology to AtESP, it does not possess epithiospecifier activity under similar conditions. Therefore, we propose to designate this protein as *A. thaliana* nitrile-specifier protein 2 (AtNSP2). Although the biological roles of AtNSP2 and related proteins are not yet known, their specificities and distinctive expression patterns indicate the presence of a fine-tuned mechanism for GSL degradation controlling the outcome of an array of biologically active molecules.

**EXPERIMENTAL PROCEDURES**

**Biological material** – cDNA clones of the *A. thaliana* genes described in this article were obtained from RIKEN (http://www.brc.riken.jp): pda06554 (*At2g33070*), pda0243 (*At3g07720*), pda01982 (*At3g16400*) and pda02960 (*At5g48180*). The cDNA of *AtESP* used in this study has been described before (22). Seeds of the *A. thaliana* ecotypes C24 (N906), Col-0 (N1092), Cvi (N1097), Ru-0 (N1496) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK; http://arabidopsis.info/). Seeds of *Brassica rapa* (cv. Per) were obtained from Svalöf-Weibull (Svalöv, Sweden).

**Standards of GSL hydrolysis products** – The GSL hydrolysis products 2-propenylisothiocyanate (purity: 95%), 3-butenylisothiocyanate (purity: 98%), benzylisothiocyanate (purity: 98%), 2-phenylacetonitrile (purity: 98%) and phenylisothiocyanate (purity: 98%) were purchased from Sigma-Aldrich (St. Louis, USA). 

**Plant growth conditions** – Seeds were surface-sterilized and sown on ½ x concentration of Murashige and Skoog Basal Salt Mixture (Sigma-Aldrich, St. Louis, USA) supplemented with 2% (w/v) sucrose and 0.6% (w/v) phytoagar (Duchefa, Haarlem, The Netherlands), which is referred to as ½ MS medium subsequently in the
text. The plated seeds were cold treated at 4°C for 3 days before being transferred to a growth room, where they were grown in a 16h photoperiod with 75 \( \mu \text{mol} \cdot \text{m}^2 \cdot \text{sec}^{-1} \) light at 21-23 °C. After 3 to 4 weeks, plants were transferred to soil. Alternatively seeds were imbibed and incubated at 4°C for 3 days before being sown directly onto soil. Plants on soil were grown in a 16h photoperiod with 100 \( \mu \text{mol} \cdot \text{m}^2 \cdot \text{sec}^{-1} \) light at 20°C until tissue was harvested for assay purposes or for the production of seeds.

In silico analysis of expression patterns of \( \text{AtESP} \) and homologous genes – Expression data represented in this article is based on a compilation of \( A. \text{thaliana} \) microarray experiment results that are publicly available at Genevestigator (31; www.genevestigator.ethz.ch; 3110 ATH1:22k arrays as of June 11, 2008). Meta-Profile Analysis at Genevestigator was used to generate the presented outputs. Denomination of organs, growth stages and stimuli is given as provided by Genevestigator, but for clarity reasons the probe identities were replaced by the identification numbers of the recognized genes. The Affymetrix probe identities are as follows: 245161_at for \( \text{At2g33070} \), 248713_at for \( \text{At5g48180} \), 259228_at for \( \text{At3g07720} \), 263174_at for \( \text{At1g54040 (AtESP)} \) and the non discriminatory probe 259381_s_at recognizing \( \text{At3g16390, At3g16400 and At3g16410} \).

Design of \( \text{AtESP}- \) and \( \text{AtNSP}- \)expression constructs and an “empty vector” control for protein expression in \( E. \text{coli} \) – Regions encompassing the open reading frames of \( A. \text{thaliana} \) ESP (\( \text{At1g54040} \)) and four homologous genes (i.e. \( \text{At2g33070, At3g07720, At3g16400 and At5g48180} \)) were amplified by PCR upon the cDNA templates described above using the primers (Invitrogen, Carlsbad, USA) listed in supplemental Table S1A and \( \text{Pfu} \) DNA polymerase (Fermentas International, Burlington, Canada) according to the supplier’s instructions. After a clean-up step (Wizard SV Gel and PCR Clean-up System; Promega Corporation, Madison, USA), the PCR-amplified fragments and the Gateway donor vector pDONR-Zeo were submitted to a BP recombination reaction using the Gateway BP clonase II Enzyme Mix (Invitrogen, Carlsbad, USA). To generate an “empty vector” control, a 98 base pair non-coding cassette, instead of the cloned ESP/NSP genes, was cloned into pDONR-Zeo (supplemental Table S1B). All cloned fragments were verified by sequencing (BigDye Terminator Cycle Sequencing kit, Applied Biosystems, Foster City, USA) and transferred into the pDEST17 (N-terminal His-tag) vector by an LR recombination reaction (Invitrogen, Carlsbad, USA). The generated protein expression cassettes were verified by restriction digestion.

Preparation of crude \( E. \text{coli} \) extracts – For expression in \( E. \text{coli} \), small-volume precultures of transformed \( E. \text{coli} \) BL21-CodonPlus (DE3)-RIPL cells (Stratagene, La Jolla, USA) were grown overnight at 37°C in Luria Bertani (LB) medium supplemented with the selection agents before inoculation of a larger volume. This culture was grown likewise at 37°C until an \( \text{OD}_{600} \) of approximately 0.8. The culture was supplemented with 0.5 mM IPTG and incubated for a further 14 hours at 21°C. Bacterial cultures expressing the “empty vector” were grown and processed the same way. Cells from 500 ml liquid culture were pelleted for 15 min at 5000g at 4°C, stored overnight at -80°C, freeze-thawed three times, resuspended in 15 ml imidazole-HCL buffer (100 mM; pH 6.5) and incubated with lysozyme (1 mg/ml) for one hour at 4°C. The supernatant was recovered after a centrifugation for 15 min at 20000g at 4°C and 5 µl of this crude extract were used in the nitrile-specifier activity assays. Accumulation of the recombinant proteins was monitored by SDS-PAGE and verified by Western blot assays with an anti-His antibody (Amersham Biosciences, Uppsala, Sweden).

Purification of recombinant \( \text{AtESP} \) and \( \text{AtNSP2} \) proteins – For the generation of purified \( \text{AtESP} \) and \( \text{AtNSP2} \) recombinant proteins, \( E. \text{coli} \) cultures were grown as described above. Upon centrifugation of the liquid culture for 15 min at 5000g at 4°C, and overnight storage at -80°C, pellets were resuspended (50 mM TrisHCL pH 7.2, 200 mM NaCl) and incubated with lysozyme (1 mg/ml) for two hours at 4°C. The supernatant was recovered after a centrifugation for 15 min at 20000g at 4°C, filtered (0.2 µm) and purified at 4°C using FPLC (Äkta FPLC System, GE Healthcare, Little Chalfont, UK) using a HisTrap FF column (GE Healthcare, Little Chalfont, UK) and the above-mentioned Tris buffer supplemented with 500 mM imidazole. The purity
of the fractions was assessed by SDS-PAGE and protein concentrations were estimated using the Bio-Rad Protein Assay (Bio-Rad, Hercules, USA) and BSA as a standard. 10 µg of purified AtESP or AtNSP2 were used in the corresponding in vitro activity assays.

**Overexpression of AtNSP2 in A. thaliana** – The AtNSP2 was transferred from pDONR-Zeo to the Gateway-compatible binary vector pEG100 (32), that allows to overexpress AtNSP2 under control of a CaMV35S promoter. This construct was then transferred to Agrobacterium tumefaciens LBA4404 by electroporation and A. thaliana plants were transformed using the "floral dip" method (33). T1 generation plants were regenerated on selection medium consisting of ½ MS medium supplemented with 20 mg/l glufosinate-ammonium (Sigma-Aldrich, St. Louis, USA) and 125 mg/l cefotaxime (Duchefa, Haarlem, The Netherlands) and their transgenic character was verified by PCR on leaf tissue after the plants had been transferred to soil. Selected lines were selfed and taken to the T3 generation. The effect of AtNSP2 overexpression on the GSL hydrolysis profile was assessed in plant tissue autolysis assays as described below.

**In vitro assays of epithiospecifier and nitrile-specifier activities** – Either crude bacterial extracts containing the recombinant proteins or FPLC-purified proteins were used to assay their epithiospecifier and nitrile-specifier activities. The specific details of each assay are indicated at the appropriate places in the text, but basically 5 µl of crude extract or 10 µg of purified recombinant protein was incubated in a total volume of 200 µl together with myrosinase - either 0,5 µg purified Sinapis alba myrosinase (34) or 3 µg recombinant Brevicoryne brassicae myrosinase (35) - and a pure GSL substrate. 2-propenylGSL was purchased (Sigma-Aldrich, St. Louis, USA) and benzylGSL was extracted from Lepidium sativum. The effect of ferrous ion (Fe2+) on nitrile-specifier activity was assessed by the addition of a (NH4)2Fe(SO4)2 solution at the onset of the assays. After incubation at room temperature for 30 min, 200 µl of dichloromethane (DCM) supplemented with phenylisothiocyanate, which served as an internal standard were added to the assay, the samples were vortexed and centrifuged. The DCM layer was recovered, dried with anhydrous MgSO4 and analysed by gas chromatography-mass spectrometry (GC-MS) as described below. All assays were run at least in triplicates and representative traces are shown in the text.

**Plant tissue autolysis assays** – Autolysis assays were performed on rosette leaves (100 mg) of 5 week old A. thaliana Cvi plants, mature seeds (100 mg) of Brassica rapa cv. Per and mature seeds (50 mg) of A. thaliana Ru-0. To test AtNSP2 activity, purified recombinant AtNSP2 protein (10 µg) or an equal volume of purification buffer (control) was added to plant tissue. Regarding autolysis assays of AtNSP2 overexpression lines, mature seeds (50 mg) of the T3 generation and rosette leaves (150 mg) of 4 week old soil-grown plants were analysed. In the latter case 2-propenylGSL (2 µl of a 200 mM solution) and benzylGSL (1 µl of a 100 mM solution) were added prior to processing the samples. When seeds were analysed, twice the amount of water (v/w) was added and in the case of A. thaliana seeds recombinant B. brassicae myrosinase (3 µg) was also added prior to processing the samples. In all cases tissue was ground manually (30 s for leaves and 1 minute for seeds) with miniature pestles and incubated at room temperature for 30 min. After incubation, 200 µl of DCM supplemented with phenylisothiocyanate that served as an internal standard were added, the samples were vortexed and centrifuged. The DCM layer was recovered, dried with anhydrous MgSO4 and analysed by gas chromatography-mass spectrometry (GC-MS) as described below.

**Gas chromatography-mass spectrometry (GC-MS) analysis of GSL hydrolysis products** – GSL hydrolysis products were analysed on a Hewlett-Packard GC 6890N linked to a 5975 inert Mass Selective Detector (Agilent Technologies, Santa Clara, USA). Injections at 200°C were made onto an Agilent HP-5MS 5% Phenylmethylsiloxane (30m x 0.25mm x 0.25µm) column in a pulsed split mode using the following temperature programme: 3 min at 35°C, ramp of 12°C · min⁻¹ until 96°C, ramp of 18°C · min⁻¹ until 240°C, 6 min hold at 240°C. A background subtraction was performed with MSD Chemstation (Agilent Technologies, Santa Clara, USA) in the traces shown in the text. Compounds were identified by comparing their mass spectra to the ones of standards or to published mass spectra (36-39). Response factors relative to
Expression of the flower sepals. Levels observed in the radicle of seedlings and in to be expressed in most organs, with the highest transcripts of these genes are predominantly discriminatory probe recognizing to be limited to seeds. The results from the non-
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Fig. S1). Expression patterns of this gene family also vary during the growth cycle of the plant (supplemental
At3g16390, At3g16400, At3g16410 and AtESP) (Fig. 2). The proteins encoded by At3g07720 and At5g48180 (AtNSP1) contain four to five Kelch repeats and show a sequence identity of approximately 45% to AtESP. The proteins encoded by At2g33070 (AtNSP2), At3g16390 and At3g16400 (AtNSP3) contain one N-terminal jacalin-like lectin domains in addition to the four to five Kelch repeats whereas the protein encoded by At3g16410 contains two N-terminal jacalin-like lectin domain. Consequently, the overall sequence similarity of these latter sequences with AtESP drops to around 40% and 30% respectively (Fig. 2).

In silico analysis of AtESP and homologous gene expression patterns – Analysis of publicly available gene expression data (31) revealed that AtESP (At1g54040) and the homologous genes At2g33070, At3g07720, At3g16390, At3g16400, At3g16410 and At5g48180 genes are differentially expressed at the transcriptional level in A. thaliana organs (Fig. 3). Expression of the At2g33070 transcript seems to be limited to seeds. The results from the non-discriminatory probe recognizing At3g16390/At3g16400/At3g16410 indicate that the transcripts of these genes are predominantly present in root tissue. The At3g07720 gene seems to be expressed in most organs, with the highest levels observed in the radicle of seedlings and in flower sepalas. At5g48180 is also most highly expressed in sepals and at a lower level in most other organs. The expression of AtESP (At1g54040) is more restricted and highest levels seem to be present in stems and stamens. Expression patterns of this gene family also vary during the growth cycle of the plant (supplemental Fig. S1). At3g07720 and At5g48180 transcripts are expressed at all stages. Expression of
At3g16390/At3g16400/At3g16410 is limited to the early growth stages such as seedlings and young plants. AtESP (At1g54040) expression is higher at later stages of the life cycle, after bolting. At2g33070 expression is limited to the mature silique stage. Transcript levels of AtESP and these homologous genes are also responsive to various biotic and abiotic stimuli, although the degree to which these changes occur often differ among the members of this family (supplemental Fig. S2).

In vitro GSL hydrolysis assays with recombinant AtESP and AtNSP proteins on 2-propanoylGSL – To assess if proteins showing sequence similarity to AtESP have a similar activity, four of them were cloned, expressed in E. coli and used in GSL hydrolysis assays in vitro. Incubation of 2-propanoylGSL as the GSL substrate with myrosinase in the presence of ferrous ions (0.01 mM Fe²⁺) leads to the formation of 2-propanoylthioisocyanate (Fig. 4A). If purified recombinant His-tagged AtESP is also present in these assays 3,4-epithiobutylnitrile is produced (Fig. 4B), as expected from earlier studies (22). When purified recombinant His-tagged AtNSP2 (At2g33070) was used instead of AtESP, no 3,4-epithiobutylnitrile was produced but the production of 3-butenylnitrile was detected (Fig. 4C). Therefore, we propose to call this protein A. thaliana nitrile-specifier protein 2 (AtNSP2). No GSL hydrolysis products were detected upon incubation with AtNSP2 in the absence of myrosinase (Fig. 4D). The activity of AtNSP2 and three of the other homologous proteins on 2-propanoylGSL was also assessed using crude bacterial extracts. Besides for AtNSP2 (Fig. 5B), 3-butenylnitrile was also detected upon incubation with AtNSP1 (Fig. 5A) and AtNSP3 (Fig. 5C), showing that these proteins also possess nitrile-specifier activity. The recombinant protein encoded by At3g07720 did however not exhibit this activity (Fig. 5D). Assays with crude extracts expressing the “empty vector” control did not lead to 3-butenylnitrile formation from 2-propanoylGSL (supplemental Fig. S3), indicating that the observed nitrile-specifier activity was due to the expressed AtNSPs and not to E. coli proteins.

In vitro GSL hydrolysis assays with purified recombinant AtNSP2 on benzylGSL – To assess whether AtNSP2 can redirect the hydrolysis of other GSLs, we substituted the alkenyl 2-propanoylGSL by the aromatic benzylGSL. In

RESULTS

Identification of genes with sequence similarity to epithiospecifier protein (ESP) in the A. thaliana genome – We identified six genes whose encoded polypeptides show primary sequence similarity and conservation to the protein domains of A. thaliana epithiospecifier protein (AtESP) (Fig. 2). The proteins encoded by At3g07720 and At5g48180 (AtNSP1) contain four to five Kelch repeats and show a sequence identity of approximately 45% to AtESP. The proteins encoded by At2g33070 (AtNSP2), At3g16390 and At3g16400 (AtNSP3) contain one N-terminal jacalin-like lectin domains in addition to the four to five Kelch repeats whereas the protein encoded by At3g16410 contains two N-terminal jacalin-like lectin domain. Consequently, the overall sequence similarity of these latter sequences with AtESP drops to around 40% and 30% respectively (Fig. 2).

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phenylisothiocyanate were experimentally determined for 2-propenylisothiocyanate, 3-butenylnitrile, benzylisothiocyanate and 2-phenylacetonitrile.
assays where benzylGSL is incubated with myrosinase in the presence of Fe^{2+} (0.01 mM) almost exclusively benzylisothiocyanate was produced, although some 2-phenylacetonitrile was also detected (Fig. 6A). When purified recombinant AtNSP2 was present in the hydrolysis assays the proportion of 2-phenylacetonitrile increased (Figs. 6B and 6D). Assays with crude bacterial extracts containing AtNSP1 (At5g48180) or AtNSP3 (At3g16400) recombinant proteins also resulted in the production of 2-phenylacetonitrile. Assays containing AtNSP2 but no myrosinase did not lead to the detection of GSL hydrolysis products (Fig. 6C). Heat-treating the purified AtNSP2 protein prior to adding it to the assay reverted the proportion of 2-phenylacetonitrile to the levels obtained in the sole presence of myrosinase (Fig. 6D), confirming that the detected nitrile-specifier activity was of a proteinaceous nature.

*Effect of ferrous ion on the in vitro activity of purified recombinant AtNSP2 protein* – We reported previously (22) that the in vitro epithiospecifier activity of ESP is ferrous ion dependent. We were therefore interested in studying the effect of ferrous ion on nitrile-specifier activity of AtNSP2. In the absence of added Fe^{2+} the incubation of 2-propenylGSL or benzylGSL with 10 µg purified recombinant AtNSP2 did not result in the formation of 3-butenylnitrile (Fig. 7A) and only trace amounts of 2-phenylacetonitrile (Fig. 7B), respectively. At the lowest added Fe^{2+} concentration (i.e. 0.01 mM) nitriles derived from both GSLs were observed in the presence of AtNSP2 and nitrile proportion increased with higher Fe^{2+} concentrations over the tested range. Increasing Fe^{2+} concentrations also led to higher nitrile proportions in the absence of AtNSP2, although at a lower rate in both cases (Fig. 7).

*Plant tissue autolysis assays with exogenous application of recombinant AtNSP2* – To further characterize how AtNSP2 impacts GSL hydrolysis, we performed plant tissue autolysis assays. Autolysis of mature seeds of the *A. thaliana* ecotype Ru-0 generated a GSL hydrolysis profile consisting of isothiocyanates and to a lesser extent nitriles derived from several aliphatic GSLs (Fig. 8A). Addition of purified recombinant AtNSP2 (Fig. 8B) to these seeds before autolysis increased the proportion of nitriles from these GSLs. Additional assays where bacterial extracts containing recombinant AtNSP2 were added to other *A. thaliana* tissues and other Brassicaceae species, such as those exemplified in supplemental Fig. S4, confirmed the nitrile-specifier activity of AtNSP2 on diverse GSLs under these conditions.

*Overexpression of AtNSP2 in A. thaliana* – To assess whether AtNSP2 is capable of nitrile-specifier activity in planta, AtNSP2 was overexpressed in the two *A. thaliana* ecotypes Col-0 and C24 and mature seeds were submitted to autolysis assays (Fig. 9). In GSL hydrolysis profiles of wild-type seeds of both genetic backgrounds isothiocyanates and/or nitriles of a range of methylthioalkyl GSLs and of 3-benzoyloxypropylGSL and 4-benzoyloxybutylGSL were detected. The relative amounts of these GSLs are however different in these two ecotypes (Figs. 9A and 9C). Analysis of transgenic seeds revealed a change in the GSL hydrolysis profile towards a higher proportion of the nitrile for each of the identified GSLs (Figs. 9B and 9D). A higher nitrile proportion was also observed in rosette leaves of AtNSP2 overexpression plants (Fig. 10), both on the endogenous 4-methylsulfinylbutylGSL and the GSLs that were exogenously applied in this case (i.e. 2-propenylGSL and benzylGSL). For these latter ones the nitrile proportion increased from 8 to 90% and from 2 to 55%, respectively.

**DISCUSSION**

*Identification of genes with sequence similarity to epithiospecifier protein (ESP) in the A. thaliana genome* – A search of *A. thaliana* genes with sequence homology to AtESP identified a group of six genes. These genes putatively encode proteins that like AtESP contain four to five Kelch repeats. Four of the six proteins contain one or two additional N-terminal jacalin-like lectin domains. Although Kelch repeats are involved in protein-protein interactions (24) and lectins are known to bind carbohydrates (40), the role of these domains in AtESP and homologous proteins is not yet known. Some of these six proteins are annotated and were earlier described as myrosinase-binding like proteins (MBPs) (19, 21, 25). This annotation is however misleading as the characterized myrosinase-binding proteins present a varying number of jacalin-like lectin
domains but do not contain Kelch repeats (41-43). Whereas ESP from *A. thaliana* and other Brassicaceae species has been the subject of several studies (21-23, 25, 44, 45), none of the six proteins mentioned above have been characterized previously.

*In vitro* GSL hydrolysis assays with recombinant AtESP and AtNSP proteins – It was previously shown that recombinant AtESP redirects myrosinase-catalysed hydrolysis of the alkenyl GSL 2-propenylGSL from 2-propenylisothiocyanate to the corresponding epithionitrile (i.e. 3,4-epithiobutylnitrile) in the presence of ferrous ion (21, 22). The *in vitro* assays performed with purified AtESP in the present study showed equivalent results, indicating that the His-tag and assay conditions did not prevent epithiospecifier activity of AtESP. However, 3,4-epithiobutylnitrile was not produced when the purified recombinant AtNSP2 protein was used. Hence, neither the relatively high sequence similarity of this protein to AtESP nor the presence of Kelch repeats is sufficient for AtNSP2 to exhibit an epithiospecifier activity under the tested conditions. However, AtNSP2 redirected myrosinase-catalysed hydrolysis of 2-propenylGSL towards the formation of the 3-butenylnitrile, hence the name of *A. thaliana* nitrile-specifier protein 2 (AtNSP2) that we propose for this protein. Two of the other three members of this protein family that we assessed *in vitro* by using crude bacterial extracts on 2-propenylGSL, also exhibited nitrile-specifier activity and were called AtNSP1 and AtNSP3. Further assays with purified AtNSP2 where the aliphatic 2-propenylGSL was replaced by the aromatic benzylGSL showed that this protein is also able to redirect the hydrolysis from isothiocyanate to nitrile for the latter GSL, which AtESP is not able of doing (25). AtNSPs show also sequence similarity, although to a lesser extent, to the thiocyanate-forming protein (TFP) from *Lepidium sativum*. However, TFP leads to the formation of thiocyanate and nitrile from benzylGSL and almost exclusively to epithionitrile from 2-propenylGSL (19). Although these structurally related proteins all divert the myrosinase-catalyzed GSL hydrolysis, the outcome differs and may be indicative of diverse biological functions. These have however not yet been identified. Interestingly, the larvae of the butterfly *Pieris rapae* and other Pieridae also produce a nitrile-specifier protein (NSP) that redirects the hydrolysis of ingested GSLs towards nitriles. This mechanism allows it to circumvent the plant defense constituted by isothiocyanates (30, 46). Surprisingly enough, neither plant ESP nor AtNSP2 and the homologous proteins described here bear any sequence similarity to this *Pieris rapae* NSP.

Enzymatic or non-enzymatic generation of nitriles from GSLs – Nitriles are produced non-enzymatically (i.e. in the absence of myrosinase) from certain GSLs, including 2-propenylGSL and benzylGSL, but this occurs at lower pH and at higher Fe$^{2+}$ concentrations than used in our assays (29 and references therein). Ferrous ion also affects the enzymatic degradation of GSLs by favouring nitrile formation even at very low Fe$^{2+}$ concentrations in the presence of myrosinase and at pH values similar to the ones used in this study (21, 22, 47). We detected indeed the formation of nitriles in the sole presence of myrosinase and ferrous ion. The facts that nitrile levels were greatly increased in the presence of purified AtNSP2 and that these levels reverted to basal ones when heat-treated AtNSP2 was used, show however that AtNSP2 is responsible for most of the nitrile formation in our *in vitro* assays.

Effect of ferrous ion on AtNSP2 activity – Epithiospecifier activity of ESP has been shown to be ferrous ion dependent, with increasing Fe$^{2+}$ concentrations leading to a higher proportion of epithionitrile and/or nitrile (22). The fact that 2-phenylacetonitrile, although at low levels, was produced upon incubation with AtNSP2 in the absence of added Fe$^{2+}$ seems to indicate that Fe$^{2+}$ is not strictly required for its nitrile-specifier activity. The addition of Fe$^{2+}$ at least in the range of 0.01 to 0.2 mM tested here, promotes however the nitrile-specifier activity of AtNSP2 from both 2-propenylGSL and benzylGSL. The promotion of nitrile formation by Fe$^{2+}$ was also reported for *L. sativum* TFP and *P. rapae* NSP (19, 25). Under our assay conditions Fe$^{2+}$ also promoted the formation of nitriles in the absence of AtNSP2, the difference in nitrile production in the presence and absence of AtNSP2 decreasing with increasing Fe$^{2+}$ concentrations.

Impact of AtNSP2 activity on hydrolysis of different GSLs – Provoked autolysis of plant tissue is widely used as an assay to determine the
potential outcome of GSL hydrolysis in plant tissue. We conducted therefore assays where we added bacterial extracts containing the recombinant AtNSP2 protein to plant tissue prior to autolysis. This allowed us to assess the nitrile-specifier activity of AtNSP2 in less artificial conditions than those employed in the in vitro assays. Moreover, plant tissues often contain a mixture of GSLs (1, 2, 4), sometimes even belonging to different classes of GSLs, and different organs of the same plant often contain different GSL profiles (7, 8). These assays therefore also allowed us to assess the action of AtNSP2 on a broader spectrum of GSLs than the limited availability of standards would have allowed us to do. The disadvantage of this approach is that the production of nitriles, either enzymatic or non-enzymatic, inherent to the plant tissue interferes with the assessment of exogenously applied AtNSP2. The autolysis assays that were performed confirmed the results of the in vitro assays and allowed us to reveal nitrile-specifier activity of AtNSP2 on an extended range of GSLs belonging to the three major classes of GSLs (aliphatic, aromatic and indolic). Interestingly, the hydrolysis of GSLs belonging to a same class seemed to be differently affected by the presence of AtNSP2, such as the various aliphatic methylthioalkyl GSLs in the seeds of A. thaliana Ru-0. Also, AtNSP2 did not or only slightly affect the hydrolysis outcome of some GSLs, such as that of the aromatic 2-phenylethylGSL present in B. rapa seeds. And 5-vinyl-2-oxazolidinethione (goitrin) was identified as the only hydrolysis product from 2-hydroxy-3-buteneGSL (progoitrin) in B. rapa seeds in the presence of AtNSP2. The reason why AtNSP2 does not seem to have an impact on the hydrolysis of some GSLs while affecting that of structurally related GSLs is presently unknown and requires further investigation.

AtNSPs and other proteins affecting GSL hydrolysis – As discussed above, AtNSP2 seems to exert nitrile-specifier activity on several but not all GSLs. TFP from Lepidium sativum presents, like AtESP, AtNSP2 and the other homologous proteins described here, Kelch repeats but has a thiocyanate-forming activity (19) that neither AtESP nor AtNSP2 have. Pieris rapae NSP, which has neither Kelch repeats nor jacalin-like lectin domains, is also able to generate nitriles from benzylGSL and various aliphatic GSLs (25, 30). To possess the structures of AtESP and AtNSP2 would be helpful in identifying the features contributing respectively to the epithiospecifier and nitrile-specifier activities of these proteins.

Cooperative actions of AtNSP2 and myrosinase – The nitrile-specifier activity of AtNSP2 is strictly dependent on the presence of myrosinase under the tested conditions as no GSL hydrolysis products were detected in the absence of myrosinase. On the other hand, AtNSP2 does not require a specific myrosinase. This has also been described for the cooperative action between ESP and myrosinases (45, 51, 52). In our experiments AtNSP2 was able to act in conjunction with plant myrosinases from three different Brassicaceae species (i.e. A. thaliana, B. rapa and S. alba). The cabbage aphid Brevicoryne brassicae also possesses a myrosinase, which is spatially separated from the ingested GSLs in the insect body (48-50), and AtNSP2 is even able to generate nitriles in conjunction with this recombinant myrosinase.

Nitrile-specifier activity of AtNSP2 in planta – The nitrile-specifier activity of AtNSP2 in planta was confirmed by overexpressing it in A. thaliana. The two A. thaliana ecotypes Col-0 and C24 were chosen for this purpose as they present different GSL profiles. In addition, C24 possesses ESP activity whereas Col-0 does not. Seeds of A. thaliana are characterized by their high amount of methylthioalkyl and benzoyloxy GSLs (4, 7, 8). While the GSL hydrolysis profile of mature Col-0 and C24 wild-type seeds exhibited both isothiocyanates and nitriles, overexpression of AtNSP2 leads to a further increase in the nitrile proportion derived from all methylthioalkyl GSLs and the two benzoyloxy GSLs that we identified. Although this increase could not be more precisely quantified due to the lack of pure standards, different GSLs seemed to be differently affected by this overexpression. Col-0 rosette leaves are characterized by the alkyl GSL 4-methylsulfinylbutylGSL (7, 8) and produce predominantly isothiocyanates upon autolysis (21). In this tissue too the expression of AtNSP2 leads to a reorientation of GSL hydrolysis from isothiocyanates towards the production of nitriles. In both seeds and leaves, this reorientation is however only partial, which may indicate that
AtNSP2 cannot compete with the default route leading to isothiocyanate formation. In the case of the GSLs exogenously applied to leaves this may also be due to an excess in the applied amount. However the overexpression of AtNSP2 confirms that the nitrile-specifier activity observed in vitro is genuinely due to the recombinant AtNSP2 and that it may also contribute to nitrile production from GSLs when A. thaliana plants are subjected to tissue damage.

Expression patterns of AtESP homologous genes in view of their putative involvement in GSL hydrolysis – One or more of the At3g16390/At3g16400/At3g16410 tandem genes seem to be expressed mostly in root tissue, but the non-discriminatory probe 259381_s_at of the Affymetrix Arabidopsis GeneChip array does not allow to discern the contribution of the individual members. A. thaliana roots are particular regarding GSL hydrolysis in that respect that they do not show ESP activity and only traces of ESP transcripts (27). In addition, A. thaliana roots express two root-specific myrosinases (called TGG4 and TGG5) whereas the myrosinases TGG1 and TGG2, responsible for the myrosinase activity in aboveground/green tissue are not expressed in roots (53-55). Whether this is related to the fact that A. thaliana roots contain a high proportion of indolic GSLs (7, 8) is not known. AtNSP2 (At2g33070) mRNA expression is limited to seeds. High amounts of benzyloxy GSLs and long-chain aliphatic GSLs are characteristics of mature seeds of A. thaliana (4, 7, 8). And although the nitrile-specifier activity of AtNSP2 does not seem to be very specific as to the type of GSL, the restricted expression patterns of the genes encoding AtNSPs, in combination with the distribution of classes of GSLs in A. thaliana organs may point towards distinct biological roles for the different members. To substantiate this, a detailed characterization of the nitrile-specifier activity of the identified AtNSPs is however required. GSLs seem to be degraded during seed germination and qualitative and quantitative changes in the GSL profile occur during the life cycle of A. thaliana (8). Although this indicates a turnover of GSLs in intact plant tissue and may involve myrosinase, AtESP and AtNSP activity, this remains speculative at the moment. The role of GSL hydrolysis products in plant defense has been documented (10) and notably the change from isothiocyanates to nitriles seem to have a deleterious effect on the plant’s ability to defend itself against some insect pests (21, 56). That AtESP and the group of six homologous proteins mentioned here may be involved in plant response to stresses is also indicated by the fact that their gene expression is responsive to a large number of biotic and abiotic stresses. In view of the specific organ expression patterns of the different members and because most of the expression data has been obtained in the ecotype Col-0, some caution should be applied when interpreting this data. In Col-0 an expression of AtESP at the transcript level has been a matter of controversy although the absence of both ESP protein and activity is generally agreed on (21, 27). Changes in AtESP transcript expression levels have been reported at several occasions but no corresponding changes in AtESP protein levels or activity have been provided (57, 58). A similar situation may be the case for AtNSP2 in some of the A. thaliana ecotypes. Evidence that the transcripts of the AtNSP2 and related genes described in this paper are however translated in planta is provided by a series of proteomic studies. Peptides corresponding to AtNSP1 and AtNSP2 were identified in the A. thaliana seed proteome (59, 60) and the protein encoded by At3g16410 was identified as major root protein in a proteomics study of eight A. thaliana ecotypes (61).

Conclusion – We described here three A. thaliana proteins that redirect GSL hydrolysis towards the production of nitriles and we therefore named A. thaliana nitrile-specifier proteins (AtNSPs). A more detailed characterization of one member, AtNSP2, reveals that it is able to redirect the hydrolysis profile of aliphatic, indolic and aromatic GSLs. AtNSP2 has no nitrile-specifier activity on GSLs on its own but requires the presence of a thioglucosidase (myrosinase). AtNSPs show sequence similarity to a protein called epithiospecifier protein (ESP)(20) that also redirects the hydrolysis of a subset of GSLs by acting downstream of myrosinase (21, 22). The quantitive contribution of AtNSPs to the generation of GSL hydrolysis products in the case of tissue rupture has not been established yet, and requires further investigation. Interestingly however, the transcript expression patterns of AtNSPs and other related genes show organ specificities, are developmentally regulated and
responsive to various stimuli. GSLs are part of a complex plant defense system whose components are spatially separated in intact plant tissue (62) and the derived isothiocyanates and nitriles are known to have biological activity (10). A detailed characterization of expression and activities of AtNSPs, analysis of corresponding overexpression and mutant lines in combination with bioassays that is in progress should allow us to discern the biological and ecological roles of these proteins.

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**FOOTNOTES**

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1Abbreviations used are: BSA, bovine serum albumin; DCM, dichloromethane; EDTA, ethylenediaminetetraacetic acid; ESP, epithiospecifier protein; ESP-L, epithiospecifier protein-like, Fe^{2+}, ferrous ion; FPLC, fast protein liquid chromatography; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; GSL, glucosinolate; His, histidine; IPTG, isopropyl β-D-thiogalactopyranoside; MS, Murashige and Skoog; NSP, nitrile-specifier protein; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; TFP, thiocyanate-forming protein

2results not shown

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**NOTE ADDED IN PROOF**

While this article was under review, an article by Burow et al. [64] [Burow, M., Losansky, A., Müller, R., Plock, A., Klieberstein, D.J., and Wittstock, U. (2009) *Plant Physiol.* **149**, 561-574] describing the same family of nitrile-specifier proteins of *A. thaliana* was published. While we put our focus on the characterization of the protein encoded by *At2g33070*, these authors chose the protein encoded by *At3g16400* as representative of this family. They characterized its substrate specificity and the role of ferrous ion on nitrile formation by this protein. They also showed that *Pieris rapae* damaged leaves of *A. thaliana* show an increase in nitrile production upon autolysis and induced expression of *At3g16400*. A T-DNA insertion mutant of *At3g16400* produced only trace amounts of nitriles from endogenous glucosinolates upon rosette leaf autolysis, and did not exhibit increased nitrile production after insect attack.
**FIGURE LEGENDS**

**Fig. 1** Simplified scheme of enzymatic GSL hydrolysis (A) and structures and names of GSLs and their hydrolysis products that are described in the text (B).

A Myrosinase acts on GSLs to form an unstable aglycone intermediate that can rearrange spontaneously to form an isothiocyanate. Hydrolysis can be diverted from this default route under certain conditions (e.g. presence of NSPs, ferrous ions or at pH<5 ) to give the corresponding nitrile. Epitiospecifier protein (ESP) is responsible for the formation of epithionitriles from alkenyl GSLs in a ferrous ion dependent mechanism.

B The general structure of GSLs, indicating the variable side-chain as R, is given as well as the three major classes of hydrolysis products (i.e. isothiocyanates, nitriles and epithionitriles). The listed GSLs are the ones described in the text and are ranged according to the class of GSLs they belong to and with an increase in chain length or complexity. The names of the respective hydrolysis products are given for a better understanding of the present article and not all were encountered during our studies.

**Fig. 2** Schematic comparison and multiple sequence alignment of AtESP and AtNSP proteins.

A Schematic representation of the major predicted pfam domains of the AtESP and AtNSP polypeptides sequences. Proteins whose activities have not yet been identified are labelled as “AtESP/NSP?” pending further investigation. The corresponding gene identification numbers (AtGID) and the overall sequence similarity of these proteins to AtESP are also given. Jacalin-like lectin domains are indicated by black ellipses and Kelch repeats are indicated by black squares. Kelch repeats that are not significant are indicated by grey squares. Diagrams are not drawn to scale.

B Multiple sequence alignment of AtESP and AtNSP proteins that are described in this study. Polypeptides are ranged in the order given in Fig. 2A and the proteins labelled as AtESP/NSP in Fig. 2A are referred to by their AtGID. The alignment was produced with the Genedoc software and manually optimized. Shading of the levels of conservation is as follows: 7 out of 7: white letters on black background; 6 out of 7: white letters on dark grey background; 4 or 5 out of 7: black letters on light grey background; fewer than 4 out of 7: black letters on white background. Gaps were introduced in order to align the jacalin-like lectin domains (Jac) and Kelch repeats (K) predicted by http://pfam.sanger.ac.uk, the position of these domains being indicated by arrows below the alignment. The dotted arrow indicates the Kelch repeat whose prediction was not significant for all sequences.

**Fig. 3** Expression patterns of *AtESP* and homologous transcripts in different organs of *A. thaliana*.

Heatmap representation of the in silico expression analysis of *AtESP* and homologous genes in different organs of *A. thaliana* based on microarray data available at Genevestigator (www.genevestigator.ethz.ch). Expression levels are indicated by blue colouring, a darker blue colour indicating a higher expression level. Organ denomination is given on the left of the heatmap and the number of arrays which this heatmap is based on is given on the right.

**Fig. 4** *In vitro* activity of purified AtESP and AtNSP2 on 2-propenylGSL.

GC traces of GSL hydrolysis products generated *in vitro* from 2-propenylGSL (2 mM) in the presence of Fe$^{2+}$ (0.01 mM) upon (A) incubation with *B. brassicae* myrosinase (3µg) (B) incubation with myrosinase and purified recombinant His-tagged AtESP (10 µg), (C) incubation with myrosinase and purified recombinant His-tagged AtNSP2 (10 µg) or (D) incubation with purified AtNSP2 on its own. Peaks identified as GSL hydrolysis products are labelled: 1 = 2-propenylisothiocyanate, 2 = 3,4-epithiobutylnitrile, 3 = 3-butenylnitrile, IS = internal standard (phenylisothiocyanate).

**Fig. 5** *In vitro* activity of AtNSP1, AtNSP2, AtNSP3 and At3g07720 on 2-propenylGSL using crude extracts.

GC traces of GSL hydrolysis products generated *in vitro* from 2-propenylGSL (10 mM) after incubation with purified *S. alba* myrosinase (0.5 µg) and crude bacterial extracts expressing either recombinant His-
tagged AtNSP1 (A), AtNSP2 (B), AtNSP3 (C) or At3g07720 (D). GSL hydrolysis product-related peaks are labelled: 1 = 2-propenylisothiocyanate, 3 = 3-butenylnitrile, IS = internal standard (phenylisothiocyanate).

**Fig. 6** In vitro activity of purified AtNSP2 on benzylGSL. A to C GC traces of GSL hydrolysis products generated in vitro from benzylGSL (1 mM) in the presence of Fe$^{2+}$ (0.01 mM) upon (A) incubation with only *B. brassicae* myrosinase (3 µg), (B) incubation with myrosinase and purified recombinant His-tagged AtNSP2 (10 µg) or (C) incubation with purified AtNSP2 on its own. Peaks identified as GSL hydrolysis products are labelled: 4 = benzylisothiocyanate, 5 = 2-phenylacetonitrile, IS = internal standard (phenylisothiocyanate).

**D** Proportion of 2-phenylacetonitrile to the total amount of benzylGSL derived hydrolysis products when assays were performed in the presence of 10 µg purified AtNSP2 (as in Fig. 6B), in the absence of AtNSP2 (as in Fig. 6A) or when AtNSP2 was boiled prior to the assay (n=3).

**Fig. 7** Effect of ferrous ions on AtNSP2 activity in vitro.

Proportion of 3-butenylnitrile (A) and 2-phenylacetonitrile (B) to the total amount of hydrolysis products derived respectively from 2-propenylGSL (2 mM) and benzylGSL (1 mM) generated in vitro upon incubation with recombinant *B. brassicae* myrosinase (3 µg) in the absence (black bars) or the presence (grey bars) of purified recombinant His-tagged AtNSP2 (10 µg) at Fe$^{2+}$ concentrations ranging from 0 to 0.2 mM.

**Fig. 8** Autolysis assay of *A. thaliana* Ru-0 seeds in the absence or presence of exogenously applied purified AtNSP2.

GC traces of GSL hydrolysis products generated upon autolysis of mature seeds (50 mg) of *A. thaliana* Ru-0 supplemented with (A) recombinant *B. brassicae* myrosinase (3 µg) or (B) myrosinase and purified recombinant His-tagged AtNSP2 (10 µg). Peaks identified as GSL hydrolysis products are labelled: 1 = 2-propenylisothiocyanate, 9 = 9-methylthiononylnitrile, 11 = 4-methylthiobutynitrile, 12 = 3-methylthiopropylisothiocyanate, 13 = 4-methylthiobutylisothiocyanate, 14 = 8-methylthiooctylisothiocyanate, 15 = 7-methylthioheptylisothiocyanate, 16 = 8-methylthiooctylisothiocyanate, 17 = 4-benzoyloxybutynitrile, 18 = 3-benzoyloxypropylisothiocyanate, IS = internal standard (phenylisothiocyanate).

**Fig. 9** Effect on the GSL hydrolysis profile of *A. thaliana* seeds by overexpressing AtNSP2.

GC traces of GSL hydrolysis products generated in autolysis assays containing *B. brassicae* myrosinase (3 µg) and 50 mg mature seeds of AtNSP2 over-expression lines in two genetic backgrounds. A: Col-0 wild-type seeds, B: AtNSP2 overexpression line in the Col-0 background, C: C24 wild-type seeds, D: AtNSP2 overexpression line in the C24 background. Peaks identified as GSL hydrolysis products are labelled: 9 = 9-methylthiononylnitrile, 13 = 4-methylthiobutylisothiocyanate, 14 = 8-methylthiooctylisothiocyanate, 15 = 7-methylthioheptylisothiocyanate, 16 = 8-methylthiooctylisothiocyanate, 17 = 4-benzoyloxybutynitrile, 18 = 3-benzoyloxypropylisothiocyanate, 26 = 4-methylsulfinylbutylisothiocyanate, 27 = 5-methylsulfinypentylisothiocyanate, 28 = 7-methylthioheptylnitrile, 29 = 5-methylthiopentylisothiocyanate, 30 = 5-benzoyloxypentynitrile, 31 = 4-benzoyloxybutylisothiocyanate, IS = internal standard (phenylisothiocyanate).

**Fig. 10** Effect on the GSL hydrolysis profile of *A. thaliana* rosette leaves by overexpressing AtNSP2.

GC traces of GSL hydrolysis products generated in autolysis assays containing 150 mg crushed rosette leaves of 4 week old AtNSP2 over-expression plants and exogenously applied 2-propenylGSL and benzylGSL. A: Col-0 control line, B: AtNSP2 overexpression line in the Col-0 background. Peaks identified as GSL hydrolysis products are labelled: 1 = 2-propenylisothiocyanate, 3 = 3-butenylnitrile, 4 = benzylisothiocyanate, 5 = 2-phenylacetonitrile, 10 = indol-3-acetonitrile, 13 = 4-methylthiobutylisothiocyanate, 24 = 4-methylsulfinylbutylisothiocyanate, 25 = 5-methylsulfinylpentynitrile, IS = internal standard (phenylisothiocyanate).
FIGURES

Figure 1

A

\[ \text{glucosinolate} \rightarrow \text{myrosinase} \rightarrow \text{unstable aglycone} \]

\[ \text{NSP Fe}^{2+} \rightarrow R = C \equiv N \quad \text{nitrile} \]

\[ \text{default} \rightarrow R - N = C = S \quad \text{isothiocyanate} \]

\[ \text{ESP/Fe}^{2+} \rightarrow \text{epithionitrile} \]
### General Structure of Glucosinolates

**General Structure of Glucosinolates**

\[
\text{R} \quad \text{Glucose} \quad \text{SO}_3^- \quad \text{N} \quad \text{R} \quad \text{C} \quad \text{S} \quad \text{N} \quad \text{C} \quad \text{S} \quad \text{R} \quad \text{O} \quad \text{C} \quad \text{N} \quad \text{CN} \quad \text{S} \quad \text{n}
\]

### Hydrolysis Products

| Glucosinolate Class          | Name of Glucosinolate | R Group | Name of Isothiocyanate | Name of Nitrile | Name of Epithionitrile |
|------------------------------|-----------------------|---------|------------------------|-----------------|------------------------|
| **Methylthioalkyl Glucosinolates** | 3-Methylthio propyl GSL | ![Methylthioalkyl](methylthioalkyl.png) | 3-Methylthio propyl ITC | 4-Methylthio butyl NIT | n.a. |
| 4-Methylthio butyl GSL | ![Methylthioalkyl](methylthioalkyl.png) | | 4-Methylthio butyl ITC | 5-Methylthio pentyl NIT | n.a. |
| 5-Methylthio pentyl GSL | ![Methylthioalkyl](methylthioalkyl.png) | | 5-Methylthio pentyl ITC | 6-Methylthio hexyl NIT | n.a. |
| 6-Methylthio hexyl GSL | ![Methylthioalkyl](methylthioalkyl.png) | | 6-Methylthio hexyl ITC | 7-Methylthio heptyl NIT | n.a. |
| 7-Methylthio heptyl GSL | ![Methylthioalkyl](methylthioalkyl.png) | | 7-Methylthio heptyl ITC | 8-Methylthio octyl NIT | n.a. |
| 8-Methylthio octyl GSL | ![Methylthioalkyl](methylthioalkyl.png) | | 8-Methylthio octyl ITC | 9-Methylthio nonyl NIT | n.a. |
| **Methylsulfinylalkyl Glucosinolates** | 4-Methylsulfinyl butyl GSL | ![Methylsulfinylalkyl](methylsulfinylalkyl.png) | 4-Methylsulfinyl butyl ITC | 5-Methylsulfinyl pentyl NIT | n.a. |
| **Alkenyl Glucosinolates** | 2-Propenyl GSL | ![Alkenyl](alkenyl.png) | 2-Propenyl ITC | 3-Butenyl NIT | 3,4-Epithiobutyl NIT |
| 3-Butenyl GSL | ![Alkenyl](alkenyl.png) | | 3-Butenyl ITC | 4-Pentenyl NIT | 4,5-Epithiopentyl NIT |
| 4-Pentenyl GSL | ![Alkenyl](alkenyl.png) | | 4-Pentenyl ITC | 5-Hexenyl NIT | 5,6-Epithiohexyl NIT |
| **Other Aliphatic Glucosinolates** | 2-Hydroxy-3-Butenyl GSL | ![Alkenyl](alkenyl.png) | 5-Vinyl-2-Oxazolidine Thione* | 3-Hydroxy-4-Pentenyl NIT | 2 Diastereoisomeric Compounds |
| 3-Benzoyloxy propyl GSL | ![Alkenyl](alkenyl.png) | | 3-Benzoyloxy propyl ITC | 4-Benzoyloxy butyl NIT | n.a. |
| 4-Benzoyloxy butyl GSL | ![Alkenyl](alkenyl.png) | | 4-Benzoyloxy butyl ITC | 5-Benzoyloxy pentyl NIT | n.a. |
| **Aromatic Glucosinolates** | Benzyl GSL | ![Aromatic](aromatic.png) | Benzyl ITC | 2-Phenyl Aceto NIT | n.a. |
| 2-Phenylethyl GSL | ![Aromatic](aromatic.png) | | 2-Phenylethyl ITC | Benzene Propane NIT | n.a. |
| **Indolic Glucosinolate** | Indolyl-3-Methyl GSL | ![Indolic](indolic.png) | Indolyl-3-Methyl ITC³ | Indol-3-Aceto NIT | n.a. |

**Abbreviations:** n.a.: not applicable; GSL: glucosinolate; ITC: isothiocyanate; NIT: nitrile.

*The isothiocyanate formed from 2-Hydroxy-3-Butenyl GSL rearranges spontaneously into 5-Vinyl-2-Oxazolidine Thione.

³Indolyl-3-Methylisothiocyanate is unstable.
Figure 2

A

| AtGID protein | similarity |
|---------------|------------|
| At1g54040 AtESP |            |
| At3g07720 AtESP/NSP? | 44% |
| At5g4180 AtNSP1 | 46% |
| At2g33070 AtNSP2 | 39% |
| At3g16390 AtESP/NSP? | 39% |
| At3g16400 AtNSP3 | 39% |
| At3g16410 AtESP/NSP? | 29% |

Jacalin-like lectin domains
Kelch repeats

B

Figure 2 continued
Figure 4

A. 2-propanyl GSL myrosinase + Fe²⁺

B. 2-propanyl GSL myrosinase + Fe²⁺ + AIESP

C. 2-propanyl GSL myrosinase + Fe²⁺ + AtNSP2

D. 2-propanyl GSL + Fe²⁺ + AtNSP2
Figure 5

A  2-propenylGSL myrosinase + AtNSP1

B  2-propenylGSL myrosinase + AtNSP2

C  2-propenylGSL myrosinase + AtNSP3

D  2-propenylGSL myrosinase + At3g07720
Figure 6

A. 

B. 

C. 

D.

Retention time (min)

Abundance

Retention time (min)

Abundance

Retention time (min)

Abundance

Retention time (min)

Abundance

benzylGSL + myrosinase + Fe^{2+}

benzylGSL + myrosinase + Fe^{2+} + AtNSP2

benzylGSL + Fe^{2+} + AtNSP2

2-phenylacetamide proportion (%)
Figure 7

A

Fe²⁺ concentration (mM)

3-butenylnitrile proportion (%)

assay without AtNSP2
assay with purified AtNSP2

B

Fe²⁺ concentration (mM)

2-phenylacetonitrile proportion (%)

assay without AtNSP2
assay with purified AtNSP2
Figure 8

A. thaliana Ru-0 seeds + myrosinase

A. thaliana Ru-0 seeds + myrosinase + AtNSP2
Figure 9

A

A. thaliana Col-0

B

AtNSP2 in Col-0

C

A. thaliana C24

D

AtNSP2 in C24
Figure 10

A. thaliana Col-0

AtNSP2 in Col-0

Retention time (min)

Abundance

0,0
2,0e+5
4,0e+5
6,0e+5
8,0e+5
1,0e+6
1,2e+6
1,4e+6
1,6e+6
**Supplemental Table S1**: Sequences of primers used for cloning *AtESP* and *AtNSP* genes (A) and sequence of cassette cloned into pDONR-Zeo to generate the “empty vector” control (B)

**A** Sequences of forward and reverse primers used for the amplifying *AtESP* and *AtNSP* genes upon template cDNA for cloning into *E. coli* expression cassettes using the Gateway technology (Invitrogen, Carlsbad, USA). Putative start codons are indicated in bold.

| Gene ID (protein name) | Forward primer sequence | Reverse primer sequence |
|------------------------|-------------------------|-------------------------|
| *At1g54040* (AtESP)    | GGGGACAAAGTTTGTACAAAAAAAGCA | GGGGACCACCTTTTGTCACAAGAAAGCTGG |
|                        | GGCTCCATGGCTCGGACTTTCGCAA | GTTTAAGCTGAATTGACCGCA |
| *At2g33070* (AtNSP2)   | GGGGACAAAGTTTGTACAAAAAAAGCA | GGGGACCACCTTTTGTCACAAGAAAGCTGG |
|                        | GGCTCCATGGCTCGGACTTTCGCAA | GTTTAAGCTGAATTGACCGCA |
| *At3g07720* (ATESP/NSP) | GGGGACAAAGTTTGTACAAAAAAAGCA | GGGGACCACCTTTTGTCACAAGAAAGCTGG |
|                        | GGCTCCATGGCTCGGACTTTCGCAA | GTTTAAGCTGAATTGACCGCA |
| *At3g16400* (AtNSP3)   | GGGGACAAAGTTTGTACAAAAAAAGCA | GGGGACCACCTTTTGTCACAAGAAAGCTGG |
|                        | GGCTCCATGGCTCGGACTTTCGCAA | GTTTAAGCTGAATTGACCGCA |
| *At5g488180* (AtNSP1)  | GGGGACAAAGTTTGTACAAAAAAAGCA | GGGGACCACCTTTTGTCACAAGAAAGCTGG |
|                        | GGCTCAATTGGTTCGCTGGTGAGAA | GTCAGCTTAGTCATATTGAC |

**B** Sequence of the 98 base pair cassette cloned into pDONR-Zeo to generate the vector used *in vitro* as “empty vector” control in nitrile-specifier assays.

5’-ggggacaagtttgtacaaaaaagcagctgctacgagcttaatttaagtcagatcttaattaaactcaagcggcagcccctttttttgttaacagtggctccc-3’
3’-cccctgttcaaacatgttttttcgtccgagcgactccgaattaattcctaggaattaatttggagtcgctgggctcgaaagaacatgttttcaccagggg-5’
Supplemental Figure S1  Expression patterns of *AtESP* and homologous genes during the growth cycle of *A. thaliana*.

**Legend to supplemental Fig. S1**

Heatmap representation of the *in silico* expression analysis of *AtESP* homologous genes at different growth stages of *A. thaliana* based on microarray data available at Genevestigator (www.genevestigator.ethz.ch). Expression levels are indicated by blue colouring, a darker blue colour indicating a higher expression level. Growth stages based on (63) are given and the number of arrays which this heatmap is based on is indicated.
At1g54040 (AtESP)
At3g07720
At3g16390/At3g16400/At3g16410
At5g48180
At2g33070 (AtNSP2)

Treatment Arrays (exp/ctrl)
Legend to supplemental Fig. S2
Heatmap representation of the \textit{in silico} expression analysis of \textit{AtESP} and homologous genes in \textit{A. thaliana} in response to biotic or abiotic stimuli based on microarray data available at Genevestigator (www.genevestigator.ethz.ch). Fold changes in expression levels in plants submitted to the stimulus versus control plants are as indicated by the coloured log$_2$-scale on top of the figure.
Supplemental Figure S3  *In vitro* nitrile-specifier activity of crude extracts expressing AtNSP2 or the “empty vector”.

Legend to supplemental Fig. S3
GC traces of GSL hydrolysis products generated *in vitro* from 2-propenylGSL (10 mM) after incubation with recombinant *B. brassicae* myrosinase (3 µg) and crude bacterial extracts expressing either the empty vector control (A) or recombinant His-tagged AtNSP2 (B). GSL hydrolysis product-related peaks are labelled: 1 = 2-propenylisothiocyanate, 3 = 3-butenynitrile, IS = internal standard (phenylisothiocyanate).
**Supplemental Figure S4** Plant tissue autolysis assay in the absence or presence of exogenously applied recombinant AtNSP2.

Legend to supplemental Fig. S4
GC traces of GSL hydrolysis products generated upon autolysis of plant tissue in the absence or presence of bacterial extracts containing recombinant AtNSP2: rosette leaves (100 mg) of 5 week old *A. thaliana Cvi* plants without (A) or with (B) addition of AtNSP2; mature seeds (100 mg) of *Brassica rapa* cv. Per without (C) or with (D) addition of AtNSP2. Peaks identified as GSL hydrolysis products are labelled: 1 = 2-propenylisothiocyanate, 2 = 3,4-epithiobutylnitrile, 3 = 3-butenynitrile, 6 = 4-pentenynitrile, 7 = 3-butenylisothiocyanate, 8 = 4,5-epithiopentylnitrile, 9 = 9-methylthiononylnitrile, 10 = indol-3-acetonitrile, 19 = 4-pentenylisothiocyanate, 20 = phenylethylisothiocyanate, 21 = 5-vinyl-2-oxazolidinethione, 22 = 5-hexenynitrile, 23 = benzenepropanenitrile, IS = internal standard (phenylisothiocyanate).
Nitrile-specifier proteins involved in glucosinolate hydrolysis in Arabidopsis thaliana
Ralph Kissen and Atle M. Bones

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