Title

desulfo-Glucosinolate sulfotransferases from Arabidopsis thaliana catalyzing the final step in biosynthesis of the glucosinolate core structure

Authors

Markus Piotrowski\textsuperscript{1}, Andreas Schemenewitz\textsuperscript{1}, Anna Lopukhina\textsuperscript{1}, Axel Müller\textsuperscript{1}, Tim Janowitz\textsuperscript{1}, Elmar W. Weiler\textsuperscript{1}, and Claudia Oecking\textsuperscript{2}

\textsuperscript{1}Department of Plant Physiology, Ruhr-Universität, Universitätsstr. 150, 44801 Bochum, Germany

\textsuperscript{2}Plant Physiology, Center for Plant Molecular Biology, University of Tübingen, Auf der Morgenstelle 5, 72076 Tübingen, Germany

Running Title

desulfo-Glucosinolate sulfotransferases from Arabidopsis thaliana

Corresponding author

Elmar W. Weiler, Department of Plant Physiology, Ruhr-Universität, Universitätsstr. 150, 44801 Bochum, Germany, (phone) ++49(0)234-3224291, (fax) ++49(0)234-3214187, (e-mail) Elmar.Weiler@ruhr-uni-bochum.de
SUMMARY

The phytotoxin coronatine is a structural analog of octadecanoid signaling molecules which are well known mediators of plant defense reactions. In order to isolate novel coronatine-regulated genes from Arabidopsis thaliana, differential mRNA display was performed. Transcript levels of CORI-7 (coronatine induced) were rapidly and transiently increased in coronatine-treated plants and the corresponding cDNA was found to encode the sulfotransferase AtST5a. Likewise, upon wounding an immediate and transient increase in AtST5a mRNA levels could be observed in both locally wounded and unwounded (systemic) leaves. Furthermore, application of octadecanoids and ethylene as compounds involved in plant wound-defense reactions resulted in AtST5a gene activation whereas pathogen-defense related signals (yeast elicitor, salicylic acid) were inactive.

AtST5a and its close homologs AtST5b and AtST5c were purified as (His)6-tagged proteins from Escherichia coli. The three enzymes were shown to catalyze the final step in the biosynthesis of the glucosinolate core structure, the sulfation of desulfo-glucosinolates. They accept a broad range of desulfo-glucosinolates as substrates. However, in a competitive situation AtST5a clearly prefers tryptophan- and phenylalanine-derived desulfo-glucosinolates while long-chain desulfo-glucosinolates derived from methionine are the preferred substrates of AtST5b and AtST5c.

Treatment of Arabidopsis plants with low concentrations of coronatine resulted in an increase in the amounts of specific glucosinolates, primarily glucobrassicin and
neoglucobrassicin. Hence, it is suggested that AtST5a is the sulfotransferase responsible for the biosynthesis of tryptophan-derived glucosinolates \textit{in vivo}.
INTRODUCTION

In plants, as compared to the animal cell, very little is known regarding structural and regulatory roles of the sulfate group. The transfer of the active sulfate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to acceptor molecules is catalyzed by sulfotransferases. Members of the superfamily of sulfotransferases are known in prokaryotes as well as eukaryotes, however, the study of enzymes that catalyze the sulfation reaction in plants considerably lags behind as compared to animal systems. Cytosolic sulfotransferases from plants have been characterized in some detail (1) and refs. therein) and some cDNAs have been identified. These fall into three subgroups, the flavonol-sulfotransferases described for Flaveria species (1), the steroid sulfotransferases identified in Brassica napus (2;3) and a hydroxyjasmonic acid specific sulfotransferase from Arabidopsis thaliana (4). An additional sulfotransferase (RaR047, At2g03760) has been cloned from Arabidopsis thaliana, and its mRNA level was found to be upregulated by pathogens and salicylic acid; its physiological substrate, however, is still unknown (5).

In plants, sulfate groups occur in a number of secondary metabolites, notably the sulfoflavonoids (6) and the glucosinolates (7). Glucosinolates (GSs) are secondary compounds found in at least 16 different plant families, 15 of which belong to the order Capparales (for review see (8)). Within this order, much interest is directed to the family of the Brassicaceae: the genus Brassica alone contains a large number of agricultural important crops like many vegetables (e.g. broccoli, brussels sprouts, cauliflower and cabbage) and one
of the most important oilseed crops, oilseed rape (*Brassica napus*), the defatted seed meal of which is fed to animals. GSs in edible species or seed meal have attracted much attention because their breakdown products were described to have anticancer but also goitrogenic and antinutritional activity.

Although many functions like sulfur- and nitrogen-storage have been assigned to GSs, defense against herbivors and pathogens seems to be their main function. Upon wounding, GSs are hydrolyzed by a thioglucosidase, called myrosinase, and the released unstable aglycons rearrange to form isothiocyanates, thiocyanates, nitriles and other compounds, production of which depends on the GS itself, reaction conditions as well as the presence of certain co-factors (review in:(9)). These compounds have antimicrobial activity and are toxic or deterrent to non-specialist herbivors.

Much progress has been made recently in identifying the gene products involved in the biosynthesis of GSs (Fig. 1): Aldoxime forming and aldoxime oxidizing cytochrome P450 enzymes characterized by different substrate specificities were identified (10-19). C-S lyase, the enzyme catalyzing the subsequent step in GS biosynthesis was recently identified as the *SUPERROOT1*-gene product (SUR1) (20), and the gene encoding for UDP-glucose:thiohydroximate glycosyltransferase was cloned from *Brassica napus* (21). The final step in the biosynthesis of the glucosinolate core structure is catalyzed by a *desulfo*-glucosinolate:PAPS sulfotransferase (dsGS-ST), transferring the sulfate moiety from PAPS to the *desulfo*-glucosinolate (dsGS). Enzymatic activity of dsGS-STs has been analyzed in partially purified protein fractions from *Brassica juncea* and cress (*Lepidium sativum*) (22;23). The enzymes have similar biochemical characteristics with respect to native
molecular mass, isoelectric point, pH- and temperature optima as well as inhibition by various SH-group reagents. Furthermore, the enzyme from cress was found to prefer desulfo-benzylglucosinolate over desulfo-sinigrin. However, the corresponding proteins have not been purified to homogeneity and until now genes encoding for dsGS-STs have not been identified. Here we report the cloning and functional expression of a small family of such desulfo-glucosinolate-specific sulfotransferases from Arabidopsis thaliana.
EXPERIMENTAL PROCEDURES

Chemicals--High quality PAPS (minimum 95%) was obtained from the German Institute of Human Nutrition (Prof. H.R. Glatt, Berholz-Rehbruecke, Germany). 12-Hydroxyjasmonic acid was a kind gift from Claus Wasternack (Institute of Plant Biochemistry, Halle a.d. Saale, Germany). 11-Hydroxyjasmonic acid was purified from suspension cultures of *Eschscholtzia californica* as described by Xia and Zenk (24). Synthesis of $^{13}$C$_2$-glucobrassicin was described in Müller and Weiler (25). Sinigrin (Allyl-glucosinolate) and benzylglucosinolate were obtained from Acros Organics (Geel, Belgium) and Merck Biosciences (Schwalbach, Germany), respectively.

Plant material--*Arabidopsis thaliana* C24 and Col-0 were grown in a greenhouse. Prior to treatment, plants were transferred to a phytotron chamber (8 h photoperiod at 20 °C and 150 µmol photons (400-700 nm) cm$^{-2}$ s$^{-1}$, 16 h darkness at 18 °C, rel. humidity 70%) for at least three days.

Treatment of plants--Wounding was done by using a hemostat and crushing across approximately 80-100% of the area of a specific leaf. The leaves of one half of a rosette were treated in this way, the opposite half of the rosette remained undamaged in order to allow differentiation of local and systemic effects. Application of compounds dissolved in 40% (v/v) acetone and 0.1% (v/v) Tween 20 was performed by spraying plant rosettes until run-
off. Control plants were treated with the solvent alone. Plant material was frozen in liquid nitrogen immediately after harvesting.

General molecular biological and biochemical procedures--If applicable, the standard protocols of Ausubel et al. (26) and Sambrook and Russel (27) were used. Plant-RNA was prepared according to Barkan (28). Ten micrograms of total RNA were separated on formaldehyde-agarose gels, transferred onto nylon membranes, and hybridized with a radiolabeled probe by using standard laboratory procedures. The probe for AtST5a mRNA corresponded to nucleotides 22 to 470 of the AtST5a cDNA. Synthesis of cDNA, PCR-amplification, DNA restriction and ligation followed the manufacturers' protocols, respectively.

Semiquantitative RT-PCR--Semiquantitative RT-PCR was done using the cMaster RTplus PCR system (Eppendorf, Wesseling-Berzdorf, Germany) following the manufacturer’s instructions. Contaminating DNA in the RNA preparations was digested with the RQ1 RNase-free DNase (Promega, Mannheim, Germany). This step was critical because the AtST5 genes contain no introns and amplification could therefore result from genomic DNA. Absence of DNA after digestion was confirmed by PCR using the RNA as template. Specificities of AtST5 primers were tested using the respective plasmid DNA as template. RT-PCR conditions were 94 °C for 5 min, 25-30 cycles of 30 sec at 94 °C, 45 sec at 55 °C, 60 sec at 72 °C, followed by a final extension at 72 °C for 10 min. After electrophoretical separation PCR products were visualized by ethidium bromide staining and quantified using
the TINA 2.0 program (Raytest, Straubenhardt, Germany). The data were normalized to signals obtained by RT-PCR of Actin1.

Cloning and bacterial expression of cDNAs encoding for selected sulfotransferases from Arabidopsis--The AtST1 cDNA (Lacomme and Roby 1996) was kindly provided by Dr. D. Roby (Castanet-Tolosan, France) (plasmid pRaR047).

The AtST5a gene was originally identified by differential mRNA display as a coronatine-induced gene (CORI-7) in A. thaliana (29). The obtained cDNA-fragment encompassed 514 bp and was identified to be part of the Arabidopsis EST-clone, OBO154 (accession F14418). Nevertheless, this 1165 bp-comprising EST-clone still displayed an incomplete 5’-end. The missing sequence was generated by 5’-RACE-PCR (30) using cDNA from coronatine-treated (5 µM, 2 h) plants as template. Meanwhile, the corresponding genomic sequence of AtST5a (At1g74100) was obtained within the scope of the Arabidopsis genome project.

For expression of Arabidopsis sulfotransferases as N-terminal RGS-(His)6-tagged proteins in E. coli M15, the corresponding cDNA regions were amplified from plasmid templates (among others the pUNI 51 clones U50569 and U50309 containing the cDNA encoding for AtST5b and AtST5c, respectively) by PCR and cloned into pQE 30 (Qiagen, Hilden, Germany) by using the KpnI/PstI (AtST5a) or BamHI/SalI (AtST1, AtST5b, AtST5c) restriction sites. Primer sequences can be obtained upon request. The recombinant RGS-(His)6-tagged proteins were purified under native conditions by using Ni-NTA agarose according to the manufacturer’s protocol. The eluted protein was immediately desalted using Sepharose
G-25 (PD-10 columns, Amersham Biosciences) pre-equilibrated with 50 mM Tris pH 7.0, 1 mM DTT, shock frozen in liquid nitrogen and stored at −80 °C.

Assay for sulfotransferase activity--Assays for sulfotransferase activity of the recombinant proteins were performed at 30 °C in a total volume of 100 µl 50 mM potassium phosphate buffer, pH 7.0, including PAPS (20-200 µM), substrate (20-200 µM desulfo-GSs) and purified protein (10 µg). Desulfo-GSs were extracted from leaves and seeds of Arabidopsis thaliana ecotypes Col-0 and C24 and purified by HPLC as described below. Controls were identical, but contained no protein. The reactions were stopped by the addition of 300 µl ice-cold methanol to precipitate the protein. At that point, protein was also added to the controls to ensure comparable conditions between samples and controls in the subsequent steps. After 2 h at −80 °C the precipitated protein was collected by centrifugation, the supernatant was taken to dryness and subsequently resuspended in 100 µl of water. Insoluble material was precipitated by an additional centrifugation and the resulting supernatant was analyzed by reverse phase HPLC. HPLC was performed on a Hyperclone 5µ BDS C18 1 (1 mm x 250 mm) (Phenomenex, Aschaffenburg, Germany) using a LaChrom Elite HPLC workstation equipped with a diode array detector (VWR International, Darmstadt, Germany). HPLC conditions are described elsewhere (31).

Preparation and analysis of glucosinolates in Arabidopsis thaliana--Extraction of glucosinolates from plant material and purification as desulfo-glucosinolates was performed as described in Brown et al. (32). For quantification, 1.25 µmol of benzylglucosinolate was
added as internal standard at the beginning of the extraction procedure. Desulfo-glucosinolates were separated by HPLC as described (31). Individual dsGS were identified by their UV-VIS spectra and quantified by comparison with the internal standard. These values were corrected for the response factors for the different dsGS. A list of these response factors is given in Brown et al. (32).
RESULTS

*Identification of a coronatine-induced sulfotransferase*--The structure of the phytotoxin coronatine is comparable to the octadecanoids 12-oxophytodienoic acid and/or jasmonic acid (33). Differential mRNA display (34) was performed in order to identify novel coronatine-regulated genes in *Arabidopsis thaliana* (29). For this purpose, mRNA from coronatine-treated (5 µM, 2 h) or solvent-treated plants was applied. Eight differentially regulated genes were studied in more detail and up-regulated genes were tentatively assigned as *CORI* (coronatine induced). The sequence of the amplified cDNA fragment of *CORI-7* (514 bp) was identical to the *A. thaliana* gene At1g74100, coding for a sulfotransferase family member. In *A. thaliana*, 18 genes for soluble sulfotransferases are known (Fig. 2 and (35)). One gene, At3g51210 (omitted from Fig. 2) seems to represent a pseudogene encoding a polypeptide that simply consists of 67 amino acids corresponding to the C-terminal region of sulfotransferases and up to now no EST clone is present in the databases. Phylogenetic analysis revealed that CORI-7 forms a small subfamily together with two other sulfotransferases encoded by the genes At1g18590 and At1g74090 (Fig. 2 and Table 1). According to the nomenclature introduced by Marsolais et al. (3;36) these sulfotransferases are called AtST5a (CORI-7), AtST5b (At1g74090), and AtST5c (At1g18590). This small subfamily is orthologous to the flavonol sulfotransferase family known from two different *Flaveria* species (Fig. 2). The closest relatives to this subfamily in *A. thaliana* are AtST2a and AtST2b (encoded by the genes At5g07010 and At5g07000, respectively). AtST2a has recently been identified as hydroxyjasmonate sulfotransferase, specifically sulfating 11- and
12-hydroxyjasmonate (4). However, hydroxyjasmonates are very unlikely substrates for the AtST5 subfamily of sulfotransferases, since even AtST2b, the closest homolog of AtST2a, is not able to sulfate these compounds (4).

Expression of sulfotransferases—RNA blot analysis of leaf tissue from untreated, healthy plants showed low CORI-7 mRNA levels (Fig. 3A). The following phytohormones, when applied exogenously at 50 µM concentration did not significantly alter the CORI-7 transcript level within two days: abscisic acid, 2,4-dichlorophenoxyacetic acid, gibberellin A3, kinetin, or salicylic acid (data not shown). However, its mRNA level specifically and transiently increased upon application of coronatine (5 µM, positive control), jasmonic acid (JA, 50 µM) or the JA precursor 12-oxophytodienoic acid (OPDA, 50 µM). In addition, the ethylene precursor 1-amino-2-cyclopropene-1-carboxylic acid (ACC, 50 µM) induced a transient increase in the CORI-7 gene product (Fig. 3A). The increase in mRNA level stimulated by coronatine, OPDA or ACC differs from that induced by JA in that the latter was delayed. While elevated mRNA levels already could be observed 1 h after coronatine-, OPDA- or ACC-treatment, the effect of JA is detectable first after 4 h. Wounding of leaves resulted in a rapid and transient local accumulation of CORI-7 mRNA and to a comparable systemic effect, i.e. accumulation in unwounded leaves (Fig. 3B). Likewise, UV-C illumination induces a transient increase, while yeast elicitor (10 mg ml⁻¹) was ineffective (data not shown). Taken together, the level of CORI-7 mRNA is strongly influenced by wounding and signaling compounds (octadecanoids, ethylene) mediating plant wound-defense reactions.
while pathogen-defense related signals (yeast-elicitor, salicylic acid) were inactive.

RT-PCR was used to analyze the expression of the other AtST5 genes after application of coronatine (5 µM) and MeJA (250 µM) (Fig. 3C). Plants were harvested two hours after spraying. The AtST5a gene was up-regulated 2.4 and 1.8 fold by coronatine and MeJA, respectively. AtST5b was only slightly – if at all - induced by coronatine (1.3 fold) and unchanged by MeJA while AtST5c showed a 2.4 fold induction by coronatine and a negligible (1.2 fold) induction by MeJA.

*Enzymes of the AtST5 subfamily are functional desulfo-glucosinolate sulfotransferases*--

The cDNAs for the AtST5 enzymes were cloned into the pQE-30 vector for bacterial expression of N-terminal (His)6-tagged proteins. The proteins could be purified under native conditions by means of Ni2+-chelate affinity chromatography (Fig. 4). For comparison, the cDNA of AtST1 (RaR047, encoded by At2g03760), a sulfotransferase also described to be up-regulated by jasmonic acid (5), was cloned and expressed in the same manner. In initial experiments, a range of known sulfotransferase substrates was tested using recombinant AtST5a. However, sulfation of neither quercetin, 17-β-estradiol, brassinolide, 24-epibrassinolide, castasterone, 24-epicastasterone, nor 11-hydroxy- and 12-hydroxyjasmonic acid could be observed. Thus, it was considered unlikely that these or structurally similar compounds are potential substrates *in vivo*. Since glucosinolate levels increase in members of the Brassicaceae after wounding and jasmonate treatment (e.g. (37-39)) we considered
desulfo-glucosinolates (dsGSs), the immediate precursors of glucosinolates (GSs), as possible substrates. Upon incubation of the AtST5 enzymes with a mixture of several dsGSs (total amount: 14 nmol) in the presence of 2 nmol 3’-phosphoadenosine 5’-phosphosulfate (PAPS), turnover of dsGSs was observed, while at the same time, the formation of new substances could be detected (Fig. 5A, the abbreviations used for the different dsGSs are given in Table II). To investigate if these products are indeed glucosinolates, the reaction product formed from indole-3-methyl-dsGS (desulfo-glucobrassicin) by AtST5a was purified by HPLC and further analyzed by ESI-TOF-MS. The relative molecular mass of the product in negative ion mode was 447.0515, which is a deviation of 3.8 ppm from the expected relative molecular mass of glucobrassicin (M-H+: 447.0532) and therefore within the specification of the mass spectrometer used (5 ppm). The CID-MSMS spectra of the product in positive and negative ion mode were in accordance (peak pattern and relative intensities) with the data obtained from synthetic 13C2-glucobrassicin (Fig. 6 A).

As a second line of evidence, the sulfation product formed from 8-methylthiooctyl-dsGS (8MTO-dsGS) by AtST5c was incubated with the enzyme myrosinase (Fig. 6B). Myrosinase is a thioglucosidase which is specific for the hydrolysis of GSs. This enzyme requires the full GS core structure and does not react with dsGSs ((40) and Fig. 6B). As can be seen from Fig. 6B, the product formed from 8MTO-dsGS by AtST5c vanished in the presence of myrosinase, a clear indication that this product is characterized by a complete glucosinolate core structure. Taken together, the three AtST5 isoforms are functional desulfo-glucosinolate sulfotransferases (dsGS-STs). While this paper was under review, similar results regarding
the identification of the AtST5 enzymes as dsGS-STs were also reported by another group (41).

AtST1 (RaR047) showed no activity towards dsGSs. AtST1 is an ortholog of the steroid sulfotransferases BnSST3 from *Brassica napus* (Fig. 2). BnSST3 is known to sulfate certain brassinosteroids (2); it seems therefore likely that AtST1 is also a steroid sulfotransferase.

**Substrate specificity of dsGS-STs**—When incubated with a mixture of dsGSs (derived from the amino acids methionine, phenylalanine, and tryptophan) in the presence of approximately equimolar amounts of PAPS (15 nmol PAPS vs. 14 nmol total dsGSs), all three dsGS-STs were able to sulfate different types of dsGSs, albeit with different preferences (Fig. 5B). These substrate specificities became more obvious on condition that limited amounts of PAPS (2 nmol) were applied (Fig. 5C): AtST5a clearly prefers dsGS derived from aromatic amino acids, namely I3M- and, to a lesser extent, Bz-dsGSs, while AtST5b and AtST5c prefer methionine-derived dsGS. The substrate specificities were further characterized by comparing substrate-pairs, in that the preferred substrate for each enzyme was incubated simultaneously with a second dsGS (Table III). Again, these data emphasize the preference of AtST5a for I3M-dsGS. The substrate specificities for AtST5b and AtST5c are similar but clearly distinguishable. The favorite substrate identified for AtST5b was 7MTH-dsGS while AtST5c prefers 8MTO-dsGS. Furthermore, additional potential substrates could be identified by incubating the enzymes with dsGSs mixtures isolated from leaves or seeds of *Arabidopsis thaliana* Col-0 (leaves) and C24 (leaves and seeds). By means of these experiments, the following dsGSs were shown to become sulfated: 3MTP-, 3MSOP-
Since the substrate specificities of the dsGS-STs are quite broad, it is not surprising that even side-chain modified dsGSs, like oxidized forms (e.g. 8MSOO-dsGS), methoxylated forms (e.g. 4MOI3M-dsGS, NMOI3M-dsGS) or more extensively modified forms like S2OH3Bn-dsGS, were also accepted as substrates. This indicates that, in principle, certain side-chain modification reactions could take place before the glucosinolate core structure is completed.

**Effect of coronatine on glucosinolate biosynthesis**--It is known that treatment of *A. thaliana, Sinapis alba, Brassica juncea, Brassica napus*, and *Brassica rapa* with jasmonic acid leads to an increase in indole-derived GSs (e.g. (38;39;42-44)). Because coronatine is proposed to be a structural analog of jasmonic acid and/or 12-OPDA, we analyzed the effect of the phytotoxin on glucosinolate biosynthesis in *A. thaliana* Col-0. The contents of the individual GSs found in control plants was in good agreement with recently published data (31;32) with the exception of glucobrassicin (I3M-GS), the content of which was consistently about twofold higher in our experiments than described in the literature (data not shown). This increase was not due to spraying (40% (v/v) acetone, 0.1% Tween 20), because untreated plants showed the same level of I3M-GS. Application of coronatine resulted in a two- to threelfold increase in the amount of I3M-GS and a five- to sevenfold increase in neoglucobrassicin (NMOI3M-GS) after 24 h and a further slight increase after 48 h, while the amount of the other glucosinolates (including the tryptophan-derived 4MOI3M-GS) was either unchanged or only marginally affected (Fig. 7). Qualitatively comparable results were
obtained after MeJA treatment (Fig. 7 and (44)) indicating that both compounds – although at different concentrations (5 µM coronatine vs. 250 µM MeJA) - affect the biosynthesis of I3M- and NMOI3M-GS in a similar manner.
DISCUSSION

Here cloning and functional characterization of a small family of desulfo-glucosinolate sulfotransferases (dsGS-STs) is described. The founding member of this family, AtST5a, was identified using the mRNA differential display technique, comparing mock treated Arabidopsis thaliana with plants sprayed with 5 µM coronatine. Phylogenetic analysis indicates that AtST5a clusters with AtST5b and AtST5c out of the 18 annotated sulfotransferases in the Arabidopsis genome (Fig. 2). The three genes are located on chromosome 1 and AtST5a and AtST5b are organized in tandem fashion. However, according to phylogenetic analysis AtST5b is more closely related to AtST5c as compared to AtST5a. Comparison with known sulfotransferases from other species revealed that the AtST5 family is orthologous to the SULT3 family of flavonol-sulfotransferases (Fig. 2 and (3)). Nevertheless, sulfated flavonoids are not known from Arabidopsis thaliana (6).

AtST5b and AtST5c are not only phylogenetically more closely related to each other as compared to AtST5a, they additionally show similar substrate specificities. Both enzymes prefer long-chain dsGSs derived from methionine, while AtST5a clearly prefers dsGSs derived from the aromatic amino acids tryptophan and phenylalanine. The tryptophan-derived I3M-GS (glucobrassicin) is present in relatively high concentrations in A. thaliana through the whole life cycle, while phenylalanine-derived GSs are only barely detectable in many ecotypes of A. thaliana (11;31;32;45). Thus, the proposed function of AtST5a in vivo is biosynthesis of I3M-GS. However, it should be noted that all three AtST5 enzymes are capable of converting a wide range of different dsGSs and hence they do not show strict
substrate specificities (Fig. 5 and Table III). Therefore, biosynthesis of specific GSs seems to be regulated at the pre-sulfotransferase level. For example, the preferred substrates for AtST5b and AtST5c, namely 7MTH- and 8MTO-dsGS, respectively, are only present in small amounts in leaves of *A. thaliana* Col-0, while the main glucosinolate in this tissue is 4MSOB-GS. 4MSOB-GS is derived from 4MTB-GS, which is a reasonably good substrate for AtST5b and AtST5c, too. Further experiments using plants in which single *dsGS-ST* genes are knocked-out will provide evidence regarding functional redundancy.

Coronatine-treatment of plants resulted in a specific increase of the tryptophan-derived I3M-GS and 4NMOI3M-GS (Fig. 7), while, at the same time, the genes coding for AtSt5a and AtST5c were up-regulated (Fig. 3C). This again points to a pre-sulfotransferase regulation of glucosinolate biosynthesis. It is already known that the aldoxime-forming enzymes CYP79B2, B3, F1 and F2, which are involved in the biosynthesis of tryptophan-derived GSs (CYP79B2 and B3) and methionine-derived GSs (CYP79F1 and F2) are up-regulated by treatment with jasmonic acid methyl ester (MeJA) (44). The fact that methionine-derived GSs are still only slightly affected by MeJA and coronatine indicates that their biosynthesis is substrate limited, very likely at the level of chain-elongation. Because tryptophan does not undergo chain-elongation before entering the GS-biosynthetic pathway, it could readily be consumed by CYP79B2 and B3.

One of the following steps in GS biosynthesis is catalyzed by a C-S lyase. Interestingly, one additional gene identified to be up-regulated by coronatine, *CORI-3*, encodes a C-S lyase, initially annotated as tyrosine aminotransferase (29;46). Although the C-S lyase involved in GS biosynthesis was identified as the *SUPERROOT1*-gene product, which is not
identical to CORI-3 (20), the possibility that CORI-3 may be involved in 13M-GS biosynthesis induced by coronatine or MeJA can not be ruled out.
Reference List


FOOTNOTES

1The abbreviations used are: ACC, 1-amino cyclopropane-1-carboxylic acid; dsGS: desulfo-glucosinolate; dsGS-ST, desulfo-glucosinolate:PAPS sulfotransferase; GS, glucosinolate; MeJA, jasmonic acid methyl ester; OPDA, 12-oxophytodienoic acid; PAPS, 3’-phosphoadenosine 5’-phosphosulfate; ST, sulfotransferase

ACKNOWLEDGEMENTS

We would like to thank D. Roby (Castanet-Tolosan, France) for providing the AtST1 cDNA (plasmid pRa047), C. Wasternack (Institute of Plant Biochemistry, Halle a.d. Saale, Germany) for the kind gift of 12-hydroxyjasmonic acid as well as D. Kreuder, L. Rößner and S. Schönfelder for excellent technical assistance. M.P and T.J. are grateful to Michael Reichelt and Jonathan Gershenzon (Max-Planck Institute of Chemical Ecology, Jena, Germany) for the introduction to glucosinolate analysis. This project was partially funded by the Deutsche Forschungsgemeinschaft (SPP1152 (M.P.) and SFB446-A18 (C.O.)).
Fig. 1: Biosynthesis of the glucosinolate core structure in *Arabidopsis thaliana*. The initial substrate is either a proteinogenic amino acid or a chain-elongated amino acid.

Fig. 2: Phylogenetic analysis of plant sulfotransferase enzymes by the maximum-likelihood method. Shown is the majority rule consensus tree of 100 bootstrap replicates. Human SULT1C2 was used as outgroup. Details for the sequences used in this analysis are given in Table I. Enzymes with known substrates are shown in bold.

Fig. 3: Expression of *AtST5* genes analyzed by Northern Blotting (A, B) and RT-PCR (C). Treatment of plants and extraction of RNA are described in Experimental Procedures. In A and B autoradiograms are shown, an 18S-rRNA probe was used as control. In C the ethidium bromide stained PCR-fragments are shown. The bands of *Actin1* were used to normalize the measured band intensities.

Fig. 4: Expression and purification of N-terminal RGS-(His)$_6$-tagged AtST5 enzymes. Ten µl each of the bacterial crude extract (CE), the column flow-through (FT) and the combined eluate fractions (EL) were loaded on a 12.5% SDS-polyacrylamide gel.

Fig. 5: Activity measurements of Arabidopsis dsGS-STs and AtST1 by HPLC. In A, typical chromatograms are shown. Arrowheads point to new peaks representing reaction
products. In B and C the quantified residual substrate amounts are shown. A mixture of each 2 nmol of purified desulfo-glucosinolates was used. For the abbreviations of the individual dsGSs, see Table II.

Fig. 6: Identification of the reaction products formed by AtST5 enzymes as glucosinolates.

A The reaction product formed by AtST5a from I3M-dsGS was purified by HPLC and analyzed by Q-TOF mass spectrometry. Shown are the CID-MSMS spectra of the reaction product and of the $^{13}$C$_2$-I3M-GS standard in negative ion mode. Possible structures of the fragments obtained are given. B Conversion of 8MTO-dsGS (10 nmol) by AtST5c and verification of a full glucosinolate core structure in the reaction product. 8MTO-dsGS was first incubated in the presence or absence of sulfotransferase enzyme (AtST5c). After completion of the reaction, myrosinase was added or not. Myrosinase did not hydrolyze 8MTO-dsGS (from bottom: second trace), but the reaction product formed by AtST5c (third trace) vanished after myrosinase treatment (fourth trace). Thus the product formed had a full glucosinolate core structure.

Fig. 7: Glucosinolate content in leaves of Arabidopsis 24 h (closed bars) or 48 h (hatched bars) after spraying with coronatine (5 µM) (gray bars), MeJA (250 µM) (black bars) or spraying solution alone (white bars). The abbreviations used are explained in Table II.
**Table I**

_Overview of sulfotransferases mentioned in this work._

<table>
<thead>
<tr>
<th>AGI code/GenBank Accession Number</th>
<th>Organism</th>
<th>Referred to as</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g18590</td>
<td><em>Arabidopsis thaliana</em></td>
<td>AtST5c</td>
<td>desulfo-glucosinolates, preferentially methionine-derived</td>
<td>this work</td>
</tr>
<tr>
<td>At1g74090</td>
<td></td>
<td>AtST5b</td>
<td>desulfo-glucosinolates, preferentially methionine-derived</td>
<td>this work</td>
</tr>
<tr>
<td>At1g74100</td>
<td></td>
<td>AtST5a</td>
<td>desulfo-glucosinolates, preferentially phenylalanine- and tryptophan-derived</td>
<td>this work</td>
</tr>
<tr>
<td>At2g03760</td>
<td></td>
<td>AtST1</td>
<td>unknown (RaR047)</td>
<td></td>
</tr>
<tr>
<td>At5g07000</td>
<td></td>
<td>AtST2b</td>
<td>unknown</td>
<td>(4)</td>
</tr>
<tr>
<td>At5g07010</td>
<td></td>
<td>AtST2a</td>
<td>hydroxyjamonates</td>
<td>(4)</td>
</tr>
<tr>
<td>AF000305</td>
<td><em>Brassica napus</em></td>
<td>BnSST1</td>
<td>unknown</td>
<td>(2)</td>
</tr>
<tr>
<td>AF000306</td>
<td></td>
<td>BnSST2</td>
<td>unknown</td>
<td>(2)</td>
</tr>
<tr>
<td>AF000307</td>
<td></td>
<td>BnSST3</td>
<td>brassinosteroids</td>
<td>(2)</td>
</tr>
<tr>
<td>U10277</td>
<td><em>Flaveria bidentis</em></td>
<td>FbFST-L</td>
<td>unknown</td>
<td>(47)</td>
</tr>
<tr>
<td>U10275</td>
<td></td>
<td>FbF3ST</td>
<td>quercetin</td>
<td>(48)</td>
</tr>
<tr>
<td>M84135</td>
<td><em>Flaveria chloraeefolia</em></td>
<td>FcF3ST</td>
<td>quercetin</td>
<td>(49)</td>
</tr>
<tr>
<td>M84136</td>
<td></td>
<td>FcF4’ST</td>
<td>quercetin-3-sulfate</td>
<td>(49)</td>
</tr>
<tr>
<td>AAC95519</td>
<td><em>Homo sapiens</em></td>
<td>SULT1C2</td>
<td>p-nitrophenol/N-hydroxy-2-acetylaminofluorene</td>
<td>(50)</td>
</tr>
<tr>
<td>-</td>
<td><em>Brassica juncea</em></td>
<td>-</td>
<td>desulfo-benzylglucosinolate</td>
<td>(23)</td>
</tr>
<tr>
<td>-</td>
<td><em>Lepidium sativum</em></td>
<td>-</td>
<td>desulfo-benzylglucosinolate &gt;&gt; desulfo-sinigrin</td>
<td>(22)</td>
</tr>
</tbody>
</table>
Table II

*Biosynthetic origin and abbreviation of several desulfo-glucosinolates used in this work.*

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Side-chain modification</th>
<th>Structure</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>Elongation</td>
<td>3-Methylthiopropyl-</td>
<td>3MTP-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-Methylthiobutyl-</td>
<td>4MTB-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-Methylthiopentyl-</td>
<td>5MTP-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-Methylthiohexyl-</td>
<td>6MTH-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-Methylthioheptyl-</td>
<td>7MTH-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-Methylthiooctyl-</td>
<td>8MTO-</td>
</tr>
<tr>
<td></td>
<td>+ oxidation</td>
<td>3-Methylsulfinylpropyl-</td>
<td>3MSOP-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-Methylsulfinylbutyl-</td>
<td>4MSOB-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ others</td>
<td>3Bn-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(S)-2-Hydroxy-3-butenyl-</td>
<td>S2OH3Bn-</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>None</td>
<td>Benzyl-</td>
<td>Bz-</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>None</td>
<td>Indole-3-methyl-</td>
<td>I3M-</td>
</tr>
<tr>
<td></td>
<td>Hydroxylation</td>
<td>4-Hydroxyindole-3-methyl-</td>
<td>4OHI3M-</td>
</tr>
<tr>
<td></td>
<td>+ methylation</td>
<td>4-Methoxyindole-3-methyl-</td>
<td>4MOI3M-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-Methoxyindole-3-methyl-</td>
<td>NMOI3M-</td>
</tr>
</tbody>
</table>
TABLE III

*Substrate specificities of Arabidopsis dsGS-STs*

Each enzyme was incubated with pairs of dsGSs. Each dsGS-mixture contained the favorite substrate (5 nmol) together with a second substrate (5 nmol). Incubation was performed under PAPS-limited conditions (2.5 nmol) until completion of the reaction. Shown is the relative percentage of activity in comparison to the best substrate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Best substrate (-dsGS)</th>
<th>Other substrates (-dsGS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtST5a</td>
<td>I3M</td>
<td>Bz &gt; 4MTB = 4MOI3M &gt; NMOI3M (59%)</td>
</tr>
<tr>
<td>AtST5b</td>
<td>7MTH</td>
<td>8MTO &gt; 4MTB = Bz &gt; I3M &gt; 8MSOC (70%)</td>
</tr>
<tr>
<td>AtST5c</td>
<td>8MTO</td>
<td>7MTH &gt; Bz H 3Bn &gt; 4MTB &gt; 8MSOC (58%)</td>
</tr>
</tbody>
</table>
Desulfo-glucosinolate sulfotransferases from Arabidopsis thaliana catalyzing the final step in biosynthesis of the glucosinolate core structure

Markus Piotrowski, Andreas Schemenewitz, Anna Lopukhina, Axel Müller, Tim Janowitz, Elmar W. Weiler and Claudia Oecking

J. Biol. Chem. published online September 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407681200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2004/09/09/jbc.M407681200.citation.full.html#ref-list-1