Biochemical and Biophysical Research Communications 434 (2013) 829–835

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification and characterization of a novel kaempferol sulfotransferase from *Arabidopsis thaliana*

Takuyu Hashiguchi [a,b], Yoichi Sakakibara [a,b,*], Yosuke Hara [b], Takehiko Shimohira [a], Katsuhisa Kurogi [a,b], Ryo Akashi [a,c], Ming-Cheh Liu [d], Masahito Suiko [a,b]

[a] Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, Miyazaki, Miyazaki 889-2192, Japan
[b] Department of Biochemistry and Applied Biosciences, University of Miyazaki, Miyazaki, Miyazaki 889-2192, Japan
[c] Frontier Science Research Center, University of Miyazaki, Miyazaki, Miyazaki 889-2192, Japan
[d] Department of Pharmacology, College of Pharmacy and Pharmaceutical Sciences, The University of Toledo, Toledo, OH 43614, USA

ARTICLE INFO

Article history:
Received 11 April 2013
Available online 20 April 2013

Keywords:
Sulfotransferase
Flavonoid
Arabidopsis thaliana

ABSTRACT

In plants, flavonoids have been shown to be subjected to conjugation modifications such as glycosylation, methylation, and sulfation. Among these modifications, sulfation is known as an important pathway in the regulation of the levels of endogenous compounds such as steroids. Although a large variety of flavonoid sulfates also exist in plants, the detailed biochemical characterization of *Arabidopsis thaliana* sulfotransferases (AtSULTs) remains to be fully clarified. We report here that uncharacterized AtSULT202E1 (AGI code: At2g03770), a SULT202E subfamily member, shows the sulfating activity toward flavonoids. The general characteristics of the enzyme were studied on the optimum temperature and pH, the effect of divalent cations, and the thermal stability with kaempferol as substrate. A comparative analysis of the sulfation of flavonoids by AtSULT202E1, AtSULT202B1 and AtSULT202A1 revealed that three AtSULTs have differential substrate specificities. Surprisingly, 3-hydroxyflavone was sulfated only by AtSULT202A1 while 7-hydroxyflavone was highly sulfated by AtSULT202E1 and AtSULT202B1. These results indicate that flavonols might be sulfated in a position specific manner. In conclusion, our studies indicate that a novel AtSULT202E1 has the sulfating activity toward flavonoids together with AtSULT202B1 and AtSULT202A1. The existence of three flavonoid sulfotransferases in *A. thaliana* suggests that sulfation of flavonoids have an important role in regulation of their functions.

1. Introduction

For plants, flavonoids have many beneficial biological functions [1]. In general, flavonoids are divided into chemically diverse subgroups: chalcone, flavanone, flavonol, flavone, isoflavone, and anthocyanidin. They differ in the position of their hydroxyl groups and in the structure of C-ring. In addition, flavonoids have structural diversity with modifications such as sulfation. Since the first discovery of flavonoid sulfates in 1937, a number of flavonoid sulfates with structural variation were reported in various plant species [2]. However, the physiological significance of flavonoid sulfates, and the responsible sulfotransferases have not been fully elucidated.

The sulfate conjugation reaction is catalyzed by a gene superfamily of enzymes called the cytosolic sulfotransferases (SULTs). Generally, the universal sulfate donor for the sulfation is 3′-phosphoadenosine 5′-phosphosulfate (PAPS). A sulfonate group (SO^{3-}) from PAPS is transferred to an appropriate hydroxyl or amino group of the substrate compounds with the parallel formation of 3′-phosphoadenosine 5′-phosphate (PAP). In animals including human, sulfation is one of the major phase II reactions and is an important pathway in the biotransformation/excretion of numerous drugs and xenobiotics, as well as endogenous compounds such as catecholamine and steroid [3]. In general, the sulfation facilitates the water solubility of acceptor substrates, thereby leading to the inactivation of biologically active compounds and the sulfated substrates are excreted from the body via urine and/or bile.

Like mammals, SULTs can be found in wide range of plant species and amino acid sequences of a large number of plant SULTs can be retrieved from public databases. However, their physiological role is less well characterized. In a model plant species *Arabidopsis thaliana*, there are 18 *SULT* genes including 1 apparent pseudogene (AGI code: At3g51210) [4]. To date, only a few SULT

Abbreviations: SULT, cytosolic sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

* Corresponding author. Address: Department of Biochemistry and Applied Biosciences, University of Miyazaki, 1-1, Gakuenkibanadai-Nishi, Miyazaki, Miyazaki 889-2192, Japan. Fax: +81 985 58 7211.
E-mail address: ysakaki@cc.miyazaki-u.ac.jp (Y. Sakakibara).

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http://dx.doi.org/10.1016/j.bbrc.2013.04.022

isoforms from *A. thaliana* have been isolated and biochemically characterized. In 1996, AtSULT202A1 (At2g03760) has been first isolated from cell suspension cultures which is induced by challenge with a pathogen and the pathogen-related signals, methyl jasmonate and salicylic acid [5]. Recent studies demonstrated that AtSULT202A1 and AtSULT202B6 (At2g14920) were specific for 24-epibrassinosteroid such as 24-epicathasterone, and for biologically active end-products including brassinolide, respectively [6]. Brassinosteroid SULTs were suggested to be involved in the loss of brassinosteroid biological activity in *Brassica napus* [7]. Flavonoid sulfotransferase, AtSULT202B1 (At3g45070), has been reported to exhibit strict regiospecificity for position 7 of flavonol and higher sulfating activity toward kaempferol 3-sulfate than kaemferol aglycone [8]. As a series of flavonol sulfotransferases which catalyze sequential sulfation of quercetin to quercetin tetrasulfate were previously isolated from *Flaveria* species [9,10], it is suggested that some flavonoid sulfotransferases other than AtSULT202B1 also exist in *A. thaliana*.

In this communication, we report an identification and characterization of a novel kaempferol sulfotransferase, AtSULT202E1 (At2g03770). Kamepferol is major flavonol aglycone found in *A. thaliana*. Firstly, in enzymatic assay, kaempferol was used as substrate candidates for sulfation, and then the biochemical characteristics were examined. Besides, flavonol sulfotransferase AtSULT202B1 and brassinosteroid sulfotransferase AtSULT202A1 were cloned and characterized by using a variety of flavonoids as substrates. This is the first report that three AtSULTs including a novel AtSULT202E1 catalyze the sulfation for flavonoids in *A. thaliana*. We discuss later in this report the existence of a position specific sulfation mechanism for flavonols in *A. thaliana*.

2. Materials and methods

2.1. Materials

Kaempferol, butein, naringenin, apigenin, quercetin, galangin, and genistein were purchased from Wako Pure Chemical Industries. Luteolin, dihydroquercetin, and 3-hydroxyflavone were products of Sigma–Aldrich Co. LLC. Chrysin was obtained from LKT Laboratories Inc. Delphinidin, cyanidin, and peralgonidin were from Extrasynthese. 7-Hydroxyflavone was purchased from Tokyo Kasei Kogyo Co., Ltd. pBluescript II SK (+) vector, XL1-Blue MRF', and BL21 *Escherichia coli* host strain were obtained from Stratagene. pGEX-4T-1 prokaryotic GST fusion vectors and glutathione Sepharose 4B were from GE Healthcare Biosciences. Cellulose thin-layer chromatography (TLC) plates were products of Merck. All other chemicals were of the highest grade commercially available.

2.2. Construction of phylogenetic dendrogram

Amino acid sequences of plant SULTs were collected from the GenBank database at the National Center for Biotechnology Information (NCBI) website. Amino acid sequences were aligned using the Clustal W program (http://www.genome.jp/tools/clustalw/) and the phylogenetic dendrogram was constructed based on the unweighted pair group method with arithmetic mean (UPGMA) method using GENETYX-MAC Version 11.1.0.

2.3. Molecular cloning of AtSULTs

A. thaliana ecotypes Col-0 were grown with a 16-h photoperiod at 22 °C in an atmosphere with 50–60% humidity. 2-Week-old *Arabidopsis* seedlings prepared were frozen in liquid nitrogen and homogenized in TRIzol Reagent (Life Technologies) for total RNA isolation according to the manufacturer's instructions. Using 1 μg of the isolated total RNA as the template and oligo (dT) as the primer, first-strand cDNA was synthesized using First-Strand cDNA Synthesis Kit (TOYOBO). PCR was subsequently carried out in 20 μl reaction mixtures under the action of KOD-Plus-Neo DNA polymerase (TOYOBO) with the first-strand cDNA as the template coupled with gene-specific primers (cf. Table 1). The PCR conditions used were: an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 10 s at 98 °C, 30 s at 55 °C, 40 s at 68 °C, and a final incubation at 68 °C for 7 min. The amplified products were restricted by *Bam*H I and *Xho* I (TOYOBO), and subcloned into pBluescript II SK (+). The cDNA inserts were subjected to nucleotide sequencing. Upon verification of their authenticity, the cDNA inserts were individually subcloned into pGEX-4T-1 prokaryotic expression vector.

2.4. Bacterial expression and purification of recombinant A. thaliana SULTs

pGEX-4T-1 harboring cloned AtSULT cDNAs were individually transformed into competent *E. coli* BL21 cells. Transformed BL21 cells were grown to OD_{600nm} = ~0.3 in 100 mL LB medium supplemented with 100 μg/mL ampicillin, and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After a 12-h induction at 24 °C, the cells were collected by centrifugation and homogenized in 15 mL of ice-cold lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) by using a French Press (Ohtake Works Co. Ltd.). The crude homogenate was subjected to centrifugation at 20,400*g* for 15 min at 4 °C. The supernatant collected was fractionated using 0.5 mL of glutathione Sepharose 4B, and the bound GST fusion protein was treated with 0.2 mL of a thrombin digestion buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 2.5 mM $CaCl_2$) containing 5 units/mL bovine thrombin. Following a 2-h incubation at 4 °C with constant agitation, the preparation was subjected to centrifugation, and the supernatant containing purified recombinant SULT was collected and used in the enzymatic assay.

2.5. Enzymatic assay

Sulfating activity of purified AtSULTs was assayed using ^{35}S-PAPS as the sulfate donor. The standard assay mixture, with a final volume of 25 μL, contained 50 mM sodium phosphate buffer, pH 7.5, 0.2 μM ^{35}S-PAPS (45 Ci/mmol), and 100 μM substrate. The reaction was started by the addition of the enzyme, allowed to proceed for 20 min at 22 °C, and terminated by heating at 98 °C for 3 min. The precipitates formed were removed by centrifugation, and the supernatant was subjected to analysis of ^{35}S-sulfated product using a previously developed TLC separation procedure [11], with *n*-butanol/isopropanol/formic acid/water (3:1:1:1; by volume) or *n*-butanol/acetic acid/water (3:1:1; by volume) as the solvent system. Afterwards, the plate was air-dried and analyzed using a Fluoro Image Analyzer FLA-3000 (Fujifilm). To examine the pH dependence, Good's buffers with a wide range of pH values instead of 50 mM sodium phosphate buffer, pH 7.5, were used in individual reactions. For the kinetic studies on the sulfation of flavonoids, the kinetic parameters were determined using the final substrate concentrations ranging from 0.01 to 100 μM. Data obtained were processed using the Excel program to generate the best fitting trendline for the Lineweaver–Burk plots. No enzyme inhibition was observed over the ranges of PAPS and flavonoids concentration used.

2.6. Miscellaneous methods

^{35}S-PAPS (45 Ci/mmol) was synthesized from ATP and ^{35}S-sulfate by using recombinant human bifunctional ATP

Table 1
Oligonucleotide primers used for cloning of three AtSULTs.

Isoform	Sense/Antisense	Sequence
AtSULT202E1	Sense	5′-CGCGGATCCATGACAAAATCCGAAACCACT-3′
	Antisense	5′-CCGCTCGAGTTAGCAAAAGAATCTAAAATC-3′
AtSULT202B1	Sense	5′-CGCGGATCCATGGAGATGAACTTGAGAATT-3′
	Antisense	5′-CCGCTCGAGTCAGAATTTCAAACCAGAGTT-3′
AtSULT202A1	Sense	5′-CGCGGATCCATGTCATCATCATCATCAGTT-3′
	Antisense	5′-CCGCTCGAGTCAAGAAGAAAATTTAAGACC-3′

Recognition sequences of restriction enzymes added to facilitate cloning are underlined.

sulfurylase/adenosine 5′-phosphosulfate kinase, as previously described [12]. Protein determination was performed based on Lowry's method with bovine serum albumin as the standard [13].

3. Results and discussion

Previous studies have revealed that eighteen *AtSULT* genes are present in *A. thaliana* genome [4]. The biochemical properties and physiological involvements of the coded AtSULT enzymes, however, remained poorly understood. It has been reported that flavonoids are sulfated only by one sulfotransferase, AtSULT202B1, which shows the strict regiospecificity for the position 7 of flavone and flavonol. In *Flaveria* species, however, four position-specific flavonol SULTs have been identified. In view of the large number of SULTs that are likely present in *A. thaliana*, we speculated that some other AtSULT(s) might also be involved in the flavonoid sulfation. As part of effort to clarify this issue, we cloned, expressed, and purified AtSULT202E1 for detailed enzymatic characterization.

3.1. Classification of plant SULTs

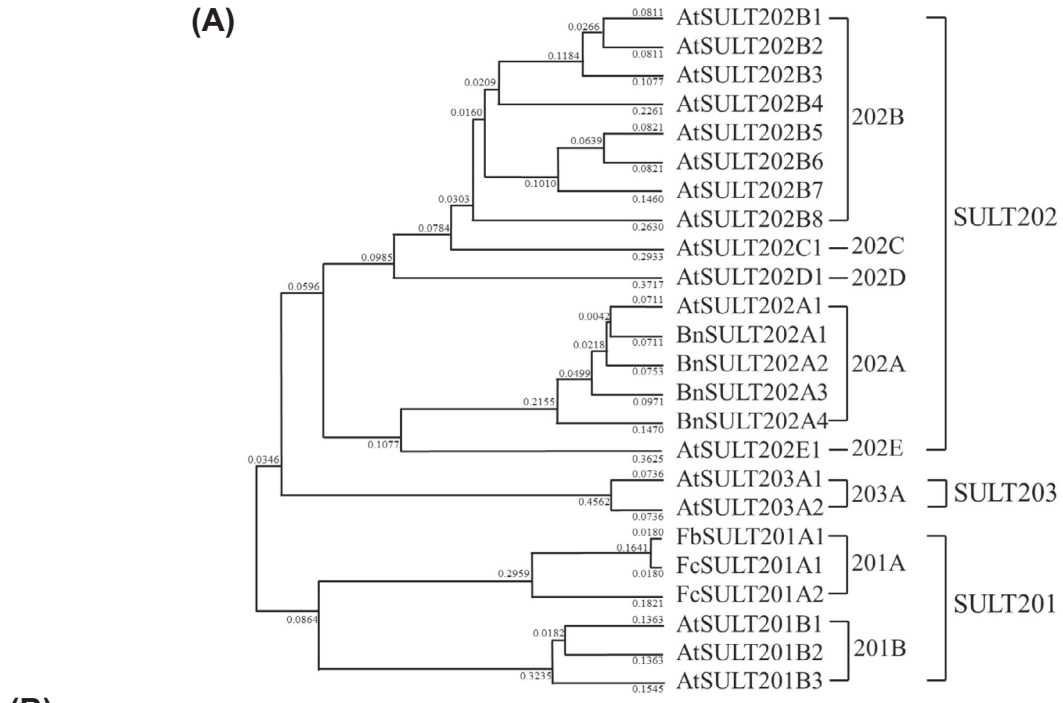
In a previous study, the fully sequenced genome of *A. thaliana* was searched for AtSULT sequences by applying the basic local alignment search tool (BLAST) with the first isolated AtSULT202A1 amino acid sequence. As a result, 18 sulfotransferase genes including one pesudogene were identified and individual SULTs were named according to the phylodendrogram generated. Unlike the consensus that have been reached for the SULTs that are present in vertebrate animals [14], there is currently no systematic nomenclature that have been proposed for plant SULTs. To address this gap, we attempted to construct a phylogenetic dendrogram for SULTs found in *A. thaliana*, *B. napus*, *Flaveria chloraefolia*, and *Flaveria bidentis*, and assigned names based on the previously proposed nomenclature system. As shown in Fig. 1A, all plant SULTs were classified into three major families, with members of each family share at least 45% amino acid sequence identity (Fig. 1B). Since the flavonol-specific SULTs from *F. chloraefolia* were the first plant SULT enzymes reported [9], the family that contains them, as well as a closely related SULT from *F. bidentis* [10], was designated the SULT201 family. Three AtSULTs were categorized into this family as a separate subfamily (designated 201B), apart from the two FcSULTs and a FbSULT that constitute another subfamily (designated 201A). For the SULT202 family, there are five subfamilies. Members of each SULT202 subfamily shares about 60% amino acid sequence identity. The first identified AtSULT (AGI code: At2g03760) [5] was designated as AtSULT202A1. Eight other AtSULTs constituted the 202B subfamily. In contrast, only single AtSULT members were classified into 202C, 202D, and 202E subfamilies. The third family, SULT203, contains the two remaining AtSULTs.

3.2. Molecular cloning of AtSULT202E1

The gene encoding AtSULT202E1 (AGI code: At2g03770) is located on chromosome 2 in tandem with the gene encoding AtSULT202A1 (AGI code: At2g03760). At the amino acid sequence level, AtSULT202E1 displays the highest homology with AtSULT202A1, showing 53.5 sequence identity. AtSULT202B1 (AGI code: At3g45070), on the other hand, shares 46.7% of amino acid sequence identity with AtSULT202E1. Fig. 2 shows the amino acid sequence alignment of AtSULT202E1 aligned with AtSULT202A1 and AtSULT202B1 using Clustal W program coupled with the Box shade software to shade identical residues in the alignments (http://www.ch.embnet.org/software/BOX_form.html). The highly conserved sequences, 5′-phosphosulfate binding loop (5′-PSB loop) in the N-terminal region and 3′-phosphate binding motif (3′-PB motif) at the center of the enzyme were found in AtSULT202E1 sequence [15]. AtSULT202E1 also contains the catalytic residue His^{134} conserved among almost all SULTs. A P-loop related motif which has been proposed to be important for either PAPS binding or catalysis is also conserved in AtSULT202E1 [16]. The presence of these functionally important sequence elements suggests that AtSULT202E1 is likely to be catalytically active. For comparison purposes, the open reading frames (ORFs) of these three AtSULTs were PCR-amplified using respective gene-specific primers (Table 1). The obtained sequences completely matched the GenBank accession number. GST fusion protein (64 kDa) was induced with IPTG, and recombinant AtSULT202E1 was purified to near homogeneity (data not shown). The molecular weight of the AtSULT202E1 was calculated by the online program Protparam (http://web.expasy.org/protparam/) and displayed the estimated molecular masses of 37.7 kDa.

3.3. Kaempferol sulfation by AtSULT202E1 and enzyme properties

We first performed the enzymatic sulfation by AtSULT202E1 with kaempferol as substrate (Fig. 3). Kaempferol is a natural flavonoid isolated from various plant sources and is known to be bioactive phytochemicals. In spite of the low sequence identity (46.7%) with AtSULT202B1, AtSULT202E1 also showed the sulfating activity for kaempferol. It seems that the low sequence identity between these two isoforms does not necessarily imply different substrate specificity. Next, we examined the temperature dependence of the activity of AtSULT202E1 using kaempferol as substrate. As shown in Fig. 4A, AtSULT202E1 exhibited the highest sulfating activity at 25 °C. There was about 50% decrease in the sulfating activity at 40 °C. In a pH dependence experiment, the AtSULT202E1 displayed a pH optimum of 6.5 (Fig. 4B). A pH optimum of flavonol sulfotransferases range from pH6.0 to 8.5 in previous studies [17], suggesting that flavonol sulfotransferases may exert the highest activity at the cytosol compartment. A thermal



(B)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
100	85.5	71.6	65.6	61.7	60.6	66.6	63.2	61.0	56.1	48.8	48.0	51.4	48.3	45.2	46.7	41.5	43.4	43.7	43.1	42.9	43.9	42.8	41.6	1 - AtSULT202B1
	100	71.2	63.7	59.8	59.2	66.5	61.6	58.3	54.0	47.3	46.2	49.1	47.2	43.5	45.6	38.7	40.4	43.2	42.5	42.5	42.0	42.0	40.7	2 - AtSULT202B2
		100	57.1	50.9	52.0	52.5	52.6	58.5	41.7	40.4	41.4	42.9	42.3	37.6	38.7	40.3	37.9	39.0	39.0	41.0	38.3	38.3	41.1	3 - AtSULT202B3
			100	62.0	62.5	63.8	64.8	61.2	55.4	44.9	44.0	45.8	42.8	42.4	46.4	42.2	41.3	40.3	40.3	42.1	42.5	41.0	42.2	4 - AtSULT202B4
				100	80.9	73.0	56.9	52.7	49.0	43.2	41.8	42.4	41.2	40.6	40.0	40.1	37.9	39.4	39.4	39.0	37.4	35.9	37.2	5 - AtSULT202B5
					100	71.0	57.8	51.4	49.4	43.2	43.0	42.4	41.7	41.3	39.2	40.0	38.5	38.8	38.8	38.9	38.3	38.7	38.7	6 - AtSULT202B6
						100	65.6	54.7	55.1	46.1	44.7	47.5	45.1	43.8	42.8	44.4	43.0	41.4	41.0	40.0	38.2	39.2	39.0	7 - AtSULT202B7
							100	55.9	55.3	46.6	44.0	46.8	41.7	39.9	46.1	42.1	42.8	40.9	39.8	38.5	40.4	39.2	39.3	8 - AtSULT202B8
								100	47.9	44.6	43.2	44.9	43.7	42.5	45.9	38.9	37.7	41.7	41.3	40.9	38.4	38.0	37.7	9 - AtSULT202C1
									100	42.4	42.5	42.7	41.3	42.3	45.9	40.5	41.4	41.3	41.6	41.8	39.3	39.4	37.8	10 - AtSULT202D1
										100	87.1	86.3	80.9	77.1	53.5	45.0	45.0	41.0	39.9	40.1	41.2	42.1	39.9	11 - AtSULT202A1
											100	87.0	82.5	81.3	54.7	42.9	43.1	39.9	38.9	38.2	40.4	41.8	41.0	12 - BnSULT202A1
												100	83.9	77.3	55.4	43.7	44.4	40.8	39.9	37.9	41.0	44.2	41.6	13 - BnSULT202A2
													100	73.8	52.4	40.4	40.9	40.2	38.8	38.1	38.9	40.8	38.9	14 - BnSULT202A3
														100	50.5	43.1	43.1	41.6	40.9	39.4	39.2	39.3	39.2	15 - BnSULT202A4
															100	40.6	42.4	40.0	38.7	42.0	41.9	41.5	41.2	16 - AtSULT202E1
																100	85.2	41.0	40.3	42.6	40.2	39.2	38.7	17 - AtSULT203A1
																	100	39.0	38.1	39.7	38.6	38.8	38.9	18 - AtSULT203A2
																		100	96.5	70.8	45.7	47.3	43.3	19 - FbSULT201A1
																			100	70.5	46.0	47.6	43.6	20 - FcSULT201A1
																				100	46.0	47.4	45.2	21 - FcSULT201A2
																					100	79.9	76.5	22 - AtSULT201B1
																						100	76.1	23 - AtSULT201B2
																							100	24 - AtSULT201B3

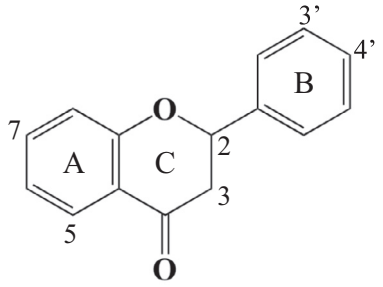
Fig. 1. Classification of AtSULT202E1 with other plant sulfotransferases on the basis of deduced amino acid sequences. (A) Phylogenetic dendrogram of plants SULTs. Divergence distances are shown on individual branches. Previously proposed nomenclature system was adopted to all plant SULTs. AtSULT201B1 (AGI code: At1g74090, Genbank ID: NP_177549, the name previously called: SOT18), AtSULT201B2 (At1g18590, NP_173294, SOT17), AtSULT201B3 (At1g74100, NP_177550, SOT16), AtSULT202A1 (At2g03760, NP_178471, SOT12), AtSULT202B1 (At3g45070, NP_190093, SOT5), AtSULT202B2 (At3g45080, NP_190094, SOT6), AtSULT202B3 (At2g27570, NP_180325, SOT4), AtSULT202B4 (At5g43690, NP_199182, SOT1), AtSULT202B5 (At1g13430, NP_172800, SOT9), AtSULT202B6 (At2g14920, NP_179098, SOT10), AtSULT202B7 (At1g13420, NP_172799, SOT8), AtSULT202B8 (At1g28170, NP_174139, SOT7), AtSULT202C1 (At4g26280, NP_194358, SOT3), AtSULT202D1 (At2g03750, NP_565305, SOT11), AtSULT202E1 (At2g03770, NP_178472, SOT13), AtSULT203A1 (At5g07010, NP_568177, SOT15), AtSULT203A2 (At5g07000, NP_196317, SOT14), BnSULT202A1 (GenBank ID: AAC63113), BnSULT202A2 (AAR14296), BnSULT202A3 (AAC63112), BnSULT202A4 (AAC63111), FbSULT201A1 (AAA61638), FcSULT201A1 (AAA33342), FcSULT201A2 (AAA33343). At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Fc, *Flaveria chloraefolia*; Fb, *Flaveria bidentis*. (B) Homology comparison of amino acid sequences of 24 plant SULTs. Analysis of aligned sequence data was carried out in GENETYX-MAC Version 11.1.0.

stability experiment was carried out using kaempferol as substrate. As shown in Fig. 4C, AtSULT202E1 was stable over the temperature range of 0–40 °C. Our previous studies had shown that divalent metal cations can exert dramatic inhibitory/stimulatory effects on various human SULTs [18]. Among 8 different divalent cations tested at 5 mM, Fe^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} rendered AtSULT202E1 virtually inactive (Fig. 4D). However, stimulatory effects of divalent cations on the sulfating activity for kaempferol were not detected in AtSULT202E1. It is therefore possible that *A. thaliana* sulfotransferases may be susceptible to heavy metal pollution.

3.4. Substrate specificity of AtSULT202E1, AtSULT202B1, and AtSULT202A1

To compare the biochemical characteristics of a novel AtSULT202E1 with those of the previously characterized AtSULTs, AtSULT202B1 and AtSULT202A1, a set of flavonoids were used for the investigation of substrate specificity. As shown in Table 2, the specific activities of AtSULT202E1 and AtSULT202A1 for flavone apigenin were weak in comparison with those for kaempferol respectively, whereas AtSULT202B1 showed high activity for both substrates. These results might be due to the absence of the

Fig. 2. Amino acid sequence comparison of AtSULT202E1, AtSULT202B1, and AtSULT202A1. Identical residues conserved among at least two of the three enzymes are drawn in black, and similar residues are in gray. 5′-PSB loop including conserved lysine residue in the N terminal, which interacts with the 5′-phosphate of PAP, and 3′-PB motif for the binding of 3′-phosphate of PAP are underlined. Conserved P-loop related motif (GXXGXXK) was also underlined. Catalytic residue histidine highly conserved in almost all known SULTs, was indicated by arrow.



Compound	C3	C5	C7	C3'	C4'	C2-C3 double bond
Naringenin	H	OH	OH	H	OH	-
Kaempferol	OH	OH	OH	H	OH	+
Quercetin	OH	OH	OH	OH	OH	+
Galangin	OH	OH	OH	H	H	+
Apigenin	H	OH	OH	H	OH	+
Luteolin	H	OH	OH	OH	OH	+
Chrysin	H	OH	OH	H	H	+
Dihydroquercetin	OH	OH	OH	OH	OH	-
3-Hydroxyflavone	OH	H	H	H	H	+
7-Hydroxyflavone	H	H	OH	H	H	+

Fig. 3. A chemical structure of some representative flavonoids used as substrates for this study.

3-hydroxyl group within apigenin. The former two AtSULTs might be more specific to 3-hydroxyl group rather than to the other positioned hydroxyl groups. Quercetin is also major flavonoids found in *A. thaliana* and has a distinct effect on root developmental process [19]. In contrast with those for kamepferol, the sulfating activities of three AtSULTs for quercetin were relatively low. AtSULT202E1 displayed no significant activity for quercetin. It is therefore possible that the position-3 of hydroxyl group of quercetin might exhibit some inhibitory effects on the sulfating activity of three SULTs. Indeed, this hypothesis is supported by the highest specific activity for galangin which does not contain any hydroxyl group in B ring. Furthermore, only AtSULT202A1 indicated the specific sulfating activity for anthocyanidins, in decreasing order, peralgonidin, cyanidin, and delphinidin. Since AtSULT202E1 and AtSULT202B1 did not show the activity toward anthocyanidin, anthocyanidin sulfation is catalyzed by AtSULT202A1 specifically. In spite of the lack of 3-hydroxyl group, flavanone naringenin, which is the key intermediate of flavonoid biosynthesis pathway, is strongly sulfated by AtSULT202A1. Unlike apigenin, naringenin has no C2′–C3′ double bond in C ring, suggesting that the C ring structure markedly influences on the sulfating activity of AtSULT202A1. While *A. thaliana* cannot produce isoflavonoid because of lacks of chalcone reductase and isoflavone synthase enzymes [20], three AtSULTs showed sulfating activity toward genistein. Hydroxycinammic acids including coumaric acid which are phenolic compounds found in almost all plants, were not sulfated by these enzymes (data not shown).

3.5. Determination of kinetic parameters of three AtSULTs for flavonoids and PAPS

The substrate specificities of three AtSULTs for flavonoids implied the existence of position specific sulfation mechanism. Therefore, we investigated whether five mono-hydroxyflavones, 3-, 5-, 7-, 3′-, and 4′-hydroxyflavone could be the substrates for three AtSULTs. Three AtSULTs exhibited no sulfating activity toward 5-, 3′-, and 4′-hydroxyflavone. Interestingly, 3-hydroxyflavone was sulfated only by AtSULT202A1 with an activity of

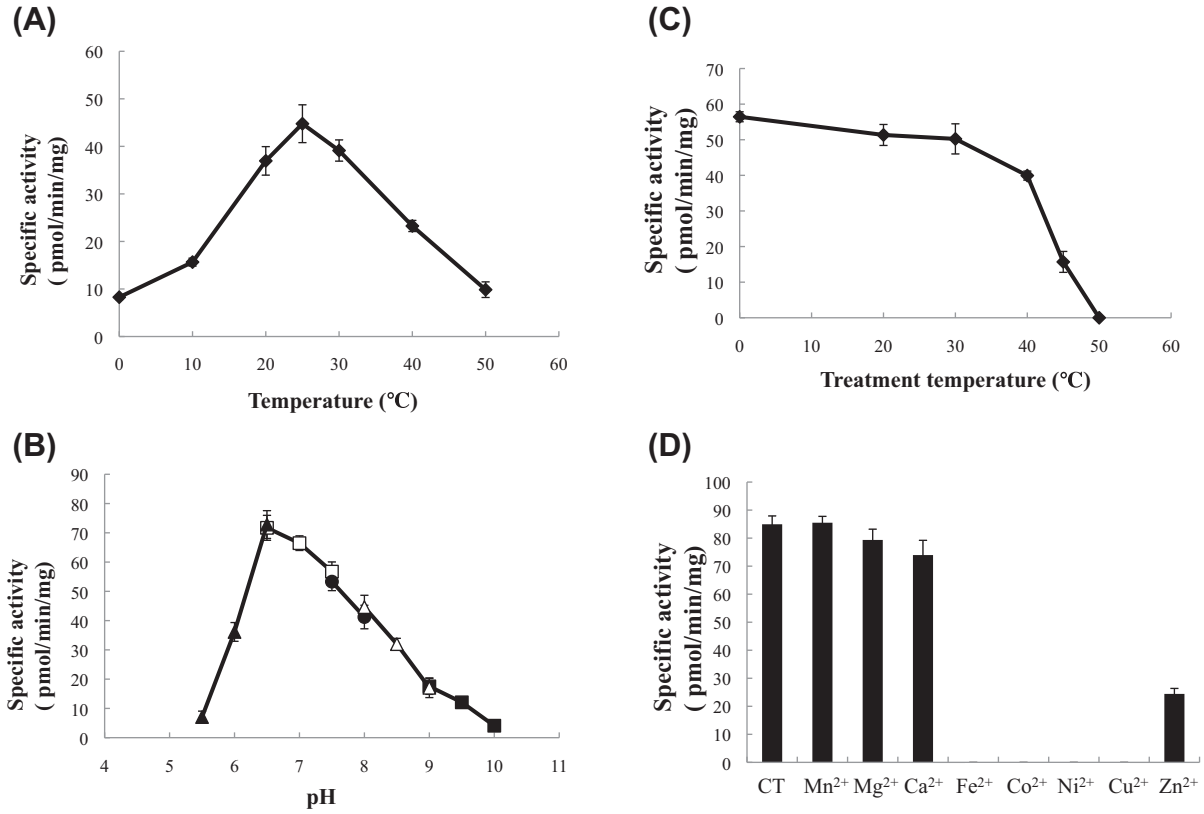


Fig. 4. Characterization of AtSULT202E1. Specific activity refers to pmol of sulfated product formed /min/mg enzyme. The data shown represent means ± SD for three determinations. (A) Temperature dependence of the sulfating activity of AtSULT202E1 with kaempferol. (B) pH dependency of the sulfating activity of AtSULT202E1 with kaempferol. The closed triangle, MES at pH 5.5–6.5; the open square, PIPES at pH 6.5–7.5; the closed circle, HEPES at pH 7.5–8.0; the open triangle, TAPS at pH 8.0–9.0; the closed square, CHES at pH 9.0–10.0. (C) Thermal stability of AtSULT202E1 with kaempferol. The enzyme was incubated for 15 min at different temperatures, followed by enzymatic assay using 100 μM kaempferol as the substrate under standard assay conditions. (D) Effects of divalent metal cations on the sulfating activity of AtSULT202E1. CT refers to control in the absence of divalent metal cations.

Table 2
Specific activities of three *Arabidopsis thaliana* SULTs with flavonoids as substrates.

Substrate compound	Specific activity (pmol/min/mg)		
	AtSULT202E1	AtSULT202B1	AtSULT202A1
Chalcone – Butein	13.5 ± 0.8	16.5 ± 2.2	25.8 ± 0.3
Flavanone – Naringenin	9.3 ± 1.5	26.7 ± 2.0	165.2 ± 5.6
Flavone – Apigenin	8.0 ± 0.5	66.1 ± 4.8	8.6 ± 0.8
– Luteolin	41.7 ± 2.9	48.1 ± 7.0	17.5 ± 1.3
– Chrysin	10.4 ± 0.4	26.1 ± 2.4	10.1 ± 0.9
Isoflavone – Genistein	13.4 ± 0.5	33.1 ± 1.3	52.9 ± 2.1
Flavonol – Kaempferol	35.5 ± 0.3	121.8 ± 7.9	101.0 ± 3.7
– Quercetin	N.D.	5.1 ± 1.0	59.5 ± 1.3
– Galangin	95.7 ± 3.0	162.9 ± 11.1	122.6 ± 3.5
Flavanonol – Dihydroquercetin	5.4 ± 0.3	5.3 ± 0.1	8.6 ± 0.1
Anthocyanidin – Delphinidin	N.D.	N.D.	22.4 ± 1.4
– Cyanidin	N.D.	N.D.	52.1 ± 3.3
– Peralgonidin	N.D.	N.D.	90.2 ± 4.1

Specific activity refers to pmol of sulfated product formed/min/mg enzyme. The data shown represent means ± S.D. from three determinations. N.D. refers to activity not detected (<5.0 pmol/min/mg).

26.2 pmol/min/mg of enzyme. On the other hand, 7-hydroxyflavone was sulfated by all three enzymes. AtSULT202E1 and AtSULT202B1 exhibited the catalytic activity for 7-hydroxyflavone of 43.0, and 38.5 pmol/min/mg of enzyme, respectively, whereas AtSULT202A1 showed a 3-fold lower activity (14.8 pmol/min/mg of enzyme). Next, kinetic parameters *V*max, *km*, and *V*max/*km* for the sulfation of kaempferol, hydroxyflavones, and PAPS by three AtSULTs were determined based on Lineweaver–Burk plots (Table 3). Surprisingly, AtSULT202A1 showed the lowest *km* value of 4.9 μM for kaempferol among three SULTs. It was found that AtSULT202E1 and AtSULT202B1 showed 4–7-fold higher *km* values for kaempferol than did AtSULT202A1. The *km* value of AtSULT202A1 for 3-hydroxyflavone was 5.7 μM while the *km* values of AtSULT202E1 and AtSULT202B1 for 7-hydroxyflavone were

Table 3
Kinetic data of three recombinant AtSULTs for the preferred flavonoids.

	km (μM)	*V*max (pmol/min/mg)	*V*max/*km*
AtSULT202E1			
Kaempferol	21.0	46.5	2.2
7-Hydroxyflavone	508.4	212.8	0.4
PAPS	0.1	107.5	1075
AtSULT202B1			
Kaempferol	35.3	192.3	5.4
7-Hydroxyflavone	781.8	243.9	0.3
PAPS	1.3	555.6	427
AtSULT202A1			
Kaempferol	4.9	117.6	24.1
3-Hydroxyflavone	5.7	32.3	5.6
PAPS	0.1	222.2	2222

The kinetic parameters of PAPS were examined with 100 μM kaempferol. Results shown represent means of three independent experiments.

relatively higher (500–800 μM), suggesting that AtSULT202A1 is the first enzyme for catalyzing the sulfation of position-3 of hydroxyl group and the generated 3-O-sulfated flavonols might become the preferred substrates for AtSULT202E1 and AtSULT202B1. AtSULT202E1 and AtSULT202B1 did not show any significant activity toward 3-hydroxyflavone. The very low activity for 7-hydroxyflavone by AtSULT202A1 prevented the determination of the kinetic constants, suggesting that AtSULT202A1 primarily catalyzes the sulfation of 3-hydroxyl group of flavonols.

To summarize, we isolated and characterized a novel *A. thaliana* sulfotransferase, AtSULT202E1. In this study, we for the first time showed that AtSULT202E1 sulfated flavonoids. However, it should be noted that *AtSULT202E1* (At2g03770) is located in tandem with *AtSULT202A1* (At2g03760) and assigned to the same family as AtSULT202A1 and *Brassinca napus* brassinosteroid sulfotransferases. It is therefore possible that AtSULT202E1 also has the sulfating activity for brassinosteroids. To date, it has been reported that AtSULT202E1 is expressed in seeds at early stage of embryonic development under publicly available microarray expression data (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). However, we have successfully cloned AtSULT202E1 from 2-week old seedlings. AtSULT202E1 might be expressed widely in stages of development and some tissues. Unexpectedly, we found for the first time that AtSULT202A1 possess the ability to catalyze the sulfation of flavonoids. This result suggested that AtSULT202A1 have a broad substrate specificity ranging from simple phenol to steroids. The studies of the substrate specificity and kinetics led to a proposition of regioselective kaempferol sulfation by three AtSULTs in *A. thaliana*. A novel AtSULT202E1 and flavonol specific AtSULT202B1 might catalyze the sulfation of 7-hydroxyl group of kaempferol, whereas AtSULT202A1 preferentially sulfates 3-hydroxyl group of it. Kaempferol- and quercetin-sulfate extracted from a root of *Argyreia speciosa* native to india was recently confirmed to show the antimicrobial activity, suggesting sulfated flavonoids might naturally play a role in plant responses to pathogen infection [21]. Additional work is warranted in order to clarify the bioactivity of sulfated flavonoids for plants.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers 23580138 (M.S.), 21580114 (Y.S.), and for JSPS Research Fellows Grant Number 24 1404 (T.H.) and National Institutes of Health Grant R03HD071146 (M.C.L.). We also thank Dr. Takehito Inaba for his support and discussions.

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