宮　崎　大　学　大　学　院

博 士 学 位 論 文

Elucidation of physiological functions of sulfotransferases in *Arabidopsis thaliana*

(シロイヌナズナ硫酸転移酵素の生理機能解明)

2014 年 3 月

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生物機能応用科学専攻

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Table of Contents

Acknowledgements

First of all, I would like to express my heartfelt gratitude to Professor Masahito Suiko at University of Miyazaki for giving me a valuable knowledge, guidance, encouragement, continuous support, and an opportunity to work on this interesting research topic.

Next, I wish to express my great appreciation Professor Yoichi Sakakibara at University of Miyazaki for constant advice, support, and unconventional idea. I have learned not only sciences but also the importance of communication between researchers.

Professor Ryo Akashi at University of Miyazaki, provided precious advice and great education for this study. I would like to sincerely thank you for warm encouragement.

I am also grateful to Professor Ming-Cheh Liu at The University of Toledo for his valuable advices to my writing article. His precious and sound advices made for a better doctoral thesis.

I got detailed advice from Dr. Katsuhisa Kurogi at University of Miyazaki. I am very appreciative of his continuous support and encouragement.

Finally, I am truly thankful to members of biochemistry laboratory at University of Miyazaki for their kind helps and encouragements during the doctoral course.

Abbreviations

APS: Adenosine 5’-phosphosulfate

AtSULT201A1: *Arabidopsis thaliana* sulfotransferase family 201 subfamily A isoform 1

BSA: Bovine serum albumin

CYP: Cytochrome P450

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

GST: Glutathione S-transferase

HEPES: 2-[4-(2-hydroxyethyl)-1-pirerazinyl]-ethanesulfonic acid

hSULT1A1: Human sulfotransferase family 1 subfamily A isoform 1

IPTG: Isopropyl β-D-thiogalactopyranoside

mSULT1A1: Mouse sulfotransferase family 1 subfamily A isoform 1

PAP: 3’-Phosphoadenosine 5’-phosphate

PAPS: 3’-Phosphoadenosine 5’-phosphosulfate

3’-PB: 3’-Phosphate binding

PBS: Phosphate-buffered saline

PMF: Peptide mass fingerprinting

PMSF: Phenylmethylsulfonylfluoride

5’-PSB: 5’-Phosphosulfate binding

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SULT: Cytosolic sulfotransferase

TLC: Thin layer chromatography

Abstract

The sulfation of xenobiotics and endogenous molecules has long been known to occur in a variety of organisms, from prokaryotes to multicellular species, and has been thought to be essential reaction for detoxification and hormone regulation. The sulfation is catalyzed by a family of cytosolic sulfotransferases (SULTs). The SULTs catalyze the transfer of a sulfonate group from active sulfate, 3'-phosophoadenosine 5'-phosphosulfate (PAPS), to an acceptor substrate compound containing hydroxyl and/or amino groups with the parallel formation of 3'-phosphoadenosine 5'-phosphate (PAP). 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 cell.

Many researchers including us have been studying mainly on the physiological function of human and mammal sulfotransferases. On the other hand, large numbers of sulfotransferase-like genes can also be found in plants, but their biological functions are relatively unknown. First of all, therefore, the author performed the comprehensive cloning and characterization of *Arabidopsis thaliana* sulfotransferases. The author could construct the expression system for nine AtSULTs, AtSULT202A1, AtSULT202B1, AtSULT202B4, AtSULT202B5, AtSULT202B6, AtSULT202B7, AtSULT202B8, AtSULT202E1, and AtSULT203A1. Among of these, four AtSULTs, AtSULT202A1, AtSULT202B1, AtSULT202B7, and AtSULT202E1 exhibited the sulfating activity toward a wide array of flavonoids. These results suggest that the function of flavonoids might be modulated by a number of sulfotransferases.

In the next study, the author conducted the characterization of a novel cloned sulfotransferase, AtSULT202E1, by using various flavonoids as substrates. Flavonoids, which are one of the secondary metabolites and widely distributed in plant, have been shown to be

subjected to conjugation modifications such as glycosylation, methylation, and sulfation in plants. 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. 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 chapter III, the author focused on AtSULT202B7 isoform. AtSULT202B7 also displayed sulfating activity specific for flavonoids (e.g., kaempferol and quercetin). The recombinant enzyme preferred flavonoid glycosides (e.g., kaempferol-3-glucoside and quercetin-3-glucoside) rather than their aglycone counterparts. Based on the comparison of HPLC retention times between sulfated kaempferol and the deglycosylated product of sulfated kaempferol-3-glucoside, the sulfation site in sulfated kaempferol-3-glucoside appeared to be the hydroxyl group of the flavonoid skeleton. In addition, by using direct infusion mass spectrometry, it was found that the sulfated product had one sulfonate group within the molecule. These results indicated that AtSULT202B7 functions as a flavonoid glycoside 7-sulfotransferase.

In a variety of plant species such as *Asteraceae* and *Oenanthe*, sulfated flavonoids have been discovered. The physiological function of these compounds, however, has not been clarified yet. For understanding of functions of flavonoid sulfates in model plant, *A. thaliana*, the author attempted to treat naringenin or naringenin sulfate, a representative flavonoid, to T87 suspension cells and performed the proteomic analysis by two-dimensional differential gel electrophoresis (2D-DIGE). The author have successfully identified for the first time the differential expression of proteins involved in basal metabolism including glycolysis and

nitrogen metabolism. Furthermore, a receptor for activated c kinase (RACK) was also identified, suggesting that sulfation might play a role in hormone response and developmental process via flavonoid sulfation in *A. thaliana*.

These studies would encourage us to investigate additional biochemical characterization of plant SULTs in the pursuit of yet-to-be-defined function.

Introduction

Our bodies have a variety of enzymes and make up intricate pathways for metabolizing xeno- and endo-biotics. These essential metabolic pathways are currently divided into three phase reactions **(Figure 1)** [Kohle, C. et al., 2007]. Firstly, exogenous organic substances including drugs and environmental chemicals or endogenous hormones are metabolized into water soluble derivatives chiefly by cytochrome P450 (CYP) enzymes in phase I pathway. CYP catalyzes the oxidation-reduction reaction of target molecules and converts it to hydrophilic compounds containing hydroxyl groups, carboxyl groups, and/or amino groups. Steroid hormones were also synthesized from cholesterol by the action of CYP enzymes through multiple stages. In phase II reaction, cytosolic sulfotransferases (SULT), glutathione S-transferases (GST), and uridine-diphosphate (UDP) -glucuronosyltransferases (UGT), which are representative phase II enzymes, transfer each polar group (sulfate, glutathione, and glucuronic acid, respectively) into the introduced functional groups of acceptor molecules, thereby eliminating target chemicals from the cytosol more rapidly or regulating the bioactivity of endogenous hormones. Finally, in phase III, conjugated products are passed out of the body, or transported to other target tissues via drug transporters. Major membrane transporters have been classified into two transporter family, ATP-binding cassette (ABC) transporter and solute carrier (SLC) transporter [Oostendorp, R.L. et al., 2009]. Little is known concerning the transport of sulfate conjugates between tissues.

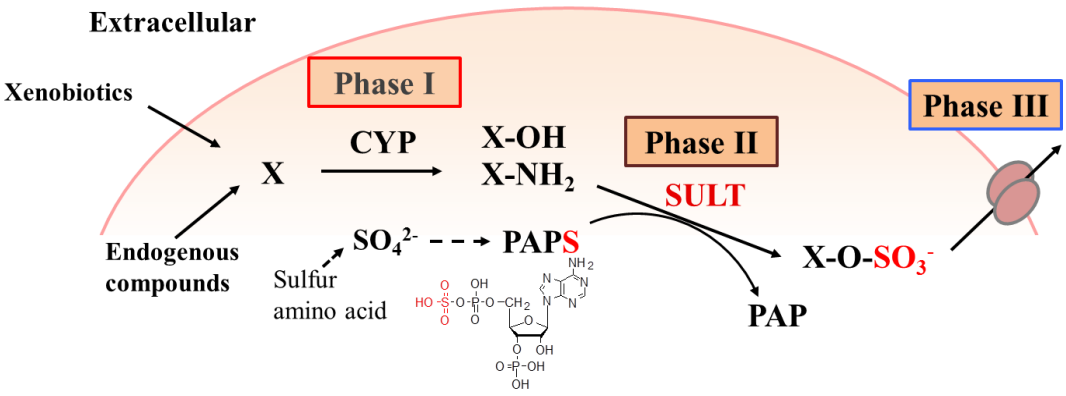


Figure 1. Metabolic pathway of xeno- and endo-biotics in a variety of organisms. Exogenous compounds such as drugs and endocrine-disrupting chemicals are detoxified into biologically inactive compounds by a series of enzymes. These enzymes also catalyze the synthesis and degradation of the endogenous compounds including steroid hormones, and consequently maintain metabolic homeostasis of organisms. The inorganic sulfate derives from dietary sulfate in foods and drinking water.

Sulfate conjugation was first reported by Baumann in 1876 [Baumann, E., 1876] and has since been shown to be an important pathway in the biotransformation/excretion of numerous drugs and xenobiotics, as well as endogenous compounds such as catecholamines, steroids, thyroid hormones, and bile acids [Falany, C.N. et al., 1997; Hemmerich, S. et al., 2004; Gamage, N. et al., 2006]. The universal sulfate donor for sulfation reaction is 3'-phosphoadenosine 5'-phosphosulfate (PAPS) [Robbins, P.W. et al., 1957]. In mammals, PAPS is synthesized from ATP and inorganic sulfate thorough two consecutive process by bifunctional PAPS synthase having ATP sulfurylase (ATPS) and APS kinase (APK) activities [Seubert, P.A. et al., 1985], while in plants, these activities are encoded by separate genes **(Figure 2)**. In plant, APS also represents the activated intermediate for reductive sulfate assimilation [Patron, N.J. et al., 2008]. There are four ATPS isoforms and APK isoforms respectively in model plants, *Arabidopsis thaliana* [Hatzfeld, Y. et al., 2000; Lee, S., 1998; Mugforf, S.G. et al., 2009]

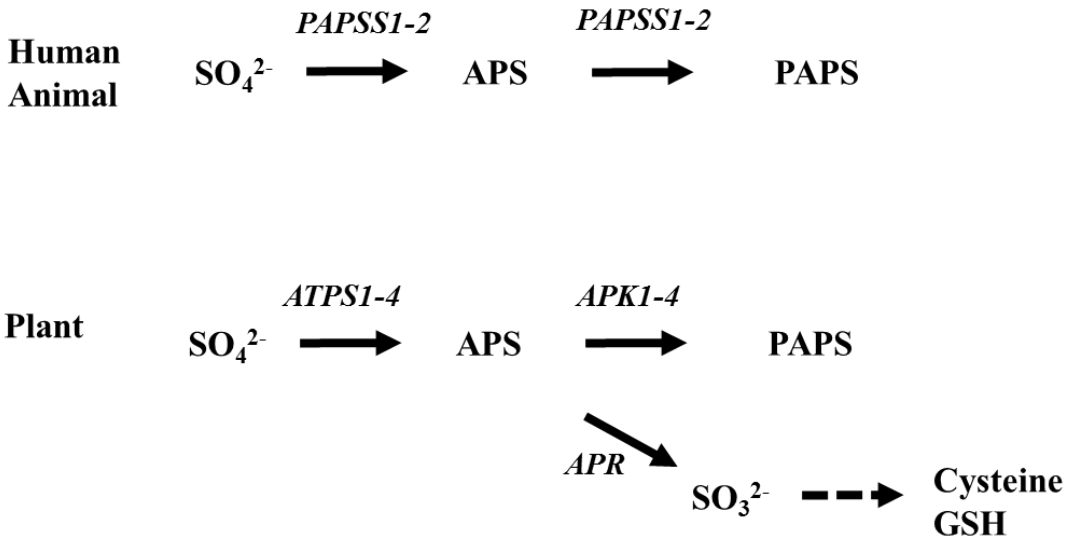


Figure 2. The PAPS synthesis pathway in organisms. PAPSS, PAPS synthase; ATPS, ATP sulfurylase; APK, APS kinase; APR, APS reductase; APS, Adenosine 5'-phosphosulfate; GSH, glutathione.

SULT catalyzes the transfer of a sulfonate group (SO_3^-) from PAPS to a hydroxyl or an amino group of the substrate compounds **(Figure 3)**. The sulfation facilitates the water solubility of acceptor substrates, thereby leading to the inactivation of biologically active compounds. However, in some cases, sulfate conjugation has been shown to result in the activation of procarcinogens such as *N*-hydroxyarylamines and heterocyclic amines [Glatt, H. et al., 1998]. In mammals, the sulfated products are generally excreted from the body via urine and/or bile. In the case of endogenous hormone metabolism, it has been known that dehydroepiandrosterone sulfate (DHEAS) is interconverted to DHEA by broadly expressed steroid sulfatase (STS) [Hobkirk, R., 1993; Purohit, A. et al., 1994]. Only desulfated DHEA is biologically active and can be converted downstream to sex steroids, thereby serving its role as a principal precursor for human sex steroid biosynthesis [Strott, C.A., 2002].

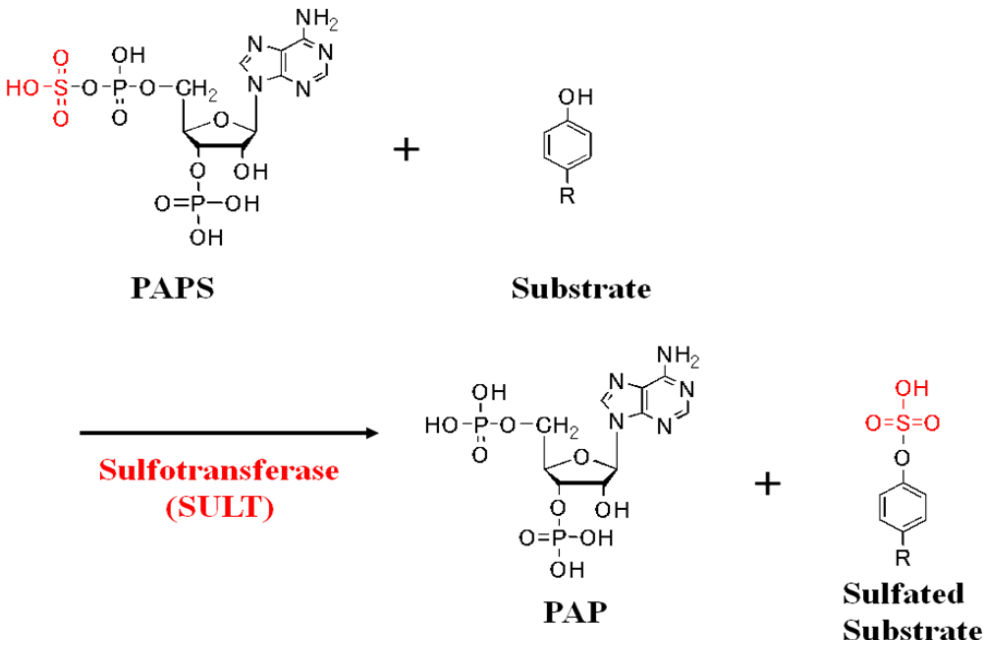


Figure 3. Schematic of sulfation catalyzed by cytosolic sulfotransferases (SULTs). SULTs catalyze the transfer of a sulfonate group (SO_3^-) from 3'-phosphoadenosine 5'phosphosulfate (PAPS), the universal sulfate donor molecule, to a hydroxyl or an amino group of acceptor substrates.

Like the other enzymes such as cytochrome P450 (CYP) enzymes, SULT comprises a supergene family. SULTs can be classified into families whose members share at least 45% amino acid sequence identity, while subfamilies share at least 60% amino acid sequence identity within each SULT gene family [Blanchard, R.L. et al., 2004]. To date, in human, 13 cytosolic sulfotransferase (hSULT) genes have been identified and they comprise four major gene family based on the amino acid sequences (SULT1, SULT2, SULT4, and SULT6), while mouse SULTs (mSULTs) contain more than 20 members and comprise seven gene family (SULT1, SULT2, SULT3, SULT4, SULT5, SULT6, and SULT7) **(Figure 4)**.

In human, the SULT1 family (SULT1) consists of four subfamilies (SULT1A, SULT1B, SULT1C, and SULT1E). SULT1A contains four functional subfamily members (SULT1A1, SULT1A2, SULT1A3, and SULT1A4). SULT1A1 members have been identified in the largest range of species including human, rat, mouse, cow, dog, rabbit, monkey, pig, and platypus [Blanchard, R.L. et al., 2004]. SULT1A1 and SULT1A2 preferentially catalyze the

sulfation of phenolic compounds such as *p*-nitrophenol, α-naphtol, and minoxidil, whereas SULT1A3 preferentially sulfates catecholamines such as dopamine, dopa, and epinephrine [Wilborn, T.W. et al., 1993; Ozawa, S. et al., 1995]. Interestingly, only SULT1A3 shows the high activity toward the D-enantiomers (as compared with the L-enantiomers) of tyrosine and dopa, and a remarkable stimulation (by more than 100-fold) of the activity by addition of millimolar levels of Mn^{2+} [Sakakibara, Y. et al., 1997]. SULT1A3 and SULT1A4 are 98.5% identical in nucleotide sequence, and encode identical SULT1A3 proteins. Therefore, the presence of a copy of SULT1A3 is thought to result from gene duplication [Hildebrandt, M.A. et al., 2004]. SULT1B1 is expressed in liver, colon, small intestine, and blood leucocytes, and appears to be involved in sulfation of dopa/thyrosine or thyroid hormones such as 3,3',5-triiodothyronine (T3), thyroxine (T4), 3,3',5'-triiodothyronine (rT3), 3,3'-diiodothyronine (T2) [Sakakibara, Y. et al., 1995; Wang, J. et al., 1998]. SULT1C1a and SULT1C1b have not been fully characterized yet [Meinl, W. et al., 2008]. SULT1C2 is expressed in the adult stomach, kidney, and thyroid, as well as in fetal kidney and liver, whereas SULT1C4 is expressed in fetal lung and kidney. They catalyze the sulfation of xenobiotics such as *p*-nitrophenol and *N*-hydroxy-2-acetylaminofluorene [Sakakibara, Y. et al., 1998]. SULT1E1 has been widely studied due to its important roles in steroid homeostasis. SULT1E1 possesses a high affinity (nM range) for 17β-estradiol (E2), estrone, and a variety of synthetic estrogens including diethylstilbestrol and 4-hydroxytamoxifen [Falany, C.N. et al., 1995; Falany, C.N., 1997]. Intriguingly, it has been reported that SULT1E1 gene knockout mouse showed placental thrombosis and spontaneous fetal loss [Tong, M.H. et al., 2005].

The SULT2 family has two genes, encoding three enzymes (SULT2A1, SULT2B1a, and SULT2B1b). SULT2A1 (also called DHEA sulfotransferase) catalyzes the sulfation of various steroids such as DHEA, androsterone, testosterone, estradiol, and many other endogenous steroids [Falany, C.N. et al., 1997; Chen, G. et al., 1996]. Steroid sulfation has

been recognized as an important process for maintaining steroid hormone levels during their metabolism in humans. Steroid sulfates are usually described as precursors of active steroids formed by enzymatic cleavage of the sulfate group by sulfatase [Hobkirk, R., 1993; Purohit, A. et al., 1994]. The SULT2B1 isoforms, in contrast to SULT2A1, show a more selective substrate preference. For example, SULT2B1a preferentially sulfates pregnenolone, whereas SULT2B1b mainly catalyzes the sulfation of cholesterol [Fuda, H. et al., 2002].

SULT3 members have been identified only in rabbit and mouse [Yoshinari, K. et al., 1998; Takahashi, S. et al., 2008]. Rabbit SULT3A1 catalyzes the sulfation of amines such as 4-phenyl-l,2,3,6-tetrahydropyridine (PTHP), aniline, and desipramine, but barely O-sulfation of typical aryl and hydroxysteroid sulfotransferase substrates. In mouse, there are two isoforms of SULT3, SULT3A1 and SULT3A2. SULT3A1 and SULT3A2 show high catalytic activity toward 1-naphthylamine and are specifically expressed only in the liver. It is suggested that the SULT3 enzymes are involved in the metabolic detoxification of xenobiotic compounds including aromatic amines in mouse liver.

SULT4A1 is most highly expressed in brain and highly conserved between species, but no endogenous or exogenous substrate has been identified for the enzyme [Falany, C.N. et al., 2000; Sakakibara, Y. et al., 2002]. In a recent pharmacogenetic study, it has been suggested that SULT4A1 polymorphisms may be linked to schizophrenia risk [Brennan, M.D. et al., 2005].

In human, SULT6B1 is expressed in the testis of primates as mRNA, but neither the protein nor its enzymatic activity has been characterized [Freimuth, R.R. et al., 2004], while mouse SULT6B1 has been characterized as the enzyme which catalyzes the sulfation of thyroxine and bithionol, but this functional role has not been fully understood [Takahashi, S. et al., 2009]

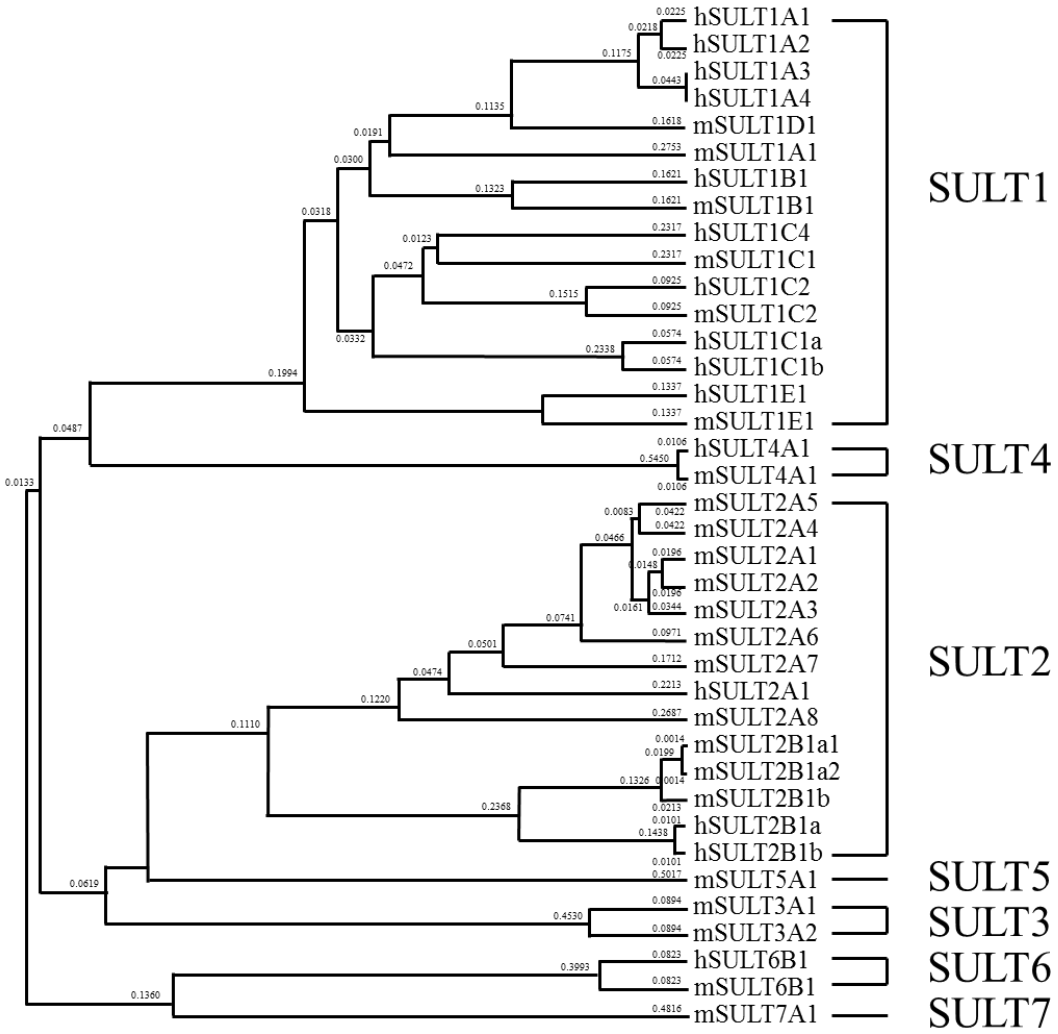


Figure 4. Phylogenetic tree of human and mouse sulfotransferases (hSULTs and mSULTs). Protein sequences were obtained from GenBank database and the respective accession numbers are as follows: hSULT1A1 (NP_001046), hSULT1A2 (NP_001045), hSULT1A3 (NP_808220), hSULT1B1 (NP_055280), hSULT1C1a (NP_001008743), hSULT1C1b (DAA01770), hSULT1C2 (NP_001047), hSULT1C4 (NP_006579), hSULT1E1 (NP_005411), hSULT2A1 (NP_003158), hSULT2B1a (NP_004596), hSULT2B1b (NP_814444), hSULT4A1 (NP_055166), hSULT6B1 (NP_001027549), mSULT1A1 (NP_598431), mSULT1B1 (NP_063931), mSULT1C1 (NP_061221), mSULT1C2 (NP_081211), mSULT1D1 (NP_058051), mSULT1E1 (NP_075624), mSULT2A1 (NP_001104766), mSULT2A2 (L27121), mSULT2A3 (NP_001171909), mSULT2A4 (NP_001095004), mSULT2A5 (NP_001095056), mSULT2A6 (NP_001074794), mSULT2A7 (NP_001171910), mSULT2A8 (AAH31851), mSULT2B1a1 (AF478566), mSULT2B1a2 (unregistered in any database bank), mSULT2B1b (NP_059493), mSULT3A1 (NP_065590), mSULT3A2 (NP_001094922), mSULT4A1 (NP_038901), mSULT5A1 (NP_065589), mSULT6B1 (NP_001157097), mSULT7A1 (NP_001138862). The SULT protein sequences were aligned using the Clustal W program. The phylogenetic tree based on the resultant alignment was constructed using UPGMA algorithm of GENETYX-MAC Version 11.1.0. The respective proteins were grouped according to their amino acid sequence similarities. Divergence distances are shown on individual branches.

Members of the SULT family have been found in most organisms investigated to date, except in archaea. In plants, SULTs comprise the supergene family similar to that found in mammals. In a model plant species *Arabidopsis thaliana*, there are 18 *SULT* genes including 1 apparent pseudogene (AGI code: At3g51210) **(Figure 5)** [Klein, M. et al., 2004]. Unlike the consensus that has been reached for the SULTs that are present in vertebrate animals [Blanchard, R.L., 2004], there is currently no systematic nomenclature that have been proposed for plant SULTs. To address this gap, the author attempted to construct a phylogenetic dendrogram for SULTs found in *Arabidopsis thaliana* (At), *Brassica napus* (Bn), *Flaveria chloraefolia* (Fc), and *Flaveria bidentis* (Fb), and assigned names based on the previously proposed nomenclature system. As shown in **Figure 5**, all plant SULTs were classified into 3 major families, with members of each family share at least 45% amino acid sequence identity. Since the flavonol-specific SULTs from *Flaveria chloraefolia* were the first plant SULT enzymes reported [Varin, L. et al., 1989], the family that contains them, as well as a closely related SULT from *Flaveria bidentis* [Varin, L. et al., 1991], 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) [Lacomme, C. et al., 1996] 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.

To date, only a few SULT isoforms from *Arabidopsis 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 [Lacomme, C. et al., 1996]. Among AtSULTs, only the three-dimensional structure of AtSULT202A1 has been solved previously [Smith, D.W. et al., 2004]. This enzyme has high structural similarity to two human steroid sulfotransferase: estrogen sulfotransferase (SULT1E1) and dehydroepiandrosterone sulfotransferase (SULT2A1). 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 [Marsolais, F. et al., 2007]. Brassinosteroid SULTs were suggested to be involved in the loss of brassinosteroid biological activity in *Brassica napus* [Rouleau, M. et al., 1999]. Interesteingly, AtSULT203A1 (At5g07010) catalyzed the sulfation of 12-hydroxyjasmonate which was initially isolated as a tuber-inducing compound from *Solanum tuberosum* [Gidda, S.K. et al., 2003]. AtSULT201B1 (At1g74090), AtSULT201B2 (At1g18590), and AtSULT201B3 (At1g74100) have been characterized as a desulfoglucosinolate sulfotransferases which catalyze the final step in the biosynthesis of the glucosinolate core structure [Piotrowski, M. et al., 2004]. 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 [Gidda, S.K. et al., 2006]. In *Flaveria* species, four flavonol sulfotransferases which catalyze sequential sulfation of quercetin to quercetin tetrasulfate were previously isolated [Varin, L. et al, 1989; Varin, L. et al., 1991].

In other plants, there are several sulfotransferases deserving to be investigated more detail. Choline sulfate accumulates in the *Limonium* species and in all other species of the salt stress-tolerant *Plumbaginaceae* family [Hanson, A.D. et al., 1994]. It has been hypothesized that choline sulfate act as osmoprotectants in response to salinity or drought stress. A choline SULT catalyzes the sulfation to produce choline-O-sulfate, which might be a part of the salt

tolerance mechanisms in plants [Rivoal, J et al., 1994; Varin, L. et al., 1997a]. In *Mimosa pudica*, a sulfotransferase that catalyzes sulfation of gallic acid glucoside was characterized from plasma membrane preparations. The sulfated product, gallic acid 4-O-(β-D-glucopyranosyl-6'-sulfate), also named periodic leaf movement factor 1 (PLMF-1) could induce the seismonastic response [Varin, L. et al., 1997b].

In chapter I, the author presents a comprehensive cloning and characterization of *Arabidopsis thaliana* sulfotransferase. In chapter II, the author describes the biochemical properties of AtSULT202E1 together with AtSULT202B1, and AtSULT202A1. In chapter III, the author focuses on AtSULT202B7 which is a unique flavonoid glycosides sulfotransferase. Finally, the physiological functions of sulfated flavonoids in plant cells are discussed in chapter IV.

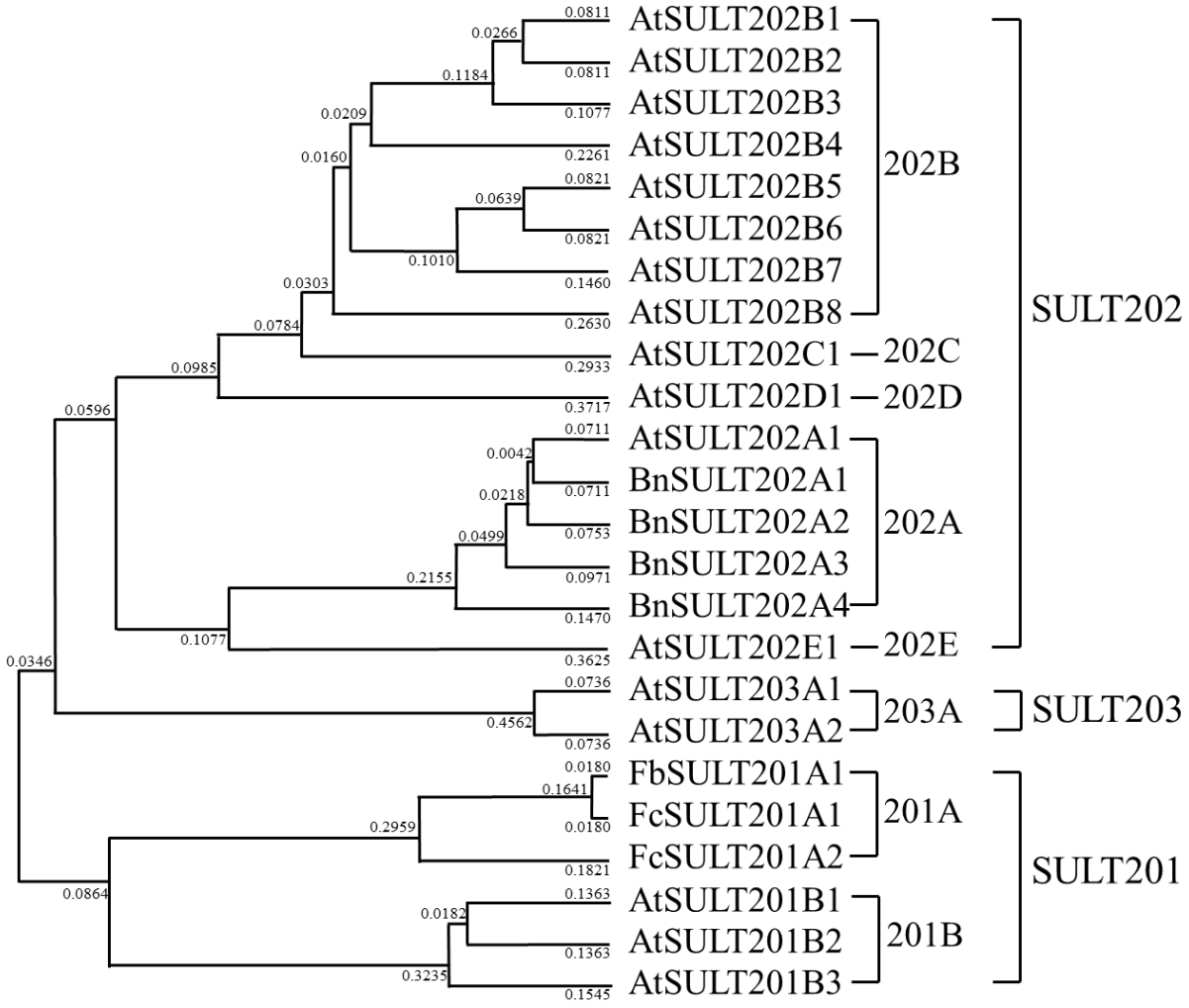


Figure 5. Classification of AtSULT202E1 with other plant sulfotransferases on the basis of deduced amino acid sequences. Phylogenetic dendrogram of plants SULTs. Divergence distances are shown on individual branches. Previously proposed nomenclature system was adopted to all plant SULTs. AtSULT201B1 (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). Construction of phylogenetic dendrogram was done based on methods as described in Figure 4. At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Fc, *Flaveria chloraefolia*; Fb, *Flaveria bidentis*.

Chapter I

Comprehensive cloning and characterization of sulfotransferases in *Arabidopsis thaliana*

Cytosolic sulfotransferases (SULTs), the gene superfamily of enzymes that catalyze sulfation, 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. In *Arabidopsis thaliana*, a popular model plant species, there are 17 SULT genes [Klein, M. et al., 2004]. However, their physiological role is less well characterized. To clarify the biochemical properties of plant SULTs, the author attempted cloning and characterization of all *A. thaliana* SULTs (AtSULTs).

Experimental Procedures

Materials. Kaempferol, butein, naringenin, apigenin, quercetin, galangin, genistein, giberellin A3, trans-zeatin, salicylic acid, 2-cyclohexenone, 4-nitrophenol, α-tocopheol, cholecarciferol, L-tyrosine, menthol, ethanol, sucrose, and glucose were purchased from Wako Pure Chemical Industries. Luteolin, dihydroquercetin, catechin, epicatechin, epigallocatechin (EGC), epigallocatechin gallate (EGCG), *p*-coumaric acid, caffeic acid, arbutin, salicin, 1-naphtylamine, 1,2-naphthoquinone, γ-tocopherol, choline, dehydroepiandrosterone (DHEA), 17β-estradiol (E2), and cholesterol were products of Sigma-Aldrich Co. LLC. Chrysin was obtained from LKT Laboratories Inc. Delphinidin, cyanidin, and peralgonidin were from Extrasynthese. Abscisic acid was purchased from Tokyo Kasei Kogyo Co., Ltd. Brassinolide, castasterone, and 24-epicastasterone were purchased from Brassino Co., Ltd. β-Sitosterol, campesterol, and stigmasterol were products of Tama biochemical Co., Ltd. Phytol was products of Molecular BioSciences, Inc. 1-Naphthol was from Katayama Chemical, 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.

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.

Molecular cloning of AtSULTs. *Arabidopsis thaliana* ecotypes Col-0 were grown with a 16-h photoperiod at 22°C in an atmosphere with 50-60% humidity. Two-week-old *Arabidopsis* seedlings 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 I-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 appropriate restriction enzymes (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.

Table I-1. Oligonucleotide primers used for cloning of AtSULTs.

Isoform	Sense/Antisense	Sequence
For cloning of *Arabidopsis thaliana* SULT cDNA		
AtSULT202A1	Sense	5'-CGCGGATCCATGTCATCATCATCATCAGTT-3'
	Antisense	5'-CCGCTCGAGTCAAGAAGAAAATTTAAGACC-3'
AtSULT202B1	Sense	5'-CGCGGATCCATGGAGATGAACTTGAGAATT-3'
	Antisense	5'-CCGCTCGAGTCAGAATTTCAAACCAGAGTT-3'
AtSULT202B2	Sense	5'-CGCGGATCCATGGATGAGAAAAAGATTACG-3'
	Antisense	5'-CCGCTCGAGTCAGAATTTCAAACCAGAGTT-3'
AtSULT202B3	Sense	5'-CGCGGATTCATGGATGATAAAAAAATGGCG-3'
	Antisense	5'-CGGAATTCTCAGAATTTCAAACCAGAATTT-3'
AtSULT202B4	Sense	5'-CGCGGATCCATGGCTGGAAAAAGTGATCTT-3'
	Antisense	5'-CGGAATTCCTAGAACTTCAAGTCCGAACCT-3'
AtSULT202B5	Sense	5'-CGGAATTCATGGATGAGAAAGATATTCTAA-3'
	Antisense	5'-CGGAATTCCTAGAATTTCAAACCGGAACCT-3'
AtSULT202B6	Sense	5'-CGCGGATCCATGGATGAAAAAGATAGACCA-3'
	Antisense	5'-CGGAATTCTTAGAATTTCAAACCGGAACCT-3'
AtSULT202B7	Sense	5´-CGCGGATCCATGGGTGAGAAAGATATTCCA-3´
	Antisense	5'-CGGAATTCCTACAATTTCAAACCAGAGCCT-3'
AtSULT202B8	Sense	5'-CGCGGATCCATGGATGAGACCAAGATCCCA-3'
	Antisense	5'-CGGAATTCTCAGAATTTCAAATCCGAACCT-3'
AtSULT202C1	Sense	5'-CGCGGATCCATGGAGAAGTGGATGAACTTG-3'
	Antisense	5'-CGGAATTCCTAGAACTTCAAGTCCGAACCT-3'
AtSULT202D1	Sense	5'-CGCGGATCCATGTTCACCTTTTTCACTATC-3'
	Antisense	5'-CGGAATTCTTATTGAAATATCAAACCGGAG-3'
AtSULT202E1	Sense	5'-CGCGGATCCATGACAAAATCCGAAACCACT-3'
	Antisense	5'-CCGCTCGAGTTAGCAAAAGAATCTAAAATC-3'
AtSULT203A1	Sense	5'-CGCGGATCCATGGCTACCTCAAGCATGAAG-3'
	Antisense	5'-CGGAATTCTTAGCTCAACCTGAAAGTGAGA-3'

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

Bacterial expression and purification of recombinant Arabidopsis 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

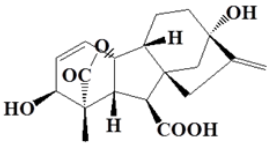
β-D-thiogalactopyranoside (IPTG). After a 12-h induction at 24°C, the cells were collected by centrifugation and homogenized in 15 mL of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) by using a French Press (Ohtake Works Co. Ltd.). The crude homogenate was subjected to centrifugation 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.

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 of various substrate candidates (**Figure I-1, I-2, I-3, and I-4**). The reaction was started by the addition of 0.5 μg 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 [Liu, M.C. et al., 1984], with *n*-butanol/isopropanol/formic acid/water (3:1:1:1; by volume) or *n*-butanol/acetic acid/water (3:1:1; by volume) or ethyl acetate/*n*-butanol (2:1; by volume) as the solvent system. Afterwards, the plate was air-dried and analyzed using a Fluoro Image Analyzer FLA-3000 (Fujifilm).

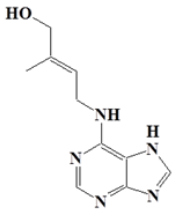
Miscellaneous methods. To prepare the solutions for use in the enzymatic assay, all substrate compounds were dissolved in dimethylsulfoxide (DMSO). ^{35}S-PAPS (45 Ci/mmol) was

synthesized from ATP and ^{35}S-sulfate by using recombinant human bifunctional ATP sulfurylase/adenosine 5'-phosphosulfate kinase, as previously described [Yanagisawa, K. et al., 1998]. SDS-PAGE was performed on 10% polyacrylamidegels using the method of Laemmli [Laemmli, U.K. et al., 1970]. Protein determination was performed based on Lowry's method with bovine serum albumin as the standard [Lowry, O.H. et al., 1951].

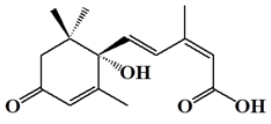
Plant hormones



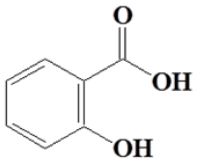
Giberellin A3



trans-Zeatin

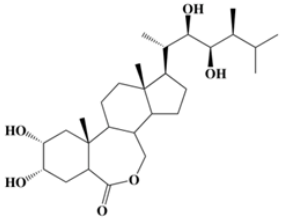


Abscisic acid

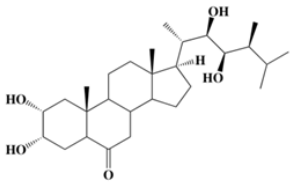


Salicylic acid

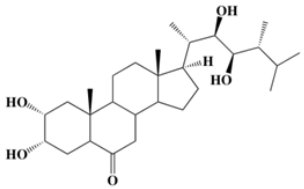
Steroids



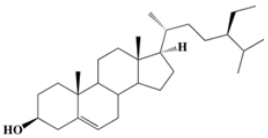
Brassinolide



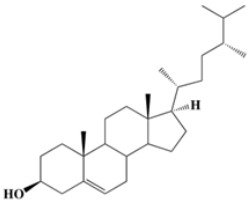
Castasterone



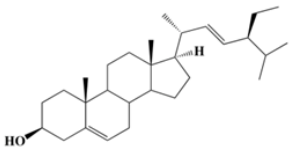
24-Epicastasterone



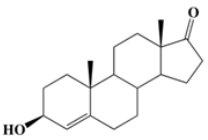
β-Sitosterol



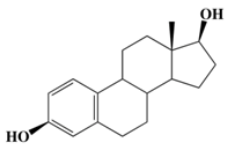
Campesterol



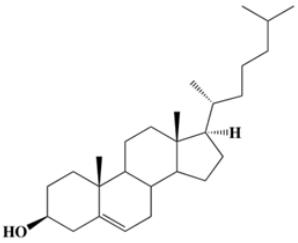
Stigmasterol



DHEA



17β-Estradiol



Cholesterol

Figure I-1. Chemical structures of substrate candidates used in this study.

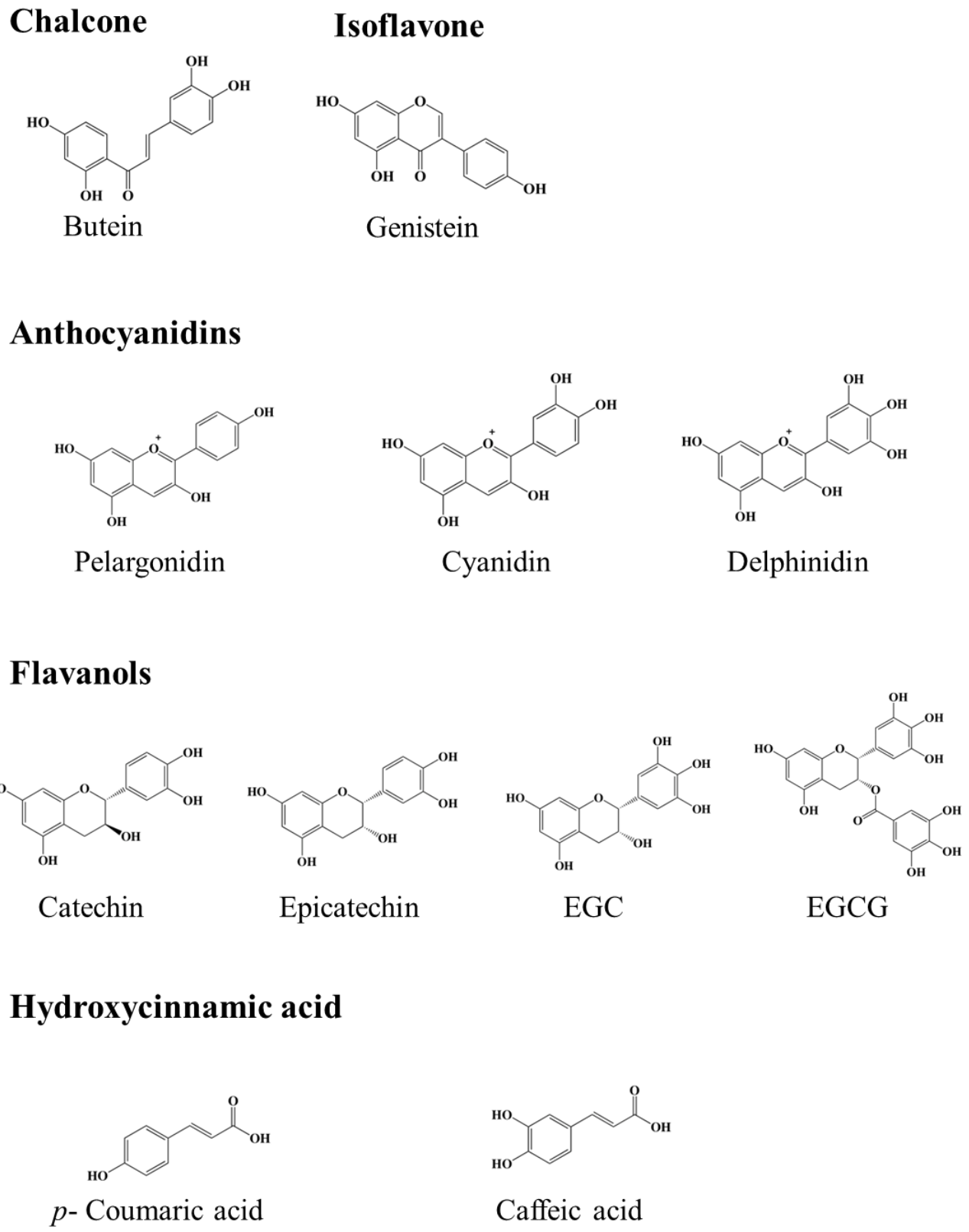
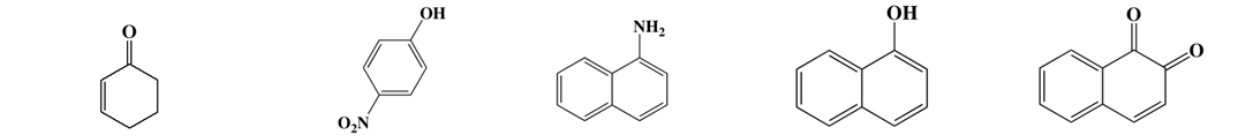


Figure I-2. Chemical structures of substrate candidates used in this study.

Xenobiotics



Miscellaneous compounds

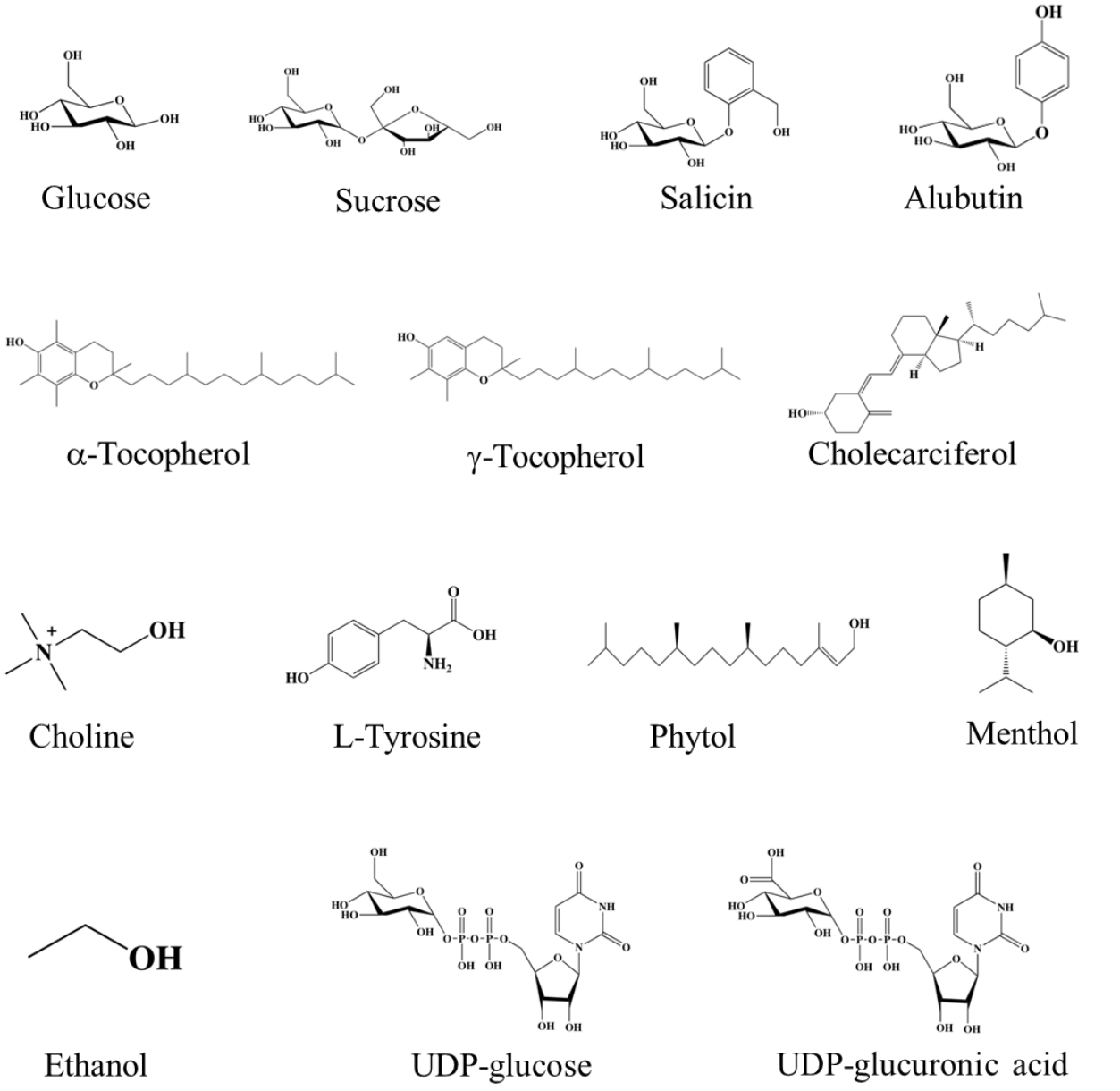
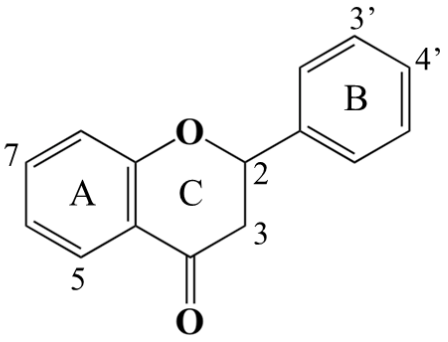


Figure I-3. Chemical structures of substrate candidates used in this study.



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	-

Figure I-4. Chemical structures of flavonoids used in this study.

Results and Discussion

Previous studies have revealed that eighteen *AtSULT* genes including one pseudogene are present in *A. thaliana* genome [Klein, M. et al., 2004]. The biochemical properties and physiological involvements of the coded AtSULT enzymes, however, remained poorly understood. Therefore, I have tried to clone all *AtSULTs* genes and construct the expression system for recombinant enzymes.

Molecular cloning of AtSULTs

Figure I-5 shows the amino acid sequence alignment of 17 AtSULTs 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 all AtSULT sequences [Negishi, M. et al., 2001]. AtSULTs also contain the catalytic His residue 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 AtSULTs [Chiba, H. et al., 1995]. The presence of these functionally important sequence elements suggests that almost AtSULTs are likely to be catalytically active. For comparison studies, the open reading frames (ORFs) of these AtSULTs were PCR-amplified using respective gene-specific primers **(Table I-1)**. The PCR product was cloned into the pBluescript II SK (+) vector and sequenced. The obtained sequences completely matched the sequences registered in GenBank. The coding sequence of AtSULTs was subsequently subcloned into pGEX-4T-1, a prokaryotic expression vector, for the expression of recombinant enzymes in *Escherichia coli*. GST fusion proteins were induced with IPTG, and recombinant AtSULTs was purified to near homogeneity. The

molecular weight of the AtSULTs was calculated by the online program Protparam (http://web.expasy.org/protparam/) and displayed the estimated molecular masses. As a result of molecular cloning, expression, and purification, the author could have successfully obtained nine AtSULTs, AtSULT202A1, AtSULT202B1, AtSULT202B4, AtSULT202B5, AtSULT202B6, AtSULT202B7, AtSULT202B8, AtSULT202E1, and AtSULT203A1 **(Figure I-6)**. Unfortunately, AtSULT202B2, AtSULT202B3, AtSULT202C1, and AtSULT202D1 enzyme could not be induced in this expression system. It might be due to the difference of the codon usage between bacteria and plants.

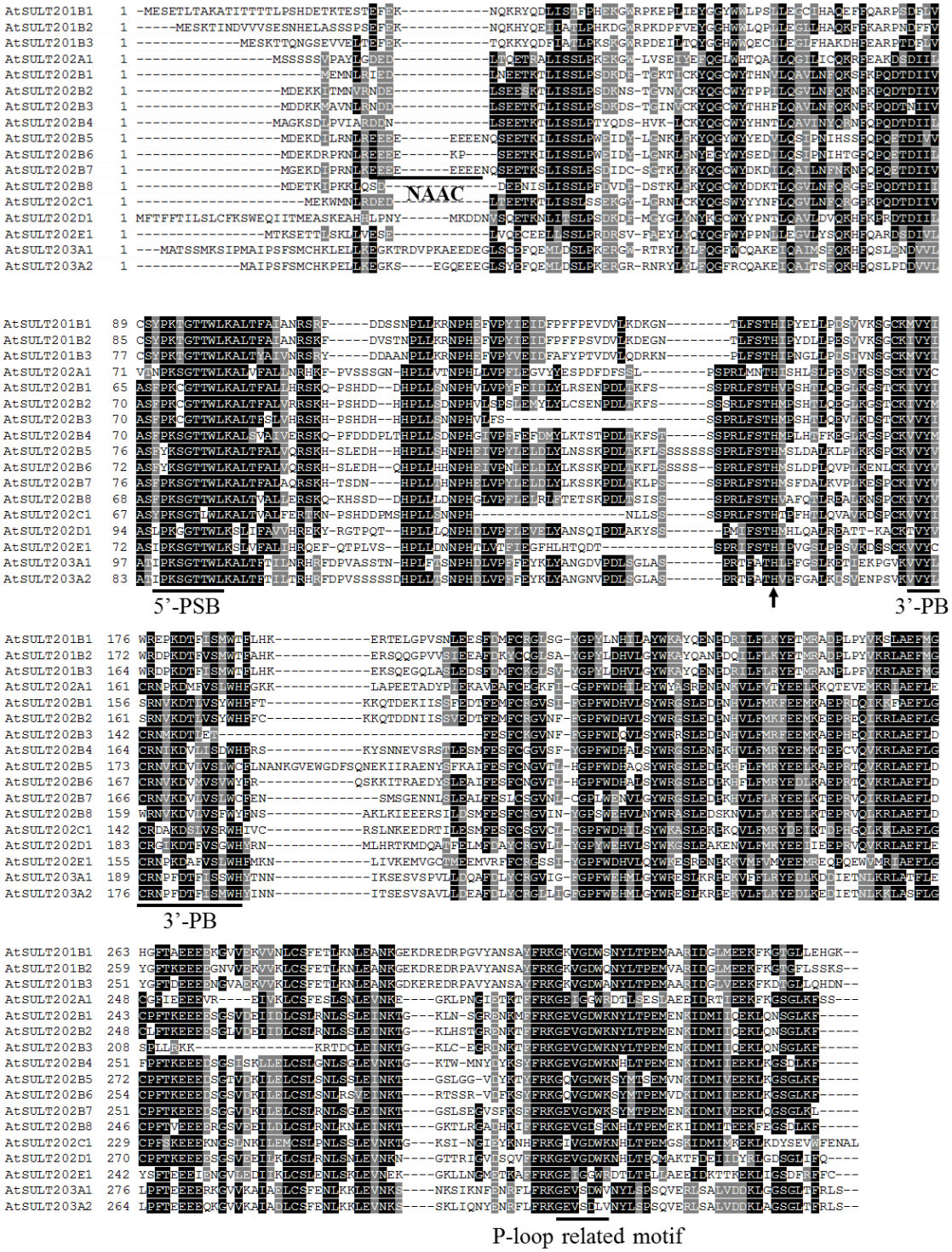


Figure I-5. Amino acid sequence comparison of AtSULTs. Identical residues conserved among at least two of the 17 enzymes are drawn in black, and similar residues are in gray. The NAAC (the N-terminal acidic amino acid cluster), which might act as a potential sorting determinant, is underlined. The 5'-PSB loop including conserved lysine residue in the N terminal, which interacts with the 5'-phosphate of PAP, and the 3'-PB motif for the binding of the 3'-phosphate of PAP are underlined. Conserved P-loop related motif (GXXGXXK) was also underlined. Highly conserved catalytic histidine residue in almost all known SULTs, was indicated by arrow.

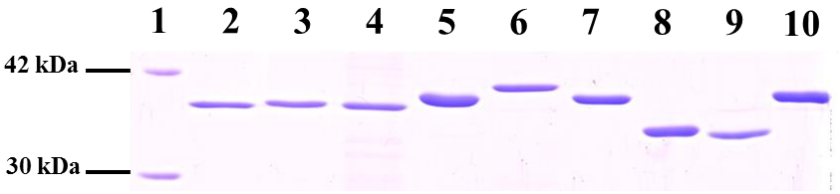


Figure I-6. SDS-PAGE image of purified nine AtSULTs. Lane 1, molecular weight markers; lane 2, AtSULT202B4 (38.2 kDa); lane 3, SULT202B1 (37.5 kDa); lane 4, SULT202B8 (37.8 kDa); lane 5, SULT202B7 (37.7 kDa); lane 6, SULT202B5 (40.7 kDa); lane 7, SULT202B6 (38.9 kDa); lane 8, SULT202A1 (37.1 kDa); lane 9, SULT202E1 (37.7 kDa); lane 10, SULT203A1 (41.4 kDa).

Substrate specificity of nine AtSULTs

Firstly, the author comprehensively investigated the substrate specificity of nine AtSULT enzymes. As a result of sulfation, flavonoids, brassinosteroids, mammalian steroids, some structurally simple phenolic compounds, and aromatic amines were sulfated by eight AtSULT enzymes **(Table I-2)**.

Table I-2. Substrate specificities of nine AtSULTs for various substrates.

Substrate compound	Specific activity (pmol/min/mg)								
	202A1	202B1	202B4	202B5	202B6	202B7	202B8	202E1	203A1
Chalcone-Butein	25.8±0.3	16.5±2.2	55.3±7.8	N.D.	N.D.	N.D.	N.D.	13.5±0.8	9.6±0.5
Flavanone-Naringenin	165.2±5.6	26.7±2.0	N.D.	N.D.	N.D.	N.D.	N.D.	9.3±1.5	N.D.
Flavone- Apigenin	8.6±0.8	66.1±4.8	N.D.	N.D.	N.D.	11.9±1.9	N.D.	8.0±0.5	N.D.
-Luteoline	17.5±1.3	48.1±7.0	36.9±1.3	N.D.	N.D.	18.3±1.3	N.D.	41.7±2.9	N.D.
-Chrysin	10.1±0.9	26.1±2.4	N.D.	N.D.	N.D.	N.D.	N.D.	10.4±0.4	N.D.
Isoflavone-Genistein	52.9±2.1	33.1±1.3	6.2±0.3	N.D.	N.D.	N.D.	N.D.	13.4±0.5	N.D.
Flavonol-Kaempferol	101.0±3.7	121.8±7.9	N.D.	N.D.	N.D.	40.0±1.7	14.0±0.8	35.5±0.3	N.D.
-Quercetin	59.5±1.3	5.1±1.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
-Quercetin-3-glucoside	18.8±0.3	17.9±0.3	N.D.	N.D.	N.D.	128.2±2.1	N.D.	12.5±0.1	N.D.
-Galangin	122.6±3.5	162.9±11.1	N.D.	N.D.	N.D.	N.D.	N.D.	95.7±3.0	N.D.
Flavanonol-Dihydroquercetin	8.6±0.1	5.3±0.1	N.D.	N.D.	N.D.	N.D.	N.D.	5.4±0.3	N.D.
Anthocyanidin-Delphinidin	22.4±1.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
-Cyanidin	52.1±3.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
-Peralgonidin	90.2±4.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Brassinosteroid-Brassinolide	N.D.	N.D.	N.D.	N.D.	89.1±9.2	N.D.	N.D.	73.5±4.6	N.D.
-Castasterone	N.D.	N.D.	N.D.	N.D.	54.8±2.0	N.D.	N.D.	97.3±4.1	N.D.
-24-Epicastasterone	6.3±0.2	N.D.	N.D.	N.D.	19.0±0.8	N.D.	N.D.	24.2±2.1	N.D.
Mammal steroids-DHEA	32.4±1.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	13.9±0.7	6.8±0.1
-17β-Estradiol	8.3±0.7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	34.7±1.7	N.D.
Xenobiotics- 4-Nitrophenol	60.6±2.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
-1-Naphtylamine	44.7±3.7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
-1-Naphtol	32.1±2.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
-Menthol	78.8±9.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	19.1±0.5	N.D.

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).

Interestingly, flavonoids were one of the best compounds that are widely recognized as substrates by AtSULTs. Not only AtSULT202B1, which is known as flavonol 7-sulfotransferase [Gidda, S.K. et al., 2006], but also AtSULT202A1, AtSULT202B4, AtSULT202B7, AtSULT202B8, AtSULT202E1, and AtSULT203A1 showed the sulfating activity toward flavonoids. Among of these, AtSULT202A1, AtSULT202B1, and AtSULT202E1 exhibited broad substrate specificities for flavonoids. These results suggest that several AtSULTs regulates the bioactivity of flavonoids via sulfation. Brassinosteroids, which are additionally recognized as plant hormone for many aspects affecting plant growth and development [Santner, A. et al., 2009], were sulfated by three SULTs, AtSULT202A1, AtSULT202B6, and AtSULT202E1. To date, only two SULTs, AtSULT202A1, AtSULT202B6, have been reported to be catalytically active for brassinosteroids [Marsolais, F. et al., 2007], while the biochemical properties of AtSULT202E1 remains unknown. However, there are two critical issues for characterization of brassinosteroid sulfotransferases. Firstly, the author could not obtain reference standard of various brassinosteroids commercially. Secondary, these steroids would cost too much. Being more easily available, the characterization of brassinosteroid sulfotransferases would proceed early. Intriguingly, AtSULT202A1 showed the enzymatic activity not only for flavonoids and brassinosteroids, but also for xenobiotics such as 4-nitrophenol, suggesting AtSULT202A1 have a broad substrate specificity ranging from simple phenol to steroids. AtSULT202A1 might be multifunctional sulfotransferases. Furthermore, the author investigated whether vitamin E such as α-tocopherol and γ-tocopherol could be the substrates for AtSULTs. Our previous studies revealed that tocopherols and the related metabolites were metabolized by hSULT1 family of enzymes and excreted into the media in A549 cells [Hashiguchi T. et al., 2011]. However, all nine AtSULTs could not show any sulfating activity toward tocopherols and their metabolites.

Chapter II

Characterization of novel kaempferol sulfotransferases in *Arabidopsis thaliana*

Flavonoids are a group of secondary metabolites distributed in a wide range of plant species. They share a common phenyl benzopyrone structure and are divided into major subclasses (e.g., flavanone, flavonol, flavone, isoflavone, and anthocyanidin) based on the numbers and positions of the hydroxyl group and the C-ring structure. Flavonoids have been implicated in a variety of physiological functions, e.g., provision of colors attractive to pollinators [Mol, J. et al., 1998]; protection of the plant body from external stress such as fungal infection and UV irradiation [Treutter, D. et al., 2005]; communication with the symbiont Rhizobia [Hassan, S. et al., 2012]; and influence in the transport of the plant hormone, auxin [Peer, W.A. et al., 2007]. To date, nearly 9,000 structural variants of flavonoids have been reported [Williams, C.A. et al., 2004]. 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 [Barron, D. et al., 1988]. However, the physiological significance of flavonoid sulfates, and the responsible sulfotransferases have not been fully elucidated.

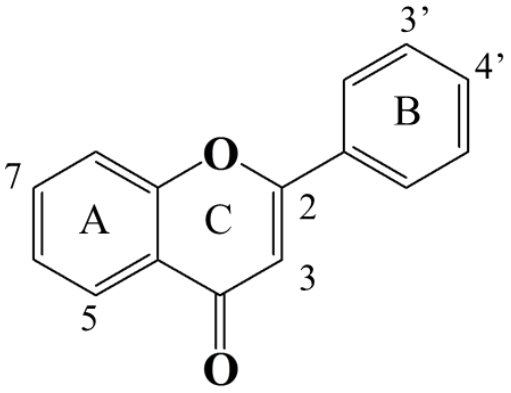
In chapter I, the author conducted briefly comprehensive characterization of nine AtSULTs. Among of these, three sulfotransferases, AtSULT202B1 (the name previously called flavonol 7-sulfotransferase), AtSULT202A1 (Brassinosteroid sulfotransferase), and AtSULT202E1 (a novel flavonoid sulfotransferase), displayed the sulfation activity toward a variety of flavonoids. In chapter II, in more detail, the author investigated the biochemical

characterization of a novel sulfotransferase AtSULT202E1, together with AtSULT202B1, and AtSULT202A1.

Experimental Procedures

Materials. 5-Hydroxyflavone, 3-hydroxyflavone were products of Sigma-Aldrich Co. LLC. 7-Hydroxyflavone was purchased from Tokyo Kasei Kogyo Co., Ltd. 3´-Hydroxyflavone, and 4´-hydroxyflavone were obtained from Indofine Chemical. The structures of hydroxyflavones were represented in **Figure II-1**. All other materials were the same as described in chapter I.

Enzymatic assay. The method for measurement of sulfating activity was basically the same as described in chapter I. To examine the pH dependence, Good's buffers with a wide range of pH values, MES at pH 5.5-6.5, PIPES at pH 6.5-7.5, HEPES at pH 7.5-8.0, TAPS at pH 8.0-9.0, CHES at pH 9.0-10.0, 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 μM 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.



Compound	C3	C5	C7	C3'	C4'
3-Hydroxyflavone	OH	H	H	H	H
5-Hydroxyflavone	H	OH	H	H	H
7-Hydroxyflavone	H	H	OH	H	H
3’-Hydroxyflavone	H	H	H	OH	H
4’-Hydroxyflavone	H	H	H	H	OH

Figure II-1. Chemical structures of hydroxyflavone used in this study.

Results and Discussion

Substrate specificity of AtSULT202E1, AtSULT202B1, and AtSULT202A1

The gene encoding AtSULT202E1 is located on chromosome 2 in tandem with the gene encoding AtSULT202A1. At the amino acid sequence level, AtSULT202E1 displays the highest homology with AtSULT202A1, showing 53.5% sequence identity. AtSULT202B1, on the other hand, shares 46.7% of amino acid sequence identity with AtSULT202E1. 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 I-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 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 major flavonoids found in *Arabidopsis thaliana* and has a distinct effect on root developmental process [Lewis, D.R., 2011]. In contrast with those for kaempferol, 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 (see the structure in **Figure I-2**). Since AtSULT202E1 and AtSULT202B1 did not show the activity toward anthocyanidin, anthocyanidin sulfation is specifically catalyzed by AtSULT202A1. 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 *Arabidopsis thaliana* cannot produce isoflavonoid because of lacks of chalcone reductase and isoflavone synthase enzymes [Aoki, T. et al., 2000], 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.

Kaempferol sulfation by AtSULT202E1 and enzyme properties

To further characterize the biochemical properties, the author performed the enzymatic sulfation by AtSULT202E1 with kaempferol as substrate. 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 **(Table I-2)**. It seems that the low sequence identity between these two isoforms does not necessarily imply different substrate specificity. Next, the author examined the temperature dependence of the activity of AtSULT202E1 using kaempferol as substrate. As shown in **Figure II-2A**, 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 **(Figure II-2B)**. A pH optimum of flavonol sulfotransferases range from pH6.0 to 8.5 in previous studies [Varin, L. et al., 1997], suggesting that flavonol sulfotransferases may exert the highest activity at the cytosol compartment. A thermal stability experiment was carried out using kaempferol as substrate. As shown in **Figure II-3C**, AtSULT202E1 was stable over the temperature range of 0°C to 30°C. Our previous studies had shown that divalent metal cations can exert dramatic

inhibitory/stimulatory effects on various human SULTs [Pai, T.G. et al., 2002]. Among 8 different divalent cations tested at 5 mM, Fe^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} rendered AtSULT202E1 virtually inactive **(Figure II-4D)**. However, stimulatory effects of divalent cations on the sulfating activity for kaempferol were not detected in AtSULT202E1. It is therefore possible that *Arabidopsis thaliana* sulfotransferases may be susceptible to heavy metal pollution.

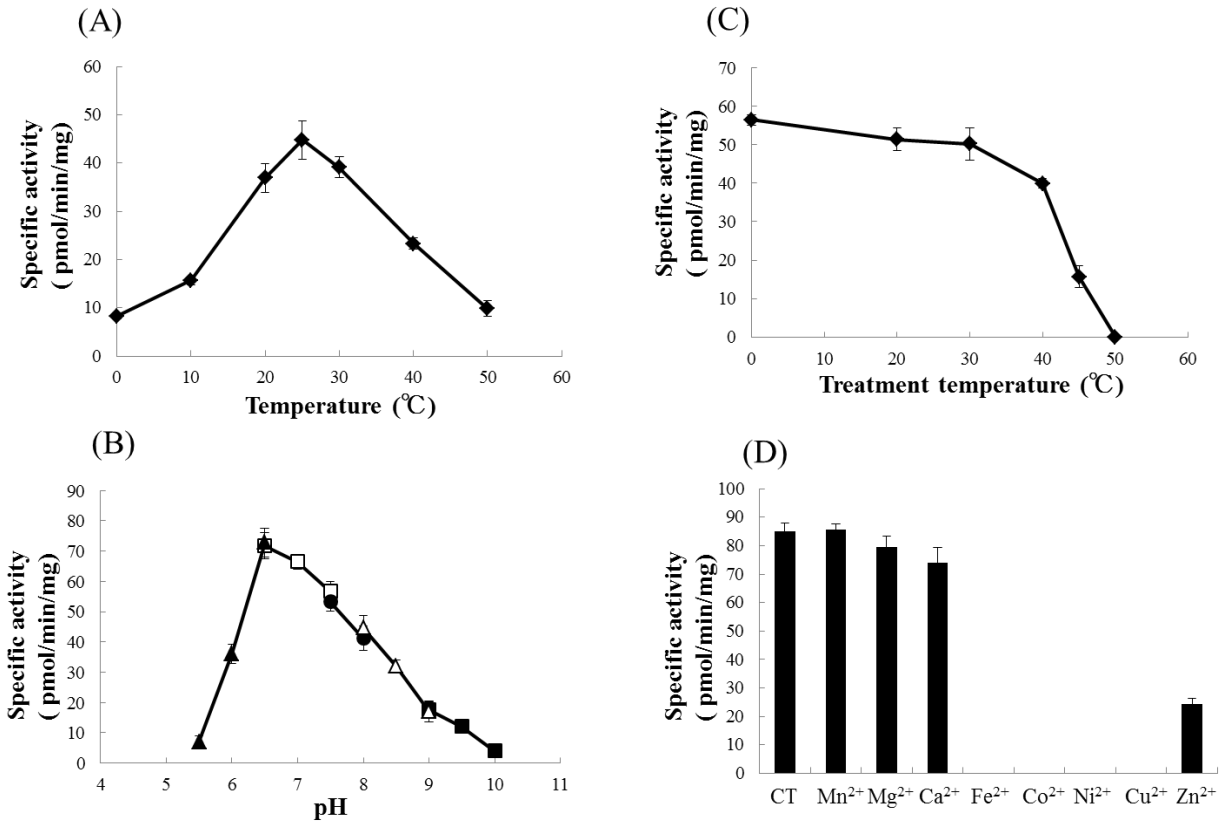


Figure II-2. Characterization of AtSULT202E1. Specific activity refers to pmol of sulfated product formed /min/mg enzyme. The data shown represent means ±SD for three determinations. (A) Temperature dependence of the sulfating activity of AtSULT202E1 with kaempferol. (B) pH dependency of the sulfating activity of AtSULT202E1 with kaempferol. (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.

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, the author investigated whether five mono-hydroxyflavones, 3-, 5-, 7-, 3'-, and 4'-hydroxyflavone could be the substrates for three AtSULTs **(Table II-1)**. Three AtSULTs exhibited no sulfating activity toward 5-, 3'-, and 4'-hydroxyflavone. Interestingly, 3-hydroxyflavone was sulfated only by AtSULT202A1 with an activity of 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).

Table II-1. Substrate specificities of three recombinant AtSULTs for hydroxyflavones.

Substrate	Specific activity (pmol/min/mg)		
	SULT202E1	SULT202A1	SULT202B1
3-Hydroxyflavone	N.D.	26.2 ± 1.8	N.D.
5-Hydroxyflavone	N.D.	N.D.	N.D.
7-Hydroxyflavone	43.0 ± 2.3	14.8 ± 0.6	38.5 ± 3.0
3'-Hydroxyflavone	N.D.	N.D.	N.D.
4'-Hydroxyflavone	N.D.	N.D.	N.D.

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).

Next, kinetic parameters *V*max, *K*m, and *V*max/*K*m for the sulfation of kaempferol, hydroxyflavones, and PAPS by three AtSULTs were determined based on Lineweaver-Burk plots **(Table II-2)**. Surprisingly, AtSULT202A1 showed the lowest *K*m value of 4.9 μM for kaempferol among three SULTs. It was found that AtSULT202E1 and AtSULT202B1 showed 4-7 fold higher *K*m values for kaempferol than did AtSULT202A1. The *K*m value of AtSULT202A1 for 3-hydroxyflavone was 5.7 μM while the *K*m values of AtSULT202E1 and

AtSULT202B1 for 7-hydroxyflavone were 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.

Table II-2. Kinetic data of three recombinant AtSULTs for the preferred flavonoids

		Km (μM)	Vmax (pmol/min/mg)	Vmax/Km
AtSULT202E1				
	Kaempferol	21.0	46.5	2.2
	7-Hydroxyflavone	508.4	212.8	0.4
	PAPS	0.1	107.5	1,075
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	2,222

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

To summarize, the author isolated and characterized a novel *Arabidopsis thaliana* sulfotransferase, AtSULT202E1. In this study, the author 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 was therefore demonstrated that AtSULT202E1 also has the sulfating activity for brassinosteroids **(Table I-2)**. Unexpectedly, the author 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 *Arabidopsis 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 [Habbu, P.V. et al., 2009]. Additional work is warranted in order to clarify the biological activity of sulfated flavonoids for plants.

Chapter III

Identification of a novel flavonoid glycoside sulfotransferase in *Arabidopsis thaliana*

To date, nearly 9,000 structural variants of flavonoids have been reported [Williams, C.A. et al., 2004]. In different plants, flavonoids occur as glycosides (e.g., glucoside, galactoside, rhamnoside, and arabinoside), which, except for flavanols such as catechins and proanthocyanidins, are generated upon glycosylation by uridine-diphosphate glycosyltransferases (UGTs) [Calderon-Montano, J.M., 2011]. Glycosylation increases the solubility and the stability of flavonoids, and reduces their reactivity [Jones, P. et al., 2001]. In addition to glycosylation, flavonoids are known to undergo sulfate conjugation in at least 32 families of plants [Barron, D. et al., 1988]. In chapter III, the author reports the identification and characterization of a novel *A. thaliana* sulfotransferase, designated AtSULT202B7. The enzymatic activity of recombinant AtSULT202B7 toward a major flavonol and its glycosides was tested. A systematic analysis of the optimum pH and kinetics parameters toward flavonols and their glycosides was performed. To the best of our knowledge, this is the first report on a sulfotransferase highly capable of catalyzing the sulfation of flavonoid glycosides in *A. thaliana*.

*A part of this chapter is a pre-copyedited, author-produced PDF of an article accepted for publication in [Identification of a novel flavonoid glycoside sulfotransferase in *Arabidopsis thaliana*] following peer review. The citation information is described in References. The definitive publisher-authenticated version is available online at: http://jb.oxfordjournals.org/content/early/2013/12/05/jb.mvt102.abstract

Experimental Procedures

Materials. Quercetin-3-glucoside, uridine 5'-diphosphoglucose (UDP-glucose), uridine 5'-diphosphoglucuronic acid (UDP-glucuronic acid) were products of Sigma-Aldrich Co. LLC. Quercetin-3-galactoside, quercetin-3-rutinoside, kaempferol-3-glucoside, kaempferol-7-glucoside, and kaempferol-3-robinoside-7-rhamnoside were from Extrasynthese. Quercetin-7-glucoside was a product of Apin Chemical Ltd. Quercetin-3-rhamnoside, were obtained from Indofine Chemical. The chemical structures of flavonoid glycosides were shown in **Figure III-1**. All other materials were the same as described in chapter I.

Enzymatic assay. The method for measurement of sulfating activity was basically the same as described in chapter I. To examine the pH-dependence, different buffers (50 mM sodium acetate buffer at pH 4.0–6.0 and 50 mM sodium phosphate buffer at 6.5–8.0) were used in the reaction mixtures. For the kinetic studies on the sulfation of flavonoids, substrates with different concentrations ranging from 0.01 μM to 40 μM were used. 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 range of the PAPS and flavonoids concentration used.

Identification of sulfated kaempferol-3-glucoside by reverse phase HPLC followed by direct infusion mass spectrometry. The sulfation was performed in a reaction mixture (with a final volume of 250 μL) containing 50 mM sodium acetate buffer, a pH of 5.5, 100 μM PAPS, 20 μM kaempferol or kaempferol-3-glucoside, and 25 μg of purified AtSULT202B7. The reaction was started by the addition of the enzyme, allowed to proceed for 12 h at 22°C, and

terminated by heating at 98°C for 5 min. The precipitates formed were removed by centrifugation, and the supernatant was subjected to the analysis using a Shimadzu Prominence HPLC system consisting of a photodiode array (PDA) detector, and the LC Solution software was used for all HPLC analysis. 5 μm Capcell PAK C18 MG column (250 × 4.6 mm; SHISEIDO), and a gradient elution of acetonitrile and ultrapure water were used for the HPLC separations. For all analyses, the injection volume was 40 μL; the flow rate was 1 mL/min; and the controlled oven temperature was 40°C. Fraction containing sulfated kaempferol-3-glucoside was lyophilized and dissolved in dimethylsulfoxide. The deglucosylation was conducted in a reaction mixture (with a final volume of 250 μL) containing 100 mM sodium acetate buffer, pH 5.0, 8 units/mL of β-glucosidase from sweet almond, and 10 μL of the redissolved sulfated kaempferol-3-glucoside. The reaction was started by the addition of the enzyme, allowed to proceed for 24-h at 37°C, and terminated by heating at 98°C for 5 min. The precipitates formed were removed by centrifugation, and the supernatant was subjected to the above described HPLC analysis. The sulfated kaempferol-3-glucoside dissolved in methanol was analyzed by Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) with a heated electrospray ionization (HESI) source through direct infusion using a syringe pump. The data was acquired using Targeted-MS2 scan event. Typical mass spectrometric conditions were: polarity, negative ionization mode; spray voltage, 3.5 kV; sheath gas flow rate, 6; auxillary gas, 0; sweep gas, 0; heated capillary temperature, 320°C. The resolution was set at 140,000. The AGC target was 2E5. The maximum ion injection time was 100 ms. The normalized collision energy (NCE) was 20%. The raw data files were analyzed using Qual Browser software in Xcalibur (Thermo Fisher Scientific).

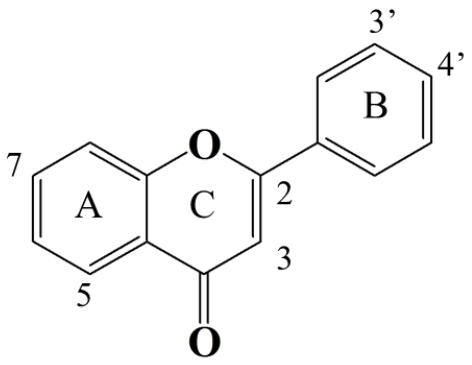
RNA extraction and RT-PCR analysis. For use as templates in RT-PCR, total RNA from diverse tissues of 3-, 7-, or 14-day old *Arabidopsis thaliana* ecotype (Col-0) was isolated using Plant RNA Purification Reagent (Invitrogen) according to manufacturer's instructions. First-strand cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO). PCR was carried out using the synthesized cDNAs as a template coupled with gene-specific primers (**Table III-1**) and Taq DNA polymerase. Reaction conditions were 3 min at 95°C for initial denaturation, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C. The products were then separated by electrophoresis on 2.0% agarose gels, stained with ethidium bromide, and visualized by UV illumination.

Table III-1. Oligonucleotide primers used for RT-PCR analysis.

Isoform	Sense/Antisense	Sequence
For RT-PCR analysis		
AtSULT202A1	Sense	5'-CCGACATTATCCTCGTCACTAATCC-3'
	Antisense	5'-GGACTCGTAGTAAACTCCTTCCAAG-3'
AtSULT202B1	Sense	5'-CGTAGCGAAAATCCTGACCTTACC-3'
	Antisense	5'-GCTGACTCCCCTACAAAACATCTC-3'
AtSULT202B7	Sense	5'-GAGAGGAAGCTTGGAAGATCCTAAG-3'
	Antisense	5'-CCAAGATCTTGTCTACACCTCCAC-3'
AtSULT202E1	Sense	5'-CCAGAGATTCAGACATCGTCCTTG-3'
	Antisense	5'-GACCCTACAGGAATGTGTGTAGAG-3'
Actin-2	Sense	5'-GAAAAGATCTGGCATCACACTTTCTA-3'
	Antisense	5'-ACATACATAGCGGGAGAGTTAAAGGT-3'

The measurement of sulfating activity of A. thaliana extracts. *Arabidopsis thaliana* seeds were sterilized and plated on 1/2 Murashige and Skoog (MS) medium (1/2 MS salts, pH 6.0, 1% sucrose, and 0.5% agarose) with a continuous light at 22°C in an atmosphere with 50-60% humidity. *Arabidopsis* plants obtained were frozen in liquid nitrogen and were ground using a mortar and pestle. Two hundred mg of the powder was lysed in 0.5 mL of

ice-cold lysis buffer, PBS with 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail (Sigma), and homogenized using a polytron type homogenizer. The homogenates thus prepared were subjected to centrifugation at 20,400 x g for 15 min to remove insoluble pellet. The protein concentrations of cell-free extracts were measured by Bradford assay [Bradford, M.M., 1976] with bovine serum albumin as the standard, and were used as crude enzyme in the following assay. Sulfating activity of protein extracts was assayed as described above. Briefly, 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 15 μg of crude enzyme, allowed to proceed for 30 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 [Liu, M.C. et al., 1984], with *n*-butanol/pyridine/formic acid/water (5:4:1:3; by volume) as the solvent system. Afterwards, the plate was air-dried and analyzed using a Fluoro Image Analyzer FLA-3000 (Fujifilm).



Compound	C3	C5	C7	C3'	C4'
Kaempferol-3-glucoside	Glucose	OH	OH	H	OH
Kaempferol-7-glucoside	OH	OH	Glucose	H	OH
Kaempferol-3-robinoside-7-rhamnoside	Robinose	OH	Rhamnose	H	OH
Quercetin-3-glucoside	Glucose	OH	OH	OH	OH
Quercetin-7-glucoside	OH	OH	Glucose	OH	OH
Quercetin-3-galactoside	Galactose	OH	OH	OH	OH
Quercetin-3-rutinoside	Rutinose	OH	OH	OH	OH
Quercetin-3-rhamnoside	Rhamnose	OH	OH	OH	OH

Figure III-1. Chemical structures of flavonoids used in this study.

Results and Discussion

Substrate specificity of AtSULT202B7 for flavonols and flavonol glucosides

To understand the substrate specificity in more detail, the author performed enzymatic sulfation of a variety of flavonoids by AtSULT202B7. The deduced amino acid sequence of AtSULT202B7 was aligned with AtSULT202B1, a flavonol 7-sulfotransferase [Gidda, S.K. et al., 2006], and AtSULT202B5, an uncharacterized sulfotransferase, using the Clustal W program (**Figure I-5**). AtSULT202B7 was shown to display 66.6% and 73.0% amino acid sequence identities to AtSULT202B1 and AtSULT202B5, respectively. In an initial experiment, a panel of flavonols and flavonol glucosides was tested as substrates for AtSULT202B7 at pH 7.5. As shown in **Table I-2**, AtSULT202B7 showed strong activities (128.2 pmol/min/mg enzyme) toward quercetin-3-glucoside. In contrast, the activity toward flavonols, such as kaempferol and quercetin, were minimal or none. These results suggested that AtSULT202B7 might be a flavonol glycoside-specific sulfotransferase. It is interesting to note that despite the high amino acid sequence similarity between AtSULT202B5 and AtSULT202B7, AtSULT202B5 showed no activity toward a variety of flavonoids, including its glycosides. Other phenolic and steroidal compounds, such as phytohormones and brassinosteroids, were also tested as substrates for AtSULT202B7. No activity, however, was detected.

Determination of pH optimum

The pH dependence of the activity of AtSULT202B7 was determined using four substrates, kaempferol, quercetin, kaempferol-3-glucoside, and quercetin-3-glucoside. AtSULT202B7 displayed the highest sulfating activity at pH 5.5 for all four substrates, and the activity started decreasing at higher pHs (**Figure III-2**). Previous studies have shown that

flavonol sulfotransferases derived from *Flaveria* species displayed optimum pH ranging 6.0–8.5 [Varin, L. et al., 1997]. Considering that the vacuolar pH of plant cells is about 5.0–5.5 [Taiz, L. et al., 1992] and that the flavonol glycosides accumulates mainly in the vacuole [Landry, L.G. et al., 1995], it is possible that AtSULT202B7 might somehow be transported into this acidic organelle and sulfate the flavonol glycosides locally as substrates. No reported vacuole sorting determinant, however, could be discerned in the amino acid sequence of AtSULT202B7. Using the computer program PSORT (http://psort.hgc.jp/), AtSULT202B7 has been reported as a cytoplasmic protein [Klein M. et al., 2004], while its exact subcellular location still remains unknown. Further examination of the amino acid sequence of AtSULT202B7 revealed an acidic amino acid cluster located in its N-terminal region of AtSULT202B7 (**Figure I-5**). It may be worthwhile mentioning that previous studies have reported that acidic amino acid cluster might serve as a basolateral sorting signal in MDCK cell [Simmen, T. et al., 1999]. The N-terminal acidic amino acid cluster in AtSULT202B7, therefore, could be proposed to function as a novel sorting determinant for a certain organelle in the cells of *Arabidopsis thaliana*. To this end, immunocytochemical analysis using specific antibody against AtSULT202B7 will be required in order to unequivocally identify the subcellular location of AtSULT202B7.

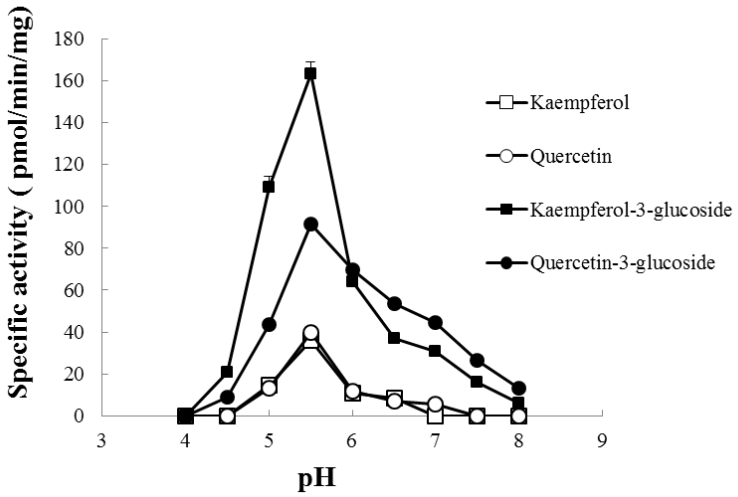


Figure III-2. pH dependency of the sulfating activity of AtSULT202B7 with flavonols and flavonol glucosides as substrates. The enzymatic assays with 10 μM of substrate compounds were carried out under standard assay conditions using different buffer systems. This figure was modified from previously described figure [Hashiguchi, T. et al., 2013].

Substrate specificity of AtSULT202B7 for a variety of flavonoids and related compounds

Under optimum pH conditions, the enzymatic activities of AtSULT202B7 toward a variety of flavonoids and related compounds were examined. As shown in **Table III-2**, AtSULT202B7 displayed considerably higher activity toward a wide range of flavonol glycosides (e.g., quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-rutinoside, quercetin-3-rhamnoside, and kaempferol-3-glucoside) than their aglycone counterparts. These results suggested that AtSULT202B7 has no strict sugar selectivity in the sulfation of flavonoid glycosides. Interestingly, no activity was detected with flavonols having a sugar at position 7 (e.g., quercetin-7-glucoside, kaempferol-7-glucoside, and kaempferol-3-robinoside-7-rhamnoside), suggesting that AtSULT202B7 may sulfate the 7-hydroxyl group of flavonols in a position-specific manner. This possibility was confirmed in a subsequent experiment using five kinds of hydroxyflavones that contain only one hydroxyl group at 3, 5, 7, 3′, and 4′ as substrates. Of the five, only 7-hydroxyflavone was

sulfated. These results supported the notion that AtSULT202B7 was active toward only flavonoids with a hydroxyl group at position 7. Moreover, no sulfation products of glucose, UDP-glucose and phenolic plant glucosides, such as arbutin and salicin, were detected. Collectively, these results suggested that sulfation by AtSULT202B7 required the presence of a flavone backbone in substrate compounds.

Table III-2. Specific activities of AtSULT202B7 with various flavonoids as substrates.

Substrate compound	Specific activity (pmol/min/mg)
Kaempferol	63.3 ± 3.4
Kaempferol-3-glucoside	343.8 ± 8.5
Kaempferol-7-glucoside	N.D.
Kaempferol-3-robinoside-7-rhamnoside	N.D.
Quercetin	47.8 ± 3.2
Quercetin-3-glucoside	311.7 ± 4.5
Quercetin-7-glucoside	N.D.
Quercetin-3-galactoside	235.0 ± 3.6
Quercetin-3-rutinoside	183.1 ± 4.5
Quercetin-3-rhamnoside	153.8 ± 3.6
3-Hydroxyflavone	N.D.
5-Hydroxyflavone	N.D.
3'-Hydroxyflavone	N.D.
4'-Hydroxyflavone	N.D.
7-Hydroxyflavone	16.9 ± 1.5
Salicin	N.D.
Arbutin	N.D.
Glucose	N.D.
UDP-glucose	N.D.

Specific activity refers to pmol of sulfated product formed/min/mg enzyme. Data shown represent means ±S.D. from three determinations. N.D. refers to activity not detected (< 5.0 pmol/min/mg). This table was cited from previously described study [Hashiguchi, T. et al., 2013].

Determination of kinetic parameters for AtSULT202B7-mediated sulfation of flavonols and flavonol-3-glucosides

Kinetic parameters (*Vmax*, *Km*, and *Vmax/Km*) for the sulfation of kaempferol, kaempferol-3-glucoside, quercetin, or quercetin-3-glucoside by AtSULT202B7 were determined. The results compiled in **Table III-3** indicate that the apparent *Km* for kaempferol was 27.6 μM, and that the *Vmax* value was 209.5 pmol/min/mg of enzyme. The *Km* value for kaempferol-3-glucoside was 14.4 μM, and the *Vmax* was 592.1 pmol/min/mg of enzyme. Based on these results, the *Vmax/Km* for kaempferol-3-glucoside was about six times higher than that for its aglycone counterpart. Similar results were also found with quercetin and its glucoside as substrates. These findings suggested that AtSULT202B7 sulfates flavonol-3-glucosides more efficiently than flavonol aglycones. The *Km* values for PAPS, determined with fixed concentration of kaempferol-3-glucoside, was 0.4 μM, which is within the range of Km values (0.2–0.4 μM) previously reported for other flavonoid sulfotransferases [Varin, L. et al., 1997].

Table III-3. Kinetic constants of the sulfation of flavonols and flavonol glucosides by AtSULT202B7.

	Km (μM)	Vmax (pmol/min/mg)	Vmax/Km
Kaempferol	27.6±2.7	209.5±16.4	7.6
Kaempferol-3-glucoside	14.4±2.7	592.1±57.0	41.1
Quercetin	42.1±8.1	224.1±25.1	5.3
Quercetin-3-glucoside	18.1±1.9	418.2±31.5	23.1
PAPS	0.4±0.1	495.4±43.4	1,238.5

The kinetic parameters of PAPS were examined with 10 μM of kaempferol-3-glucoside. Results shown represent means of three independent experiments. This table was cited from previously described study [Hashiguchi, T. et al., 2013].

HPLC detection and mass spectrometry of sulfated kaempferol-3-glucoside

To further determine the structure of sulfated flavonol glucoside, deglucosylated products of sulfated kaempferol-3-glucoside, generated upon treatment with β-glucosidase, were subjected to HPLC analysis. The retention time and the wavelength of maximum absorption (λ max) of the deglucosylated products were found to be almost identical to those of sulfated kaempferol (**Figure III-3**). These results suggested that AtSULT202B7 sulfates the hydroxyl group of the flavone backbone. To date, 3-sulfatoglucoside and 3-sulfatorhamnoside of flavonols have been detected in some plant species [Barron, D. et al., 1988]. To clarify whether the glucose moiety of kaempferol-3-glucoside can indeed be sulfated by AtSULT202B7, the author performed mass spectrometry of sulfated kaempferol-3-glucoside in negative ion mode. The parent ion, the highest relative abundance of ion in full scan mode was at m/z 527.05. As shown in **Figure III-4**, further selected ion monitoring (SIM) of the parent ion and the following high-energy collision dissociation (HCD) fragmentation of the parent ion produced authentic kaempferol-3-glucoside lacking one SO_3^- group (m/z 79.96) at m/z 447.09. These results strongly suggests that sulfated kaempferol-3-glucoside by AtSULT202B7 contains no sulfonate group in glucose moiety.

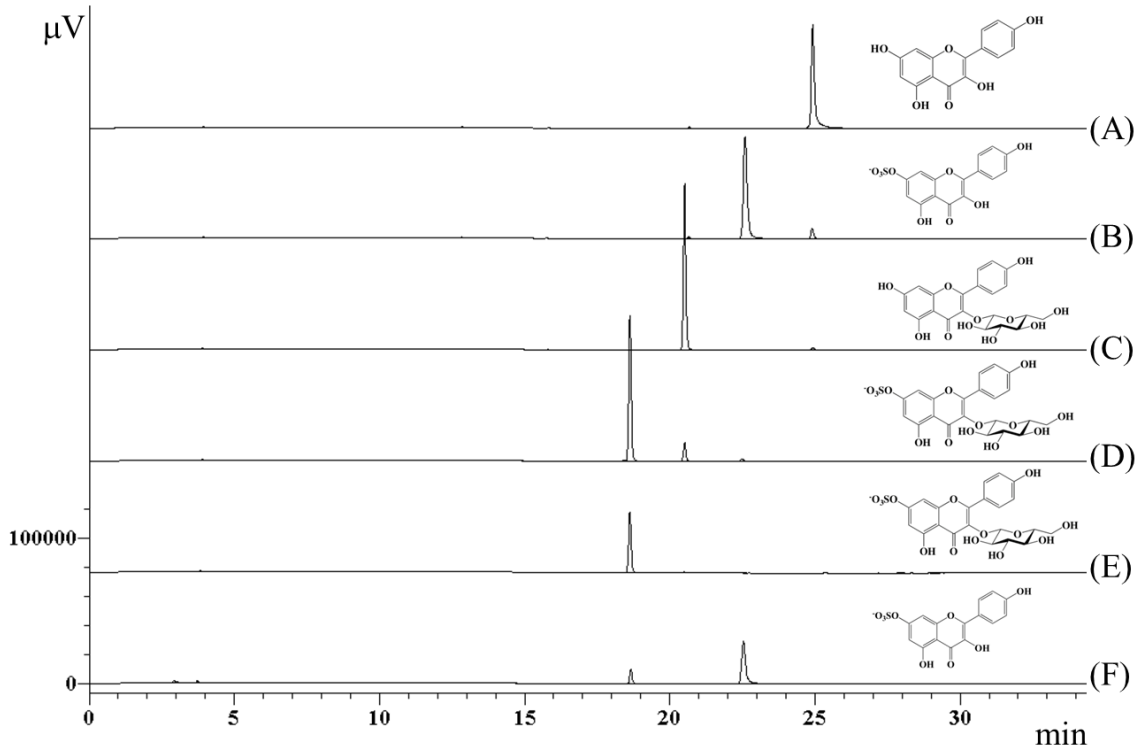


Figure III-3. HPLC detection of sulfated kaempferol-3-glucoside. (A) Kaempferol; (B) Sulfated kaempferol generated using AtSULT202B7; (C) Kaempferol-3-glucoside; (D) Sulfated kaempferol-3-glucoside generated using AtSULT202B7; (E) Sulfated kaempferol-3-glucoside; (F) Deglucosylated products of sulfated kaempferol-3-glucoside generated using β-glucosidase. This figure was cited from previously described figure [Hashiguchi, T. et al., 2013].

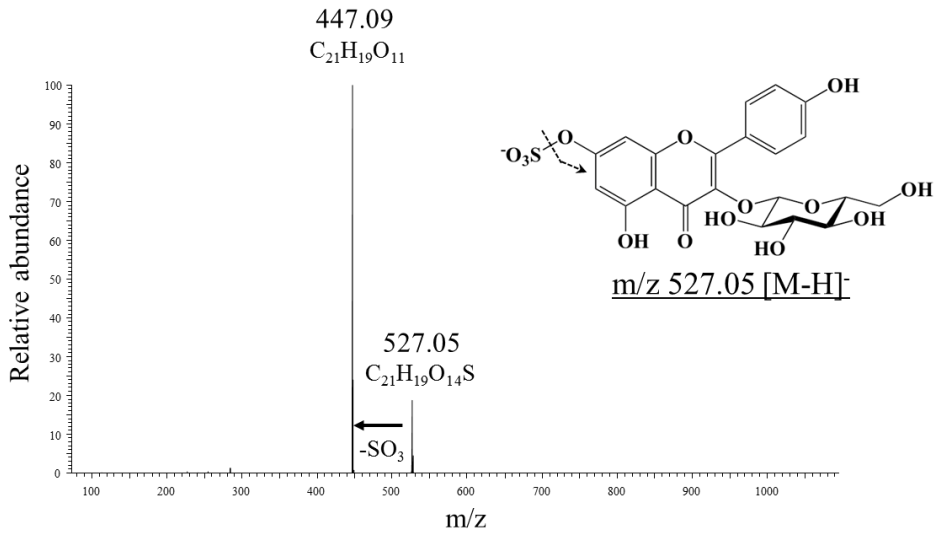


Figure III-4. MS2 spectrum of the sulfated kaempferol-3-glucoside. MS2 data were obtained from the 527.05 m/z ion as the precursor for high energy collisional dissociation. This figure was cited from previously described figure [Hashiguchi, T. et al., 2013].

Expression pattern and potential sulfating activity of AtSULT202B7 in A. thaliana

The *Arabidopsis* eFP browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) was informative tool that is easily adaptable to microarray or other large-scale data sets from several sources [Winter, D. et al., 2007]. According to the NASCArrays data (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl), it has been reported that AtSULT202B7 and AtSULT202B1 were expressed in root specifically. To confirm the specific expression of AtSULT202B7 and AtSULT202B1 in root, the author conducted the RT-PCR analysis of flavonoid sulfotransferases in *A. thaliana* (**Figure III-5**). The author found that AtSULT202B7 and AtSULT202B1 were actually expressed predominantly in root, whereas AtSULT202A1 was expressed in both aerial part and root. AtSULT202E1 expression was not detected at all in the present study. In microarray expression data, is has been reported that AtSULT202A1 is broadly found in various tissues with the highest expression in sepals, and AtSULT202E1 is expressed in seeds at early stage of embryonic development. These differential expression patterns of SULTs suggest that the expression of distinct AtSULT isoforms is regulated by different transcriptions factors in respective tissues.

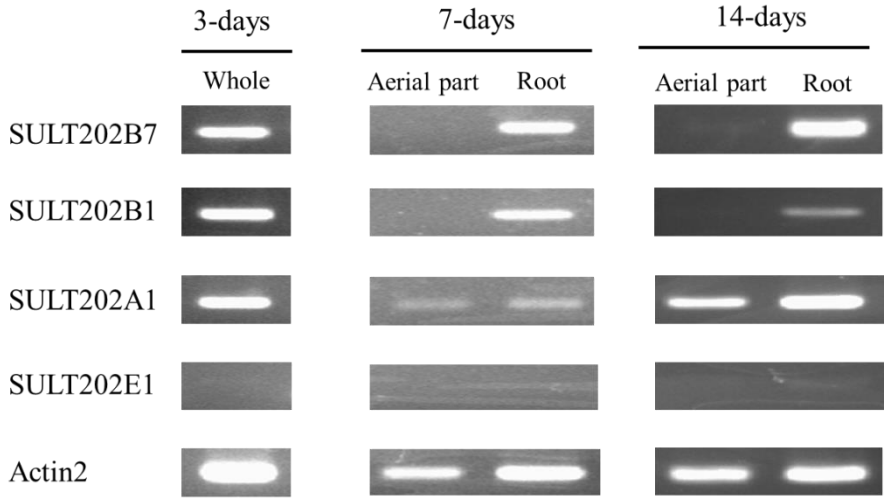


Figure III-5. RT-PCR analysis of four flavonoid sulfotransferases in *Arabidopsis thaliana*. Seedlings were corrected 3, 7, and 14 days after germination for RT-PCR analysis. Gene specific primers for PCR were designed by Primer 3 Plus software based on information from GenBank. Actin 2 was used as a positive control. Non-specific products of five genes were not observed with respective primer pairs.

In order to confirm the sulfating activity of proteins extracted from plant, the author carried out the protein extraction of 14-day old *Arabidopsis* seedlings and measured the enzymatic activity for sulfation of flavonoid and its glucoside. As shown in **Table III-4**, crude enzyme collected from *A. thaliana* indicated the sulfating activity for kaempferol and kaempferol-3-glucoside. The existence of sulfating activity in *A. thaliana* suggests that flavonoid sulfotransferases are certainly expressed in protein levels, and function via flavonoid sulfation not only in root, but also in aerial parts.

Table III-4. Sulfation activities of crude enzymes extracted from 14-day old seedlings with flavonoids as substrates.

Substrate compound	Specific activity (fmol/min/mg)	
	aerial part	root
Kaempferol	51.3±6.8	80.3±4.8
Kaempferol-3-glucoside	54.7±4.5	88.1±7.1

Specific activity refers to fmol of sulfated product formed/min/mg of protein. Data shown represent means ±S.D from three determinations.

To help researchers to understand the function and regulation of particular genes and gene networks, an ATTED-II database (http://atted.jp/data/locus/837902.shtml) that provides co-regulated gene relationships based on co-expressed genes deduced from microarray data and the predicted cis elements, was constructed [Obayashi, T. et al., 2007]. By using ATTED-II, the author searched for co-expressed gene with flavonoid sulfotransferases (**Table III-5**). The author found that some phase I- and phase II-enzymes such as cytochrome P450 (CYP) and UDP-glycosyltransferases (UGT) are co-expressed with AtSULTs. This result suggests that the three enzymes coordinately function for the synthesis of sulfated flavonoid glycosides.

Table III-5 ATTED co-expressed gene network connecting the expression of AtSULTs.

SULT isoform	Mutual rank	Pearson's correlation	Locus	Function
SULT202B7	1.0	0.70	At2g25160	Cytochrome P450, family 82, subfamily F, polypeptide
	2.5	0.59	At4g11210	Disease resistance-responsive family protein
	2.5	0.56	At2g22930	UDP-glycosyltransferase superfamily protein
	3.2	0.54	At2g25150	HXXXD-type acyl-transferase family protein
	3.5	0.53	At3g50300	HXXXD-type acyl-transferase family protein
	4.2	0.53	At5g04120	Phosphoglycerate mutase family protein
SULT202A1	1.0	0.68	At2g41730	Unidentified
	2.5	0.58	At1g05680	UDP-glycosyltransferase 74E2
	2.5	0.54	At5g43450	2-oxoglutarate and Fe (II)-dependent oxygenase superfamily protein
SULT202E1	3.5	0.68	At1g47810	F-box and associated interaction domains-containing protein
	7.2	0.68	At2g31760	RIN/U-box superfamily protein
	10.5	0.68	At2g26320	Agamous-like 33

The co-expression network of SULT202B7, SULT202A1, and SULT202E1 was computed and retrieved from ATTED-II database (http://atted.jp/data/locus/837902.shtml). Unfortunately, there is no identified gene information about AtSULT202B1. Mutual rank and pearson's correlation indicate strength of transcriptional coordination. Low mutual rank and high pearson's correlation mean stronger coexpression relationship.

To summarize, the author isolated and characterized a novel *A. thaliana* sulfotransferase, AtSULT202B7 in the present study. AtSULT202B7 was shown to exhibit higher affinity toward flavonoid glucosides than its aglycone. AtSULT202B7 showed the sulfating activity only for 7-hydroxyflavone and could not sulfate flavonol-7-glucosides. Moreover, the deglucosylated product of sulfated kaempferol-3-glucoside is more likely to be the sulfated kaempferol, and the product lost the sulfonate group to be kaempferol-3-glucoside by HCD fragmentation, suggesting that AtSULT202B7 might be a flavonol glucoside 7-sulfotransferase. The high content of flavonoid glycosides in plants and the high specific activity of AtSULT202B7 for these compounds might imply the presence of sulfated flavonoid glycosides in plants. Attempting to detect sulfated flavonoid glycoside *in*

vivo, the author performed an LC-MS/MS analysis of the methanol extracts of mature *A. thaliana* plants. Repeated experiments, however, failed to detect the presence of sulfated flavonoid glycoside. Moreover, extensive metabolomic database search yielded no evidence for the existence of sulfated flavonoid glycoside in *A. thaliana*. It is therefore likely that the abundance of those compounds may be extremely low, or that the sulfated flavonoid glycosides might be produced only under certain conditions, such as at a particular developmental stage or under environmental stress. In the present study, the author could identify the expression site of AtSULT202B7 as being in the root. Flavonoids are involved in the polar auxin transport in roots [Lewis, D.R. et al., 2011], suggesting that SULT202B7 might regulate the growth and development of the root via flavonoid glycosides sulfation.

Chapter IV

Effect of flavonoid sulfate on suspension cultured cells in *Arabidopsis thaliana*

For plants, flavonoids have many beneficial biological functions [Treutter, D., 2005]. 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 [Barron, D., 1988]. In chapter II, the author also revealed that there were some flavonoid sulfotransferases in *Arabidopsis thaliana*. However, the physiological significance of flavonoid sulfates has not been fully elucidated yet. In human, sulfation is not only just a simple detoxification and regulation of hormone levels, but also could play a role in the addition of novel functions. Isoflavone, daidzein-sulfate affects transcriptional and antiproliferative activities of estrogen receptor-β in cultured human cancer cells [Totta, P. et al., 2005]. Human metabolic transformation of quercetin to sulfate blocks its capacity to decrease eNOS expression and endothelin-1 secretion by human endothelial cells [Tribolo, S. et al., 2013].

Naringenin, one of the flavanones, is a key intermediate and a precursor of most flavonoids in flavonoid biosynthetic pathway. In *Arabidopsis thaliana*, naringenin treatment exerts inhibitory activity for polar auxin transport [Brown, D.E. et al., 2001]. In root, the growth and gravitropism were inhibited by higher concentrations of naringenin. Recently, it has also been reported that naringenin inhibits seed germination [Hernandez, I. et al., 2012], suggesting that naringenin is notable for the bioactivity against plant cells.

In this study, to clarify the differences of the effects of flavonoids and flavonoids

sulfate on plant cells, the author synthesized the naringenin sulfate by previously developed metabolic engineering methods [Shimohira, T. et al., in preparation]. The produced naringenin sulfate was purified by RP-HPLC, and treated to *Arabidopsis thaliana* T87 suspension cells. The effects of sulfated naringenin on T87 cells were analyzed by two dimensional fluorescence difference gel electrophoresis (2D-DIGE), and differentially expressed proteins between naringenin- and naringenin sulfate- treated cells were identified by mass spectrometry.

Experimental procedures

Materials. *Arabidopsis thaliana* T87 cells (Resource No. rpc00008) were obtained from RIKEN BioResource Center (Tsukuba, Japan) and were cultured at 22°C under continuous illumination with shaking at 120 rpm. Cells were subcultured every 10-14 days by adding 2 ml of the cell suspension to a 300-mL flask containing 80 mL of JPL medium [Axelos, M. et al., 1992]. IC-dyes (IC3-OSu and IC5-OSu) were purchase from Dojin Chemicals, Ltd. All the other chemicals were of the highest grade commercially available.

The synthesis of sulfated naringenin by using metabolic engineering. In order to synthesize a large amount of naringenin sulfate, *E. coli* BL21 cells transformed with hSULT1A3 were inoculated into fresh 1 L of LB media containing 100 μg/mL of ampicillin and were grown to OD_{600nm}=~1.0. The cells were induced with 0.25 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 12 h, and then the media were converted to 500 mL of M9 media supplemented with 200 μM naringenin, 20 mM glucose, 2 mM $MgSO_4$, and 100 μg/mL ampicillin. After a 24-h culture at 24°C, the media were centrifuged and the supernatant were then fractionated with Diaion HP20 (Rensui) by batch method. The methanol eluates from beads were concentrated by evaporation using speed vac and were subjected to HPLC analysis. A Shimadzu Prominence HPLC system consisting of a PDA detector, and the LC Solution software was used for HPLC analysis. 5 μm Capcell PAK C18 MGII column (250 × 10.0 mm; SHISEIDO), and a gradient elution of ultrapure water (A) and methanol (B) were used for the HPLC separations. The injection volume was 200 μL; the flow rate was 1 mL/min; and the controlled oven temperature was 40°C. The gradient program for the fractionation was 0-30% B at 0-5 min, 30% B at 5-35 min, 30-80% B at 35-60 min, 80-100% B at 60-65 min, with a liner gradient. A fraction containing sulfated naringenin was adjusted to the

neutral pH, lyophilized, and then measured by weight. The synthesized naringenin sulfate was dissolved in dimethylsulfoxide (DMSO) for the next study.

Identification of sulfated naringenin by direct infusion mass spectrometry. The sulfated naringenin dilluted in methanol was analyzed by Q Exactive hybrid quadrupole-Orbitrap mass spectrometer with a heated electrospray ionization (HESI) source through direct infusion using a syringe pump. The data was acquired using Targeted-MS^2 scan event. Typical mass spectrometric conditions were: polarity, negative ionization mode; spray voltage, 3.5 kV; sheath gas flow rate, 6; auxillary gas, 0; sweep gas, 0; heated capillary temperature, 320°C. The resolution was set at 140,000. The AGC target was 2E5. The maximum ion injection time was 100 ms. The normalized collision energy (NCE) was 20%. The raw data files were analyzed using Qual Browser software in Xcalibur (Thermo Fisher Scientific).

Measurement of cell survival by TTC assay. The cells were treated with various concentrations of naringenin and naringenin sulfate, and 0.5% of DMSO for 24 h. TTC reduction viability assay was performed according to a previously described procedure [Towill, L.E. et al., 1974]. 2,3,5-Triphenyltetrazolium chloride (TTC) was dissolved in pH 7.5 sodium phosphate buffer (50 mM). One mL of the TTC solution was added to the 0.5 mL of culture medium containing T87 cells and incubated without shaking for 18-22 hr in the dark at 22°C. After incubation, the cells were pelleted and the red formazan was extracted with 1 mL of 98% ethanol for 15 min at 60°C. Absorbance of the extract was read at 485 nm with Smartspec 3000 spectrophotometer (Bio-Rad).

Comparative analysis of uptake of naringenin or naringenin sulfate into T87 cells. About

300 mg of T87 cells were treated with 100 μM of naringenin or naringenin sulfate. After 3-h treatment, the cells were collected and washed with PBS buffer, and then extracted with 100% methanol. After a sonication for 5 min, the solvent were evaporated, and the extracts were dissolved in dimethylsulfoxide (DMSO). The samples were subjected to HPLC analysis as described above.

Protein extraction. The cells (150 mg/ml) were treated with 100 μM of naringenin or 100 μM of naringenin sulfate. The final concentration of DMSO in both treatments was 0.5%. After 24-h treatment, the media were removed by filtration and the cells were ground in liquid nitrogen with a mortar and pestle. Five hundred mg of the powder was lysed in 1 mL of ice-cold lysis buffer, PBS with 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail (Sigma), and homogenized using a polytron type homogenizer. The homogenates thus prepared were subjected to centrifugation at 20,400 x g for 15 min to remove insoluble pellet. The protein concentrations of cell-free extracts were measured by Bradford assay [Bradford, M.M., 1976] with bovine serum albumin as the standard.

Two-dimensional differential gel electrophoresis (2D-DIGE) of T87 cells. Fifty μg of each protein sample were denatured with an equal volume of 1 mM guanidine chloride for 3 min at 98°C. The denatured protein samples were covalently labeled with a 400 pmol of IC3-OSu for naringenin-treated sample and IC5-OSu for naringenin sulfate-treated protein at 4°C for 1 hr in the dark. The reaction was quenched by adding 2 μL of 10 mM lysine for 15 min. IC3-OSu- and IC5-OSu-labeled protein samples were mixed with in 1:1 ratio. The mixed samples were subjected to TCA-acetone precipitation procedures and resuspended in a solubilization buffer composed by 7 M urea, 2 M thiourea, 75 mM DTT, 0.2 % Bio-Lyte Ampholytes pH3-10, 2 % *n*-dodecyl-β-D-maltoside, 2 % CHAPS. IC-Dye labeled samples

were loaded onto rehydrated IPG (Immobilized pH gradient) strip gel (Ready Strip^{TM} IPG strip: Bio-Rad), and then IEF (isoelectric focusing) procedure was performed with the following settings: 250 V (60 min), 4000 V (60 min), and 4000 V (10,000 Vhrs). The strips were equilibrated with equilibration buffer A [6 M Urea , 375 mM Tris-HCl (pH8.8) , 20% Glycerol , 2% DTT, 2% SDS] for 30 min and, subsequently with equilibration buffer B [6 M Urea, 375 mM Tris-HCl (pH8.8), 20% Glycerol, 2% IAA, 2% SDS] for 30 min. The equilibrated IPG strips were transferred onto 10% SDS-PAGE gels and separated in the second dimension. The 2D gels were scanned with a Typhoon FLA9500 (GE Healthcare Life Science) at different excitation wavelengths (IC3-OSu: λex, 550 nm, λem, 570; IC5-OSu: λex, 640 nm, λem, 660 nm). Images were imported into Prodigy same Spot (Nonlinear Dynamics) for spot identification and normalization of spot intensities within each gel. ANOVA (analysis of variance) was used to compare the percentage spot volume of proteins from naringenin- (n=3) and naringenin sulfate-treated cells (n=3). Statistical analysis is performed using the F-test in one-way analysis of variance (ANOVA). Spots that showed a significant difference (the $P<0.05$ level) in percentage of spot volume between the two groups were selected for the following protein identification.

Protein identification by peptide mass fingerprinting (PMF) analysis. For ingel digestion, spots were excised from CBB-stained 2D gels and incubated in 100 μL of 25 mM ammonium bicarbonate with 50% acetonitorile for 10 min at 20°C for destaining. One hundred μL of 100% acetonitrile was added to dehydrate the gel pieces for 5 min and removed, and then incubated for 12 hr at 37°C in 5 μL of 10 μg/mL trypsin enzyme (Promega) in 50 mM ammonium bicarbonate. Proteins were extracted with 30 μL of extraction buffer (5% TFA, 50% acetonitorile) for 30 min at 20°C. The extracted protein samples were concentrated with a speed vac. For PMF analysis, a layer of matrix (α-cyano-4-hydroxy-cinnamic acid, CHCA)

was prepared in advance onto a MTP Anchorchip^{TM} 600/384 TF (Bruker Daltonics), and then 1 μL of the proteins were applied. MALDI spectra in positive ion mode were obtained using autoflex III TOF/TOF mass spectrometer (Bruker Daltonics). External calibration was carried out using the peptide calibration standard II (Bruker Daltonics). Mass spectra were analyzed by a MASCOT search (http://www.matrixscience.com/) against the fragment ions theoretically derived from proteins in Swiss Prot database.

Results and Discussion

Naringenin is a key intermediate of flavonoid biosynthesis (**Figure IV-1**). Kaempferol and quercetin, the major flavonol aglycones found in *A. thaliana*, are biosynthesized by some enzymes through the intermediate, naringenin. The mature tt4 mutant plants lack naringenin, have reduced apical dominance, and a reduced primary inflorescence length as compared with wild type plants [Brown, D.E. et al., 2001]. Based on this perspective, the author decided to use a naringenin as a model compounds for elucidation of physiological function of flavonoid sulfate.

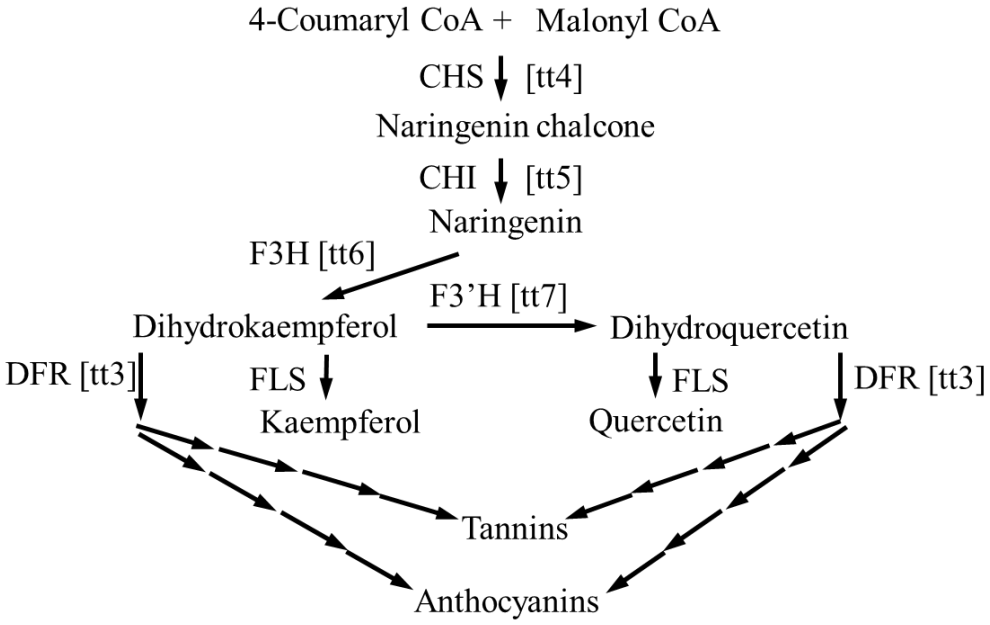


Figure IV-1. Flavonoid synthetic pathway. tt means transparent testa mutants, which are deficient in flavonoid synthesis genes. CHS, Chalcone synthase; CHI, Chalcone isomerase; F3H, Flavanone 3-hydroxylase; F3'H, Flavonoid 3'-hydroxylase; FLS, Flavonol synthase; DFR, Dihydroflavonol 4-reductase. This pathway is modified from previously described figure [Lewis, D.R. et al., 2011]

The synthesis of naringenin sulfate by metabolic engineering

Currently, sulfated flavonoids are mainly prepared by chemical or enzymatic synthesis [Jones, D.J. et al., 2005; Nakano, H. et al, 2004; Vaidyanathan, J.B. et al, 2002]. However, both preparation methods have problems to produce sulfated derivatives; it is difficult for organic synthesis to prepare regiospecific sulfated flavonoids, whereas enzymatic synthesis is too expensive due to valuable cofactor PAPS. To resolve these problems, in the previous study, the author developed novel synthetic approach which is based on metabolic sulfation by *E. coli* cells [Shimohira, T., in preparation]. For the synthesis of large amount of naringenin sulfate, naringenin was sulfated by *E. coli* BL21 cells which transformed pGEX vector harboring hSULT1A1, hSULT1A3, hSULT1B1, hSULT1C4, hSULT1E1, and AtSULT202A1 cDNAs respectively. Among these cells, the cells harboring cloned hSULT1A3 cDNAs exhibited the sulfating activity toward naringenin (**Figure IV-2**). On the other hand, the BL21 cells transformed hSULT1A1, hSULT1B1, hSULT1C4, and hSULT1E1 hardly showed the activity toward naringenin (data not shown). Sulfated-naringenin produced by hSULT1A3 equals to sulfated-naringenin from AtSULT202A1 in terms of the same retention time and the wavelength of maximum absorption (λmax). These results lead us to use the BL21 cells harboring hSULT1A3 for the quantity synthesis of naringenin sulfate.

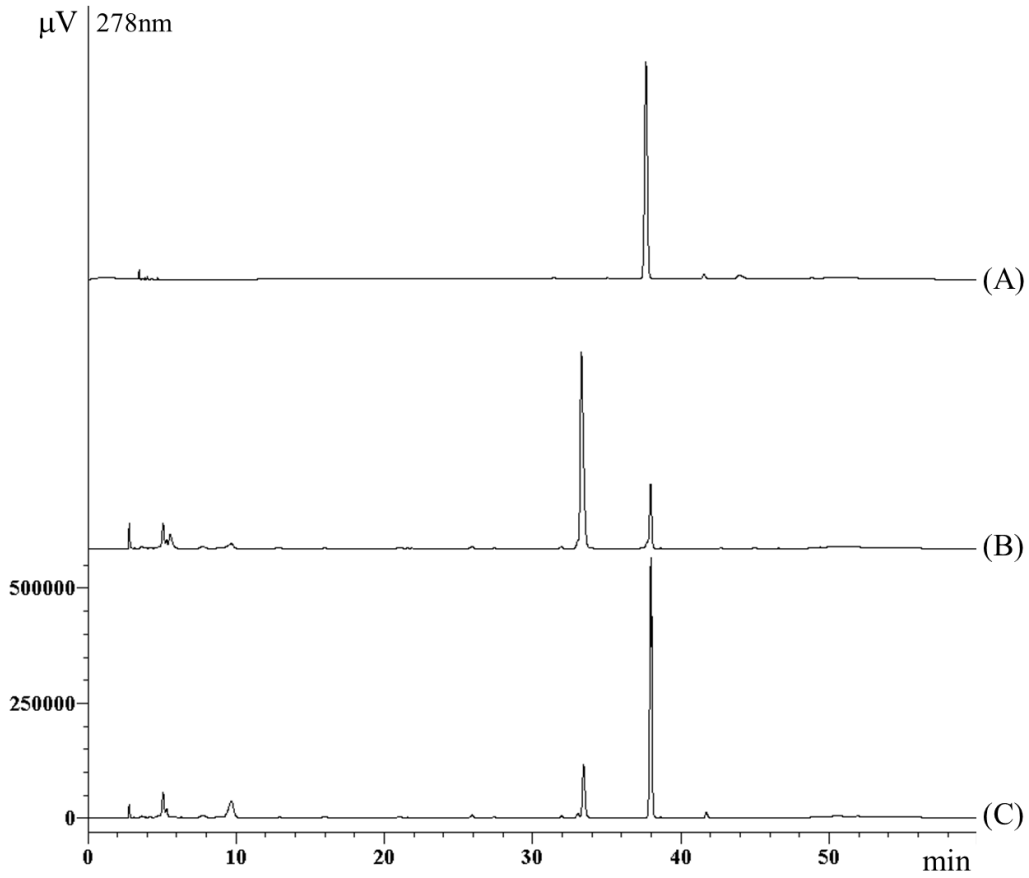


Figure IV-2. HPLC chromatogram showing the production of naringenin sulfate by BL21 cells transformed with respective SULTs. Sulfated naringenin were separated with a water/methanol gradient at a flow rate of 1 mL/min under the following condition: 0-75% methanol at 5.0-42.5 min, 75-100% methanol at 42.5-45.0 min, with a liner gradient. (A) Naringenin; (B) Sulfated naringenin using hSULT1A3-expressing BL21 cells; (C) Sulfated naringenin using AtSULT202A1-expressing BL21 cells.

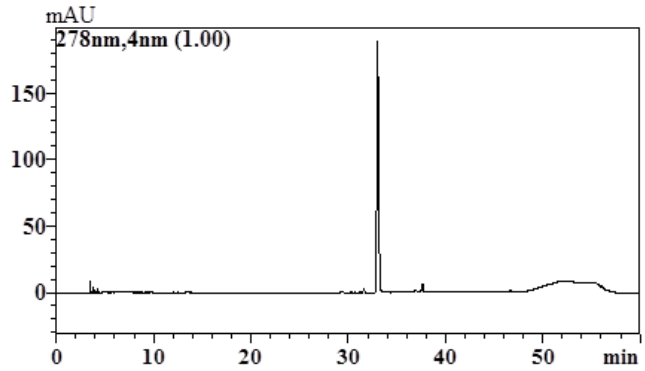


Figure IV-3. HPLC chromatogram of purified naringenin sulfate. Sulfated naringenin were separated with a water/methanol gradient at a flow rate of 1 mL/min under the following condition: 0-75% methanol at 5.0-42.5 min, 75-100% methanol at 42.5-45.0 min, with a liner gradient.

A large volume of the media containing naringenin sulfate was purified and concentrated by Diaion HP-20 beads, and the obtained naringenin sulfate was re-subjected to HPLC analysis (**Figure IV-3**).

Next, to clarify how many hydroxyl groups of naringenin can indeed be sulfated by hSULT1A3, the author performed mass spectrometry of naringenin sulfate in negative ion mode. The parent ion, the highest relative abundance of ion in full scan mode was at m/z 351.02. As shown in **Figure IV-4**, further selected ion monitoring (SIM) of the parent ion and the following high-energy collision dissociation (HCD) fragmentation of the parent ion produced authentic naringenin lacking one SO_3^- group (m/z 79.96) at m/z 271.06. These results strongly suggests that sulfated naringenin by hSULT1A3 contains one sulfonate group within the structure. In previous studies, it has been reported that hSULT1A3 exclusively mediates sulfation at the 7-hydroxyl group position of flavonoids [Meng, S. et al., 2012]. Therefore, the sulfated naringenin by hSULT1A3 is more likely to be naringenin-7-sulfate.

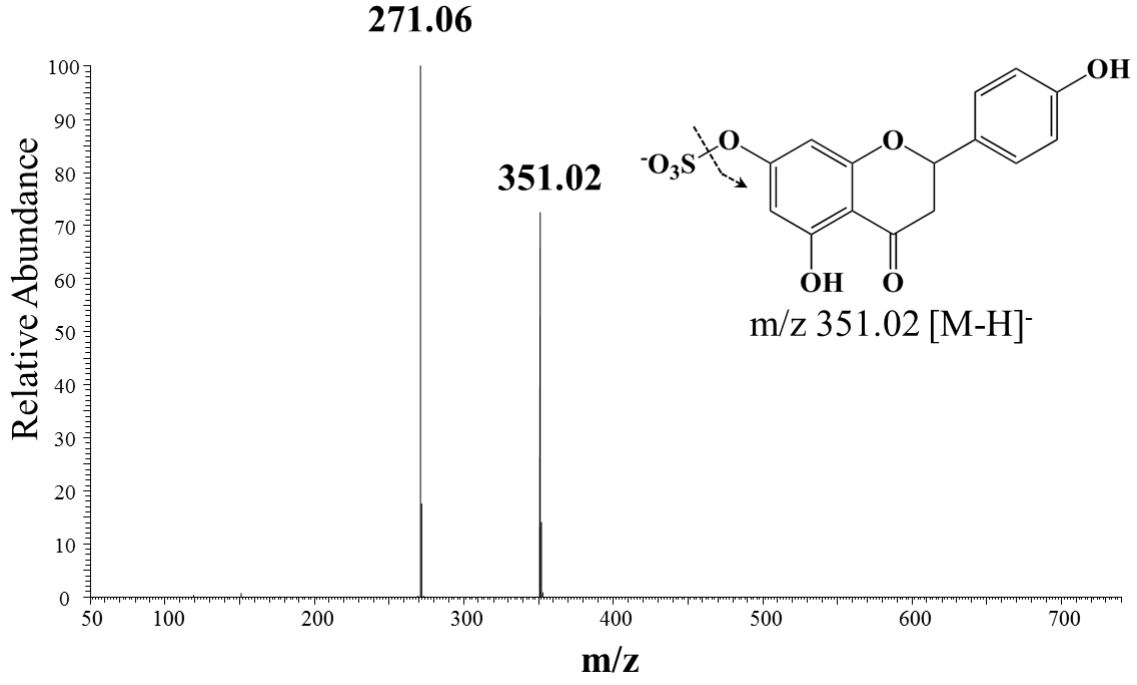


Figure IV-4. Infusion MS2 spectrum of sulfated naringenin. MS2 data were obtained from the 351.02 m/z ion as the precursor for high energy collisional dissociation.

Cell viability of T87 cells treated with naringenin or naringenin sulfate

Prior to flavonoids treatment to cells for proteomic analysis, the cytotoxicity of these two flavonoids against T87 cells was examined by TTC assay (**Figure IV-5**). T87 cells were previously established cell line from ecotype Columbia plant and were used as model cultured cells of plants [Axelos, M. et al., 1992]. One hundred μM of naringenin and naringenin sulfate showed no cytotoxicity for the cells, and thus this concentration of flavonoids were used in the following study.

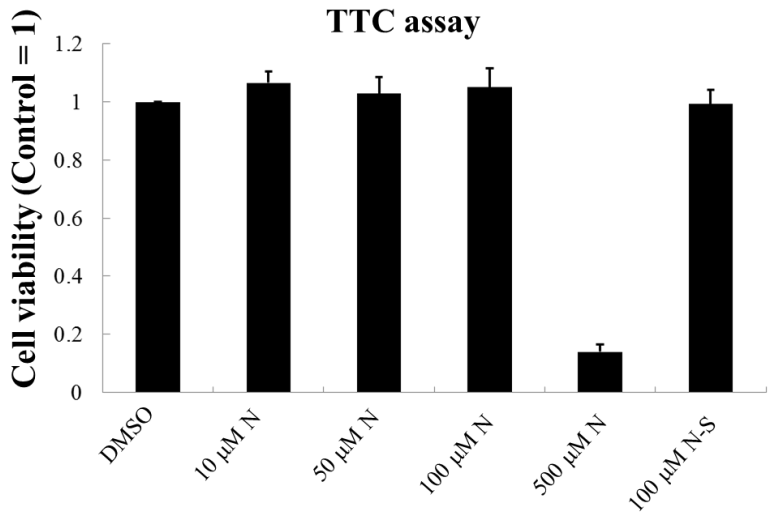


Figure IV-5. Cell viability of T87 cells after exposure to naringenin or sulfated naringenin. N and N-S indicate naringenin and naringenin sulfate, respectively. The data represent the means of three separate experiments. The results are the means ± SD.

Transport of substrates by T87 suspension cultured cells

To confirm the uptake of substrates from the media into T87 cells, methanol extracts of T87 cells after 3-h treatment were analyzed with HPLC methods. As shown in **Figure IV-6**, naringenin and the corresponding sulfate were detected in cell lysates, suggesting that these substrates were indeed transported into intracellular fraction. In plants, the mechanism of transport of sulfated flavonoids into the cells has not been characterized yet. In general, ionic products such as sulfated flavonoids could be taken up inside the cell by carrier-mediated transport in human cell line [Wong, C.C. et al., 2012]. The capability of uptake of sulfated

products in T87 cells suggests that naringenin sulfate transporter might exist in the plasma membrane.

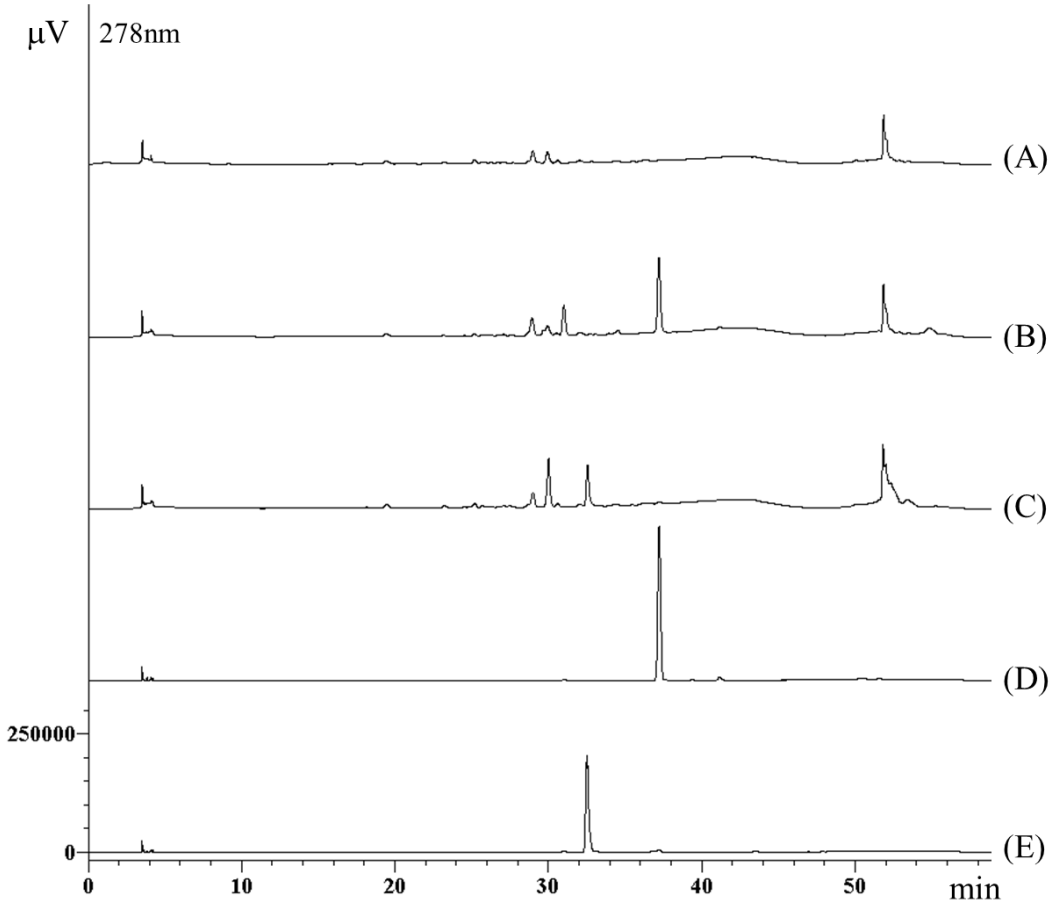


Figure IV-6. Uptake of naringenin (N) or naringenin sulfate (NS) into T87 cells. Intracellular metabolite extracted from T87 cells were separated with a water/methanol gradient at a flow rate of 1 mL/min under the following condition: 0-75% methanol at 5.0-42.5 min, 75-100% methanol at 42.5-45.0 min, with a liner gradient. (A) Methanol extracts of DMSO-treated T87 cells; (B) Methanol extracts of naringenin-treated T87 cells; (C) Methanol extracts of sulfated naringenin-treated T87 cells; (D) Naringenin; (E) Sulfated naringenin

In order to investigate the physiological significance of flavonoid sulfation, protein profiles of naringenin- and naringenin sulfate-treated T87 cells were analyzed by 2D-DIGE and the differential expression of proteins among the two groups was examined (**Figure IV-7**). Four spots were found to be increased in expression by at least 1.2-fold and these proteins were identified by MALDI-TOF-MS analysis (**Table IV-1**). Receptor for activated c

kinase 1 (RACK1) is ubiquitously expressed proteins ranging from human to plants [Adams, D.R. et al., 2011]. The *Arabidopsis* genome contains three RACK1 genes, designated as RACK1A, RACK1B, and RACK1C respectively. It has been reported that loss-of-function mutations in RACK1A confer defects in multiple hormone responsiveness and developmental processes including seed germination, leaf production, and flowering [Chen, J.G. et al., 2006]. These results provided the evidence that RACK1A is involved in multiple signal transduction pathways. Subsequently, it was also found that RACK1 is a critical negative regulator of ABA responses in *Arabidopsis thaliana* [Guo, J. et al., 2009]. Naringenin sulfation by AtSULTs might modulate hormone responses and developmental process. GAPC is a key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde-3-phosphate into 3-phospho-D-glyceroyl phosphate. It has been reported that the steady-state mRNA level of the GAPC increased when *Arabidopsis* plants were transferred from normal growth condition to heat-shock, anaerobiosis, or increased sucrose supply stresses [Yang, Y. et al., 1993]. Naringenin sulfate increased the expression levels of GAPC1 and GAPC2 compared with naringenin treatment, suggesting the sulfated naringenin might cause some environmental stress to the cells. In plants, aspartate aminotransferase is involved in nitrogen metabolism. The five ASP genes of *Arabidopsis* are predicted to encode isoenzymes for cytosolic AAT2 (ASP2 and ASP4), chloroplastic AAT3 (ASP5), and mitochondrial AAT1 (ASP1) [Schultz, C.J. et al., 1995; Wilkie, S.E. et al., 1995], of which cytosolic ASP2 is the major isoenzyme controlling the synthesis of aspartate and asparagine. Interestingly, it was reported that *Arabidopsis* mutants defective in ASP2 showed reduced root length and dramatic decreases in aspartate and asparagine levels in leaves and/or siliques [Miesak, B.H. et al., 2002]. Sulfated naringenin by AtSULTs might be involved in nitrogen metabolism through unknown pathway.

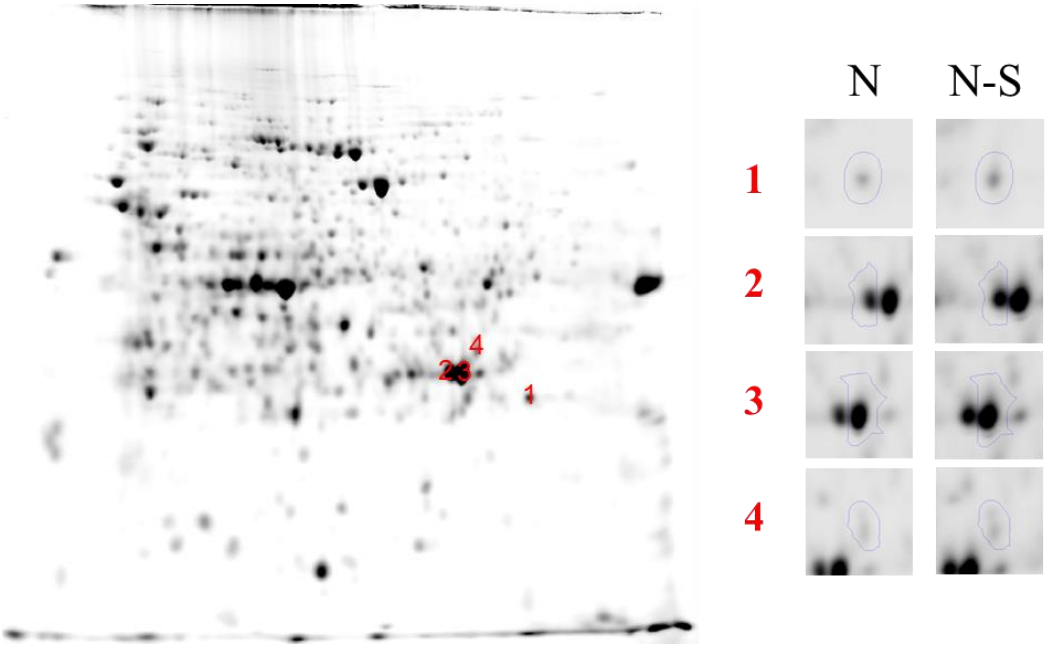


Figure IV-7. Representative 2D-DIGE image of T87 treated with narigenin and naringenin sulfate. Protein samples were labeled with IC-Dyes and analyzed 2D-DIGE. The number indicates the differentially expressed protein spots with their respective fold differences. Protein Spots were excised and identified using MALDI-TOF MS and the identified proteins are listed in Table IV-1. N and N-S indicate naringenin and naringenin sulfate, respectively.

Table IV-1. List of proteins identified by MALDI-TOF MS analysis.

Spot No.	Accession number	Protein name	Function	Sequence coverage (%)	Mascot score	Fold	Anova (p)
1	NP_173248	Receptor for activated c kinase 1A (RACK1A)	Hormone response Developmental process	35	113	1.4 (+)	0.011
2	NP_187062	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (GAPC1)	Glycolysis	31	131	1.3 (+)	0.011
3	NP_172801	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (GAPC2)	Glycolysis	31	96	1.3 (+)	0.042
4	NP_197456	Aspartate aminotransferase cytoplasmic isozyme 1 (ASP2)	Nirtogen metabolism	47	226	1.2 (+)	0.032

The proteins which were differentially expressed in sulfated naringenin-treated cells compared with naringenin-treated cells, were identified by PMF analysis as described in Experimental procedures. In this study, the author could not identify the significantly decreased protein spots in expression level.

To summarize, the author investigated for the first time the effects of sulfated flavonoids on plant cells. The physiological function of flavonoid sulfates in plant cells has not been completely elucidated because the sulfated flavonoids are commercially not obtainable. The author therefore prepared the sulfated naringenin by recently developed production method using metabolic engineering [Shimohira, T. et al., in preparation]. Next, the author identified target proteins whose expression levels were increased by narigenin sulfate compared with naringenin. Naringenin sulfates increased the expression of RACK1 protein, suggesting flavonoids would acquire the bioactivity for hormonal and developmental signaling by accepting sulfation. Naringenin sulfates also increased expression levels of three proteins, GAPC1, GAPC2, and ASP2. These results suggest that flavonoid sulfation might be key modification involved in basal metabolism such as glycolysis and nitrogen metabolism. From these results, the author speculates that AtSULTs might play a role in these important processes via flavonoid sulfation in *A. thaliana* (**Figure IV-7**). With the further elucidation of function of flavonoid sulfates, the physiological function of AtSULTs would be increasingly clarified, and the control of SULT activities might be one of the approaches for the regulation of plant growth, development, and adaptation to stress.

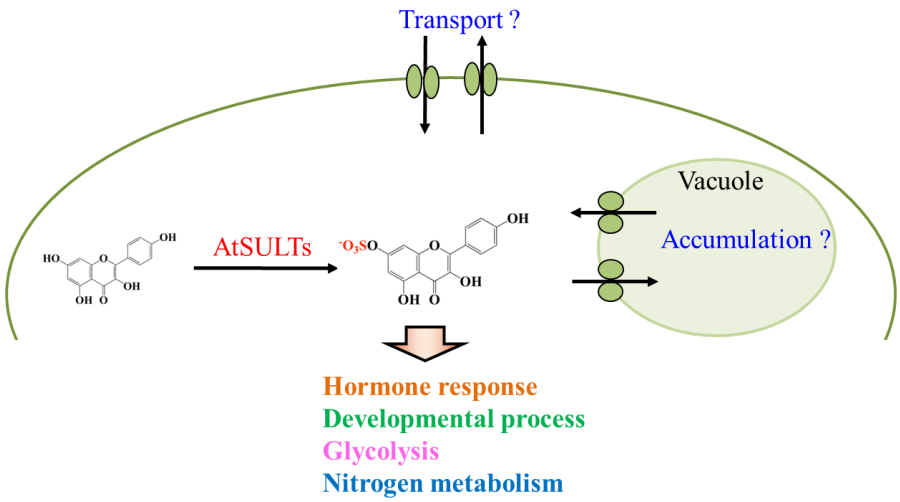


Figure IV-7. Proposed physiological functions of sulfotransferases in *A. thaliana*.

Concluding remarks

In chapter I, the author found for the first time that several SULTs exhibited the sulfating activity toward flavonoids. The existence of a number of flavonoid SULTs in *A. thaliana* suggests the regulation of flavonoid levels might play an important role in the bioactivity of flavonoids. Although no obvious phenotypes can be observed in mutants of individual SULTs [Kopriva, S., et al., 2012], probably because of the overlapping substrate specificity, the knock-out mutant of all AtSULT isoforms might cause serious damages for processes which are associated with plant growth, development, and responses to environmental stress. Unfortunately, there are some AtSULT enzymes, which the author could not determine their substrates. Therefore, it would be further required to be analyzed for the presence of unconventional substrates.

In chapter II, the author investigated the biochemical properties of a novel kaempferol sulfotransferase, AtSULT202E1, together with AtSULT202B1 and AtSULT202A1 in detail. These enzymes indicated the position specific sulfating activity toward flavonoids as reported in other plant species. The physiological significance of the position specific manner remains to be defined yet, but the author assume that the different types of sulfated flavonoids might have diverse bioactivity.

In chapter III, the author discovered the high affinity SULT, AtSULT202B7, for flavonoid glycosides compared with for aglycones. The biological significance of the high affinity for glycosides remains a matter of research. As another important property, with AtSULT202B1, AtSULT202B7 expressed in root, not in aerial part. These two genes are flavonoid specific SULT, and flavonoid regulates the auxin polar transport in root, suggesting these enzymes might regulate the root length and formation.

In chapter IV, the author conducted the proteome analysis to elucidate the function of

sulfated flavonoids in plant cells. T87 suspension cultured cells derived from *Arabidopsis thaliana*, were treated with naringenin aglycone or sulfated naringenin, and protein samples were analyzed by 2D-DIGE analysis. As a result, some differentially expressed proteins were confirmed, and were identified as proteins which are involved in glycolysis, nitrogen metabolism, plant hormone responses, and developmental processes. These results suggest that flavonoids might play a role in these important processes via sulfation.

In conclusion, the author represented the new findings on plant sulfotransferases. For the application of plant SULT studies, researchers should also perform the biochemical characterization of model plant SULTs, for example, *Lotus japonicas*, *Oryza sativa*, *Triticum aestivum*, and investigate a possible implication of plant growth, development, and adaptation to stress.

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