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Molecular and Biochemical Characterization of Hydroxyjasmonate and Flavonoid Sulfotransferases from *Arabidopsis thaliana*

Satinder Kaur Gidda

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in

The Department

of

Biology

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ABSTRACT

Molecular and biochemical characterization of hydroxyjasmonate and flavonoid sulfotransferases from *Arabidopsis thaliana*.

Satinder Kaur Gidda, Ph.D.

Concordia University, 2001

Recently, we initiated a functional genomics project with the objective of characterizing the biological function of all the sulfotransferase (ST) coding genes of *A. thaliana*. Based on amino acid sequence alignment with previously characterized soluble STs, we have identified 17 genes coding for putative STs in the genome of this plant. Prior to this work, only one ST-coding gene (*AtST1*) has been characterized from *A. thaliana*. AtST1 was shown to sulfonate brassinosteroids and was proposed to be involved in the modulation of their biological activity. This thesis presents the characterization of two more ST-coding genes from *A. thaliana*, namely *AtST2a* and *AtST3a*.

The recombinant AtST3a protein was found to exhibit strict specificity for position 7 of flavonoids. In contrast with the previously characterized flavonol 7-ST from *Flaveria bidentis* that sulfonates only flavonol disulfates, AtST3a was found to accept a number of flavonols and flavone aglycones, as well as their monosulfate derivatives. The *AtST3a* is expressed only at the earlier stage of seedling development. In contrast, the expression pattern of the flavonol 3-ST from *Flaveria* species is detectable at all stages of plant development, with highest activities found in the terminal buds and first pair of leaves. The natural occurrence of a ST exhibiting high specificity for flavonoids in *A.*

thaliana suggests that sulfated flavonoids may be of more common occurrence in the plant kingdom than once thought.

In this study, we demonstrate that the *AtST2a* from *A. thaliana* encodes a sulfotransferase specific for 11- and 12-hydroxyjasmonic acid. Jasmonic acid and its derivatives, commonly named jasmonates, are of ubiquitous occurrence in the plant kingdom and they play an important role in the plant response to biotic and abiotic stresses. More recently, it has been demonstrated that jasmonates are also involved in the control of key developmental processes such as anther development. *AtST2a* is not expressed in plants growing in the light but is induced 8 hours after their transfer to the dark. Overexpression of *AtST2a* in transgenic *Arabidopsis* leads to a delayed flowering phenotype observed only when the plants are growing under long-days. In contrast, decreasing *AtST2a* expression by expressing antisense *AtST2a* RNA leads to an early flowering phenotype observed only in short day-grown transgenic plants. Our results suggest that the function of AtST2a is to sulfonate hydroxylated jasmonic acids under growth conditions that do not favor flowering. This data also suggest that hydroxylated jasmonic acids act as signals that promote the transition from vegetative to reproductive growth when *A. thaliana* is exposed to an inductive photoperiod.

To Harjit

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TABLE OF CONTENTS

	Page
LIST OF FIGURES	xii
LIST OF TABLES	xv
LIST OF ABBREVIATIONS	xvii
INTRODUCTION	1
CHAPTER 1: REVIEW OF LITERATURE	4
Introduction	4
Sulfotransferases	4
Membrane bound mammalian sulfotransferases	5
Membrane bound plant sulfotransferases	6
Gallic acid glucoside sulfotransferase	6
Phytosulfokine α sulfotransferase	7
Soluble sulfotransferases	9
Mammalian soluble sulfotransferases	10
Plant soluble sulfotransferases	11
Flavonol sulfotransferases	11
Glucosinolate sulfotransferase	12
Choline sulfotransferase	13
Steroid sulfotransferases	14
Sulfated compounds found in plants	15
Sulfolipids	15
Riboflavin sulfates	19
12-hydoxysulfonyloxyjasmonic acid	19
Flavonols and Flavonol sulfates	20
Physiological roles of flavonols	20
UV protection	20

Plant fertility 21
Plant microbe interaction 21
Auxin transport 21
Flavonoid biosynthesis 22
Flavonols of *Arabidopsis thaliana* 23
Flavonoid sulfates 23
Jasmonic acid (JA) 24
Physiological roles of jasmonates 24
Defense response 25
Vegetative sinks and storage proteins 26
Tuberization 26
Senescence and growth inhibition 27
Plant reproduction 27
Biosynthesis of jasmonic acid 28
Octadecanoid pathway 28
Hexadecanoid pathway 31
Oxylipin signature 32
Comparison of jasmonates and mammalian prostaglandins 33
Jasmonate signal transduction 34
Conjugates of jasmonic acid 35
Tuberonic acid 36
Concept of flowering time 39
Florigen hypothesis 40
The multifactorial model for flowering time 43
CHAPTER 2: MATERIALS AND METHODS 45
Materials 45
Cloning of *AtST2a* and *AtST3a* 45
DNA sequence analysis 46
Expression of recombinant *AtST2a* and *AtST3a* 47
Preparation of anti-AtST2a antibodies 47

SDS-Polyacrylamide Gel Electrophoresis 48
Sulfotransferase assay 48
Preparation of *Arabidopsis thaliana* extracts to detect AtST3a substrate 49
Reverse phase HPLC to identify flavonol sulfate 50
Detection and quantification of JA, 12-OH-JA and 11-OH-JA from *Arabidopsis thaliana* 50
Detection of 12-OH-JA sulfate by LC-MS/MS 52
Northern blot analysis 53
Plasmid constructs for *AtST2a* and *AtST3a* transformation in *Arabidopsis thaliana* 54
Agrobacterium tumefaciens transformation 55
Arabidopsis thaliana transformation 55
Southern blot analysis 56
Detection of AtST2a protein in transgenic lines 56
Reverse Transcriptase- Polymerase Chain Reaction 57
Oligonucleotides used for amplification in the RT-PCR experiments 57
Plant growth conditions 59
CHAPTER 3: ANALYSIS OF *ARABIDOPSIS THALIANA* SULFOTRANSFERASE GENE FAMILY 60
Identification of sulfotransferase genes from *Arabidopsis thaliana* 60
Sequence homology and phylogenetic analysis 65
Mapping 67
CHAPTER 4: BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF A FLAVONOID 7-SULFOTRANSFERASE FROM *ARABIDOPSIS THALIANA* 70
Sequence analysis of *AtST3a* 70
Biochemical characterization of AtST3a 72
Enzyme kinetics 75
Identification of enzyme reaction product formed *in vitro* 78
Regulation studies 78

Analysis of transgenic *Arabidopsis thaliana* expressing the *AtST3a* in the antisense orientation 78
Phenotype of *Arabidopsis thaliana* plants with reduced levels of *AtST2a* transcript 82
Identification of enzyme substrate found *in vivo* 85
Discussion 87
CHAPTER 5: BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF 12-HYDROXY JASMONATE SULFOTRANSFERASE FROM *ARABIDOPSIS THALIANA* 91
Cloning of *AtST2a* 91
Substrate specificity 91
Enzyme kinetics 94
Properties of AtST2a 97
Identification and quantification of jasmonates in *Arabidopsis thaliana* and tobacco 97
Identification of enzyme reaction product formed *in vitro* and *in vivo.* 100
Regulation Studies 100
Regulation of expression by 12-OH-JA 103
Quantification of jasmonates and 12-OH-JA sulfate in Me- JA and 12-OH-JA treated plants 108
Expression of *AtST2a* in response to photoperiod 108
Comparison of jasmonate levels in *Arabidopsis* plants grown under long-day conditions as compared to those grown in the dark 112
Regulation of Me-JA inducible defense response gene, *Thi 2.1* 112
Construction of transgenic *Arabidopsis thaliana* expressing the *AtST2a* under the control of *CaMV: 35S* promoter 115
Phenotype of *Arabidopsis thaliana* plants overexpressing *AtST2a* 115
Quantification of jasmonates in wild type and *35S: AtST2a* sense plants 122

Analysis of transgenic *Arabidopsis thaliana* expressing the *AtST2a* in the antisense orientation 122
Phenotype of *Arabidopsis thaliana* plants with reduced levels of *AtST2a* transcript 126
Quantification of jasmonates in the *35S: AtST2a* antisense plants 126
Effect of 12-OH-JA treatment on flowering time of *Arabidopsis thaliana* plants 133
Discussion 133
CONCLUSIONS AND PERSPECTIVES FOR FUTURE WORK 144
REFERENCES 148

LIST OF FIGURES

Page

Chapter 1

Figure 1.1. Structures of some sulfated compounds found in plants. 8

Figure 1.2. Biosynthesis of jasmonic acid via the octadecanoid and hexadecanoid pathways. 29

Figure 1.3. Chemical structures of 12-hydroxyjasmonic acid and 11-hydroxy jasmonic acid. 38

Chapter 3

Figure 3.1. Amino acid sequence alignment of all the putative full length STs from *Arabidopsis thaliana* with the flavonol 3-ST from *Flaveria chloraefolia* 62

Figure 3.2. Phylogenetic tree of the *Arabidopsis thaliana* STs and the flavonol 3-ST from *Flaveria chloraefolia* 66

Figure 3.3. Localization of the17 ST coding genes on the 5 chromosomes of *Arabidopsis thaliana.* 68

Chapter 4

Figure 4.1. Amino acid sequence alignment of AtST3a, AtST3b and flavonol 3-ST from *Flaveria chloraefolia.* 71

Figure 4.2. SDS-PAGE of fractions collected during purification of recombinant AtST3a . 73

Figure 4.3. Structural formulae of flavonoids used as substrates for AtST3a. 74

Figure 4.4. Analysis of kinetic mechanism of AtST3a- catalyzed conversion of kaempferol 3-sulfate to kaempferol 3, 7-disulfate. 77

Figure 4.5. Photograph of an autoradiogram of the chromatographed reaction products of AtST3a with flavonoid substrates. 79

Figure 4.6. Elution profile of kaempferol 7-sulfate when chromatographed on C18 reverse phase HPLC column. 80

Figure 4.7. RT-PCR of RNA samples from *Arabidopsis thaliana* plants at different developmental stages, flowers, siliques and roots. 81
Figure 4.8. Characterization of *35S:AtST3a* antisense transgenic lines. 83
Figure 4.9. Phenotype of plants expressing *35S: AtST3a* in the antisense orientation grown under long day conditions. 84
Figure 4.10. Photograph of an autoradiogram of the chromatographed reaction products of AtST3a when acid hydrolyzed methanolic extract of *Arabidopsis thaliana* was used as a substrate. 86

Chapter 5

Figure 5.1. Amino acid sequence alignment of AtST2a, AtST2b and flavonol 3-ST of *Flaveria chloraefolia* 92
Figure 5.2. SDS-PAGE and Western blot of fractions collected during purification of recombinant AtST2a. 93
Figure 5.3. Chemical structure of some compounds used as substrates with the recombinant AtST2a protein. 95
Figure 5.4. Analysis of kinetic mechanism of AtST2a catalyzed conversion of 12-OH-JA to 12-OH-JA sulfate. 96
Figure 5.5. Typical mass spectrum profiles of jasmonates obtained from the GCQ Finnigan instrument. 98
Figure 5.6.A) Mass spectrum of 12-OH-JA sulfate obtained by collision induced dissociation with 30 eV energy. 101
B) HPLC profile obtained during selective ion monitoring of 12-OH-JA sulfate. 102
Figure 5.7. Northern blot analysis of *AtST2a* mRNA in 15 days old *Arabidopsis thaliana* plants treated with salicylic acid, methyl jasmonate, or in response to wounding and to fungal infection. 104
Figure 5.8. Dose response and kinetic of *AtST2a* mRNA accumulation following treatment of *Arabidopsis thaliana* plants with Me-JA 105

Figure 5.9. A) Northern blot analysis of *AtST2a* in 15 days old *Arabidopsis thaliana* plants treated with 12-*O*-PDA, JA, 12-OH-JA, JA-Isoleucine conjugate, sorbitol, coronatine and hydroxyindanoyl-Isoleucine-methyl ester or water. 106
B) Chemical structure of compounds used for the regulation studies of *AtST2a* expression. 107
Figure 5.10. Kinetic of *AtST2a* mRNA accumulation during treatment of 15 days old *Arabidopsis thaliana* plants with 100μM 12-OH-JA. 109
Figure 5.11. RNA gel blot analysis of *AtST2a* expression in the dark. 111
Figure 5.12. RT-PCR of mRNA from *Arabidopsis thaliana* plants treated with 12-OH-JA, Me-JA or water using *Thi 2.1* specific primers. 114
Figure 5.13. Southern blot of *Arabidopsis thaliana 35S: AtST2a* transgenic lines and Col0 plants. 117
Figure 5.14. Comparison of 20 days old wild type and *35S: AtST2a Arabidopsis thaliana* plants grown under long day conditions. 119
Figure 5.15. Effect of overexpression of *AtST2a* on flowering time in *Arabidopsis thaliana.* 121
Figure 5.16. Analysis of *Arabidopsis thaliana* transgenic lines expressing *AtST2a* in the antisense orientation 125
Figure 5.17. Phenotype of *Arabidopsis thaliana* plants expressing *AtST2a* in the antisense orientation grown under short day conditions. 127
Figure 5.18. Phenotype of *Arabidopsis thaliana* plants treated with 12-OH-JA. 132
Figure 5.19. Proposed model for the role of *AtST2a* in the control of flowering time in *Arabidopsis thaliana.* 140

LIST OF TABLES

Page

Chapter 1

Table 1.1. Sulfated compounds known to occur in different plant species. 16

Chapter 3

Table 3.1. Accession numbers and coordinates of the *Arabidopsis thaliana* sulfotransferases 61

Chapter 4

Table 4.1. Kinetic parameters of AtST3a for different flavonoids. 76

Chapter 5

Table 5.1. Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in *Arabidopsis thaliana* and mature tobacco plants. 99

Table 5.2. Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in two ecotypes of *Arabidopsis thaliana* 110

Table 5.3. Quantification of JA, 12-OH-JA, 11-OH-JA in *Arabidopsis thaliana* grown under long-days or grown in the dark for 48 hours and transferred back to light for 12 hours. 113

Table 5.4. Characterization of *35S: AtST2a* independent transgenic lines. 116

Table 5.5. Time of flowering of 7 independent *35S: AtST2a* lines as compared to Col0 grown under long day conditions. 120

Table 5.6. Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in 18 days old *Arabidopsis thaliana* Col0 and *35S: AtST2a* transgenic line S9. 123

Table 5.7. Characterization of *35S: AtST2a* antisense transgenic lines. 124

Table 5.8. Flowering time of *35S: AtST2a* antisense lines compared to wild type *Arabidopsis thaliana* (C24) grown under short day conditions. 128

Table 5.9. Flowering time of *35S: AtST2a* antisense lines compared to wild type *Arabidopsis thaliana* (C24) grown under long day conditions. 129

Table 5.10. Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in 18 days old *Arabidopsis thaliana* compared to transgenic line 7-2-5 expressing *AtST2a* in the antisense orientation. 130

LIST OF ABBREVIATIONS

Ac	Acetyl
AMV	Alfalfa mosaic virus
BAC	Bacterial artificial chromosome
BAW	Butanol-acetic acid-water
bp	base pairs
BSA	Bovine serum albumin
CID	Collision induced dissociation
COI1	Coronatine insensitive 1
CTAB	Hexadecyltrimethyl ammonium bromide
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetate
EST	Estrogen sulfotransferase
GC	Gas chromatography
GUS	ß-Glucuronidase
HPLC	High performance liquid chromatography
HSST	Hydroxysteroid sulfotransferase
JA	Jasmonic acid
JIP	Jasmonate inducible protein
kDa	kilo daltons
LC	Liquid chromatography
Me-JA	Methyl jasmonate
MES	2-[*N*-Morpholino]ethane sulfonic acid
MS	Mass spectrometry
12-OH-JA	12-hydroxyjasmonic acid
11-OH-JA	11-hydroxyjasmonic acid
12-OH-JA sulfate	12- hydroxysulfonyloxyjasmonic acid
OPDA	Oxo-phytodienoic acid

PAGE	Polyacrylamide gel electrophoresis
PAPS	3'-Phosphoadenosine 5'-phosphosulfate
PCR	Polymerase chain reaction
PFB	Pentafluorobenzyl
PST	Phenol sulfotransferase
R_t	Retention time
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
TA	Tuberonic acid
TAG	Tuberonic acid glucoside
TLC	Thin layer chromatography
VSP	Vegetative storage protein

INTRODUCTION

Sulfotransferase- mediated sulfate conjugation constitutes an important reaction in the transformation of xenobiotics, as well as the modulation of biological activity of steroid hormones and neurotransmitters in mammals (reviewed in Falany, 1997). For example, it is generally accepted that the estrogen sulfotransferase inactivates the biological activity of estrogenic steroids in specific tissues. In plants, sulfation appears to play an important role in signaling processes as indicated by the requirement of a sulfate moiety for the biological activity of gallic acid glucoside sulfate in the seismonastic movements of *Mimosa pudica* (Schildknecht and Meier-Augenstein, 1990). Recently, a brassinosteroid sulfotransferase was characterized from *Brassica napus* and it was demonstrated that the sulfation of brassinosteroids abolishes their biological activity (Rouleau et al., 1999). These results suggest that plants, like mammals utilize the sulfation reaction to control the level of biologically active molecules. In view of the important role of the sulfation reaction in plants, we initiated a functional genomics project to characterize all the sulfotransferase coding genes from *A. thaliana*. The sequencing of the complete *A. thaliana* genome has enabled us to identify 18 putative sulfotransferase coding sequences.

This study deals with the biochemical and molecular characterization of two novel sulfotransferases designated as AtST2a and AtST3a. The substrate specificity of the recombinant enzymes was characterized using a variety of compounds derived from plants and mammals.

The determination of substrate specificity of AtST3a indicates that this enzyme sulfonates flavonoids. Flavonoids constitute one of the largest groups of naturally

occurring phenolic compounds. Flavonoids have generally been reported to play important roles as flower pigments, stress metabolites formed in response to UV, in pollen germination and polar auxin transport (reviewed in Shirley, 1996). Flavonoid sulfates are of common occurrence in plants, especially in the Asteraceae, however they have yet to be identified in *Arabidopsis*. Due to the lack of information regarding the factors regulating their accumulation, the exact role of flavonoid sulfates remains to be elucidated. AtST3a exhibits a distinct substrate specificity, as compared to previously characterized flavonol sulfotransferases from *Flaveria* species. In order to determine the biological function of ATST3a, transgenic plants constitutively expressing the *AtST3a* RNA in the antisense orientation were analyzed and attempts were made to identify the endogenous enzyme substrate.

AtST2a exhibits a novel enzyme activity as it was found to sulfonate 12-hydroxyjasmonic acid. Jasmonic acid and its derivatives, commonly named jasmonates, are of ubiquitous occurrence in the plant kingdom and play important roles in plant development, plant response to wounding, defense against fungal pathogens, tuberization, maturation and release of pollen (reviewed in Wasternack and Parthier, 1997; Sanders et al., 2000). A number of jasmonate conjugates, including glycosylated, hydroxylated and amino acid conjugates have been identified, some of which have been proposed to possess biological activity (reviewed in Sembdner et al., 1994). For example, 12-OH-JA has been proposed to induce tuber formation in potato (Yoshihara et al., 1989). The expression pattern of *AtST2a* under normal growth conditions and on treatment with its substrate was also studied. Regulation studies and the analysis of transgenic plants

overexpressing AtST2a, as well as plants constitutively expressing *AtST2a* antisense RNA, allowed us to propose that this enzyme might control flowering time in *A. thaliana*.

Chapter 1

REVIEW OF LITERATURE

Introduction

This thesis presents the results of the characterization of two sulfotransferase enzymes from *Arabidopsis thaliana* at the biochemical and molecular level. The literature review begins with an overview of mammalian and plant sulfotransferases, followed by a brief description of sulfonated compounds found in plants. The characterization of the first sulfotransferase shows that this enzyme accepts flavonoids as substrates. This is the first report of such an enzyme activity in *Arabidopsis*. In view of this discovery, an overview of the physiological roles of flavonoids is presented in the second section. Given that the second enzyme sulfonates hydroxy jasmonates, the subsequent section of the literature review comprises an overview of the biosynthesis and biological function of jasmonic acid and its hydroxylated metabolites. Results presented in this thesis indicate that this enzyme sulfonates a metabolite that may have a role to play in the determination of flowering time in *A. thaliana*. In view of these results, the final section comprises a brief overview of what is known about the control of flowering time, particularly in *A. thaliana*.

Sulfotransferases

Sulfotransferases (STs) are the enzymes that catalyze the transfer of a sulfonate group from an activated sulfonate donor to an appropriate alcoholic or phenolic hydroxyl group of an acceptor molecule. The universal sulfonate donor is 3'- phosphoadenosine 5'-phosphosulfate (PAPS). The major sulfonate acceptor groups are aromatic or aliphatic

hydroxyls, however sulfotransferases are also involved in the conjugation of primary amines, N- oxides and hydroxyl-amines.

STs are widely distributed in plants and animals. Based on their sub-cellular localization, the STs can be classified into two groups. The first group consists of membrane bound enzymes that are involved in the sulfonation of glycosaminoglycans, glycoproteins and protein tyrosines (Bowman and Bertozzi, 1999; Niehrs et al., 1994). The second group consists of soluble STs that commonly sulfonate small organic molecules such as flavonoids, steroids, catecholamine neurotransmitters and xenobiotics (Varin et al., 1997a, Weinshilboum et al., 1997).

Membrane bound mammalian sulfotransferases

Protein tyrosine O-sulfation is a post-translational modification that occurs in many secretory and membrane bound mammalian proteins (reviewed by Kehoe and Bertozzi, 2000). This reaction is catalyzed by tyrosyl protein sulfotransferases (TPSTs), integral membrane glycoproteins residing in the trans golgi network. Both human and mouse TPST cDNA have been cloned. Tyrosine O-sulfation has been shown to be important in protein-protein interactions in several systems. For example, sulfation of a tyrosine residue in the leukocyte adhesion molecule P-selectin glycoprotein ligand I (PSGL-1) is required for binding to P-selectin on activated endothelium (reviewed by Kehoe and Bertozzi, 2000).

Similar to TPSTs, carbohydrate STs are also transmembrane, resident enzymes of the Golgi network that recognize glycans attached to lipids and proteins passing through the secretory pathway. In contrast to soluble STs, carbohydrate STs play a role in

extracellular signaling and adhesion by generating unique ligands from a carbohydrate scaffold (reviewed by Bowman and Bertozzi, 1999). For example, high affinity binding of heparin sulfate to antithrombin, an inhibitor of the clotting cascade, requires the presence of 3-O-sulfated glucosamine. Therefore the glucosamine 3-ST could be an important blood clotting regulator.

Sulfonation of the glycoprotein hormones produced in the pituitary gland such as lutropin and thyrotropin, reduces their serum half-life thereby regulating their bioactivity. These sulfonated hormones are specifically recognized by receptors on the liver cells that result in clearance of the hormones from the medium (Fiete et al., 1991). The corresponding sulfotransferase therefore plays a role in regulating hormone activity by creating a unique ligand for liver clearance receptors.

Membrane bound plant sulfotransferases

Though a number of genes encoding mammalian membrane-bound STs have been cloned and characterized in recent years, there are very few examples of membrane-bound STs known to occur in plants.

Gallic acid glucoside sulfotransferase

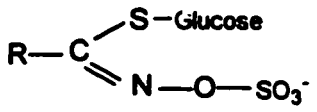
Mimosa pudica (Leguminosae) performs both nyctinastic and seismonastic movements. The leaves of this plant decline and leaflets close a few seconds after the plant is touched and in the absence of further stimulus, the leaves will recover their original position within 5 minutes. It was demonstrated that this response of *Mimosa* is under the control of electrical and chemical signals. In 1916, it was reported that extracts from Mimosa or other plants that exhibit nyctinastic movements were able to induce leaf

closure when applied to the cut end of a *Mimosa* stem. A substance that could induce leaf closure was purified and identified to be gallic acid 4-*O*- (β-D-glucopyranosyl)-6'-sulfate (Figure 1.1) and was named Periodic Leaf Movement Factor 1 (PLMF- 1) (Schildknecht et al., 1981). Structure-activity relationship studies of PLMF-1 demonstrated that, in addition to other structural requirements, the sulfonate group is indispensable for biological activity.

A sulfotransferase that specifically catalyses the transfer of a sulfonate group to gallic acid glucoside has been characterized from plasma membrane preparations of *Mimosa pudica* (Varin et al., 1997b). Using anti-flavonol 3-ST antibodies, a cross-reacting polypeptide of apparent molecular mass of 44 kDa was detected in Mimosa protein extracts. Indirect immunogold labeling of resin-embedded sections of primary and tertiary pulvini showed specific localization of gold particles on sieve tube plasma membranes.

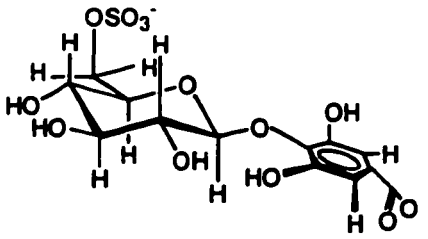
Phytosulfokine α sulfotransferase

Phytosulfokine α is a disulfated pentapeptide (Tyr (SO_4H)- Ile- Tyr (SO_4H)- Thr-Gln) that was originally isolated from conditioned medium derived from *Asparagus officinalis L* mesophyll culture (Matsubayashi and Sakagami, 1996). It has since been demonstrated to exist in both monocot (rice) and dicot plant cell cultures. Phytosulfokine α exhibits mitogenic activity and strongly stimulates colony formation of rice protoplasts. Plasma membranes of rice suspension cultures were shown to have binding sites for recombinant phytosulfokine α. However, desulfated phytosulfokine α loses its mitogenic activity and ligand binding ability (Matsubayashi and Sakagami, 1999). The sulfonation

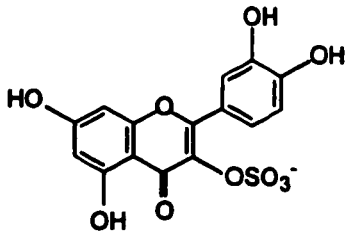


R= Allyl-, indolyl- or benzyl-

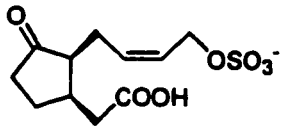
Glucosinolates



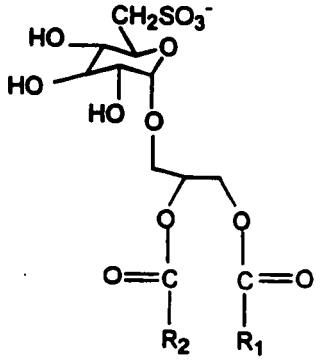
Gallic acid 4-O- (β-D-glucopyranosyl)-6'-sulfate



Flavonol sulfate (quercetin 3-sulfate)

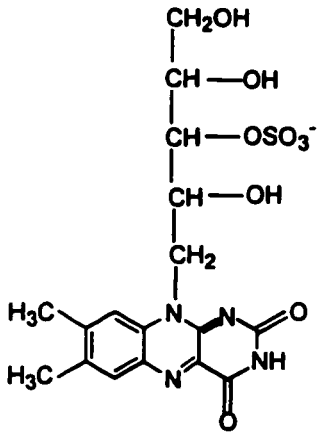


12- hydroxysulfonyloxy jasmonic acid



R_1 and R_2 = Acyl chains of different length

Sulfoquinovosyl diacyl glycerol



Riboflavin 3'-sulfate

Figure 1.1

Structures of some sulfated compounds found in plants

of the pentapeptide is catalyzed by a tyrosine protein sulfotransferase. Similar to mammalian tyrosine protein sulfotransferases, phytosulfokine α sulfotransferase is a membrane-bound enzyme of the trans golgi network (Hanai et al., 2000).

Soluble sulfotransferases

Over the past five to ten years, a large number of cDNAs encoding STs have been isolated and characterized. The comparison of amino acid sequences of sulfotransferases of plant and animal origin reveal distinctive features. Though mammalian and plant soluble STs share only 25-30% amino acid sequence identity, they share four regions of conserved amino acid residues (Varin et al., 1992). Amino acid residues that are involved in co-substrate binding and catalysis have been identified and were localized in these conserved regions. Conserved region I, with the sequence YPKSG(T/N)W, is located in the N-terminal portion of the ST. The conserved region IV is located on the C-terminal portion of the STs and its sequence is RK(G/A)XXGDWK(N/T)XFT. Conserved regions II and IV contain arginine residues that have been shown to be involved in PAPS binding (Marsolais et al., 1999). Residues corresponding to these amino acids co-ordinate with the 3'- phosphate of PAP in the estrogen ST crystal structure (Kakuta et al., 1997). Site-directed mutagenesis studies of the flavonol 3-ST from *Flaveria chloraefolia* revealed that a conserved lysine (Lys^{59}) and histidine (His^{119}) are involved in catalysis (Marsolais and Varin, 1995). The presence of these conserved amino acid residues involved in PAPS binding and catalysis can be used to identify new ST coding sequences.

Amino acid residues and protein domains conferring substrate specificity have also been determined (reviewed by Marsolais et al., 2000). Analysis of chimeric flavonol 3-ST and flavonol 4'-ST indicate that the region designated as domain II, spanning from amino acid 93 to 195 in flavonol 3-ST, contains all the determinants of substrate and position specificity (Varin et al., 1995). Within this domain, two subdomains of high divergence between positions 99-111 and 153-171 of flavonol 3-ST were identified by amino acid sequence comparison of all known plant STs. It has been proposed that these subdomains may participate in acceptor substrate binding in all soluble plant STs. In agreement with this proposal it has been shown by site-directed mutagenesis and from the crystal structure of the mouse EST, that residues corresponding to these subdomains are involved in substrate specificity (Petrotchenko et al., 1999).

Mammalian soluble sulfotransferases

Conjugation of many xenobiotics, drugs and endogenous metabolites with a sulfonate moiety is an important reaction in their biotransformation. Sulfonation of these compounds generally results in decreased biological activity and increased urinary excretion. Soluble STs involved in the detoxification of xenobiotics and/or endogenous compounds are generally characterized by their broad substrate specificity (Falany, 1997). However, soluble STs also include enzymes exhibiting high substrate specificity and are involved in important metabolic processes such as steroid transport or inactivation (Falany, 1997).

According to the molecular classification of STs, which is solely based on amino acid sequence comparison, the mammalian soluble STs fall in two groups, the phenol STs

(PST) and hydroxysteroid STs (HSST). The PSTs are further sub-divided into two subfamilies, the PSTs and the estrogen sulfotransferases (ESTs). The HSSTs have been demonstrated to catalyze the sulfonation of alcohol hydroxyl groups of hydroxysteroids. The phenol STs are important enzymes for the detoxification of drugs and xenobiotics (Mulder and Jacoby, 1990). In addition to this detoxification function, mammalian PSTs are also involved in the metabolic pathways of steroid and thyroid hormones as well as catecholamine neurotransmitters. For example, ESTs are involved in estrogen inactivation during the luteal phase of menstrual cycle in humans, when the proliferative stimulus of estradiol on the endometrium is not required. Sulfated steroids may also provide a soluble inactive transport form that can be regenerated into its active form at the target tissue by a sulfatase activity (reviewed by Falany, 1997).

Plant soluble sulfotransferases

The study of enzymes that catalyze the sulfonation reaction in plants has lagged behind as compared to mammalian systems. This may be due to the fact that the function of plant sulfated metabolites is difficult to predict, given that their accumulation is often restricted to a limited number of species. However in recent years, the identification and cloning of a number of plant STs has revealed that they participate in the sulfonation of important metabolites. The first plant STs that were characterized are the flavonol STs.

Flavonol sulfotransferases

In order to elucidate the role of flavonol sulfation in plants, four position-specific STs were isolated and characterized from *Flaveria chloraefolia* and *Flaveria bidentis*

(reviewed in Varin et al., 1997a). Flavonol 3-ST exhibits strict specificity for position 3 of flavonol aglycones, while the flavonol 3'-ST and the flavonol 4'-ST sulfonate hydroxyl groups at positions 3' and 4', respectively of flavonol monosulfates (quercetin 3-sulfate; figure 1.1). The flavonol 7-ST purified from *Flaveria bidentis*, accepts flavonol 3, 3' or 3, 4' disulfates preferentially. The strict substrate specificity of the above enzymes lead to the proposal of sequential enzymatic synthesis of the end product, quercetin 3, 7, 3', 4'-tetrasulfate beginning with the quercetin aglycone. The flavonol 3- and 4'-STs from *Flaveria chloraefolia* were the first plant STs for which cDNA clones were isolated and characterized (Varin et al., 1992) thereby allowing the establishment of the general structure of sulfotransferases. Structure-function studies of flavonol 3-ST identified the specific amino acids involved in catalysis, as well as cosubstrate and substrate binding. Despite extensive knowledge of the biochemistry of flavonol sulfates, information regarding their biological function is limited.

Glucosinolate sulfotransferase

Glucosinolates (GIs) are hydrophilic thioglucosides present in all members of the Brassicaceae (Figure 1.1). These molecules are composed of two parts, a common glycone moiety and a variable aglycone side chain derived from α-amino acids. Glucosinolates can be divided into three major classes depending on the nature of the side chains, which may be derived from aliphatic, indolyl, or benzyl amino acids (Poulton and Moller, 1993). After tissue damage or food processing, the GIs are hydrolyzed by a thioglucosidase enzyme to yield glucose and a variety of reactive products such as isothiocyanates, organic nitriles and thiocyanates (Poulton and Moller, 1993). The

isothiocyanates released are toxic to some microorganisms and pests (Mithen, 1992). It has been demonstrated that some glucosinolates may serve as feeding deterrents to some insect species but may attract others (Blau et al., 1978). The final steps in the biosynthesis of GIs are catalyzed by thiohydroximate glucosyltransferase and desulfo-GI ST. Two research groups have reported on the extensive purification of the desulfo-GI ST from two different species. Glendening and Poulton (1990) reported the purification of desulfoglucosinolate ST from light-grown cress (*Lepedium sativum*) seedlings. Jain et al (1990) described a 230-fold purification of a desulfo-GI ST with 58% yield from *Brassica juncea* cell cultures through gel filtration and ion exchange chromatography. Desulfoglucosinolate ST activity has been demonstrated in purified extracts in a number of cruciferous plants including *Brassica campestris, Sinapis alba, Tropaeolum majus* and *A. thaliana* (Glendening and Poulton, 1990).

Choline sulfotransferase

Choline sulfate ($(CH_3)_3$-N^+-CH_2-CH_2-CH-O-SO_3^-) accumulates in the *Limonium* species and has been detected in all other salt stress-tolerant plants from the Plumbaginaceae family that have been investigated (Hanson et al., 1994). Salinization was found to induce two to threefold increase in the levels of choline sulfate in Limonium. It has therefore been hypothesized that choline sulfate and other betaines act as osmoprotective compounds in response to salinity and drought stress. Feeding experiments showed that in Limonium plants, [^{14}C]-choline was converted to [^{14}C]-choline sulfate suggesting the presence of a choline sulfotransferase. Choline sulfotransferase activity was first observed in the root protein extracts of *Limonium sinuatum* (Rivoal et al., 1994). The choline sulfotransferase activity was also found in

other members of genus Limonium and could not be detected in plants such as maize, sunflower and Brassica, which do not accumulate choline sulfate. However, the purification to homogeneity or the isolation of a cDNA clone encoding a choline sulfotransferase has yet to be reported.

Steroid sulfotransferases

Three genes coding for brassinosteroid STs, designated BnST1 to BnST3 were isolated from *Brassica napus* genomic library using RaRO47 as a probe (Rouleau et al., 1999). RaRO47 was the first cDNA clone encoding a putative ST to be characterized from *A. thaliana*. In order to simplify the nomenclature of ST-coding genes, RaRO47 was renamed AtST1. The BnST sequences share 85 to 87% amino acid sequence identity with AtST1, suggesting that BnSTs represent orthologs of AtST1 in *B. napus*. Biochemical analysis of recombinant BnST1 and BnST3 revealed enzymatic activity with estrogenic steroids but did not accept structurally related compounds such as androgens, phytosterols or ecdysteroids (Rouleau et al., 1999). In contrast with mammalian estrogen ST that catalyses the transfer of the sulfonate group to phenolic hydroxyl at position 3 of estrogens, BnST3 sulfonates the hydroxyl group at position 17 of estradiol. The enzymatic activity of BnST3 with plant brassinosteroids was extensively characterized. Based on these studies, it has been proposed that 24-epicathasterone may be sulfated *in vivo* at position 22 of the steroid side chain. It has been proposed that brassinosteroid sulfonation results in the inactivation of their biological activity.

Brassinosteroids (BRs) are naturally occurring polyhydroxysteroids found in plants. Sulfate conjugates of brassinosteroids have not yet been identified in plants. However, indirect evidence suggests that steroid sulfates may exist in plants. Feeding

experiments with [^{3}H]-castasterone, a precursor of brassinosteroid biosynthesis, to mung bean explants showed that, in addition to the glucoside conjugate, a polar non-glycosidic conjugate was formed that could not be released by enzymatic hydrolysis (Yokota et al., 1991). Similar experiments were done with rice seedlings and based on the chromatographic behavior and susceptibility to solvolysis, it was proposed that these conjugates may be sulfate esters (Yokota et al., 1992).

Sulfated compounds found in plants

There exists an extensive diversification in the types of compounds that accumulate in various plant species. Some of the sulfated metabolites like glucosinolates have long been known, however they occur only in the Brassicaceae. Other compounds like sulfolipids are present in most photosynthetic organisms. An extensive literature search revealed that a number of sulfated metabolites with diverse structures accumulate in a variety of plant species (Table 1.1). The following section describes briefly these compounds.

Sulfolipids

The sulfolipid, sulfoquinovosyl diacylglycerol (SQDG) (Figure 1.1), is the most abundant sulfur-organic compound found in higher plants and other photosynthetic organisms (Haines, 1973). It is characterized by its unique sulfonic acid head group, a 6-deoxy-6-sulfo-glucose, referred to as sulfoquinovose. The final assembly of the sulfolipid occurs with the transfer of the sulfoquinovosyl moiety from UDP-sulfoquinovose to diacylglycerol (reviewed by Benning, 1998). Despite its abundance, very little is known

Table 1.1: Sulfated compounds known to occur in different plant species.

Compound	Compund type	Source	Reference
Corynoline-11-O-sulfate	alkaloid	*Corydalis Incisa*	Iwasa et al., 1979
Betanin-3'-sulfate	betacyanin	*Rivinia Humilis*	Imperato. F, 1975
Prebetanin	betacyanin	*Beta vulgaris.L.*	Wyler et al., 1967
Wedeloside-4'-sulfate	diterpenoid	*Wedelia asperrima*	Calanasen et al., 1998
Ramnan sulfate	polysaccharide	*Monostroma latissimum*	Lee et al., 1998
Rutaretin-1-1"-sulfate	coumarin	*Seseli libanotis*	Lemmich et al., 1984
Lomatin-3-sulfate	coumarin	*Seseli libanotis*	Lemmich et al., 1984
Khellactone-3'-sulfate	coumarin	*Seseli libanotis*	Lemmich et al., 1984
Emodin-1-glucoside sulfate	anthraquinone	*Rumex pulcher*	Harborne et al., 1977
Cardiospermine sulfate	cyanogenic glucoside	*Cardiospermum grandiflorum*	Hübel et al., 1979
Passicoccin	cyanogenic glycoside	*Passiflora coccinea*	Spencer et al., 1985

Table 1.1: Sulfated compounds known to occur in different plant species.

Compound	Compund type	Source	Reference
Malvidin 3-glucoside-5-(2"- sulphatoglucoside)	anthocyanin	*Babiana sticta*	Toki et al., 1994
Malvidin 3-glucoside-5-(2"-sulphato-6"-malonylglucoside)	anthocyanin	*Babiana sticta*	Toki et al., 1994
1-*p*-Coumarylglucose 2-sulfate	hydroxycinnamic acid	*Adiantum capillus*	Imperato. F, 1982
1-Caffeylgalactose 6-sulfate	hydroxycinnamic acid	*Adiantum capillus*	Imperato. F, 1982
2-O-*p*-Coumarylglucose 6-sulfate	cinnamic acid	*Asplenium fontanum*	Cooper-D. et al., 1976
1-Caffeylglucose 3-sulfate	cinnamic acid	*Ceterach officinarum*	Cooper-D. et al., 1976
15-deoxylactucin-8-sulfate	sesquiterpene lactone	*Lactuca sativa*	Sessa et al., 2000
15-*p*-hydroxyphenyl-acetyl lactucin-8-sulfate	sesquiterpene lactone	*Lactuca sativa*	Sessa et al., 2000
3β-sulfate ester of gypsogenin-trisaccharide	saponin	*Gypsophila bermejoi*	Acebes et al., 1998

Table 1.1: Sulfated compounds known to occur in different plant species.

Compound	Compund type	Source	Reference
23-sulfate ester of hederagenin-trisaccharide	saponin	*Gypsophila bermejoi*	Acebes et al., 1998
Benzyl sulfate	phenol	blackberry, blueberry, Vine cv. Muscat etc	Boss et al., 1999
2-Phenylethyl sulfate	phenol	Blackberry, blueberry, cress, rosehip, strawberry	Boss et al., 1999
α-Ionol sulfate	norisoprenoid	blueberry, elder, raspberry redcurrent, strawberry	Boss et al., 1999
Vomifoliol	norisoprenoid	blueberry, cress, strawberry, Vine cv. Shiraz	Boss et al., 1999
p-Coumaric acid sulfate	phenolic acid	*Zostera marina*	Todd et al., 1993

about the biosynthesis of the precursor UDP-sulfoquinovose. It has been proposed that the sulfate group is added to UDP-glucose. However, there is no evidence of such an enzyme activity.

Riboflavin sulfates

Riboflavin sulfates (Figure 1.1) were identified for the first time in the roots of iron deficient sugar beet (Susin et al., 1993). Riboflavin 3'-sulfate and riboflavin 5'-sulfate levels account for 82% and 15% of total flavin concentration in iron deficient roots of B*eta vulgaris*. The localization of riboflavin sulfates in iron deficient roots was found to be similar to that of iron reducing activity. Therefore, based on similar localization and the fact that the rates of Fe(III) reduction by the roots is 10-20 times higher in iron deficient sugar beet as compared to control, the authors suggested a relationship between the presence of flavin sulfates at high concentrations and elevated iron reducing capacity. Thus riboflavin sulfates may be playing an integral role in the plant response to iron deficiency.

12-hydroxysulfonyloxy jasmonic acid

In 1994, Achenbach et al. reported the isolation and identification of 12-hydroxysulphonyloxyjasmonic acid (Figure 1.1) from *Tribulus cistoides* (Zygophyllaceae) when they were trying to isolate saponins from this plant. There are no reports about the enzymes involved in the biosynthesis of this compound from its precursor JA or its physiological role in plants. However the unsulfated compound 12-

hydroxyjasmonic acid, was shown to have tuber-inducing activity in *in vitro* assays (Yoshihara et al., 1989)

Flavonols and flavonol sulfates

Flavonoids are phenolic compounds composed of two aromatic rings linked by a C3 unit. Based on the degree of oxidation of the heterocyclic ring, flavonoids are divided into flavones, flavonols, flavanones, isoflavonoids and anthocyanins. Each type of flavonoid can undergo further modifications such as hydroxylation, methylation, acylation, glucosylation and/or rhamnosylation, resulting in the enormous diversity of flavonoids found in nature. The flavonoids are widely distributed in the plant kingdom, which along with isoprenoids and alkaloids, form a major category of plant secondary products.

Physiological roles of flavonoids

UV-B protection

Flavonoids absorb light over a wide range of the light spectrum. It has been proposed that flavonoids act as 'sunscreens' for plant tissues. Flavonoid biosynthesis is induced by exposure to UV-B radiation (Li et al., 1993). Direct evidence of flavonoids serving as UV-protectants has been provided by studies with *Arabidopsis* mutants exhibiting a reduced flavonoid content, resulting in hypersensitivity to UV-B radiation (Li et al., 1993). Consistent with this report, UV-sensitive *Arabidopsis* mutants have been identified that are defective in flavonoid biosynthesis (Lois and Buchanan, 1994). The

UV- sensitive phenotype in this mutant appears to be directly related to the absence of kaempferol glycosides in these plants.

Plant fertility

The connection between flavonoids and fertility was first proposed from the studies of a maize mutant that is self-sterile (Coe et al., 1981) and deficient in chalcone synthase (enzyme in flavonoid biosynthetic pathway). Similarly, transgenic petunia plants in which chalcone synthase activity was disrupted by antisense suppression, exhibited reduced fertility (Van der Meer et al., 1992). The infertile phenotype could be complemented by the addition of flavonols allowing the mutant pollen to germinate. However, *A. thaliana tt4* mutant, defective in chalcone synthase activity, lacks flavonoids in the reproductive organs and is fertile, suggesting that flavonols are not essential for fertilization in *A. thaliana* (Ylstra et al., 1996; Burbulis et al; 1996).

Plant microbe interactions

Flavonoids have been proposed to function as defense factors against fungi. A variety of antifungal flavonoids, including flavans, flavanones, 3-hydroxy flavonones and flavonols have been identified in the sap wood of trees (Kemp and Burden, 1986). The role of flavonoids in the interaction of legumes with symbiotic bacteria during nodulation has been demonstrated (Clarke et al., 1992). Flavonoids released by the host plant roots induce bacterial nodulation genes that are involved in determining the specificity of the interaction.

Auxin transport

Auxin is known to be transported in a polar manner from the shoot tip and the root tip in a number of plant species. The herbicide 1, *N*-napththylphthalamic acid (NPA)

can block auxin efflux from plant cells. This results in continued uptake and accumulation of the hormone. Flavonols have been found to displace NPA from its binding site blocking polar auxin efflux, thereby stimulating auxin accumulation (Jacob and Rubery, 1988; Faulkner and Rubery, 1992). Therefore, polar auxin transport may be regulated *in vivo* by endogenous flavonoids. Recent evidence supports this role for flavonoids. [^{14}C]-Indole acetic acid transport studies showed that the flavonoid deficient *tt4* mutant seedlings exhibit altered patterns of auxin distribution as compared to wild type plants. Less auxin was retained in some tissues along the polar transport pathway, for example, the cotyledonary node and root tip. Treatment of mutant plants with the flavonoid intermediate naringenin, resulted in normal auxin distribution in the seedling and retention by the roots (Murphy et al., 2000).

Flavonoid biosynthesis

Flavonoids are derived from the phenylpropanoid pathway that mediate the conversion of phenylalanine to pigments, lignins and phytoalexins. The branch pathway leading flavonoid synthesis begins with the condensation of three acetate units from malonyl-CoA with *p*-coumaryl-CoA. In *Arabidopsis*, 11 loci required for flavonoid biosynthesis have been identified. The loci are called *transparent testa* (*tt*), because the altered seed color is due to an absence of pigments in the seed coat (Koornneef et al., 1981). These mutants have contributed to the elucidation of the various physiological roles of flavonols. However, analysis of mutants of the same enzyme in different plant species do not produce similar phenotypic consequences (for example, chalcone synthase mutant of petunia and *Arabidopsis*). This suggests that flavonoids are not universally

required for similar functions in different plant species. It has been suggested that, some flavonoid functions may have been lost during evolution in certain lineages or could have arisen in others.

Flavonols of Arabidopsis thaliana

A wide body of literature is available on the molecular biology and biosynthesis of flavonoids in *Arabidopsis*. Nevertheless, the structure of phenolics that occur in *Arabidopsis* have not been studied in detail. To date kaempferol, quercetin, myricetin and apigenin and their glycoside derivatives are the only flavonols that have been identified in *Arabidopsis* (Shirley et al., 1995; Burbulis et al., 1996; Bhushan and Dhiman, 1984). Recent studies have shown that quercetin and kaempferol occur as glycosylated compounds in *Arabidopsis* with glucose and/or rhamnose residues linked at the positions 3 and 7 of the flavonol ring system (Veit and Pauli, 1999).

Flavonoid sulfates

The first flavonoid sulfate was reported in 1937, but it was not until 1975 that flavonoid sulfates were considered to be of common occurrence in a number of plant species. Most of the compounds are sulfate esters of hydroxyflavones and hydroxyflavonols and their methyl esters. The most common site for flavonol sulfonation is position 3. In addition, sulfonation at position 7 is also frequent, after 3-sulfonation. Since flavonoids are polyhydroxylated compounds, upto tetrasulfated flavonols are known to occur specially in the *Flaveria* species. The 3'- and 4'-sulfated flavonols have also been identified, but these are usually di- or tri-sulfates where positions 3 and/or 7

have already been sulfonated. In flavonol glycosides, since position 3 is most commonly substituted with sugar, the sulfate group is found at position 7. Some flavonol glycosides are also sulfonated on the sugar moiety, in most cases at the 3"- or 6"- position. (Barron et al., 1988). Flavones are also known to occur as their sulfated conjugates. Flavones lack the hydroxyl group at position 3, therefore sulfonation at position 7 is most common in this group of flavonoids. Today more than 80 different sulfonated flavonoids are known to occur in a number of plant families (Williams and Harborne, 1994). There is no report on the identification of flavonol sulfates from *A. thaliana* to date.

Jasmonic acid (JA)

Jasmonic acid is a signaling compound, derived from linolenic acid or hexadecatrienoic acid. It is involved in the regulation of different processes in plants such as maturation and release of pollen, tuberization, response to wounding and defense against fungal pathogens. The methyl ester of jasmonic acid, methyl jasmonate, was first identified by Demole et al. (1962) as the aroma of the essential oil from *Jasminum grandiflorum*. JA was later isolated from the culture filtrate of the fungus, *Botryodiplodia theobromae* (Aldridge et al., 1971).

Physiological roles of jasmonates

Jasmonates have been proposed to influence numerous aspects of plant growth, development and response to stress. They can modulate fruit ripening, root growth, tendril coiling, production of viable pollen and plant resistance to pathogens (reviewed by Sembdner and Parthier, 1993). In the following section, I will briefly describe some of

physiological roles of jasmonates that have gained interest in recent years and that are relevant to my study.

Defense response

Numerous lines of evidence support the idea that JA is involved in the wound and pathogen induced plant defense response of plants. JA accumulates in plants upon wounding and following elicitor treatment or pathogen infection (Creelman et al., 1992). In addition, JA activates the expression of protease inhibitors that participate in the protection of plants from insect damage (Johnson et al., 1989) and activates expression of antifungal proteins like thionins and osmotin (Xu et al., 1994). Fungal elicitors induce transient accumulation of JA, as well as synthesis of several classes of phytoalexins in cell suspension cultures of a number of plant species (Blechert et al., 1995).

Exogenous application of jasmonates induces expression of some antimicrobial compounds. The defensin gene *PDF1.2* of *Arabidopsis*, is strongly induced systemically by pathogen challenge and Me-JA but not by salicylic acid (Penninckx et al., 1996). Plant defensins are cysteine-rich basic proteins that are structurally related to antimicrobial insect defensins. A number of plant defensins are potent inhibitors of fungal growth. The fungal pathogen *Alternaria brassicola* does not induce expression of *PDF1.2* in the jasmonate response mutant *coi1*, suggesting that *PDF1.2* expression is dependent on the jasmonate response. Similarly, the expression of the *Arabidopsis Thi2.1* that codes for an antimicrobial peptide thionin, is specifically upregulated by both pathogen infection and JA (Epple et al., 1995). JA deficient mutants have been shown to be more susceptible to pathogens as compared to wild type plants. For example, the tomato *def1* mutant that is deficient in JA, is more susceptible to damage by tobacco hornworm larvae (Howe et al.,

1996). Similarly, the *Arabidopsis fad3-2 fad7-2 fad8* (McConn and Browse., 1996) triple mutant which does not accumulate JA, is more susceptible to the soil borne pathogenic fungus, *Pythium mestophorum*, as compared to wild type plants (Vijayan et al., 1998). In addition, some defense responses to UV are also mediated by alterations in the levels of JA through the accumulation of defense response genes like the protease inhibitors, *PIN1* and *PIN2* (Reviewed in Creelman and Mullet, 1997).

Vegetative sinks and storage proteins

JA levels are high in vegetative sink tissues, such as soybean axes, plumules and hypocotyl hooks and in young growing leaves (Creelman and Mullet, 1995). High levels of JA in these tissues correlates well with vegetative storage protein (VSP) expression. High levels of JA are also found in developing reproductive tissues, suggesting that JA plays a role in the formation of flowers, fruits and seeds (Creelman and Mullet, 1995). Soybean *VSP* genes and *Arabidopsis AtVSP* are highly expressed in flowers and developing fruit (Berger et al., 1995). VSPs in these tissues may therefore, provide a temporary storage form of carbon and nitrogen. The *coi1* (jasmonate response mutant) and the *fad3-2 fad7-2 fad8* (fatty acid desaturase deficient) mutants do not express *AtVSP*, indicating a requirement for JA for their upregulation (reviewed in Creelman and Mullet, 1997).

Tuberization

Jasmonic acid has been proposed to induce tuberization in yam and also in Jerusalem artichoke (*Helianthus tuberosus L.*) plants (Koda and Kikuta, 1991). Using potato stem segment assays it has been demonstrated that jasmonic acid, and its

metabolite 12-hydroxyjasmonic acid, can induce tuber formation in potato (Koda, 1992). This biological function of JA, will be discussed in detail in subsequent sections.

Senescence and growth inhibition

JA treatment has been shown to cause a loss of chlorophyll, which is a marker of senescence in leaves and cell cultures (Weidhase et al., 1987). The mRNA of *A. thaliana* chlorophyllase (*AtCLH1*), the first enzyme involved in chlorophyll degradation is induced by Me-JA (Tsuchiya et al., 1999). This ability of Me-JA to promote senescence of oat leaves and chlorophyll degradation in barley leaves led to the hypothesis that jasmonates play a role in senescence (Ueda et al., 1981; Weidhase et al., 1987). However, this phenomenon may be the result of treatment at higher toxic levels of Me-JA than normally found *in vivo*. On the other hand, elevated levels of JA are found endogenously in zones of cell division, young leaves and reproductive structures. Thus although, JA induces senescence-like symptoms, the physiological role and mode of action in leaf senescence is still speculative (reviewed in Creelman and Mullet, 1997).

Plant reproduction

The critical requirement of jasmonate in plant reproduction was suggested by the analysis of the *Arabidopsis* triple mutant *fad3-2 fad7-2 fad8*, that lacks the jasmonate biosynthetic precursors, hexadecatrienoic acid and linolenic acid. The triple mutant plants are male sterile and the phenotype can be reversed by treatment with JA (McConn and Browse, 1996). Scanning electron microscopy showed that the pollen on the mutant anther appeared morphologically normal, however the mutant plants lacked proper pollen dehiscence (release of pollen from anther). Recently two groups almost simultaneously reported on the characterization of 12-oxophytodienoate reductase (OPR3), an enzyme in

the JA biosynthetic pathway (Stintzi and Browse, 2000; Sanders et al. 2000) and demonstrated that, OPR3 mutants are male sterile. It was also shown that JA and not OPDA is required for pollen fertility and the OPR3 isoform of *Arabidopsis* codes for the protein that can reduce the correct stereoisomer of OPDA to produce JA. Exogenous JA as well as overexpression of wild type OPR3 in the mutant rescued the mutant phenotype. Sanders et al. (2000), called this gene *delayed dehiscence1* and demonstrated that in this mutant the anthers release pollen too late for pollination to occur, concluding that jasmonic acid plays a role in controlling the timing of pollen release in the flower. The *Arabidopsis coi1* mutant which is unresponsive to jasmonate is also male sterile, indicating the role of JA signal transduction in plant reproduction (Feys et al., 1994).

Biosynthesis of jasmonic acid

At least two pathways for the synthesis of JA have been proposed (Figure 1.2), the established octadecanoid pathway from linolenic acid (18:3) and the newly proposed hexadecanoid pathway from hexadecatrienoic acid (16:3).

Octadecanoid pathway

A phospholipase releasing unsaturated fatty acids from membranes has been proposed to initiate JA biosynthesis from α-linolenic acid (LA). This fatty acid is converted to 13-hydroperoxy LA by lipoxygenase (LOX) and involves incorporation of molecular oxygen. In *A. thaliana*, two different lipoxygenase genes, *AtLOX1* and *AtLOX2* have been identified (Bell and Mullet, 1993; Melan et al., 1993). *AtLOX2* is a

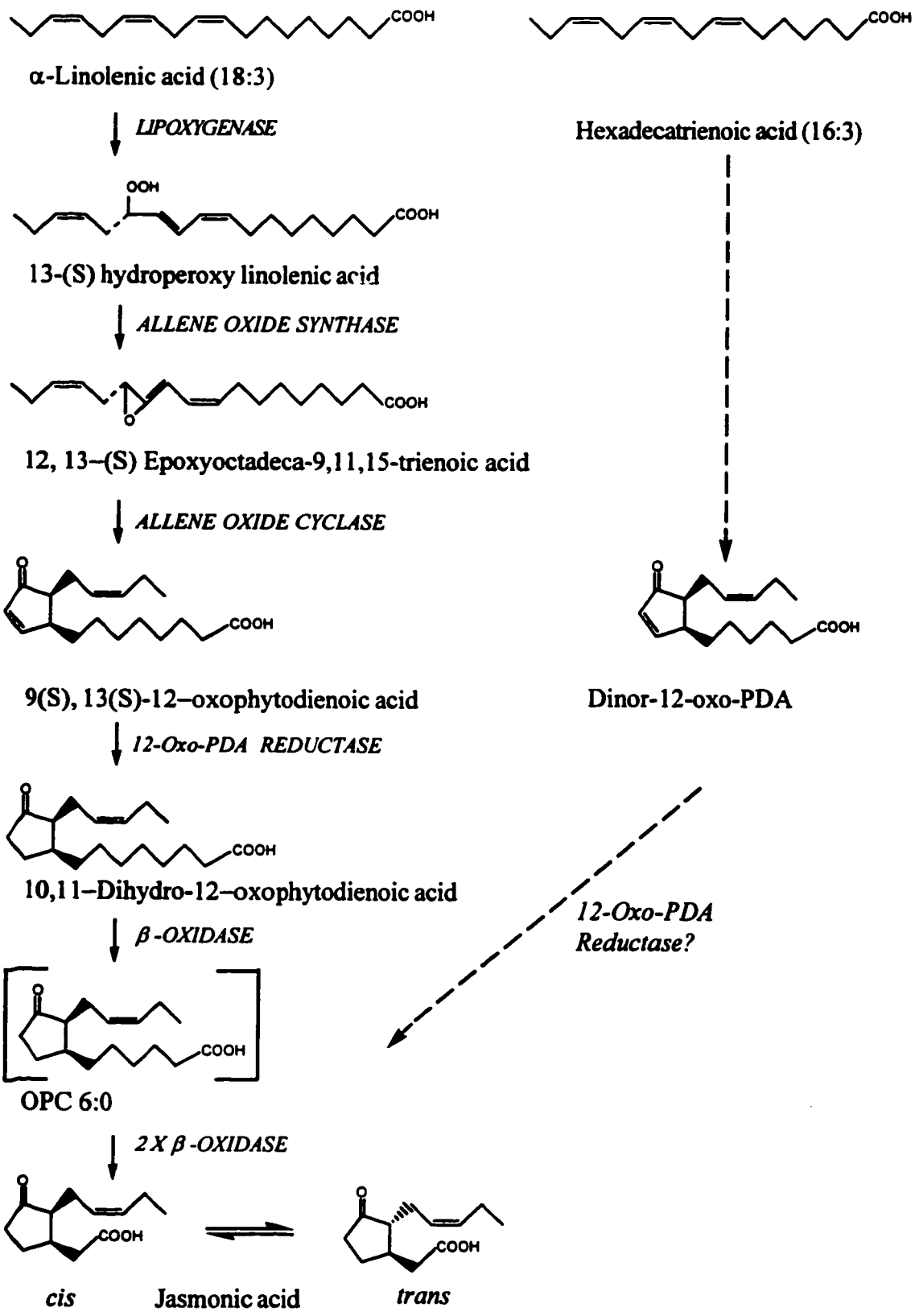


Figure 1.2: Biosynthesis of jasmonic acid via the Octadecanoid and Hexadecanoid pathways (Adapted from Hedden and Andrew, 2000)

chloroplastic lipoxygenase whose transcript levels increase after jasmonic acid treatment. JA synthesis was stimulated several-fold in wounded wild type *Arabidopsis* but was not detected in transgenic plants with reduced levels of AtLOX2. (Bell et al., 1995). The JA levels in unwounded transgenic plants were similar to wild type plants, indicating that *AtLOX2* is involved in *de novo* wound-induced JA synthesis, whereas basal JA levels are maintained by a different lipoxygenase isoform, which is not affected in the *AtLOX2*-cosupressed plants. Similarly in potato, the LOX gene family is comprised of three members (LOX1, LOX2 and LOX3) that differ in expression patterns, enzymatic properties and organ-specific expression, *LOX1* being expressed mostly in tubers and roots, *LOX2* in leaves, and *LOX3* in leaves and roots.

13-Hydroperoxy LA formed by LOX activity is converted to an unstable allene oxide by allene oxide synthase (AOS). cDNA coding for AOS have been cloned from flax (Song et al., 1993), *Arabidopsis* (Laudert et al., 1996), tomato (Howe et al., 2000) and barley (Maucher et al., 2000). Both the flax and the *Arabidopsis* enzymes contain chloroplast targeting sequences. Overexpression of flax AOS in transgenic potato plants increased JA levels (Harms et al., 1995), indicating that AOS protein may be the rate-limiting step in JA biosynthesis. Overexpression of *Arabidopsis* AOS in *Arabidopsis* and tobacco did not alter the basal levels of JA, suggesting the limiting amounts of AOS substrate under normal conditions (Laudert et al., 2000).

Allene oxide cyclase (AOC) catalyses the cyclization of the unstable allene oxide to (9S, 13S)-12-oxo-phytodienoic acid (OPDA). This enzyme has been recently cloned from tomato and has been shown to be chloroplast-localized (Ziegler et al., 2000). Wounding

of tomato leaves transiently increased the AOC mRNA, indicating its upregulation during the wound response.

12-oxo-phytodienoic acid reductase (OPR) catalyses the reduction of the 10, 11-double bond in the cyclopentanone ring of 12-OPDA to form dihydro-12-OPDA. The enzyme activity has been demonstrated from seed and seedling of corn by Vick and Zimmerman (1986). Three 12-OPDA reductases have been identified and cloned from *Arabidopsis* (Schaller and Weiler, 1997; Biesgen and Weiler, 1999; Müssig et al., 2000). Biochemical studies performed on purified OPR1 and OPR2 showed that these two isoforms were able to reduce 9*S*, 13*S*-OPDA very poorly, whereas OPR3, a third isoform was highly effective in reducing 9*S*, 13*S*-OPDA, the naturally occurring isomer in plants (Schaller et al., 2000). The initial steps in JA biosynthesis upto the formation of OPDA are catalyzed by chloroplastic enzymes. The *OPR3* has a SRL peptide sequence at the carboxy-terminal indicating that the enzyme is probably peroxisome-localized. However, the OPR1 and OPR2 lack this peroxisome targeting sequence. The final step in JA biosynthesis involves removal of six carbons from the carboxyl side chain by three successive β-oxidation reactions. The enzyme(s) and the compartment in which these transformations take place are not known.

Hexadecanoid pathway

Analysis of plant extracts of *Arabidopsis* and potato revealed a new compound very similar in structure to 12-OPDA (Weber et al., 1997), called dinor-oxo-phytodienoic acid (Dn-OPDA). Dn-OPDA cannot be detected in the *Arabidopsis* mutant *fad5*, that cannot synthesize 7,10,13-hexadecatrienoic acid (16:3). Dn-OPDA can be synthesized in

vitro from hexadecatrienoic acid (16:3). This suggests that dn-OPDA is synthesized directly from plastid 7,10,13-hexadecatrienoic acid (16:3) and not from α-oxidation of the 18-carbon, 12-OPDA (Figure 1.2). How the octadecanoid and hexadecanoid biosynthetic pathways regulate the levels of jasmonates has not been characterized in detail, however analysis of the *fad5* mutant, which is defective in 16:0 desaturase provides a preliminary answer to this question. Levels of JA, OPDA and dn-OPDA increase in wounded *Arabidopsis* and potato leaves. Surprisingly, unwounded leaves of *fad5* mutant had reduced levels of OPDA as compared to unwounded wild type leaves. However, upon wounding, both kinds of plants accumulated similar amounts of OPDA (Weber et al., 1997). This suggests that OPDA levels are differentially regulated in wounded and unwounded tissues. The dn-OPDA pathway may be involved in regulating OPDA levels in unwounded tissues.

Oxylipin signature

The jasmonate family is comprised of JA and cylcopentenones like OPDA and dn-OPDA. The levels of these compounds and their biosynthetic precursors vary among species. Weber et al., (1997) referred to the relative and absolute concentration of these compounds as 'oxylipin signature'. The term 'oxylipin signature' has been used to indicate the complexity of jasmonate signaling where single or multiple members derived from the hexadecanoid and octadecanoid pathways may contribute to signal generation. The cyclopentenones are powerful *in vivo* signals and in some cases their biological

activities are greater than JA. For example, OPDA is a far more effective inducer of tendril coiling in *Bryonia dioica* than JA (Weiler et al., 1994).

Comparison of jasmonates and mammalian prostaglandins

12-OPDA is structurally very similar to mammalian prostaglandins. Biosynthesis of both types of compounds initiates with the introduction of molecular oxygen into unsaturated fatty acids by similar enzymes, such as lipoxygenases (reviewed by Mueller, 1997). Similar to the role of plant jasmonates in triggering the defense response, one of the role of prostaglandins in mammals is to mediate inflammation or defense locally in the tissue where they are produced.

However there are important differences between the plant and mammalian syntheic pathways and in their signal transduction. The first lipid metabolizing enzymes in mammals, such as lipoxygenase and cycloxygenase are localized in the nucleus or ER membrane (Chilton et al., 1996). In contrast, the plant lipoxygenase and AOS are localized in the chloroplast. It is well established that prostaglandins exert their actions extracellularly via G-protein-coupled receptors on the cell membrane of target cells. However, they may also activate transcription factors (Devchand et al., 1996). Jasmonates are thought to act intracellularly to activate signaling pathways that alter gene expression. Protein phosphorylation has been shown to be involved in or downstream of JA perception necessary for gene activation (Rojo et al., 1998).

Jasmonate signal transduction

It is presumed that jasmonate receptors interact with intracellular targets to trigger changes in transcription and/or translation of genes participating in the jasmonate response. Analysis of promoters of two jasmonate-inducible genes, *PIN2* and *VSPB* (Kim et al., 1992; Mason et al., 1993) has resulted in the identification of the jasmonate response element that contains a G box sequence (CACGTG), which serves as a potential binding site for bZIp transcription factors. However, mutagenesis of this G box in *PIN2* promoter did not prevent JA-mediated induction.

Four classes of jasmonate insensitive mutants have been identified: *jar1, coi1, jin1 and jin4* (Staswick et al., 1992; Benedetti, 1995; Berger et al., 1996). The *jar1, jin1 and jin4* mutants were recovered by screening for plants growing on 10 to 100 μM Me-JA, that would otherwise inhibit wild type root growth. The *jar1-1* mutant is severely affected by the soil fungus, *Pythium irregulare* (Staswick et al., 1998), suggesting that it is defective in the defense response to pathogen infection.

The *coi1* mutant was identified by virtue of its resistance to coronatine, a chlorosis-inducing toxin (Feys et al., 1994). *Coi1* is insensitive to Me-JA, male sterile and exhibits decreased expression of VSPs, thionins and plant defensins. The predicted amino acid sequence of the COI1 protein contains 16 leucine-rich repeats and an F-box motif and may function by targeting repressor proteins for removal by ubiquitination. Using a yeast two-hybrid system, it was shown that COI1 interacts with the components of the ubiquitination complex. This indicates that selective proteolysis is required for jasmonate action in *Arabidopsis* (Rostro et al., 2000).

Using a VSP: luciferase transgenic *Arabidopsis* construct, a mutant, constitutive expresser of *VSP1* (*cev1*) was isolated. The mutant exhibited stunted growth, constitutively expressed *VSP2* and *THI 2.1* and exhibited enhanced resistance to powdery mildew as compared to wild type plants indicating that it participates in the jasmonate signal transduction pathway (Ellis and Turner, 2001).

Wound inducible gene expression in tobacco has been postulated to involve protein phosphorylation (Seo et al., 1995). No receptors have yet been reported for jasmonates in plants and their subcellular localization is also unknown.

Conjugates of jasmonic acid

Biotransformation of JA involves four major reactions (Sembdner and Parthier, 1993): a) hydroxylation of C-11 or C-12 to form 11-hydroxyjasmonic acid (11-OH-JA) or 12-hydroxyjasmonic acid (12-OH-JA) b) reduction of the C-6 keto group to form cucurbic acid which can also be hydroxylated on the side chain, c) the above hydroxylated compounds can undergo O-glycosylation, d) conjugation of JA or its hydroxylated derivatives with amino acids. 9,10-Dihydrojasmonic acid (DJA) has been found in broad bean and [^{14}C]-labeled feeding experiments demonstrated that DJA forms similar hydroxylated conjugates as those with JA (Meyer et al., 1989).

Generally, hormone conjugates act as reversible deactivated storage forms or are involved in irreversible inactivation of the hormone (Sembdner et al., 1994). However, some of the jasmonic acid conjugates have been proposed to exert physiological effects. The 12-O-β-D-glucopyranoside of 12-OH-JA induces tuber formation *in vitro* (Koda,

1992). In barley leaf segments, a JA-isoleucine conjugate was found to be active in inducing jasmonate-inducible proteins (JIPS) (Herrmann et al., 1987). Me-JA could be considered as the methyl ester conjugate of JA. Me-JA has physiological potencies equal to or more than that of free JA. Since methyl jasmonate is volatile and more hydrophobic due to the methyl group, the difference in physiological potencies may be due to the more efficient uptake of methyl jasmonate. None of the enzymes involved in the synthesis of JA conjugates have been identified. It has been proposed that 12-OH-JA is synthesized by direct hydroxylation of JA. However so far there is no report of such an enzyme activity.

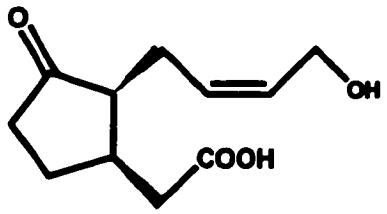
Tuberonic acid

Tuber producing species will form tubers under tuber-inducing conditions. Day length and night temperature are among the most important factors that affect the formation of tubers in *Solanum* species. Certain lines of *Solanum tuberosum* ssp. *Andigena* are strictly photoperiodic, forming tubers only under short day conditions and flowering only under long-days. As a result of grafting experiments, Gregory (1956) and Chapman (1958) demonstrated the occurrence of a tuberization stimulus, which is formed in the leaves under short day conditions and transmitted to the underground parts of the plant to induce tuberization. Although many attempts were made to identify the stimulus (reviewed by Ewing, 1995), most efforts were concentrated on the effects of known plant hormones. Using single-node potato stem segment assay, an *in vitro* bioassay for tuber inducing activity, Koda et al., (1988) demonstrated the occurrence in the leaves of *Solanum tuberosum* L. cv. Irish Cobbler of two acidic compounds that have tuber-inducing activity. The tuber-inducing activity increased under short day conditions and

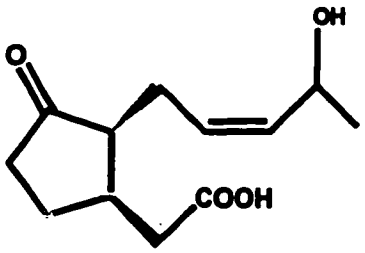
remained constant under long-days. The structure of one of the active compound was determined to be 3-oxo-2- (5'-β-D-glucopyranosyloxy-2'-Z-pentenyl)-cyclopentane-1-acetic acid (Yoshihara et al., 1989). The aglycone of this glycoside is 12-hydroxyjasmonic acid (12-OH-JA), which the authors named tuberonic acid (TA, Figure 1.3).

Helder et al. (1993) reported the effect of photoperiod on hydroxylation of JA. Under short day conditions both 12-OH-JA (TA) and 11-OH-JA (Figure 1.3) were found in the leaves of wild type *Solanum demissum* that had formed tubers. Under long-day conditions tuberization did not occur and these compounds were undetectable. 11-OH-JA, identified from potato plants grown under tuber inducing conditions is structurally very similar to 12-OH-JA, and could be a potential tuber-inducing compound. However, there are no reports on testing the biological activity of this compound as a tuber inducing substance.

When 2-[^{14}C]-JA was applied to *Solanum tuberosum* leaves under short-day and long-day conditions, it was converted to tuberonic acid glucoside (TAG) within 10 days and radioactivity migrated throughout the plant, irrespective of day length (Yoshihara et al., 1996). In addition, the radioactive TAG in the stolons of plants grown under short-day conditions, was found to be ten times higher than that of plants grown under long-day conditions. Surprisingly, the radioactivity per gram tissue under long-day conditions (non-inducing conditions for tuberization but inducing for flowering) was considerably higher in the flower buds. The authors concluded that JA is metabolized to TAG, transported to all parts of the plant and photoperiod affects the transport and localization



12-hydroxy jasmonic acid (tuberonic acid)



11-hydroxy jasmonic acid

Figure 1.3:
Chemical stuctures of 12-hydroxy jasmonic acid and 11-hydroxy jasmonic acid

of TAG (Yoshihara et al., 1996). A high accumulation of TAG in tubers and flower buds may induce tuber and flower bud formation. However, it is not clear whether accumulation of TAG in tubers and flower buds is a cause or consequence of tuberization and flowering. These experiments provide evidence that TA and TAG are synthesized by the direct hydroxylation and then glycosylation of JA.

JA, like TA, exhibits a strong potato tuber inducing activity (Koda et al., 1991). JA is capable of inducing *in vitro* tuberization in yam plants (*Dioscorea batatas*, Koda and Kikuta, 1991) and Jerusalem artichoke. However in contrast to JA, TA and TAG do not exhibit inhibitory effects on plant growth, such as promotion of leaf senescence of oat leaves, inhibition of soybean callus growth and inhibition of seedling growth of lettuce (Koda, 1992). Treatment of barley leaf segments with JA and its metabolites revealed that JA induces expression of JIP6 (6 kD jasmonate induced protein that codes for thionin) and JIP23 in barley leaves (Miersch et al., 1999). In contrast, treatment with TA does not induce JIP6 and JIP23. This suggests that TA and TAG may be synthesized for a specific function in potato plants, which may be related to tuber formation.

Concept of flowering time

The life of higher plants can be broadly divided into a vegetative phase and a reproductive phase. The shoot apical meristem (SAM) plays a central role in the floral transition. SAM is a group of stem cells that initiate structures such as leaves during vegetative growth. On floral induction, the SAM forms primordia that develop into flowers. Plants have evolved complex regulatory pathways to control when this floral transition occurs. The timing of flowering is a result of interactions between

environmental cues that signal the favorable conditions for flowering and endogenous developmental competence of the plant. Environmental factors affecting flowering time include day length, light quality, vernalization and water availability. For example, some plant species have evolved a photoperiod response pathway (some species are induced to flower under long-days, whereas others flower earlier in response to short-days). The endogenous developmental control of flowering, takes into account that plants must pass a juvenile phase during which they are not competent to flower.

Florigen hypothesis

Studies of the photoperiod-responsive plants led to the development of the florigen hypothesis which states that a flower inducer synthesized in the leaves is translocated to the shoot apex to induce the development of the floral meristem (Chailakhyan, 1936). This signal has been referred to as 'florigen'. Grafting experiments suggest that translocatable signals regulate flowering (Zeevart, 1984). For example, exposing inductive photoperiods only to the leaves causes flowering at the SAM. In addition, the flowering signal is graft transmissible from a photoperiodically induced shoot or a leaf to a non-induced graft partner (Lang et al., 1977). Although the term florigen implies a single compound, in fact the flowering signal may be a mixture of several components (Bernier et al., 1993). Despite considerable efforts, the search for the hypothetical 'florigen' hormone was unsuccessful. Molecules such as cytokinins, gibberellins and carbon assimilates have been proposed as candidates for the florigen (Bernier et al., 1993). However, these molecules did not exhibit the same effect in different species (reviewed in Levy and Dean, 1998).

Gibberellins (GAs) have been considered to be strong candidates as the flowering promoting hormone. In *Arabidopsis*, the signaling pathway mediated by GAs, promotes flowering under non-inductive photoperiods (Langridge, 1957). In support of this hypothesis it has been demonstrated that the *ga1* mutant does not flower unless provided with exogenous GAs under non-inductive photoperiod (Wilson et al., 1992). It has been proposed that GAs promote flowering by upregulating the floral identity gene, *LEAFY* (Blázquez et al., 1998). Recent analysis of the *LEAFY* promoter shows that it contains independent regulatory elements which respond to GAs and to photoperiod (Blázquez and Weigel, 2000). While there is strong evidence that GAs generally induce flowering in rosette plants like *Arabidopsis*, they inhibit flowering in many other plants (Bernier, 1988). GAs are rarely effective at inducing flowering in short day plants. In long-day plants on the other hand, the same GA can have flower promoting activity in one species but not in others (Bernier et al., 1993). In summary, the precise role of GAs in flowering does not appear to be universal. Evidence that carbon assimilates like sucrose may function as long distance signaling molecules during floral induction is provided by studies on *Sinapis alba* (Bernier et al., 1993). In addition, several *Arabidopsis* mutants defective in carbon metabolism are shown to have altered flowering time, however a comprehensive analysis of their genetic interactions has not been performed (Levy and Dean, 1998).

Recently, an unsaturated fatty acid when incubated with norepinephrine has been demonstrated to have strong flower-inducing activity in *Lemma paucicostata* (Yokoyama et al., 2000a and b). The chemical structure of the unsaturated fatty acid was determined

to be 9-hydroxy-10-oxo-12(Z), 15(Z)-octadecadienoic acid which is an α-ketol derivative of the jasmonate biosynthetic precursor, linolenic acid. However the physiological significance of 9-hydroxy-10-oxo-12(Z), 15(Z)-octadecadienoic acid remains to be examined since this compound was not detectable in fresh *Lemma* plants but only detectable under drought, heat and osmotic stress.

Recent evidence supporting the florigen hypothesis came from the characterization of the pea *GIGAS* late flowering mutant (Beveridge et al., 1996). Using grafting experiments, it was shown that this mutant is impaired in the production of a graft transmissible flower-inducing signal. Furthermore, the characterization of the maize *INDETERMINATE* (*ID1*) by Colastani et al. (1998) strongly support the florigen hypothesis. In this mutant, the terminal SAM continues to display vegetative growth indicating that ID1 is involved in some aspect of flower induction. The authors showed that *ID1* expression was not detectable in the SAM but expressed in immature leaves. The fact that ID1 is required for floral transition at the meristem, but expressed only in the leaves implies the existence of a signal mechanism between the two tissues. The mutant allele of *id1* analyzed in this study was the result of the insertion of a transposable element. The authors excised the transposable element during development to generate clonal sectors of wild type ID1 function against the overall mutant background. It was found that chimeric *id1* plants flowered earlier than the fully mutant *id1* plants, even though the SAM of these chimeric plants remained mutant.

The multifactorial model for flowering time

The key features of flowering time control include the perception, transfer and integration of multiple endogenous and environmental factors. To take into account inter-species variability and the multiplicity of putative signals, a multifactorial model was proposed (Bernier et al., 1993). In this model, growth regulators such as cytokinins and gibberellins, as well as carbon assimilates, act as promoters or inhibitors of flower formation which is triggered only when adequate levels of the stimulating and repressing molecules are present. The results of genetic studies support this more complex multifactorial model. The characterization of early- and late- flowering mutants of *A. thaliana* led to the proposal of the most favored 'multifactorial control' model for flower induction (reviewed in Simpson et al., 1999). The response of *Arabidopsis* flowering time mutants to vernalization and photoperiod combined with genetic and expression analysis has established the existence of four pathways that control flowering time in *A. thaliana.* Two of these pathways mediate signals from the environment. The photoperiod promotion pathway integrates day-length into flowering decision through a number of genes that sense and respond to regular day-to-night transition. Mutants in this pathway flower late under long-day conditions. The vernalization promotion pathway promotes flowering in many late-flowering ecotypes in response to an extended period of cold temperature. Therefore, mutants in this pathway do not show accelerated flowering following cold treatment. The third pathway termed the autonomous promotion pathway, promotes flowering independent of environmental signals. Plants carrying mutations in genes of this pathway flower late both under long-day and short-day conditions. Members of this group probably monitor various endogenous cues such as the developmental stage

of the plant. The gibberellin pathway includes genes of the gibberellin biosynthetic pathway and signal transduction, which when mutated result in early or late flowering *Arabidopsis* plants.

In addition to genes determining the correct timing of flowering, the flowering process also requires genes that switch the fate of SAM from vegetative to floral and this class of genes are called floral meristem identity genes. Therefore, we can expect that the genes that control flowering time would interact with floral meristem identity genes, which in *Arabidopsis* include *LEAFY* (*LFY*) and *APETALA1* (AP1) to mention a few. In long-days, the flowering time genes appear to interact with the meristem identity genes at LFY. The quantitative upregulation of LFY is the main event in floral transition (Nilsson et al., 1998; Weigel and Nilsson, 1995). Analysis of transcriptional activation of LFY has revealed that members of the gibberellin, photoperiod and autonomous promotion pathway function to upregulate LFY transcription (Nilsson et al., 1998). Strong evidence supporting the idea that flowering time signals converge at floral meristem identity genes comes from the recent analysis of the LEAFY promoter. LEAFY promoter deletion analysis has led to the identification of independent regulatory elements that respond to gibberellins and photoperiod (Blázquez and Weigel, 2000).

Several flowering time genes have been cloned and appear to encode regulators of gene expression. The identification of the upstream and downstream targets of these gene products will help to define the various signaling pathways.

Chapter 2

MATERIALS AND METHODS

Materials

The flavonoids used in this study were obtained from the collection of Dr. R. K. Ibrahim, Concordia University, Montreal. 11-OH-JA, 12-OH-JA, coronatine, 12-oxophytodieonic acid, 1-hydroxyindanoyl isoleucine conjugate, jasmonic acid isoleucine conjugate and cucurbic acids were provided by Dr. O. Miersch, Liebniz Institute of plant Biochemistry, Halle, Germany. Methyl jasmonate and jasmonic acid were purchased from Bedoukian Research Inc. USA and Sigma Chemicals respectively. Wild type *A. thaliana* seeds, ecotype Col-0 and C24 were obtained from Lehle seeds, USA. All other reagents were of analytical or molecular biology grade.

Cloning of *AtST2a* and *AtST3a*

cDNA clones of *AtST2a* (Genebank accession number: T43254) and *AtST3a* (Genebank accession number: T75675) were obtained from the *A. thaliana* Biological Resource Center (ABRC). Sequencing of the cDNA was performed using the dideoxy chain termination method and the ^{T7}Sequencing kit from Pharmacia. Oligonucleotide 5'-CGGGATCCATGGCTACCTCAAGCATGAAG-3' was designed to introduce a *Bam*H1 site at the 5'-end of *AtST2a*. *AtST2a* was amplified by the Polymerase Chain Reaction with Vent DNA polymerase (New England Nuclear) using the above primer and M13 -20 primer. Oligonucleotide 5'-CGGGATCCATGAACTTGAGAATTGAAG-3' was designed to introduce a *Bam*H1 site at the 5'-end of *AtST3a*. *AtST3a* was amplified by the Polymerase Chain Reaction using the above primer and M13 reverse primer.

The amplified products were digested with *Bam*H1 and ligated into the *Bam*H1 site of the bacterial expression plasmid, pQE30 (Qiagen). Clones containing *AtST2a* and *AtST3a* in the proper orientation were determined by restriction enzyme analysis and by sequencing the 5' junction. All enzymes used for cloning were from New England Biolabs and were used under the conditions recommended by the manufacturer.

DNA sequence analysis

DNA and protein sequence alignments were performed using the ClustalW program (http://dot.imgen.bcm.tmc.edu:/multialign/multi-align.html) and the similarity/identity values determined from the pairwise comparisons of all the ST genes. Boxshade was used to shade identical residues in the alignments (http://www.ch.embnet.org/software/BOX_form.html).

Parsimony trees have been generated from alignments of complete ST sequences using PILEUP of the GCG program and PROTPARS of the PHYLIP program (Felsenstein, 1993). Bootstrap values indicate the number of times that a particular node was found in trees generated from 100 replicates. The ST containing BAC clones have been localized on the chromosomes using the physical map of the genome of *A. thaliana* (http://*Arabidopsis*.org/servlets/mapper).

Expression of recombinant *AtST2a* and *AtST3a*

A culture of *E. coli*, strain XL1-blue harboring AtST2a or AtST3a ($O.D_{600}$ = 0.7) was induced with 1mM isopropylthio-β-D-galactopyranoside for 10 hours at 22°C.

Bacterial cells were collected by centrifugation, resuspended in 50mM sodium phosphate buffer (pH 8.0) containing 0.3M NaCl and 14 mM 2-mercaptoethanol (buffer A). The cells were lysed by sonication and the recombinant proteins recovered in the soluble fraction by centrifugation at 12000Xg for 15 min at 4^0C. The supernatant was applied to a nickel-nitrolotriacetic acid agarose matrix (Qiagen) equilibrated in buffer A. The resin was washed with 50 mM sodium phosphate (pH 6.0), 0.3 M NaCl and 14 mM 2-mercaptoethanol (buffer B) and the proteins were eluted with the same buffer containing 0.5M imidazole. The Ni-agarose purified protein was desalted on PD-10 (Pharmacia Biotech) column prequilibrated in 25 mM bis-Tris, pH 6.5 (buffer C). The desalted proteins were chromatographed on a 3'-phosphoadenosine 5'-phosphate (PAP) agarose column (0.5 X 10 cm) previously equilibrated in buffer C. The bound proteins were eluted with a linear salt gradient of 0 to 1M NaCl in buffer C. Affinity chromatography was performed on a Waters 625 LC HPLC system and the protein absorbance was monitored at 280 nm. AtST2a eluted at approximately 450 mM NaCl and AtST3a eluted at 700 mM NaCl. Protein concentration was estimated using the Bradford Reagent (Bio Rad) and BSA as reference protein.

Preparation of anti-AtST2a antibodies

Anti-AtST2a polyclonal antibodies were raised in rabbits using PAP agarose purified recombinant enzymes expressed in *E. coli*. 100 μg of purified protein was injected subcutaneously in a rabbit in Freund's complete adjuvant. The rabbit was injected 3 more times 21, 42, 70 days after the first injection, with 50 μg of purified protein in Freund's incomplete adjuvant. The rabbit was bled 10 days after the last

injection and serum extracted. This procedure was performed by Hélène Ste-Croix at the McGill University animal house facility.

SDS-Polyacrylamide Gel Electrophoresis

In order to verify the solubility and evaluate the level of purity of the recombinant protein after chromatography on nickel-agarose and PAP-agarose, aliquots of the recombinant enzyme were subjected to 12% polyacrylamide gel electrophoresis according to the method of Laemmli (1970). The proteins were visualized by Coomassie Blue staining.

Sulfotransferase assay

Analysis of substrate specificity was performed by testing enzymatic activity with three different concentrations of acceptor substrates: 1, 10 and 100 μM. The reaction mixture (50 μl) contained 50 pmol [^{35}S] PAPS (New England Nuclear) and approximately 0.25 μg of PAP-agarose purified recombinant AtST2a or AtST3a in 50 mM Tris pH 7.5. For kinetic analysis, a PAPS concentration of 5 μM was used. The reactions were allowed to proceed for 10 min at 25°C. The AtST2a-sulfated reaction product was extracted with 1-butanol saturated with water and an aliquot was counted for radioactivity in scintillation fluid. To identify the reaction product formed by recombinant AtST2a by LC-MS, the enzyme reaction was carried out using unlabeled PAPS.

The AtST3a-catalyzed reaction product was extracted in 0.1% (w/v) tetrabutylammonium dihydrogen phosphate (TBADP) and ethyl acetate and an aliquot

was counted for radioactivity in scintillation fluid (Varin et al., 1987). The remaining fraction was used for identification of reaction products by co-chromatography with reference compounds. TLC was carried out on cellulose plates using 1- butanol /acetic acid /water (3:1:1, v/v/v) as solvent. Developed chromatograms were visualized under UV light (360 nm) and then autoradiographed.

Preparation of *Arabidopsis thaliana* extracts to detect AtST3a substrate

15 days old and flowering (25 days old) *A. thaliana* plants (Col0) were ground in liquid nitrogen, and the powder was extracted in 50% methanol (approximately 10 ml/g of tissue). Methanol was evaporated, and the aqueous phase extracted with hexane. The resulting aqueous layer was passed through a DEAE Sephadex A25 (Pharmacia Biotech) column. The column was washed with 50 mM Tris, pH 7.5 and the unbound compounds were collected. Polar compounds bound to the column were eluted with 2M NaCl in 50 mM Tris, pH 7.5. Both the bound and unbound fractions were extracted in 10mM TBADP, 0.5% acetic acid and ethyl acetate. The ethyl acetate fraction was evaporated and resuspended in methanol. Part of this fraction was acid hydrolyzed in 2N HCl and boiled for 2 min, followed by extraction in ethyl acetate and resuspension in methanol. Both the acid hydrolyzed and non hydrolyzed fractions were tested as substrates for AtST3a. Reaction mixtures (50 µl) contained 5 µM [^{35}S]-PAPS (NEN Life Science Products) and 1-5 µg of purified recombinant sulfotransferase in 50 mM Tris-HCl, pH 7.5. Reactions were allowed to proceed for 10 min at 25 °C. Extraction of the [^{35}S]-labeled sulfate product was performed according to a standard assay (Varin et al., 1987).

After extraction with ethyl acetate, sulfated products were lyophilized and resuspended in methanol.

Reverse phase HPLC to identify flavonol sulfate

The retention time of kaempferol 7- sulfate synthesized *in vitro* by AtST3a was compared with that of authentic kaempferol 7-sulfate. Reverse phase HPLC was performed on a Novapack C18 column (Waters), equilibrated with solvent A (0.5% acetic acid, 10 mM TBADP in water). Approximately 100,000 dpm of [^{35}S]-labeled sulfate product was co-injected with authentic flavonol 7-sulfate for chromatography. The column was washed for 5 min in solvent A. Sulfated products were eluted with a linear gradient of solvent A into solvent B (100% methanol, 0.5% acetic acid, 10 mM TBADP) in 50 min, followed by 10 min isocratic period at a flow rate of 0.8 ml/min. Elution of flavonol 7-sulfate was monitored by measuring the absorbance at 340 nm using a Waters 486 tunable absorbance detector and fractions of 800 µl were collected. Elution of labeled sulfate products was monitored by measuring the amount of [^{35}S] present in individual fractions. 100 µl of the total 800 µl was counted for radioactivity by liquid scintillation counting. The chromatography experiments were performed with a Waters 625 LC HPLC system.

Detection and quantification of JA, 12-OH-JA and 11-OH-JA from *Arabidopsis thaliana*

Fresh plant material (1g) was homogenized with 10 ml methanol and 100 ng of ($^{2}H_{6}$) JA, 12-($^{2}H_{3}$)OAc-JA and 11-($^{2}H_{3}$)OAc-JA (prepared by Dr. O. Miersch) were added as

internal standards. The filtrate was evaporated and acetylated with Pyridine/Acetic acid anhydride (2:1) at 20° C overnight. The reaction mixture was evaporated, resuspended in ethyl acetate and loaded on a silica (SiOH) column (500mg; Machery- Nagel). The flow-through containing JA and acetylated forms 11-OH-JA and 12-OH-JA was collected and evaporated. The evaporated mixture was resuspended in 5 ml methanol and loaded on a 3 ml DEAE-Sephadex A25 column (acetylated-form in methanol). The column was washed with 3 ml of methanol followed by 3 ml of 0.1 M acetic acid in methanol. The jasmonates were eluted with 5 ml of 1 M acetic acid in methanol (Fraction A), evaporated and separated on preparative HPLC for GC-MS analysis.

The SiOH column was washed with methanol and the flow through (Fraction B) was collected for analysis of 12-OH-JA sulfate. Fraction B was evaporated, resuspended in 10% acetonitrile and chromatographed by reverse phase HPLC (Method gradient. 10% to 90% acetonitrile in 15 min at a flow rate of 1 ml/min). Fractions were collected from 4.5 to 7 min, evaporated and resuspended in 50 µl methanol and analyzed by LC-MS.

Preparative HPLC: Fraction A eluted from the DEAE-Sephadex A25 column was subjected to preparative HPLC column, Eurospher 100-C18 (5 µm, 250 x 4 mm). Jasmonates were eluted with methanol-0.2 % acetic acid in H_2O (1:1) at a flow rate of 1 ml/min and UV detector at 210 nm. Fractions between R_t 9.15 and 11 min containing 11-OAc-JA and 12-OAc-JA and between 12 and 13.30 min containing JA were collected and evaporated. The samples were dissolved in 200 µl chloroform/*N*, *N*-diisopropylethylamine (1:1) and derivatized with 10 µl pentafluorobenzylbromide at 20 °C overnight. The evaporated derivatized samples were dissolved in 5 ml *n*-hexane and passed through a SiOH-column (500mg; Machery- Nagel). The pentafluorobenzyl esters

were eluted with 7 ml of *n*-hexane/diethylether (2:1), evaporated, dissolved in 100 µl acetonitrile and analyzed by GC-MS.

GC-MS: (GCQ Finnigan, 70 eV, NCI, ionization gas NH_3 , source temperature 140°C, column Rtx-5 (30 m x 0.25 mm, 0.25 µm film thickness), injection temperature 250°C, interface temperature 275°C; Helium 40 cm s^{-1}; splitless injection; column temperature program: 1 min 60°C, 25° min^{-1} to 180° C, 5° min^{-1} to 270°C ,1 min 270°C, 10° min^{-1} to 300°C, 25 min 300°C).

Retention time of 12-($^2H_3$)OAc-JA-pentafluorobenzyl ester: 20.61 min, 12-OAc-JA-pentafluorobenzyl ester: 20.66 min, using fragments *m/z* 270 (standard) and *m/z* 267 for quantitation.

Retention time of 11-($^2H_3$)OAc-JA-pentafluorobenzyl ester: 18.40 min, 11-OAc-JA-pentafluorobenzyl ester: 18.38 min, using fragments *m/z* 270 (standard) and *m/z* 267 for quantitation.

Retention time of ($^2H_6$) JA -pentafluorobenzyl ester: 14.66 min, JA-pentafluorobenzyl ester: 14.72 min, using fragments *m/z* 215 (standard) and *m/z* 210 for quantitation.

Detection of 12-OH-JA sulfate by LC MS/MS

The negative ion electrospray (ES) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4 kV; heated capillary temperature 220°C; sheath gas: nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a RP18-column (4 µm, 1x100 mm, Ultrasep). For the HPLC, a gradient was used starting from H_2O: acetonitrile (90:10; containing 0.2% acetic acid) to 10:90 in

15 min followed by a 10 min isocratic period at a flow rate of 70 µl/min. The collision-induced dissociation (CID) mass spectra during the HPLC run were performed with a collision energy of 30 eV (collision gas: argon, collision pressure: 1.8 x 10^{-3} Torr). All mass spectra are averaged and background substracted. The following results were obtained:

12-Hydroxysulfonyloxyjasmonic acid: Rt (LC-MS), 12.32 min, negative electrospray MS m/z (rel. int.): 305 ($[M-H]^-$, 100); CID spectrum: 225 (93), 147 (9), 97 (100), 59 (58).

For the determination of 12-hydroxysulfonyloxyjasmonic acid in plant material the daughter ions at m/z 225, 97 and 59 were measured in the selected ion monitoring (SIM) mode. The CID spectrum of 12-hydroxysulfonyloxyjasmonic acid displays significant ions at m/z 225, 97 and 59 reflecting the typical structural features of the compound. The ion at m/z 97 represents a key ion in the negative CID mass spectra of sulfated compounds (Boss et al., 1999).

Northern blot analysis

A. thaliana plants were pulverized in liquid nitrogen and extracted in buffer/phenol/chloroform as described (Cashmore, A. R., 1982) to isolate total RNA. 10 g of total RNA was used for agarose gel electrophoresis after denaturing with glyoxal and DMSO (Mc Master and Carmicheal, 1977). Northern blot analysis of total RNA was achieved under high stringency conditions according to standard procedures (Sambrook, 1989) using the [^{32}P]- labeled coding region of *AtST2a* as a probe.

Plasmid constructs for *AtST2a* and *AtST3a* transformation in *Arabidopsis thaliana*

The cassette of the pBI525 and pBI526 constructs (Dalta et al., 1993) were removed by digestion with *Hind*III and *Eco*RI, and religated in the corresponding sites of the binary vector pBI101 (Clontech). The resulting vectors called pBI101-525 or pBI 101-526 contained two *CaMV 35S* minimal promoters in tandem followed by an AMV translational enhancer, a NOS terminator and a kanamycin resistance gene.

A *Bam*HI site was introduced by PCR at the 5' end of the *AtST2a* coding sequence using oligonucleotide 5'-CGGGATCCATGGCTACCTCAAGCATGAAG-3' (translation initiation codon of *AtST2a* underlined). The 3' *Bam*H1 site was part of the multiple cloning site of the vector and was amplified along with *AtST2a* during PCR. The PCR product obtained using the above primer and M13 primer was digested with *Bam*HI, and ligated into the corresponding site of the pBI101-526 polylinker. In the resulting construct, the translation initiation codon of *AtST2a* is in frame with the ATG present at the *Nco*I site of the polylinker, resulting in the production of a protein having six additional amino acids at its NH_2-terminus. The 5'-end junction of the *AtST2a* coding sequence was verified by sequencing. *AtST2a* cDNA was also cloned in the antisense orientation at the *Bam*H1 site of pBI101-525.

Oligonucleotide 5'-CGGGATCCATGAACTTGAGAATTGAAG-3' was designed to introduce a *Bam*H1 site at the 5' end of *AtST3a*. *AtST3a* was amplified by PCR using the above primer and M13 reverse primer. The amplified product was digested with *Bam*H1 and ligated into the *Bam*H1 site of the pBI101-526 polylinker. The orientation of the *AtST3a* cDNA insertion in the pBI101-526 vector was verified by sequencing.

Agrobacterium tumefaciens transformation

The pBI101-526- *AtST2a* sense construct was transformed in *Agrobacterium tumefaciens* GV3101 pmp90 and the pBI 101-525-*AtST2a* antisense, pBI 101-526-*AtST3a* antisense constructs were transformed in *Agrobacterium tumefaciens* strain LBA4404 using a freeze-thaw method (An et al., 1988).

Arabidopsis thaliana transformation

A. thaliana plants of ecotype Columbia (Col0) were transformed with the *Agrobacterium* strain containing the *AtST2a* in the sense orientation by the vacuum infiltration method as described previously (Benchtold *et al.*, 1993).

A. thaliana plants of ecotype C24 were transformed with the *Agrobacterium* strain containing the *AtST2a* in the antisense orientation and the *AtST3a* in the antisense orientation by the root explant method as described in Valvekans *et al.* (1988). Seeds were collected from the T_0 plants, surface sterilized and transformants were selected on MS salt medium containing vitamins and supplemented with 50 μg/ml of kanamycin. Segregation analysis was performed by plating T_1 seeds on MS media supplemented with 50 μg/ml of kanamycin. Cotyledons of resistant plants appear green and have well developed root system as compared to non-transformed plants. The Kan_R: Kan_S ratio was determined by counting plants that were resistant to kanamycin as opposed to those were not.

Southern blot analysis

To determine the number of inserts present in independent transgenic lines, genomic DNA from pools of T_2 plants was analyzed by Southern blot. For genomic DNA extraction, tissue was ground in liquid nitrogen and dissolved in extraction buffer (3% CTAB, 1.4 M NaCl, 52 mM ß-mercaptoethanol, 20 mM EDTA and 100mM Tris-HCl, pH 8.0) preheated at 60 °C. The extract was incubated for 30 min at 60 °C, with occasional shaking. The aqueous phase was extracted twice with chloroform, and precipitated with isopropanol. The precipitate was washed with 95% ethanol, containing 10 mM ammonium acetate, for 20 min. Genomic DNA was further purified by RNase and proteinase K digestions, followed by phenol-chloroform extraction. For Southern blot analysis, 10 µg of genomic DNA was digested with *Eco*RI. The *AtST2a* sequence, was used as a probe. The blot was hybridized under stringent conditions according to standard procedures (Sambrook et al., 1989).

Detection of the AtST2a protein in transgenic lines

For the analysis of AtST2a expression in independent transgenic lines, T_2 plants were pooled, ground in liquid nitrogen, and the powder was boiled in 2X SDS sample buffer to extract total proteins. To confirm the integrity of the proteins and equal loading of each sample, protein extracts were also run on SDS-PAGE and stained with Coomassie blue. Protein extracts were separated by SDS-polyacryamide gel electrophoresis on a 12% polyacrylamide gel and transferred onto nitrocellulose membrane. AtST2a was immunodetected using anti-AtST2a polyclonal antibodies (dilution 1:1000) and goat anti-

rabbit secondary antibodies conjugated with alkaline phosphatase (dilution 1:3000; Bio Rad).

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

2.5 µg of total RNA was treated with 20U of DNase I (Roche Molecular Biochemicals) in 50 µl of 0.1 M sodium acetate, 5mM $MgSO_4$, pH 5.0 for 10 min at 37°C. Dnase I was heat inactivated at 95°C for 5 min and RNA was ethanol precipitated. cDNA was synthesized using Moloney Murine Leukemia virus reverse transcriptase (New England Biolabs) in 25 µl reaction volume as recommended by the manufacturer. 1 µl of RT reaction product was used for PCR with Ex Taq DNA polymerase (Takara Biochemicals).

Oligonucleotides used for amplification in the RT-PCR experiments

Oligonucleotides were designed using the OLIGO software (Molecular Biology Insights Inc.). The sequence of the oligos used in different experiments are presented below.

Oligos used to amplify the *ACTIN* of *A. thaliana.*

(Genebank Acc. No. U39449)

(Actin 5') 5'- GCTGATGGTGAAGACATTCAAC-3' and

(Actin 3') 5'- CATAGCAGGGGCATTGAAAG-3'

Oligos used to amplify the *AtST2a* in RT-PCR experiments.

(Genebank Acc. No. T43254)

(119-Bam) 5'- CGGGATCCATGGCTACCTCAAGCATGAAG-3' and

(119-800) 5'- CGTCTTTGAGATCCTCGTACC-3'

Oligos used to amplify the *AtST3a* in RT-PCR experiments.

(Genebank Acc. No. T75675)

(AtST3a-Bam5') 5'- ACGCGGATCCATGGAGATGAACTTGAGAATT-3' and

(AtST3a-Kpn3') 5'- ACGGGGTACCTTATTACAATGCACAAACTCA-3'

Oligos used to amplify the *Thi2.1* of *A. thaliana*.

(Genebank Acc. No. L41244)

(Thi2.1 5') 5'- GGTGTATGCAAGTGAGTGGATG-3' and

(Thi2.1 3') 5'- GACACATGCACACACACACAC-3'

Oligos used to amplify the *AtST2a* antisense transcript form *A. thaliana* plants expressing the gene in the antisense orientation.

(AMV) 5'- AATTTTCTTTCAAATACTTCCACCATG-3' and 119-Bam.

Oligos used to amplify the *AtST3a* antisense transcript form *A. thaliana* plants expressing the gene in the antisense orientation.

(AMV) 5'- AATTTTCTTTCAAATACTTCCACCATG-3' and

(AtSTX-210) 5'- CCCTAAATGCGGCACCAC-3'.

Plant growth Conditions

A. thaliana plants were grown in soil in a growth chamber during a 16-hour photoperiod, at a day-time temperature of 24° C and a night-time temperature of 20° C. For some experiments, the plants were grown in magenta boxes under sterile conditions according to the following protocol. Seeds of *A. thaliana* were sterilized for 5 minutes in a solution containing 1.5% sodium hypochlorite and 0.02% SDS, and washed five times in sterile water. The sterilized seeds were vernalized for four days at 4 °C. Seeds were then spread on agar-solidified medium containing Murashige and Skoog salts, 1% sucrose and vitamins.

For phenotypic analysis of the transgenic plants, the T_2 or T_3 seeds were vernalized for four days at 4 °C. Seeds were then spread on agar-solidified medium containing Murashige and Skoog salts, B5 vitamins, 1% sucrose, 0.5g/l MES. Alternatively, the vernalized seeds were planted in soil and grown in a growth chamber under long day conditions (16-hour light) or under short day conditions (8-hour light), at a day temperature of 24 °C and a night temperature of 20°C.

Chapter 3

ANALYSIS OF *ARABIDOPSIS THALIANA* SULFOTRANSFERASE GENE FAMILY

Identification of sulfotransferase genes from *Arabidopsis thaliana*

Recently, we initiated a functional genomic project with the objective of characterizing the biological function of all ST-coding genes from one flowering plant. This project was made possible by the international effort to sequence the genome of *A. thaliana*. When we initiated this project, only one ST (*AtST1*, previously named *RaR047*) was characterized from this plant (Lacomme and Roby, 1996). Using *AtST1* as a query sequence, database mining allowed us to identify 17 novel ST-coding genes (Table 3.1). In contrast with the mammalian ST-coding genes which contain introns at conserved positions, the *Arabidopsis* ST-coding genes are intronless. The absence of introns seems to be a common feature of plant ST-coding genes since the three STs characterized from *Brassica napus* and the STs of *Flaveria* species were also found to be intronless. (Rouleau et al., 1999 and Varin, L., unpublished results). The protein sequences deduced from these genes contain the conserved domains and amino acid residues present in all plant and mammalian STs (Varin et al, 1992) (Figure 3.1). These include the two highly conserved stretches of sequences, located at the amino-terminal (PKSGTXW) and carboxy-terminal (RKGXXGDWK) of the ST proteins (Figure 3.1).

The length of *A. thaliana* ST protein sequences vary from 323 to 359 residues. Protein alignment of the *Arabidopsis* STs reveal identity values ranging from 34% to 85%, suggesting significant diversification of the members of this gene family.

Table 3.1:

Accession numbers and coordinates of the *Arabidopsis thaliana* sulfotransferases

Arabidopsis ST Designation	EST (if known)	Acc. No. of genomic sequence	Coordinates on BAC clone	Protein size (amino acids)
AtST1 (F19B11.21)	RaRO47	AC006836	70622 - 71642	326
AtST2a (MOJ9.18)	119G6T7 230H6T7	AB010697	53938 - 55015	359
AtST2b (MOJ9.19)	AV442439 AV441090	AB010697	50629 - 51670	347
AtST3a (T14D3.10)	142G17T7	AL138649	1145 - 2116	323
AtST3b (T14D3.20)	-	AL138649	3483 - 4472	329
AtST4a (I26I20.8)	217L1T7	AC005396	38691 - 39728	333
AtST4b (T6J4.16)	-	AC011810	82981 - 83976	331
AtST4c (T6J4.16 #2)	-	AC011810	84640 - 85695	351
AtST5a (F2P9.3)	OBO154 152H10T7	AC016662	7673 - 8831	338
AtST5b (F2P9.4)	AV525685	AC016662	9123 - 10175	350
AtST5c (F25I16.7)	AV525959 AV540478	AC026238	21629 - 22669	356
F19B11.20	GBGe166 AV439900	AC006836	69115 - 70164	331
MQD19.2	-	AB026651	1164 - 2159	331
F3H9.17	-	AC021044	102756 - 103736	326
F19B11.22	-	AC006836	72018 - 72992	324
T25K17.90	-	AC049171	48831 - 49832	314
AJ006409	AJ006409	-	-	-
F15K20.33	-	AC005824	13848 -15000	325

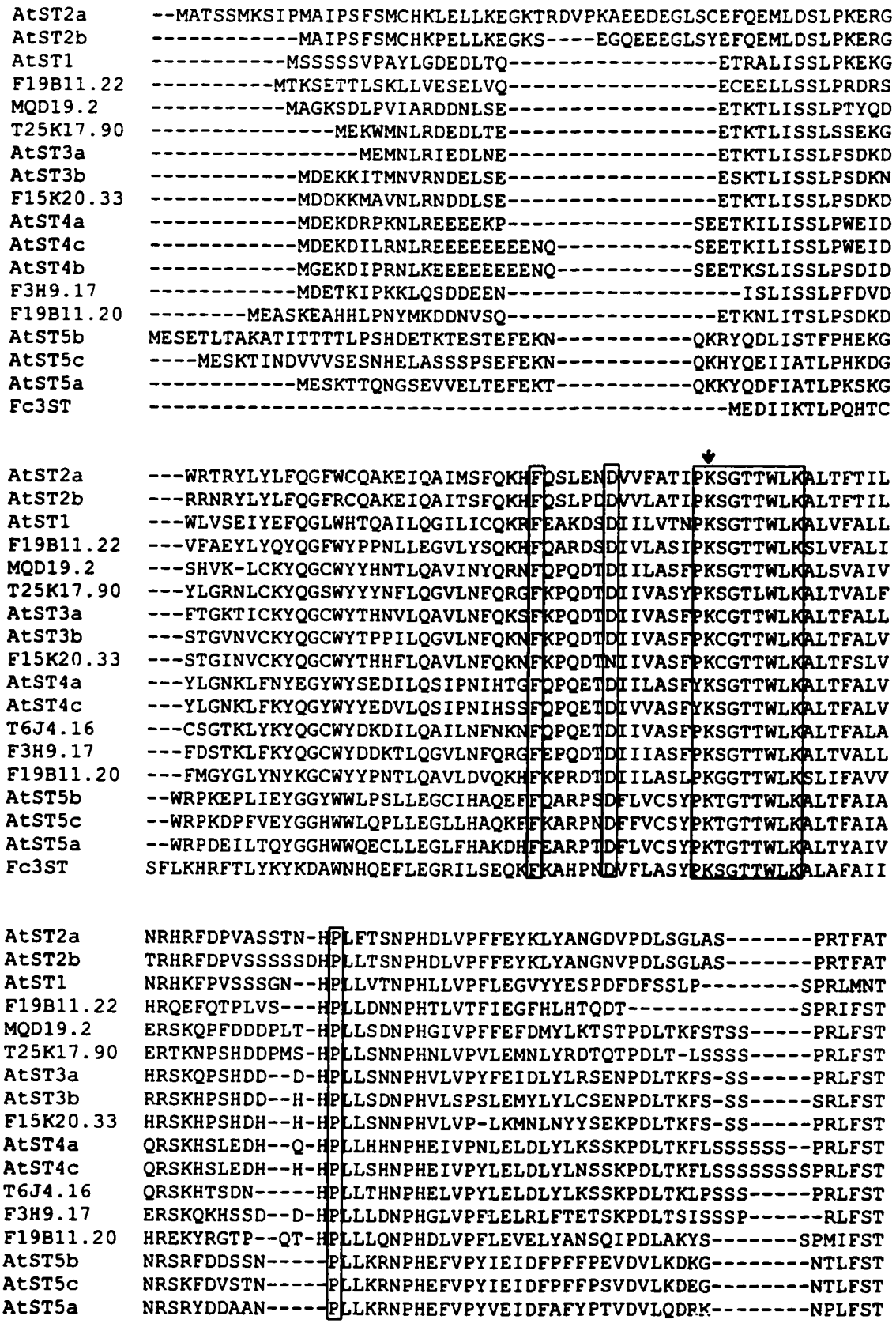


Figure 3.1 (page 1 of 3)

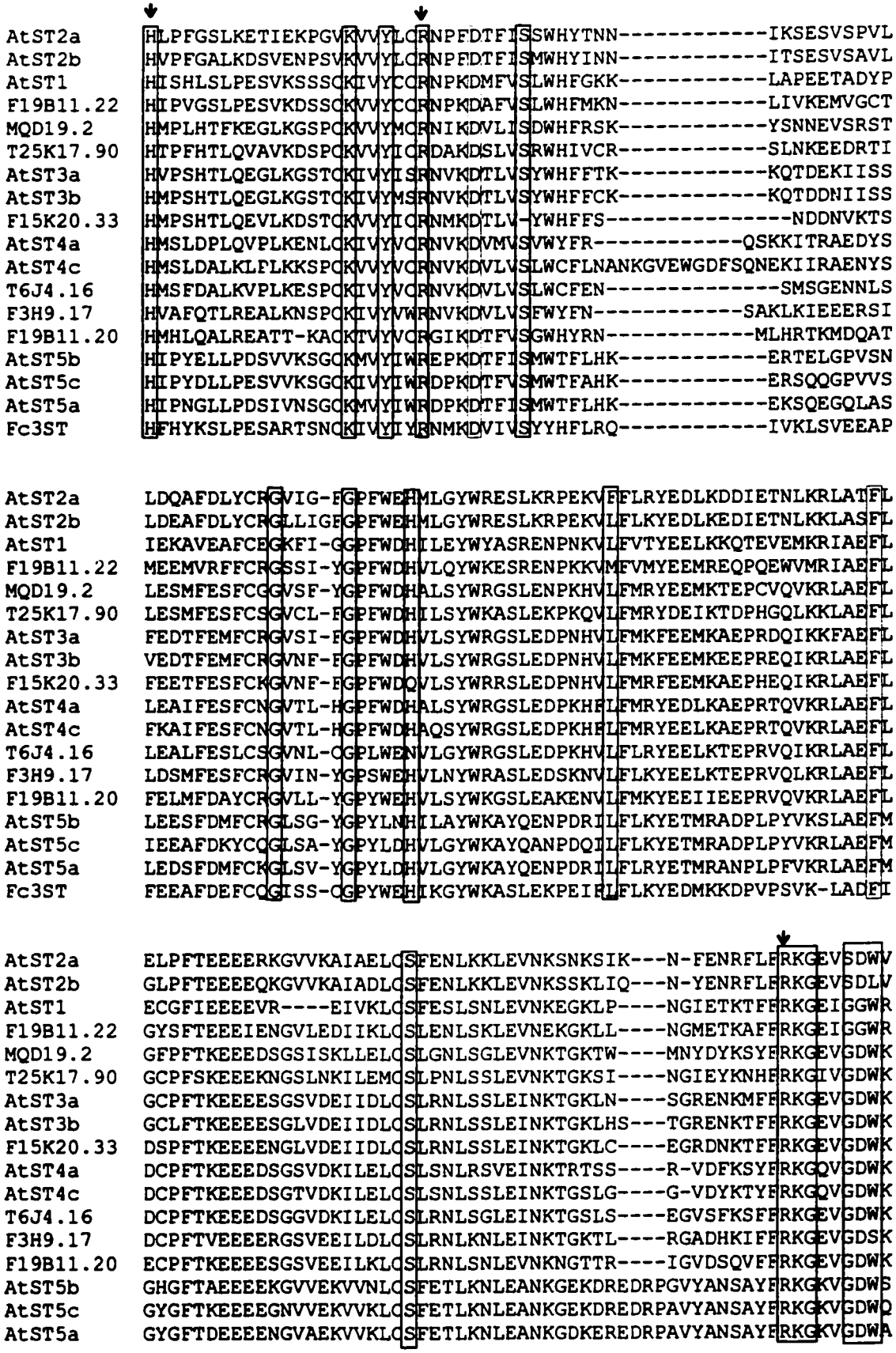


Figure 3.1 (page 2 of 3)

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AtST2a      NYLSPSQVERLSALVDDKLGGSGLTFRLS--
AtST2b      NYLSPSQVERLSALVDDKLAGSGLTFRLS--
AtST1       DTLSESLAEEIDRTIEEKFKGSGLKFSS---
F19B11.22   DTLTPLLAEEIDKTTKEKLIGSDFRFFC---
MQD19.2     NHLTPEMENKIDMIIEEKLKGSDLKF-----
T25K17.90   NHLTPEMGSKIDMIMKEKLKDYSEVWFENAL
AtST3a      NYLTPEMENKIDMIIQEKLQNSGLKF-----
AtST3b      NYLTPEMENKIDMIIQEKLQNSGLKF-----
F15K20.33   NYLTPEMENKIDMIIQEKLQNSGLKF-----
AtST4a      SYMTPEMVDKIDMIIEEKLKGSGLKF-----
AtST4c      SYMTSEMVNKIDMIVEEKLKGSGLKF-----
T6J4.16     SYMTPEMENKIDMIVEEKLQGSGLKL-----
F3H9.17     NHLTPEMEKIIDMITEEKFEGSDLKF-----
F19B11.20   NHLTPQMAKTFDEIIDYRLGDSGLIFQ----
AtST5b      NYLTPEMAARIDGLMEEKFKGTGLLEHGK--
AtST5c      NYLTPEMVARIDGLMEEKFKGTGFLSSKS--
AtST5a      NYLTPEMAARIDGLVEEKFKDTGLLQHDN--
Fc3ST       NYFTDEMTQKIDKLIDEKLGATGLVLK----
```

Figure 3.1

Amino acid sequence alignment of all the putative full length STs from *Arabidopsis thaliana* with the flavonol 3-ST from *Flaveria chloraefolia* (Accession Number M8413). The boxes indicate conserved regions found in all eukaryotic soluble STs. Residues critical for catalysis and PAPS binding are indicated by an arrow. The alignment was obtained using the multiple sequence alignment package ClustalW 1.5 program (http://dot.imgen.bcm.tmc.edu:/multialign/multi-align.html).

Out of the 18 ST-coding genes of *Arabidopsis*, one is represented by a cDNA partial sequence (AJ006409). This cDNA has been identified from *A. thaliana* ecotype Landsberg erecta and was shown to exhibits altered expression upon CaMV infection (Geri et al., 1999). So far, the genomic sequence corresponding to this cDNA cannot be found in the *Arabidopsis* database. The gene F15K20.33 on the BAC clone AC005824 is annotated as a pseudogene in the *Arabidopsis* Information Resource (TAIR) database since the open reading frame contains two stop codons. However, detailed analysis of the deduced amino acid sequence and alignment with the other *Arabidopsis* STs indicates that these are probably introduced by sequencing errors.

Sequence homology and phylogenetic analysis

A parsimony analysis using the alignment of the complete ST protein sequences from *Arabidopsis*, produced a single unrooted tree showing the most probable succession of duplication events in the ST family (Figure 3.2).

The *A. thaliana* ST subfamily I includes *AtST2a* (hydroxyjasmonate ST) which exhibits 85% aa sequence identity with its paralogue *AtST2b*. The ST subfamily II represents a less homogenous group of sequences from *A. thaliana*. The level of amino acid sequence identity among its members is generally lower than 60%. Furthermore, this subfamily can be subdivided in two distinct subgroups. The first one contains *AtST4a*, *4b* and *4c* which exhibit 72% sequence identity among each other. The second subgroup includes *AtST3a* and *3b*, which exhibit 85% sequence identity. The subfamily III includes *AtST1* and the gene F19B11.22. *AtST1* is proposed to be the ortholog of BnST3 from *Brassica napus*, which was shown to sulfonate brassinosteroids (Rouleau et al, 1999).

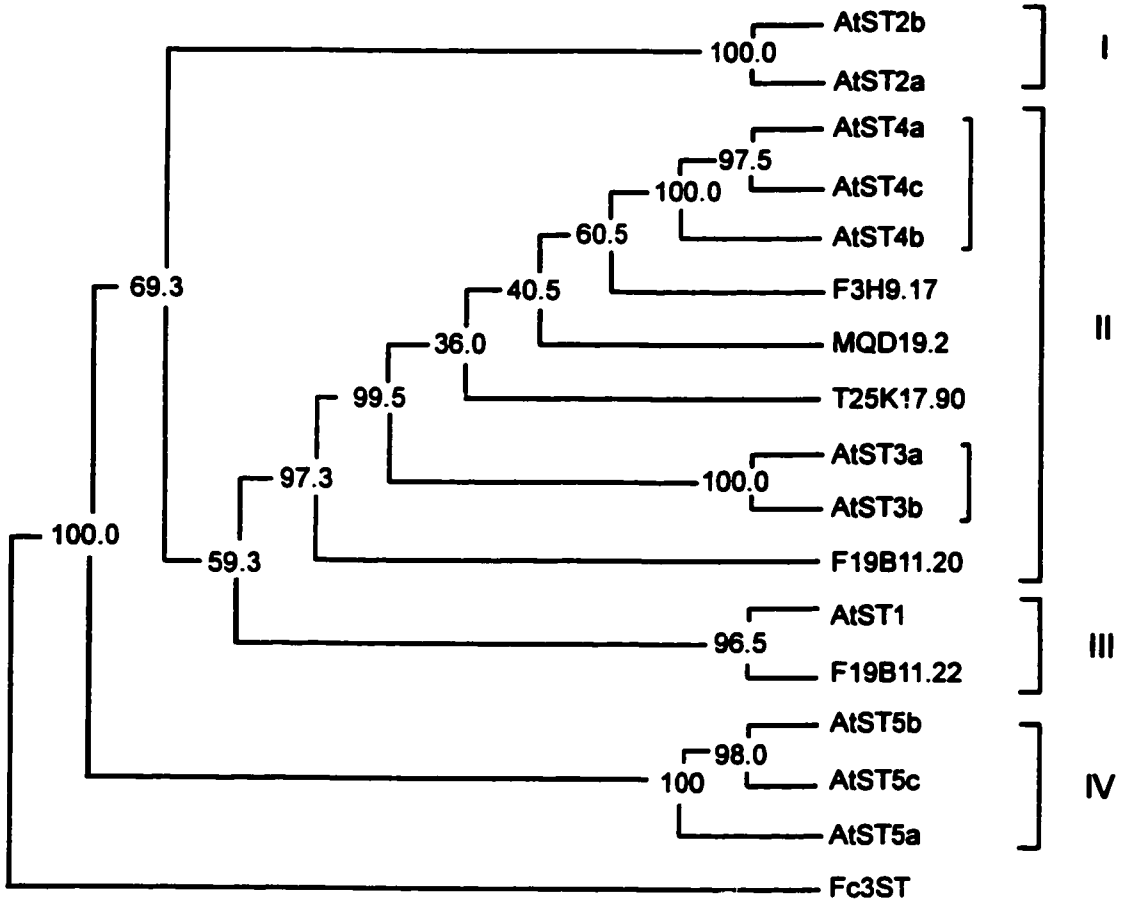


Figure 3.2

Phylogenetic tree of the *Arabidopsis thaliana* STs and the flavonol 3-ST from *Flaveria chloraefolia (*Accession number M84135). Numbers indicate bootstrap values of branches from 100 replicates. I, II, III and IV designate the proposed subfamilies defined by a minimal level of 45% amino acid identity among members. The gene subfamily II includes two subgroups as indicated. The parsimony tree has been generated from an alignment of full length STs with the ClustalW 1.5 program using PILEUP of GCG and PROTPARs of PHYLIP (Felsentein, 1993). The Gene bank Accession numbers of the *Arabidopsis thaliana* STs are presented in Table 1.

AtST5a, *5b* and *5c* form the fourth subfamily and share at least 72% amino acid sequence identity.

Transcription has been shown for 10 members of the family either by identification of expressed sequence tags present in the database or by northern and/or RT-PCR experiments done in our laboratory (Table 3.1). The expression patterns of *AtST3a* and *AtST2a* are presented in chapter 4 and chapter 5, respectively.

Mapping

Figure 3.3 shows the distribution of the *Arabidopsis* STs on the five chromosomes. The 17 STs whose complete sequences and chromosomal localization are available are presented. Five ST gene clusters are found in the *Arabidopsis* genome. Most of the clusters include arrays of phylogenetically related genes that are also associated with their sister sequence or close relative on the sequence tree. These clustered genes share at least 75-85% sequence identity among themselves. This high similarity between closely linked genes may be the result of a recent duplication event. The only exception is the cluster containing *ATST1*, F19B11.20 and F19B11.22, which exhibit relatively low amino acid identity (41-53%) among each other. Gene duplication appears to be frequent in *A. thaliana*. Analysis of the *Arabidopsis* genome reveals that 4140 individual genes are present in tandem arrays ranging from 2-23 adjacent members (The *Arabidopsis* Genome Initiative, 2000). Furthermore, Bevan (1998) estimated that 70% of the characterized genes in *A. thaliana* have at least one paralogue.

It is probable that the *A. thaliana* ST gene family arose as a result of a combination of 3 events during evolution: duplication of individual genes, chromosome

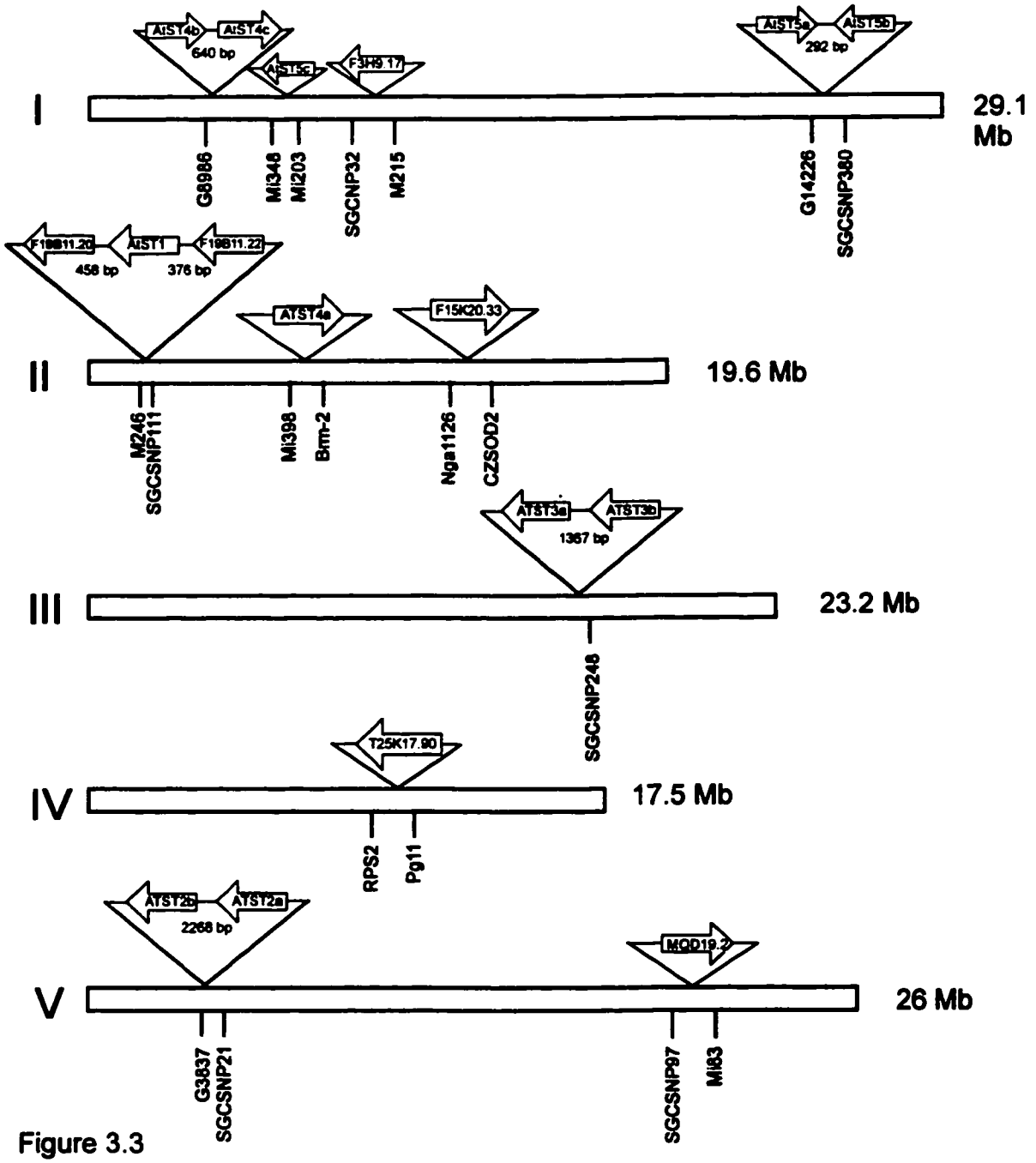


Figure 3.3

Localization of the 17 ST coding genes on the 5 chromosomes of *Arabidopsis thaliana*. Each gene is localized on the physical map of *Arabidopsis thaliana* according to the approximate position of the corresponding BAC clone in the *Arabidopsis thaliana* database. The nearest markers are shown on both sides of the genes. Arrows indicate 5'- to 3'- orientation of the STs on the BAC clone. Numbers indicate the distance in basepairs between closely linked STs.

segmental duplication and divergence. Genomic sequencing projects of prokaryotes, yeasts, plants and human are beginning to reveal the presence of gene families and their importance both in number and in size. It has been proposed that gene families arise by a combination of gene duplication of ancestral genes followed by divergence of copies (Fryxell, 1996). This process leads to specific structural and functional characteristics, and results in altered recognition properties or modified function (Henikoff et al., 1997). Different functional and biochemical properties have been attributed to the different STs identified in mammals. The same is probably true for the *A. thaliana* ST gene family, where the 18 different members might code for proteins with different biological functions. The STs so far characterized at the biochemical level from *A. thaliana* reveal that they accept structurally diverse compounds as substrates which include: flavonoids, jasmonates, brassinosteroids, and glucosinolates. This indicates that the plant sulfotransferases are involved in sulfonating a diverse range of compounds belonging to different metabolic pathways.

Chapter 4

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF A FLAVONOID 7-SULFOTRANSFERASE FROM *ARABIDOPSIS THALIANA*

RESULTS

Sequence analysis of *AtST3a*

The *AtST3a* cDNA was obtained from the *A. thaliana* Biological Resource Center (Genebank accession number: T75675). A search of the *A. thaliana* database indicates that *AtST3a* (bases 1145 to 2116) is located on chromosome III (BAC clone T14D3, Accession Number AL138649). This BAC clone also contains another open reading frame encoding a putative ST (*AtST3b*, bases 3483 to 4472) separated by 1367 bp from the *AtST3a* coding sequence. DNA sequence analysis revealed that the *AtST3a* and *AtST3b* open reading frames code for proteins of 323 and 329 amino acids which correspond to molecular masses of 37.5 and 38.06 kD, respectively (Figure 4.1). The AtST3a and AtST3b enzymes exhibit 86% amino acid sequence identity and 91% sequence similarity, suggesting that they might represent isoenzymes sharing similar substrates. The AtST3a and AtST3b proteins share 43% identity with the flavonol 3-ST and flavonol 4'-ST of *Flaveria* species and 44-48% identity with the *Brassica napus* steroid STs and with AtST1. The AtST3a and AtST3b deduced protein sequences contain the conserved domains present in all eukaryotic cytosolic sulfotransferases (Varin et al., 1992) (Figure 4.1).

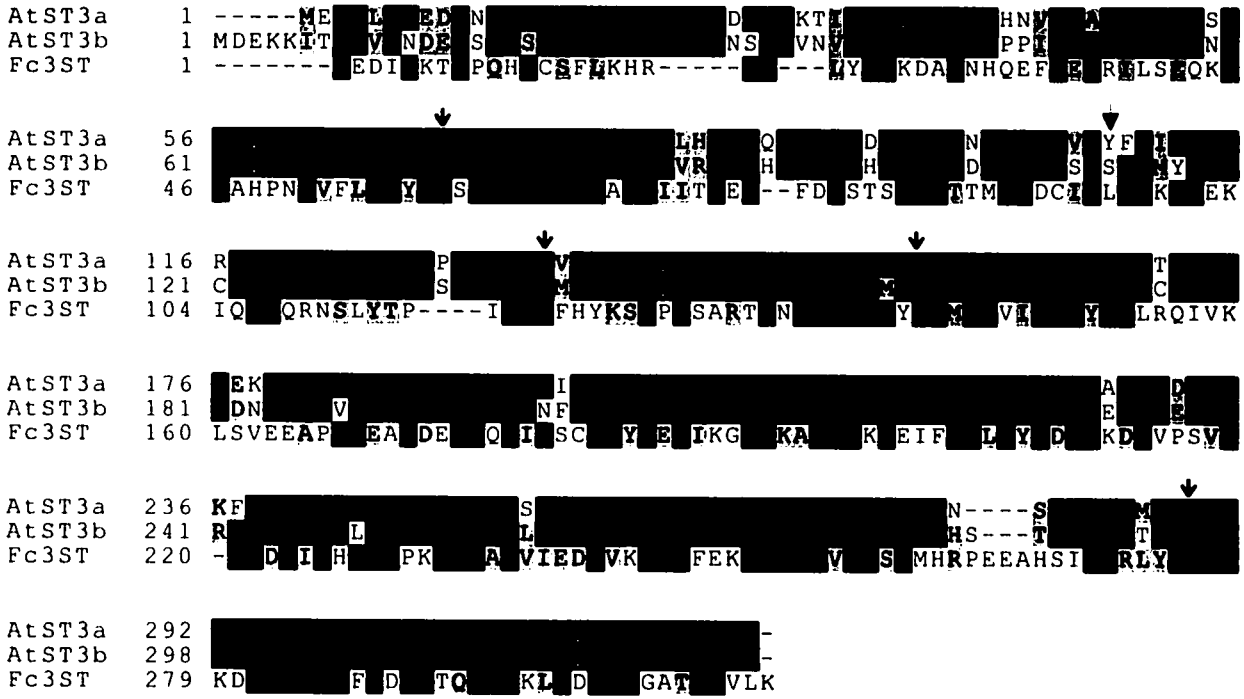


Figure 4.1

Amino acid sequence alignment of AtST3a, AtST3b and flavonol 3-ST from *Flaveria chloraefolia* (Accession number M84135). Deduced amino acid sequences were aligned with the CLUSTALW 1.8 program. Identical amino acids are boxed in black and conservative changes are boxed in gray. Hyphens indicate gaps introduced to maximize alignment. Open arrows indicate amino acid residues that are involved in catalysis in the flavonol 3-ST (Marsolais and Varin, 1997). Black arrows identify amino acids corresponding to those involved in determining substrate specificity in flavonol 3-ST (reviewed in Marsolais et al, 2000).

Biochemical characterization of AtST3a

In order to define the biochemical function of AtST3a, its coding sequence was expressed in *E. coli*. The histidine- tagged recombinant AtST3a enzyme could be purified to apparent homogeneity by Ni-agarose and PAP-agarose affinity chromatography (Figure 4.2). The PAP- agarose affinity chromatography removes inactive proteins that are purified by Ni-agarose chromatography, resulting in enzyme preparations with higher turnover number. The purified AtST3a enzyme was used to test different putative sulfate acceptor compounds including phenolic acids (gallic acid, sinapic acid, sinapoyl choline and sinapoyl glucose), brassinosteroids, desulfoglucosinolates, salicylic acid, gibberellic acid, phenylpropanoids (*p*-coumaric, caffeic and ferulic acids), hydroxy jasmonates, flavones, flavonols and coumarins. AtST3a specifically catalyzed the transfer of the [^{35}S]-labeled sulfonate group from PAPS to a number of flavones, flavonols and their monosulfates. AtST3a accepts as substrates flavones and flavonols having a hydroxyl group at position 7 (Figure 4.3). The inability of AtST3a to accept flavonols already substituted at position 7, such as apigenin-7-sulfate, kaempferol 7-sulfate or rhamnetin, suggests that it is specific for this position. Taken together these results indicate that AtST3a exhibits strict specificity for position 7 of flavone and flavonol compounds.

Substitution at position 3, as in rutin (quercetin 3-rutinoside), does not affect activity. Flavonols hydroxylated at position 3', 4' and/or 5' (kaempferol, quercetin, myrecetin), dihydroquercetin and genistein (5, 7, 4'- trihydroxyisoflavone) are also accepted as substrates. The fact that AtST3a does not accept quercetagetin (6-hydroxy quercetin) and accepts gossypetin (8-hydroxy quercetin) with low efficiency indicates that an additional hydroxyl group at position 6 or 8 interferes with the sulfonation

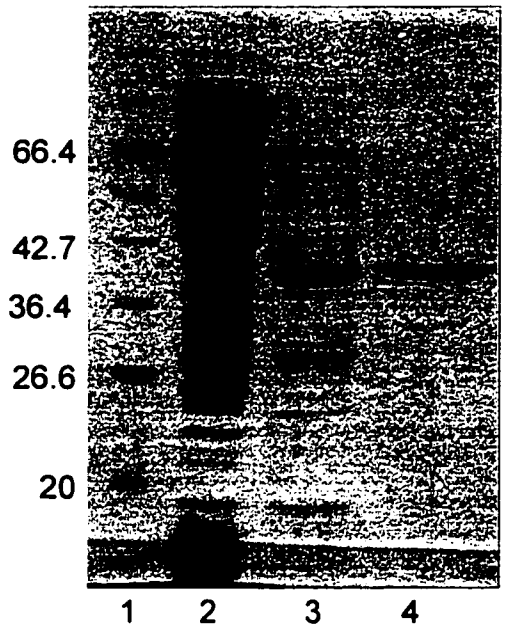
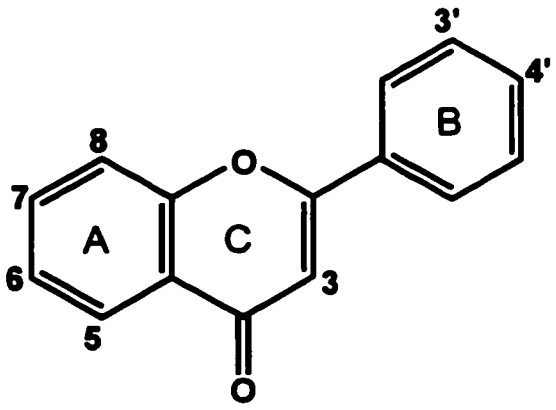


Figure 4.2

SDS-PAGE of fractions collected during purification of recombinant AtST3a. Lane1, Molecular weight markers (kDa); Lane 2, Crude *E. coli* extract; Lane 3, Ni-agarose purified extract; Lane 4, PAP-agarose purifed extract. Proteins were visualized by Coomassie staining.



Flavonoid numbering system

Compound	Position 3	Position 5	Position 6	Position 7	Position 8	Position 3'	Position 4'
Kaempferol 3-sulfate	OSO_3^-	OH	H	OH	H	H	OH
Kaempferol	OH	OH	H	OH	H	H	OH
Isorhamnetin	OH	OH	H	OH	H	OCH_3	OH
Rhamnetin	OH	OH	H	OCH_3	H	OH	OH
Galangin	OH	OH	H	OH	H	H	H
Quercetagetin	OH	OH	OH	OH	H	OH	OH
Gossypetin	OH	OH	H	OH	OH	OH	OH
Apigenin 4'-sulfate	H	OH	H	OH	H	H	OSO_3^-
Apigenin	H	OH	H	OH	H	H	OH
Quercetin	OH	OH	H	OH	H	OH	OH
Chrysin	H	OH	H	OH	H	H	H
7-hydroxyflavone	H	H	H	OH	H	H	H
3- hydroxyflavone	OH	H	H	H	H	H	H

Figure 4.3

Structural formulae of flavonoids used as substrates for AtST3a.

reaction at position 7 (Figure 4.3). The fact that coumarins (umbelliferone, esculentin, daphnetin) are not sulfonated by AtST3a, suggests that the enzyme is specific for the flavonoid structure.

The affinity of AtST3a for different flavones and flavonols was determined using purified recombinant enzyme at 5 μM PAPS (5 fold K_m) concentration and different substrate concentrations. Double reciprocal plots of initial velocity versus the substrate concentration were used to determine the apparent K_m and V_{max} for that particular substrate (Table 4.1). Of all the compounds tested, kaempferol 3-sulfate was by far the preferred substrate of the enzyme followed by isorhamnetin and kaempferol.

Enzyme kinetics

Since AtST3a exhibited the highest affinity for kaempferol 3-sulfate, this monosulfate was used to determine the kinetic parameters of the enzyme. Double reciprocal plots with kaempferol 3-sulfate as the variable substrate at several fixed concentrations of PAPS resulted in an intersecting pattern (Figure 4.4). A similar pattern was obtained with PAPS as the variable substrate. The secondary slope and intercept replots were linear. As calculated from the replots, the V_{max} for the conversion of kaempferol 3-sulfate to kaempferol 3,7-disulfate was 285.7 pkatal/mg protein. The K_m values for kaempferol 3-sulfate and PAPS were found to be 2 μM and 1 μM, respectively.

Table 4.1:

Kinetic parameters of AtST3a for different flavonoids

Compound	Km (μM)	Vmax (pkatal/mg)	Vmax/Km
Kaempferol 3-sulfate	2	286	137
Isorhamnetin	6	232	38
Kaempferol	3.2	116	36
Galangin	11	270	24
Quercetin	1.5	24	16
Apigenin 4'- sulfate	5.1	45	8.8
Apigenin	51	370	7.2
Chrysin	40.6	156	3.8
7-hydroxyflavone	26	91	3.4

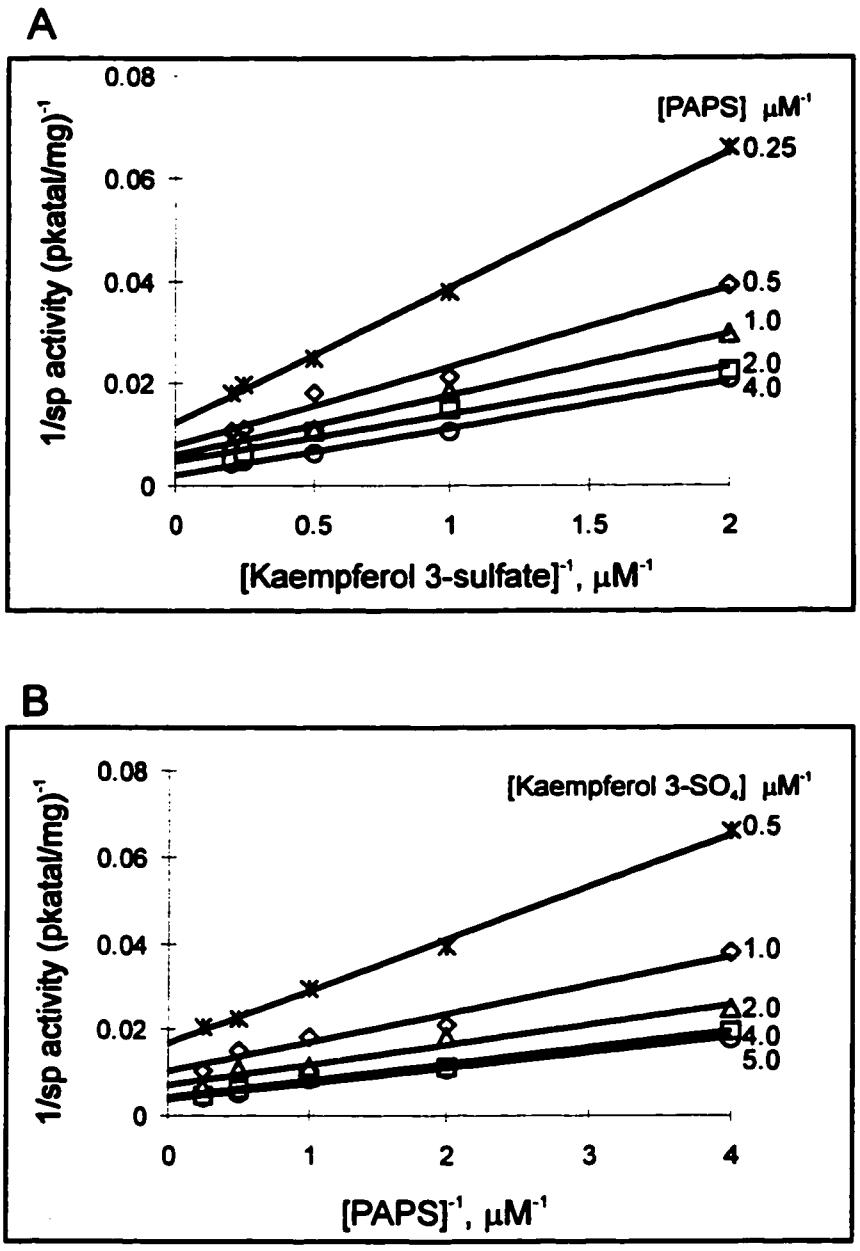


Figure 4.4

Analysis of kinetic mechanism of AtST3a- catalyzed conversion of kaempferol 3-sulfate to kaempferol 3,7- disulfate A) Double reciprocal plot of initial velocity versus kaempferol 3- sulfate concentration at different concentrations of PAPS. B) Double reciprocal plot of initial velocity versus PAPS at different concentrations of kaempferol 3- sulfate.

Identification of enzyme reaction product formed *in vitro*

Analysis of the reaction products on TLC indicated that only one reaction product was made. As expected, the R_f values of the reaction products on cellulose TLC in BAW decreases with an additional sulfate group on the flavonol (Figure 4.5). The [^{35}S]-labeled reaction product when kaempferol was used as a substrate, co-chromatographed with authentic kaempferol 7-sulfate on C18-reverse phase HPLC column (Figure 4.6).

Regulation studies

In order to study the pattern of expression of *AtST3a*, RNA was extracted from *A. thaliana* plants grown for 5 days, 15 days, 20 days and 30 days. RNA was also extracted from roots, flowers and siliques from 30 days old plants and all samples were subjected to RT-PCR. Figure 4.7 shows that the *AtST3a* transcript was detectable only from 5- day old seeding extracts. 15 days old *Arabidopsis* plants were also subjected to a number of physical and chemical treatments. *AtST3a* transcript is not detectable in plants subjected to cold, heat, hypoxia, salt stress or treatment with salicylic acid, methyl jasmonate, gibberellins, auxins and cytokinins (data not shown). Furthermore, the *AtST3a* transcript could not be detected in wounded or etiolated *A. thaliana* mRNA extracts.

Analysis of transgenic *Arabidopsis thaliana* expressing the *AtST3a* in the antisense orientation

To assess the biological function of AtST3a, we constructed transgenic *35S*: *AtST3a* plants expressing the gene in the antisense orientation. When transforming *Arabidopsis* with the 35S- antisense *AtST3a* construct, we produced 11 independent lines,

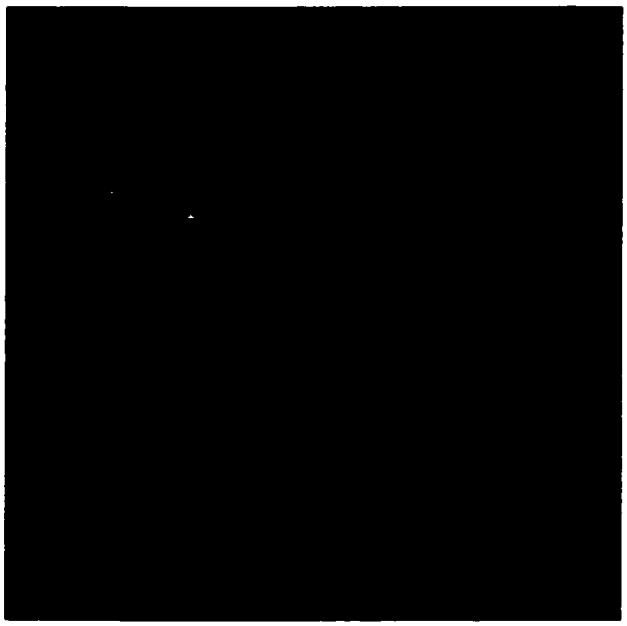


Figure 4.5

Photograph of an autoradiogram of the chromatographed reaction products of AtST3a with flavonoids substrates. (A) PAPS (B) apigenin (C) apigenin 4'-sulfate (D) quercetin (E) quercetin 3- sulfate (F) quercetin 3, 3'- disulfate(G) kaempferol (H) kaempferol 3- sulfate. TLC was carried out on cellulose plates using butanol/ acetic acid/water (3:1:1, v/v/v) as solvent. Compounds B through H were used as substrates for recombinant AtST3a.

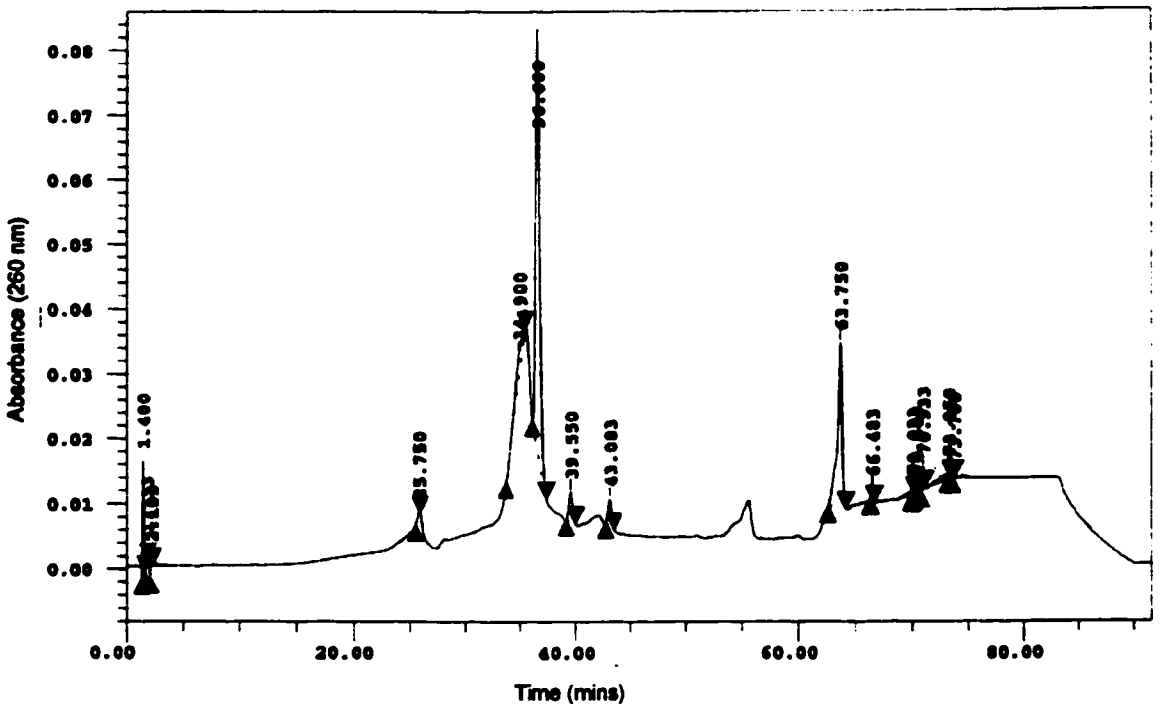


Figure 4.6

Elution profile of kaempferol 7- sulfate when chromatographed on C18 reverse phase HPLC column (See methods). The ^{35}S labeled product when kaempferol was used as a substrate co-eluted with kaempferol 7- sulfate at a retention time of 36 to 37 mins.

Figure 4.7

RT-PCR of RNA samples from *Arabidopsis thaliana* plants at different developmental stages (5, 15, 20 and 30 days old) and flowers (F), siliques (S) and roots (R). g-DNA represents amplification from the genomic DNA. 2.5 μg of total RNA was reverse transcribed and amplified with *AtST3a* 5' and 3' primer (See materials and methods). The amplification products were loaded on ethidium bromide- stained agarose gel. The RT products were also amplified with *ACTIN*- specific primers to confirm that equal amounts of RT product were used in each amplification reaction.

exhibiting resistance to kanamycin. The transgenic lines were analyzed for the segregation ratio of the selectable marker for kanamycin resistance at the T_2 generation. Based on the segregation ratios (Kan_r: Kan_s::3:1), 2 lines were shown to have a single insertion of the transgene (Figure 4.8A). The levels of AtST3a antisense transcript in the T_3 plants of four independent transgenic lines were analyzed by RT-PCR (Figure 4.8B). All the lines tested were found to express the *AtST3a* antisense RNA transcript.

Phenotype of *Arabidopsis thaliana* plants with reduced levels of *AtST3a* transcript

The growth of the transgenic plants expressing *AtST3a* was compared with that of the wild-type under various growth conditions. Plants were grown under both long-day (16 h light, 8 h dark) and short-day conditions (8 h light, 16 h dark). There was no difference between the growth of the transgenic lines and that of the wild type under these conditions. Figure 4.9 shows the phenotype of wild type *Arabidopsis* plants of ecotype C24 as compared to transgenic plants expressing the *AtST3a* in the antisense orientation under the control of the *CaMV: 35S* promoter. Several growth parameters were measured 5 weeks after germination, including the height of the main inflorescence, the number of inflorescence, the number of rosette leaves, and the number of flowers and siliques (data not shown). Root growth and root morphology was also compared. In all instances, there was no significant difference between the transgenic lines expressing *AtSt3a* in the antisense orientation and the wild type.

A

Line	Kan_R : Kan_S	Copy number of transgene by southern blot
3-3	3:1	1
3-4	3:1	1
3-7	6:1	3
3-8	100% Kan_R	nd
3-9	6:1	4
3-11	100% Kan_R	5
3-12	6:1	nd
3-17	4:1	nd
3-22	5:1	2
3-24	5:1	0
3-32	16:1	nd

B

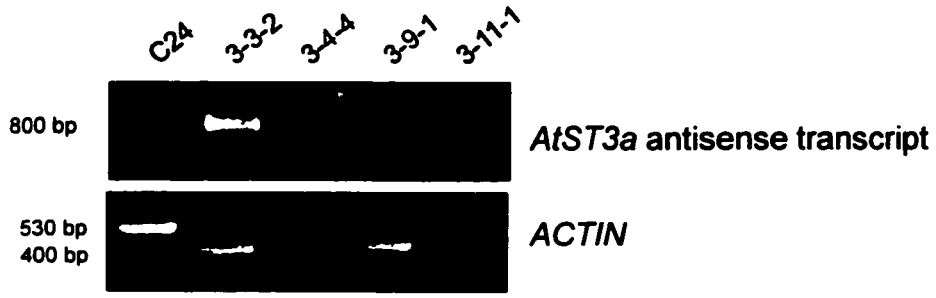


Figure 4.8

Characterization of *35S: AtST3a* antisense transgenic lines. A) Segregation ratio of the kanamycin resistance marker and copy number of transgene in independent transgenic lines. nd= not determined. B) RT-PCR of RNA samples from *A. thaliana 35S*: antisense transgenic lines and wild type, C24 plants. 3-3-2, 3-4-4, 3-9-1 and 3-11-1 represent four independent lines. 2.5 μg of total RNA was reverse transcribed and amplified with the AtSTX-210 primer and the AMV primer (See materials and methods). The products of amplification were loaded on ethidium bromide stained agarose gel. The RT products were also amplified with *ACTIN*-specific primers to confirm that equal amounts of RT product were used in each amplification reaction.

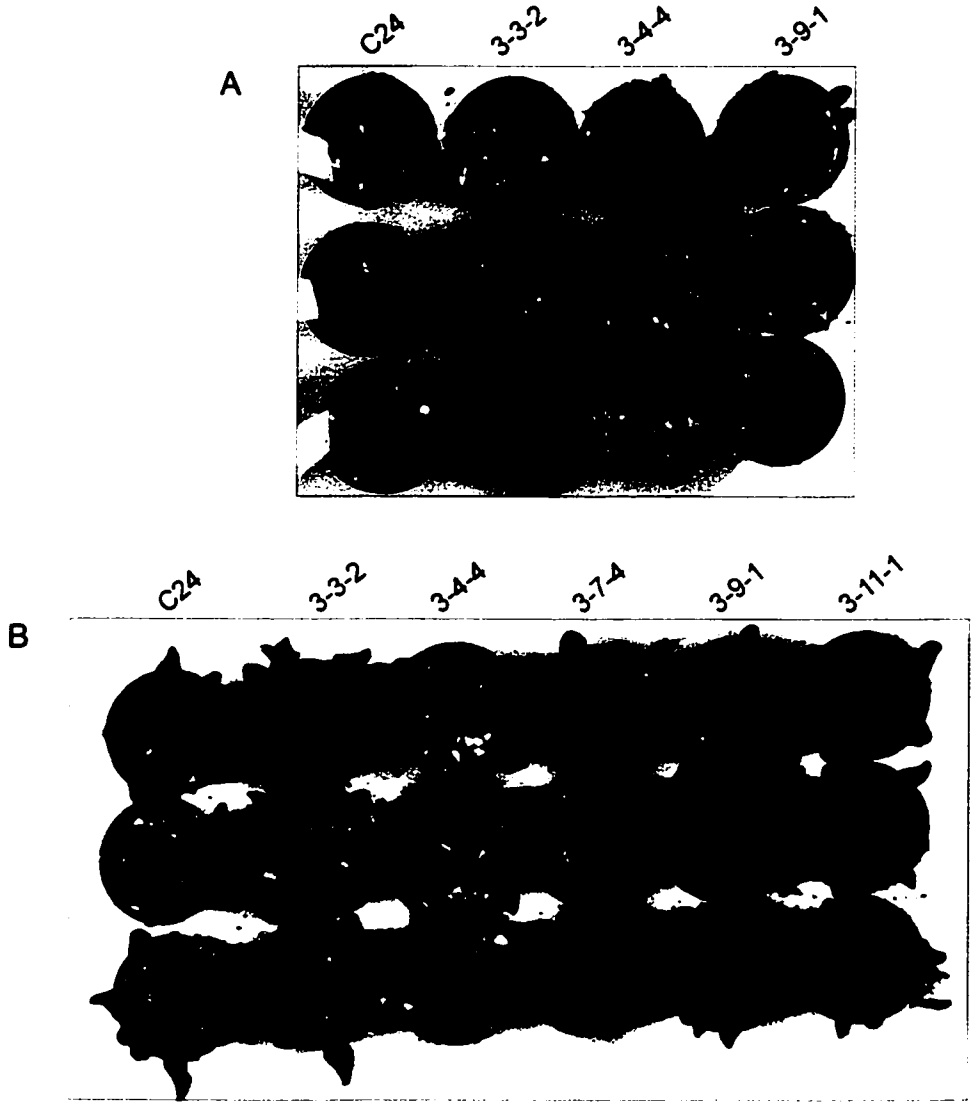


Figure 4.9

Phenotype of *Arabidopsis* plants expressing *35S: AtST3a* in the antisense orientation grown under long day conditions (16 hours light). A) Comparison of 16 days-old wild type (C24) and transgenic plants. 3-3-2, 3-4-4, 3-7-4, 3-9-1 and 3-11-1 represent five independent lines. At least 5 plants from each line were examined. B) Comparison of 25 days old wild type and transgenic plants under similar growth conditions as in A.

Identification of the enzyme substrate found *in vivo*

The absence of conclusive results from the experiments with the transgenic plants prompted us to search for endogenous substrates of AtST3a present in *A. thaliana* extracts. As a preliminary experiment, a methanolic extract of *A. thaliana* was passed through a DEAE Sephadex A25 column (Pharmacia Biotech) to bind polar compounds including flavonol sulfates to the column. The eluant (bound fraction) and the wash (unbound fraction) from this column were acid hydrolyzed to release flavonol aglycones. Enzymatic activity of AtST3a with this acid hydrolyzed extracts was tested in enzyme assays. Enzyme activity was detected in both the bound and unbound, acid hydrolyzed fractions but not in unhydrolyzed fractions, indicating that the position of sulfation was occupied and could be hydrolyzed by acid treatment. The [^{35}S]- labeled products formed were run on TLC using water as solvent. Two radioactive spots were visible with an R_f value of 0.26 and 0.77 (Figure 4.10). The R_f value of the major [^{35}S]- labeled product (R_f =0.26) was found to be in the range of that for kaempferol 7-sulfate and quercetin 7-sulfate (Barron et al., 1988). When the products of the reaction were co-chromatographed on TLC with the unhydrolyzed fraction, and the TLC sprayed with diphenylborinate, the major radioactive spot co-migrated with a blue spot, which is the color observed for kaempferol 7-sulfate under these conditions (Barron et al., 1988). In addition, the retention time of the major [^{35}S]- labeled product on HPLC was identical to that of authentic kaempferol 7- sulfate (data not shown).

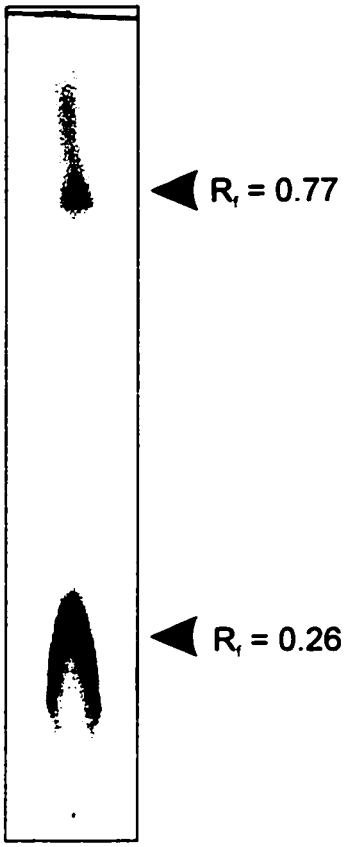


Figure 4.10

Photograph of an autoradiogram of the chromatographed reaction products of AtST3a when acid hydrolyzed methanolic extract of *Arabidopsis thaliana* was used as a substrate. TLC was carried out on cellulose TLC plates using water as solvent.

Discussion

AtST3a and AtST3b proteins share 40% amino acid sequence identity with the flavonol STs of the *Flaveria* species. Therefore they are below the 45% amino acid sequence identity threshold value to be considered members of the *Flaveria* flavonol ST gene family, suggesting that they could have evolved independently. This might explain the differences in their enzymatic properties, the specificity of AtST3a being broader as compared with the highly specific flavonol 3- and 4'-STs.

Site- directed mutagenesis studies of the flavonol 3- and 4'-STs indicate that Leu96 of flavonol 3-ST and Tyr106 of flavonol 4'-ST are critical in determining the substrate and position specificities of the individual enzymes (Marsolais and Varin, 1997). In addition, it has been demonstrated that in the mouse estrogen ST, the mutation of a tyrosine to a leucine at the position corresponding to residue 96 of the flavonol 3-ST, gives rise to a mutant enzyme which exhibits the same substrate preference as the hydroxysteroid ST (Petrotchenko EV et al., 1999). Amino acid sequence alignment of AtST3a, AtST3b and *Flaveria chloraefolia* flavonol 3-ST shows that AtST3a has a Tyr at the position corresponding to Leu96 of flavonol 3-ST and Tyr106 of flavonol 4'-ST, while ATST3b has a serine residue at the same position. The presence of different amino acids at this critical position suggests that AtST3a and AtST3b might have different substrate and/or position preferences.

When expressed in *E. coli*, the enzyme encoded by the *AtST3a* exhibits specificity for flavones, flavonols and their monosulfates. The results presented in Table 4.1 clearly indicate that AtST3a prefers flavonols over flavones. Furthermore, among the flavonols that were tested, AtST3a was shown to exhibit a higher affinity for flavonol monosulfates

as compared to their aglycones. The 3.8 fold greater V_{max}/K_m value observed for kaempferol 3-sulfate as compared to kaempferol suggests that kaempferol 3- sulfate may be the endogenous substrate for the enzyme. In addition, this suggests the existence of another flavonol ST from *Arabidopsis* that could sulfonate the hydroxyl at position 3 of the flavonol ring. The fact that AtST3a accepts as substrates, flavones (apigenin, chrysin, 7-hydroxyflavone), flavonols with different substitution patterns on B ring (kaempferol, isorhamnetin, quercetin, myricetin (3, 5, 7, 3', 4', 5' hexahydroxyflavone)) and an isoflavone (genistein) suggests that the enzyme binding site is not specific for defined ring B and C structures. However, the position specificity exhibited by AtST3a suggests that the enzyme active site interacts with the A ring of flavonoids. The two isoforms of the previously characterized flavonol 7-ST from *Flaveria bidentis* (Varin and Ibrahim, 1991) exhibit strict position and substrate specificities. The *F. bidentis* flavonol 7-STs only accept quercetin 3, 3'- or 3, 4'-, disulfates and isorhamnetin 3-sulfate as substrates, indicating the requirement of a sulfate or methoxy group at position 3- or 4'- of the flavonoid ring. The fact that AtST3a exhibits broad specificity indicates that sulfonation of flavonols in *Arabidopsis* probably does not follow a similar stepwise sequential order as in *Flaveria* species. *AtST3b* has also been cloned, however we were unsuccessful in obtaining the soluble form of the enzyme in *E. coli*. It remains to be determined if this enzyme has overlapping or different substrate and position preferences as compared with AtST3a.

Kaempferol, quercetin and myricetin are the flavonols identified so far in *A. thaliana* (Burbulis IE et al., 1996, Shirly BW et al., 1995). The substrate specificity of AtST3a is consistent with the accumulation of these compounds in *A. thaliana*. However,

the absence of any reports on the occurrence of flavonol sulfates in *Arabidopsis* suggests that these compounds might be present in very low quantities in the plant and possibly expressed in specific tissues and during specific developmental stages. The biochemical characterization of an endogenous substrate for AtST3a is critical in addressing the biological function of this enzyme. Preliminary experiments were performed, in order to purify the endogenous substrate of AtST3a present in the acid hydrolyzed methanolic extract of *A. thaliana*. The first step of purification was performed on an ion-exchanger to enrich for the presence of the sulfated acidic compounds. This approach is commonly used to purify the acid sulfated glucosinolates (Prestera et al., 1996). The retained compounds are then hydrolyzed to liberate the aglycones and used in enzyme assays. On cellulose TLC, using water as solvent, the R_f value of the [^{35}S]- labeled product (R_f: 0.26) of AtST3a with the hydrolyzed fraction was in the range of those determined for kaempferol 7- sulfate (R_f: 0.28) and quercetin 7- sulfate (R_f : 0.24) (Barron et al., 1988). Furthermore, [^{35}S]- labeled product and kaempferol 7- sulfate exhibited identical retention time on reverse phase HPLC. These results suggest that the substrate of AtST3a is probably kaempferol and that its sulfated derivative might accumulate in vivo. Since the acid hydrolyzed extracts were used as substrates, the glycosidic and sulfate esters were converted to their respective aglycones. The experimental approach aimed at the purification of a substrate for AtST3a from *A. thaliana* extracts has few limitations. In the crude metabolite fractions that were used, the presence of inhibitors may not allow the detection of enzymatic activity with the preferred substrate. In addition, the enzymatic activity observed with a substrate purified from the plant does not prove that this

substrate is in fact sulfated *in vivo*. Therefore, we are presently developing LC-MS/MS methods to identify the flavonol sulfate esters present in *A. thaliana.*

RT- PCR experiments indicate that the *AtST3a* is expressed only at the early stage of seedling development. It has been shown previously that the accumulation of flavonol sulfates in *F. bidentis* is also developmentally regulated (Hannoufa et al., 1991). In *Flaveria*, the highest flavonol ST activity was detected in terminal buds and the first pair of leaves. In addition, the synthetic auxin, 2,4- dichlorophenoxyacetic acid was found to upregulate the flavonol 3-ST activity in *Flaveria bidentis* cell cultures (Ananvoranich et al., 1994). It has been proposed that sulfated flavonols might play a role in the regulation of polar auxin transport (Jacob and Rubery, 1988). Flavonol aglycones like quercetin and kaempferol bind to the naphthylphthalamic acid receptor and thus inhibit polar auxin transport from the basal end of stem cells. In contrast, sulfated flavonols act as antagonists of quercetin and thus allow auxin efflux from tissues where it is produced (Faulkner and Rubery, 1992). The absence of a visible phenotype in transgenic *Arabidopsis* expressing AtST3a in the antisense orientation suggests that flavonol sulfates might not participate in the control of auxin polar transport. The results obtained with *A. thaliana* transgenic plants did not allow us to reach a final conclusion on the biological function of *AtST3a.* It is possible that other flavonol STs, for example, AtST3b which exhibits 86% sequence identity with AtST3a might compensate for the loss of AtST3a activity in the transgenic plants.

Chapter 5

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF 12-HYDROXYJASMONATE SULFOTRANSFERASE FROM *ARABIDOPSIS THALIANA*

RESULTS

Cloning of *AtST2a*

The *AtST2a* cDNA (Genebank acc. No. T43254) was obtained from the *Arabidopsis* Biological Resource Center. *AtST2a* is localized on chromosome V and can be retrieved from the Genbank database under the accession number AB010697 from nucleotides 53936 to 55015. It is flanked by an open reading frame encoding another putative ST, *AtST2b* from nucleotides 50627 to 51670. DNA sequence analysis revealed that the *AtST2a* and *AtST2b* open reading frames code for proteins of 359 and 347 amino acids which correspond to molecular masses of 41.3 and 39.6 kDa, respectively. The *AtST2a* and *AtST2b* deduced protein sequences contain all the regions known to be involved in the binding of the sulfonate donor PAPS and in catalysis (Figure 5.1). Amino acid sequence alignment indicates that they share 85% amino acid sequence identity and 92% similarity, suggesting that they might be isozymes with similar substrate specificity.

Substrate specificity

In order to characterize the biochemical function of AtST2a, we studied the activity of purified recombinant AtST2a expressed in *E. coli*. The histidine-tagged AtST2a could be purified to apparent homogeneity by PAP-agarose chromatography (Marsolais and Varin, 1995)(Figure 5.2). Therefore the PAP-agarose purified protein was

Figure 5.1

Amino acid sequence alignment of AtST2a, AtST2b and flavonol 3-ST from *Flaveria chloraefolia* (Accession number M84135). Deduced amino acid sequences were aligned with the CLUSTALW 1.8 program. Identical amino acids are boxed in black and conservative changes are boxed in gray. Hyphens indicate gaps introduced to maximize alignment. Open arrows indicate amino acid residues that are involved in catalysis in the flavonol 3-ST. Black arrows identify amino acids corresponding to those involved in determining substrate specificity in flavonol 3-ST (Marsolais et al, 2000).

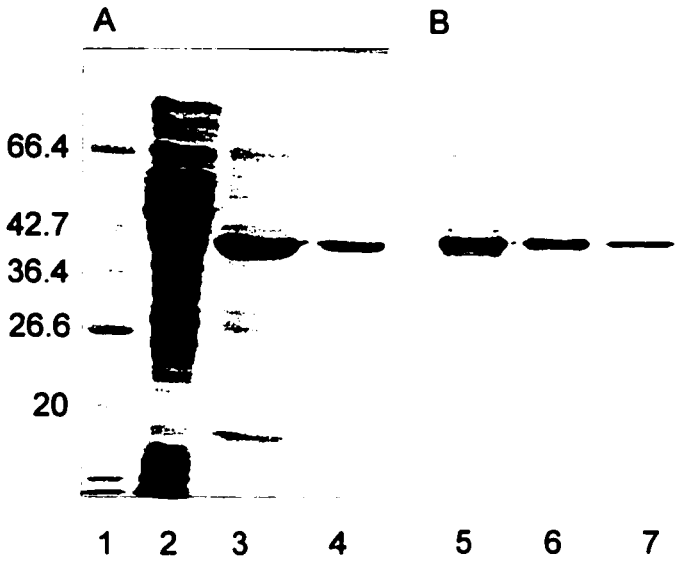


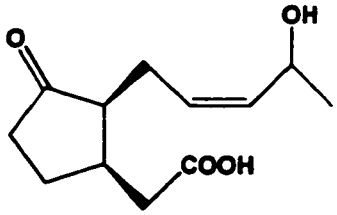
Figure 5.2

SDS-PAGE (A) and Western blot (B) of fractions collected during purification of recombinant AtST2a. Lane1, molecular weight markers in kDa; Lane 2 and 5, crude *E. coli* extract; Lane 3 and 6, Ni-agarose purified extract; Lane 4 and 7, PAP-agarose purified extract. Proteins were visualized by Coomassie staining. The proteins were probed with anti- AtST2a antibodies as described in materials and methods.

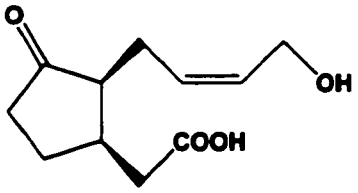
used to prepare rabbit anti-AtST2a antibodies. The purified recombinant AtST2a enzyme was used to test a variety of acceptor molecules of plant and mammalian origin. These compounds include phenolic acids, desulfoglucosinolates, flavonoids, steroids, gibberellic acids, phenylpropanoids and coumarins. AtST2a was found to exhibit strict specificity for 11- and 12-hydroxyjasmonic acid (Figure 5.3). It did not accept structurally related compounds such as, 12-hydroxy jasmonate methyl ester, cucurbic acid (CA)(Figure 5.3), 7-iso CA, 6-epi CA, 6-epi 7-iso CA and their methyl esters (Figure 5.3). All compounds tested were (+/-) enantiomers. In addition, compounds similar in structure to jasmonic acid that are found in mammals like prostaglandin E2, arachidonyl alcohol and 11-eicosenol (Figure 5.3) were not accepted as substrates by AtST2a.

Enzyme kinetics

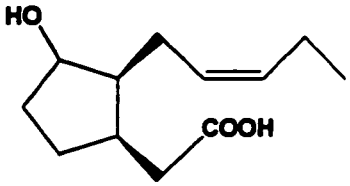
The kinetic parameters of AtST2a were determined from the results of substrate interaction kinetic experiments. AtST2a exhibited preference for 12-hydroxyjasmonate (12-OH-JA) over 11-hydroxyjasmonate (11-OH-JA). Double reciprocal plots with 12-OH-JA as the variable substrate at several fixed concentrations of PAPS resulted in an intersecting pattern (Figure 5.4). A similar pattern was obtained with PAPS as the variable substrate and the secondary slope and intercept replots were linear. As calculated from the replots, the V_{max} for the conversion of 12-OH-JA to 12-hydroxysulfonyloxyjasmonic acid was 37.5 pkatal/mg protein. The K_m values for 12-OH-JA and PAPS were found to be 10 μM and 0.06 μM, respectively. The Km value for 11-OH-JA was found to be 50 μM.



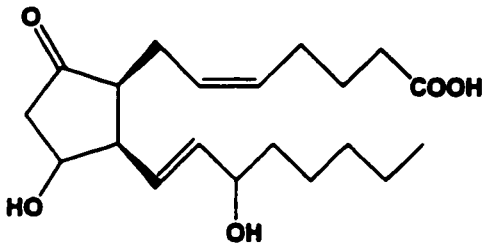
11-hydroxyjasmonic acid



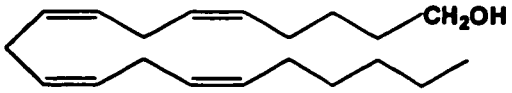
12-hydroxyjasmonic acid



Cucurbic acid



Prostaglandin E2



Arachidonyl alcohol

11-Eicosenol

Figure 5.3

Chemical structure of some compounds used as substrates with the recombinant AtST2a protein.

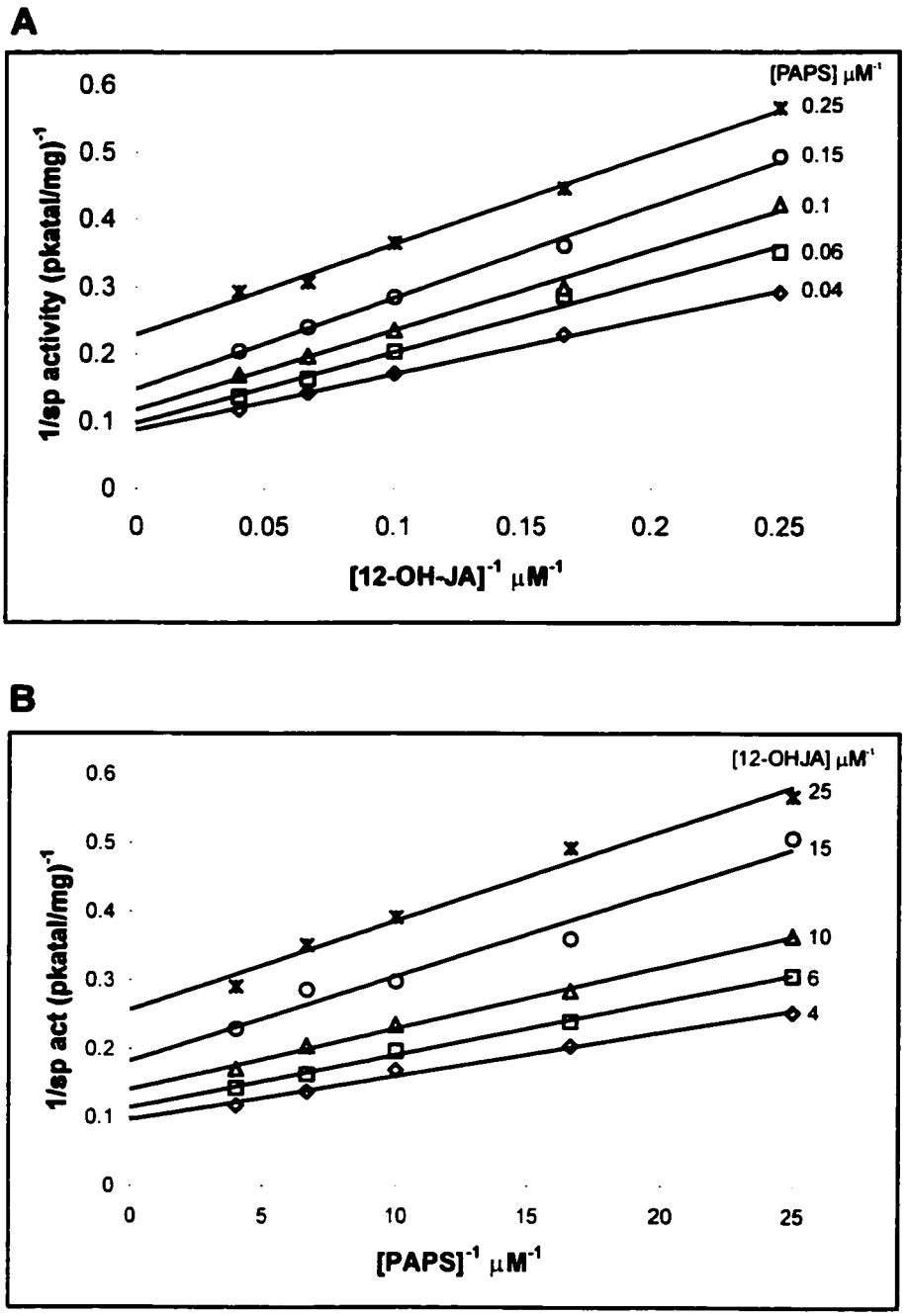


Figure 5.4

Analysis of kinetic mechanism of AtST2a- catalyzed conversion of 12-OH-JA to 12-OH-JA sulfate. A) Double reciprocal plot of initial velocity versus 12-OH-JA concentration at different concentrations of PAPS. B) Double reciprocal plot of initial velocity versus PAPS at different concentrations of 12-OH-JA.

Properties of AtST2a

To determine the pH optimum of AtST2a enzyme activity, the sulfonation of 12-OH-JA was carried out at fixed substrate and PAPS concentration in buffer ranging from pH 6 to 10. The pH optimum for AtST2a was found to be 7.5. The enzyme is active in a broad pH range and retains approximately 80% enzyme activity at pH 9.5.

Since JA is structurally similar to 12-OH-JA, we tested if it was acting as a competitive inhibitor of 12-OH-JA in enzyme assays. The assays were performed with fixed concentration of 12-OH-JA (100 μM) and variable concentrations of JA (0.1 μM to 100 μM). Addition of JA did not affect the amount of product formed (data not shown), indicating that JA does not interfere with the binding of 12-OH-JA at the enzyme active site.

Identification and quantification of jasmonates in *Arabidopsis thaliana* and Tobacco

To date, the presence of 11- and 12-OH-JA has been reported to occur only in a few plant species (Yoshihara et al., 1996). In order to validate the results of our *in vitro* studies, methanolic extracts of *A. thaliana* were analyzed for the presence of hydroxylated jasmonates using GC-MS (Figure 5.5). The method developed by Dr. Otto Miersch (Liebniz Institute of Plant biochemistry, Halle, Germany) allows the simultaneous identification and quantification of JA, 11- and 12-OH-JA. 11- and 12-OH-JA and JA were detected and quantified in the two ecotypes of *A. thaliana* that were analyzed (Table 5.1). JA and 11-OH-JA were found to be present in equivalent amounts in *Arabidopsis*. The quantities of 12-OH-JA are approximately 4 fold less than those of

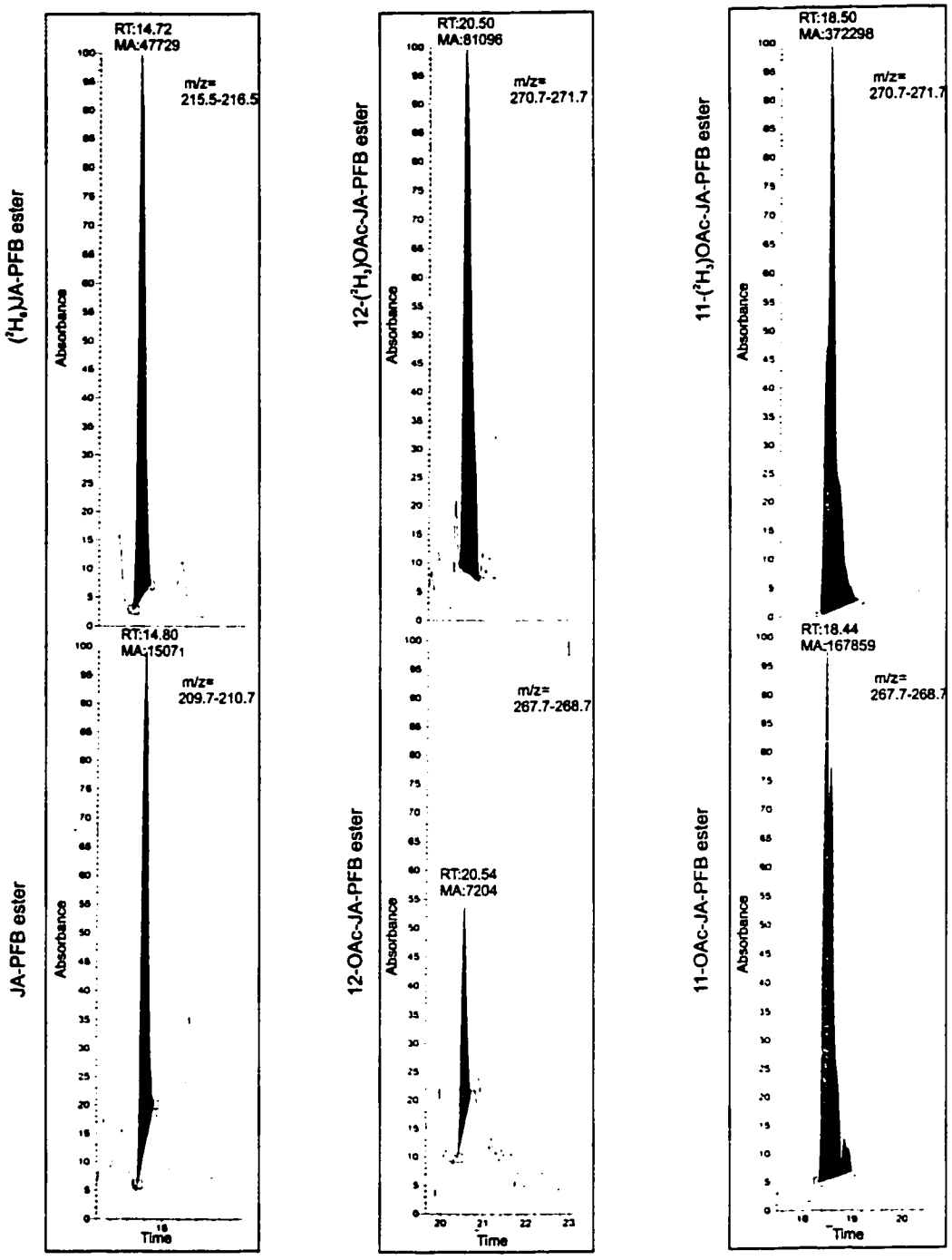


Figure 5.5

Compiled GC-MS profiles of jasmonates obtained from the GCQ Finnigan instrument. RT: retention time; MA: peak area. The content of JA, 12-OH-JA and 11-OH-JA were calculated on the basis of peak area of internal deuterated standards. The co-elution of the deuterated internal standards and the jasmonates is confirmed by the presence of molecular ion of mass indicated on the top right corner of each column.

Table 5.1.

Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in *Arabidopsis thaliana* (18 days old) and mature tobacco plants.

Sample	JA (pmol/g)	12-OH-JA (pmol/g)	11-OH-JA (pmol/g)	12-OH-JA SO_4 (peak area/g)
Col0	186	56	285	211
C24	418	34	405	990
Tobacco	278	182	105	+

Values represent the results of a single experiment in which a pool of 30-50 plants grown in magenta boxes on MS media was analyzed. In the case of tobacco, a young leaf of a mature tobacco plant grown in soil under green house conditions was analyzed. + = detected but not quantified

JA and 11-OH-JA. In addition these compounds could also be detected in tobacco plants. However, in tobacco plants the levels of 12-OH-JA are higher than that of 11-OH-JA.

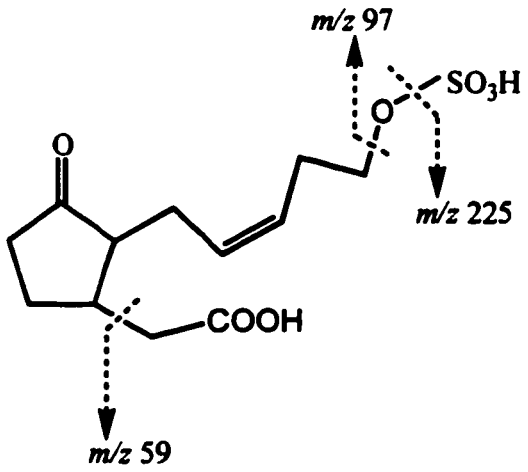
Identification of the enzyme reaction product formed *in vitro* and *in vivo*

In order to identify the reaction product formed by recombinant AtST2a using LC-MS/MS, the enzyme reaction was carried out using unlabeled PAPS. After extraction of the product in butanol, the solvent was evaporated and the product resuspended in methanol. The reaction product eluted with the same retention time and gave identical ion fragmentation patterns as authentic 12-OH-JA sulfate (Figure 5.6A and B).

So far 12-OH-JA sulfate has only been reported to occur in *Tribulus cistoides* of the Zygophylaceae family (Achenbach et al., 1994). In order to determine if 12-OH-JA sulfate is present in *A. thaliana,* methanolic extracts of *Arabidopsis* plants (Col0 and C24 ecotypes) were subjected to LC-MS methods and 12-OH-JA sulfate was identified as an endogenous compound in these plants (Table 5.1). The lack of internal standard precluded the quantification of this compound in plant extracts.

Regulation studies

In order to study the pattern of expression of *AtST2a*, RNA was extracted from rosette leaves, roots, stem (inflorescence excluding flowers and siliques), flowers and siliques of *A. thaliana* plants. RNA was also extracted from 15 days old *A. thaliana* plants treated with salicylic acid and methyl jasmonate. Analysis of the different RNA samples by northern blots using *AtST2a* as a probe revealed no detectable *AtST2a* transcript under normal growth conditions in any of the tissues analyzed (data not



12-hydroxyjasmonate sulfate

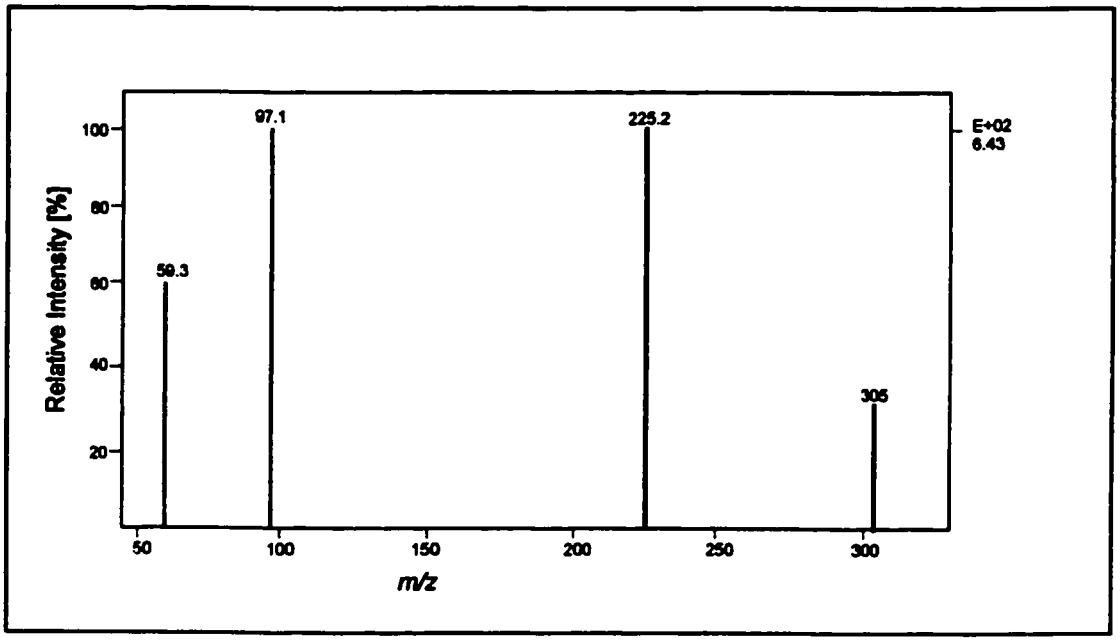


Figure 5.6A

Mass spectrum of 12-OH-JA sulfate obtained by collision- induced dissociation (CID) with 30 eV energy.

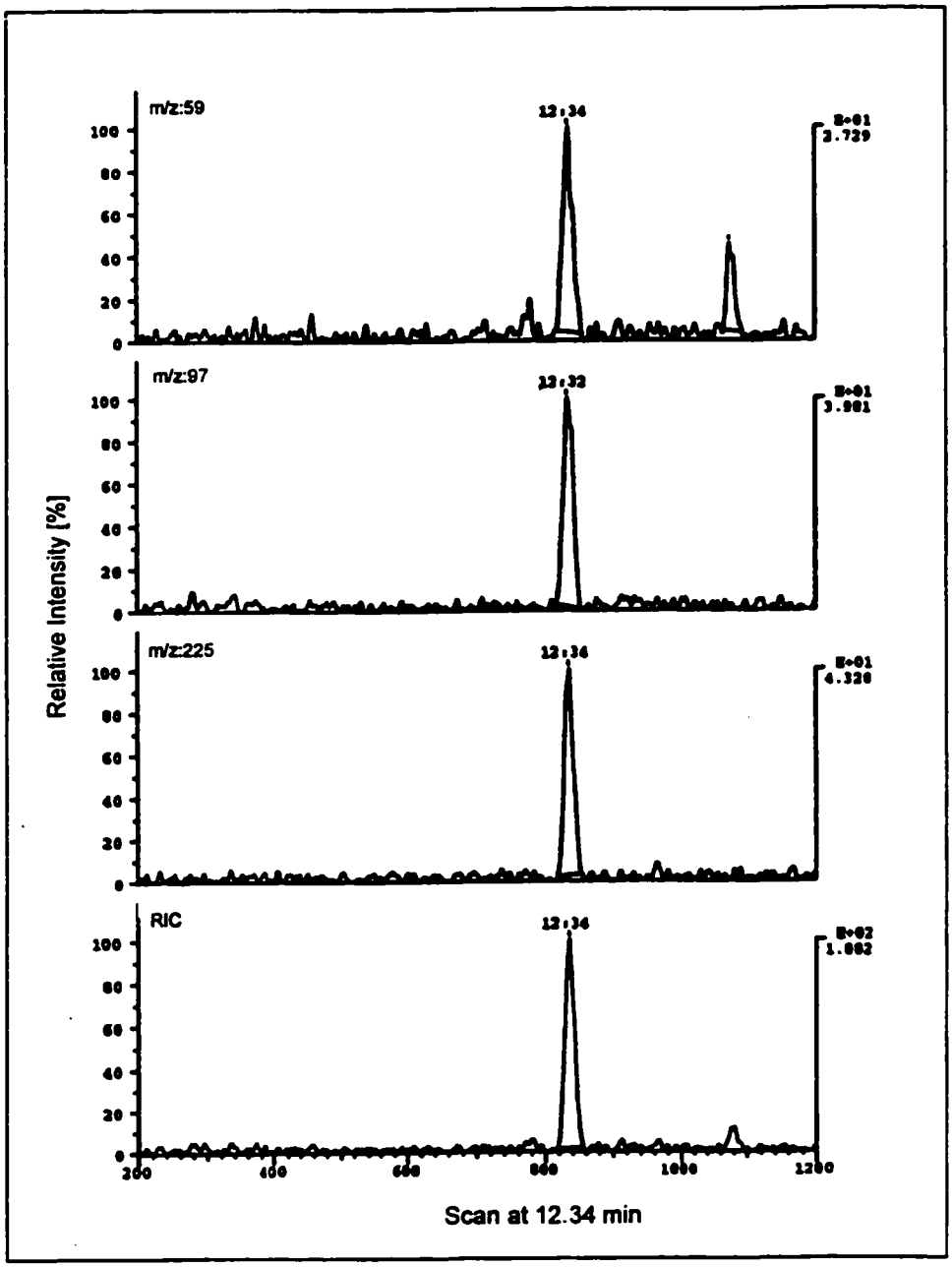


Figure 6B

HPLC profile obtained during selective ion monitoring of 12-OH-JA sulfate. The chromatogram shows traces of 12-OH-JA sulfate ion and the characteristic daughter ions of m/z 59, 97 and 225 obtained after collision induced dissociation.

shown). However a significant increase of *AtST2a* mRNA was obtained in plants treated with methyl jasmonate. *AtST2a* expression was also detectable in wounded plants and plants infected with a fungus (Figure 5.7). Treatment of 15 days old *Arabidopsis* plants with 0.1, 1, 10, 100 μM Me-JA, indicates that as low as 10 μM Me-JA strongly induces the expression of *AtST2a* mRNA (Figure 5.8A). In addition, the kinetics of induction by Me-JA treatment reveals rapid induction of *AtST2a*. The steady state mRNA levels increased within 30 minutes following Me-JA treatment and reaches a maximum in approximately 2 hours (Figure 5.8B). To test if the accumulation of *AtST2a* transcript is specific to methyl jasmonate or a general stress response, *Arabidopsis* plants were subjected to a number of physical and chemical treatments. *AtST2a* transcript is not detectable in plants subjected to cold, heat, hypoxia or salt stress. In addition, the treatment of plants with phytohormones like gibberellins, abscisic acid, zeatin and indole 3- acetic acid did not significantly increase *AtST2a* transcript level (data not shown).

Regulation of expression by 12-OH-JA

Since *AtST2a* expression is upregulated by Me-JA, the expression pattern of *AtST2a* following treatments with jasmonic acid biosynthetic precursors or metabolites derived from JA was studied. Figure 5.9A shows a northern blot of *Arabidopsis* plants treated with (+/-)JA, (+/-)12-oxo-PDA, (+/-)12-OH-JA, (-)-JA- (S)- Isoleucine conjugate, coronatine and 1-hydroxyindanoyl-(S)- Ile-methyl ester at indicated concentrations (Figure 5.9A and 5.9B). Since sorbitol treatment is known to induce JA biosynthesis, it was added in this study (Lehmann et al., 1995). Most of the compounds tested have an ability to upregulate *AtST2a* expression at 100 μM concentration. Interestingly, *AtST2a*

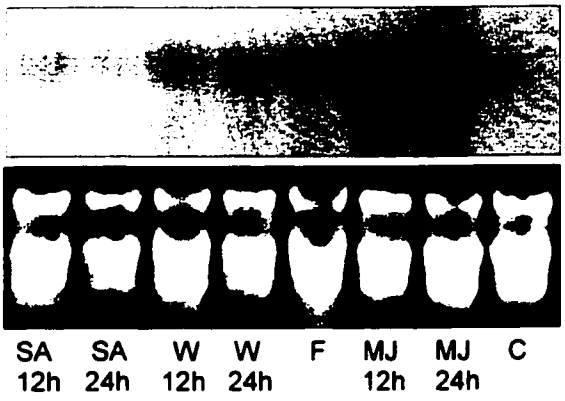


Figure 5.7

Northern blot analysis of *AtST2a* in 15 days old *Arabidopsis thaliana* plants treated with salicylic acid (SA), methyl jasmonate (MJ) or in response to wounding (W) and to fungal infection (F). Plants were treated with100 μg of SA or MJ for indicated time periods. 10 μg of total RNA was run on 1% agarose gel, transferred to nylon membrane and hybridized with ^{32}P labeled *AtST2a* cDNA as a probe. Ethidium bromide staining was used to confirm equal loading.

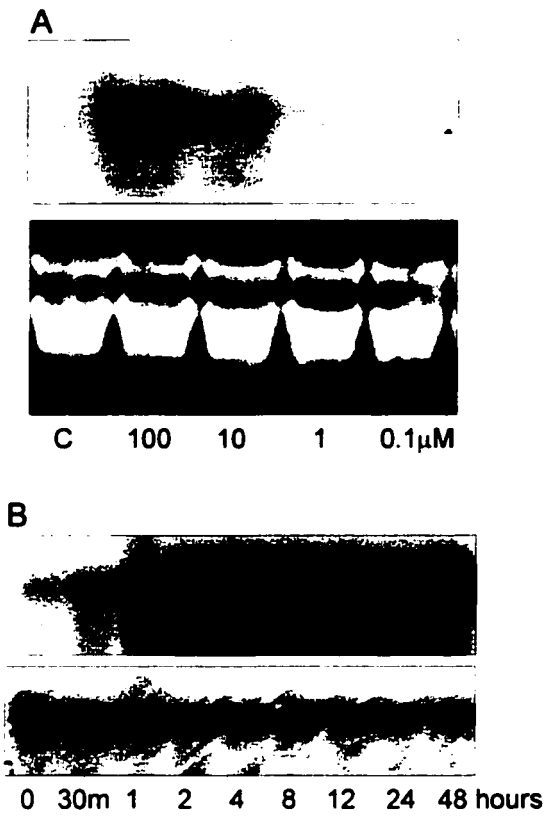


Figure 5.8

Dose response and kinetic of *AtST2a* mRNA accumulation during treatment of *Arabidopsis thaliana* plants with Me-JA. A) 15 days old *A. thaliana* plants grown on MS media in magenta boxes were treated with 100, 10, 1, 0.1 μM each of Me-JA or water (C). Total RNA was extracted 12 hours after treatment. 10 μg of total RNA was run on 1% agarose gel, transferred to nylon membrane and hybridized with ^{32}P labeled *AtST2a* cDNA as a probe. Ethidium bromide staining was used to confirm equal loading. B) Kinetic of *AtST2a* mRNA accumulation during treatment of 15 days old *A. thaliana* plants with 100 μM Me-JA. The plants were germinated on MS media in magenta boxes and total RNA was extracted at indicated time periods.10 μg of total RNA was treated as in A. The same nylon membrane was hybridized with the *ACTIN* probe to confirm equal RNA loading.

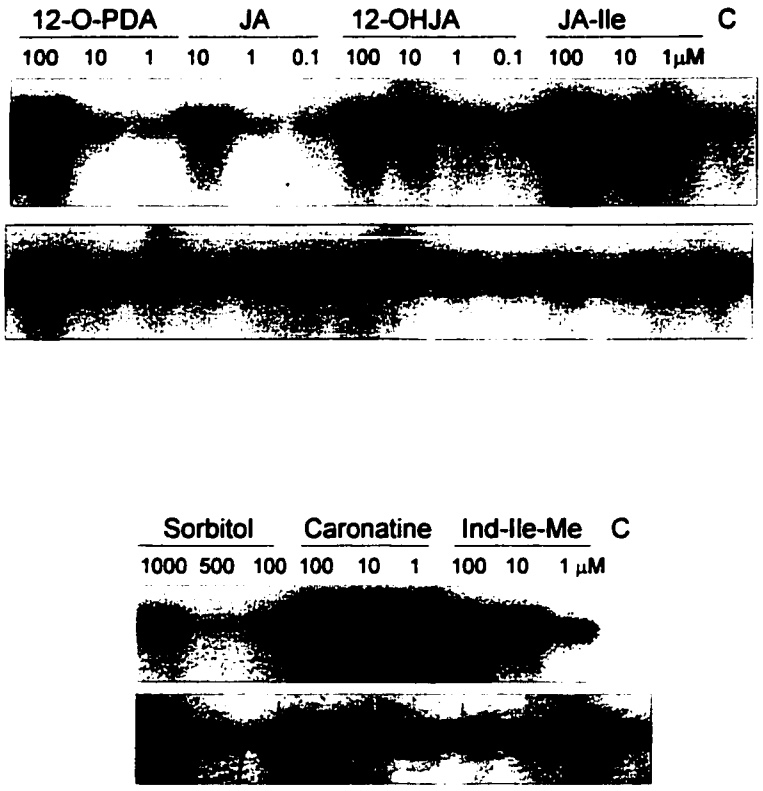
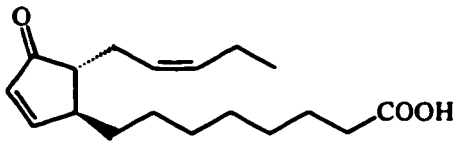
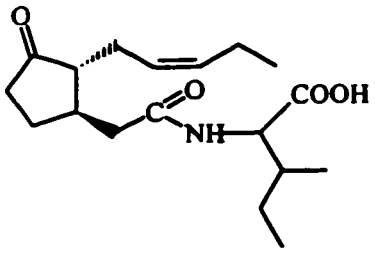


Figure 5.9A

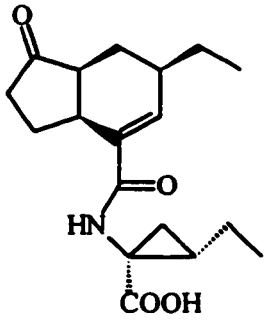
Northern blot analysis of *AtST2a* in 15 days old *Arabidopsis thaliana* plants treated with 12-OPDA (12-Oxo-phytodieonic acid), JA (jasmonic acid), 12-OH-JA, JA-Ile conjugate, sorbitol, coronatine,1-hydroxyindanoyl-Ile-methyl ester (Ind-Ile-Me) and water (C). The plants were germinated on MS media in magenta boxes and total RNA was extracted 12 hours after treatment. 10 μg of total RNA was run on 1% agarose gel, transferred to a nylon membrane and hybridized with ^{32}P labeled *AtST2a* cDNA as a probe. The same nylon membrane was hybridized with the *ACTIN* cDNA probe to evaluate the RNA loading.



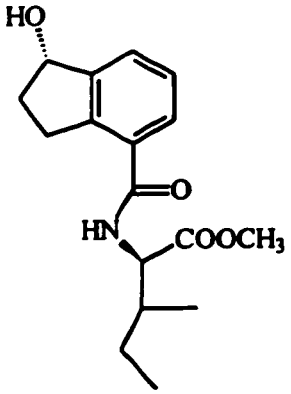
trans-12-Oxophytodienoic acid



(-)-Jasmonic acid-(*S*)-Isoleucine



Coronatine



1-Hydroxyindanoyl-(*S*)-Ile methyl ester

Figure 5.9B

Chemical structure of compounds used for the regulation studies of *AtST2a* expression.

expression is induced in a dose dependent manner by its substrate, 12-OH-JA. The steady state mRNA levels increased within 30 minutes following 12-OH-JA treatment (Figure 5.10). Coronatine, a fungal phytotoxin is also a strong inducer of *AtST2a* expression. The specific upregulation of *AtST2a* by 12-OH-JA was confirmed by RT-PCR using AtST2a-specific oligonucleotides (Figure 5.13).

Quantification of jasmonates and 12-OH-JA sulfate in Me-JA and 12-OH-JA treated plants

In order to determine if the upregulation of *AtST2a* expression by Me-JA and 12-OH-JA leads to increased endogenous levels of 12-OH-JA sulfate, the later compound was quantified in *Arabidopsis* plants treated with Me-JA and 12-OH-JA. Table 5.2 shows the comparison of the levels of jasmonates in *Arabidopsis* plants treated for 36 hours with Me-JA or 12-OH-JA as compared to untreated plants. Me-JA treatment resulted in increased endogenous levels of 12-OH-JA sulfate in the two ecotypes of *Arabidopsis* that were tested. Treatment with Me-JA increases endogenous levels of JA, 12-OH-JA and 11-OH-JA. Surprisingly, the increase observed for each compound differed significantly. 12-OH-JA levels increased by 20 fold while 11-OH-JA levels increased by 250 fold. Treatment with 12-OH-JA did not significantly affect the levels of 11-OH-JA and JA.

Expression of AtST2a in response to photoperiod

A low transcript level is observed in plants grown in presence of light (Figure 5.11A). However, when the plants are transferred to dark, *AtST2a* transcript levels start to increase in approximately 6 hours reaching a maximum after 48 hours (Figure 5.11A).

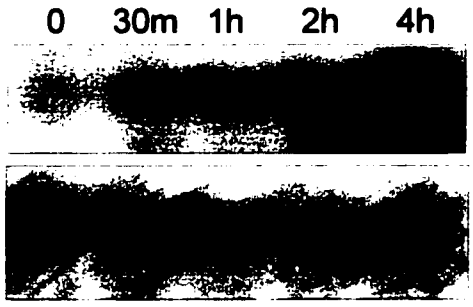


Figure 5.10

Kinetic of *AtST2a* mRNA accumulation during treatment of 15 days old *Arabidopsis thaliana* plants with 100 μM 12-OH-JA. The plants were germinated on MS media in magenta boxes and total RNA was extracted at the indicated time period after treatment. 10 μg of total RNA was run on 1% agarose gel, transferred to a nylon membrane and hybridized with ^{32}P labeled *AtST2a* cDNA as a probe. The same nylon membrane was probed with *ACTIN* cDNA to confirm equal RNA loading.

Table 5.2.

Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in two ecotypes of *Arabidopsis thaliana*.

Sample	JA (pmol/g)	12-OH-JA (pmol/g)	11-OH-JA (pmol/g)	12-OH-JA SO_4 (peak area/g)
Col0	186	56	285	211
Col0 Me-JA	25428	1152	72354	945
Col0 12-OH-JA	210	77482	398	1842
C24	418	34	405	990
C24 Me-JA	19663	985	35785	1778

18 days old plants were treated with 20 μM of Me-JA or 12-OH-JA for 36 hours. Values represent the results of a single experiment in which a pool of 30-50 plants in magenta boxes grown on MS media was analyzed.

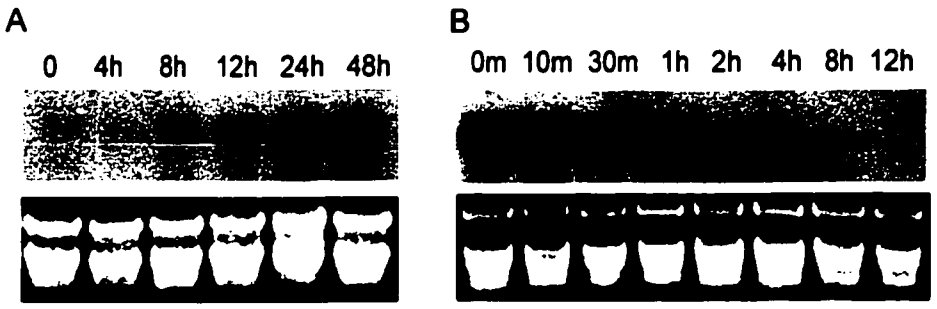


Figure 5.11

RNA gel blot analysis of *AtST2a* expression in the dark. A) 15 days old *A. thaliana* plants were kept in the dark for the indicated time periods. 10 μg of total RNA was resolved on 1% agarose gel, transferred to a nylon membrane and hybridized with ^{32}P labeled *AtST2a* cDNA as probe. B) *Arabidopsis* plants kept in the dark for 48 hours were brought back to light and total RNA extracted at indicated time intervals. Gel blots were performed as in A. The lower panels show the amount of RNA loaded as revealed by ethidium bromide staining of the rRNA bands.

When the dark-grown plants are re-exposed to light, *AtST2a* transcript levels start to decrease in approximately 30 minutes and return to the basal level observed in light-grown plants within two hours (Figure 5.11B). The specific upregulation of *AtST2a* transcript was confirmed by RT-PCR experiments using *AtST2a* specific oligonucleotides (data not shown).

Comparison of jasmonate levels in Arabidopsis plants grown under long-day conditions as compared to those growth in the dark

Since *AtST2a* expression is induced in the dark, the levels of jasmonates in light grown plants and dark grown plants was determined (Table 5.3). Approximately 2 fold decrease in the levels of JA and 12-OH-JA is observed when plants are transferred to the dark. However, the levels of 11-OH-JA are not affected by the dark treatment. 12 hours after transfer of the dark grown plants to light, a slight increase in the levels of JA and 12-OH-JA is observed. More time may be required for the plants to accumulate levels, similar to light grown plants.

Regulation of the Me-JA inducible defense response gene, Thi 2.1

Thi2.1 is a marker gene for Me-JA treatment or wounding (Epple et al., 1995; Bohlman et al., 1998), and we wanted to test if 12-OH-JA treatment affects the expression of this gene. RNA was extracted from *Arabidopsis* plants treated with 100, 10, 1 and 0.1 μM concentration of 12-OH-JA and 100 μM concentration of Me-JA. The RNA samples were reverse transcribed and the product amplified with *Thi 2.1*- and *AtST2a*-specific primers. Figure 5.12 shows that *Thi2.1* is not induced when plants are treated

Table 5.3.

Quantification of JA, 12-OH-JA and 11-OH-JA in 18 days old *Arabidopsis thaliana* plants grown under long-day conditions, grown in the dark for 48 hours and transferred back to light for 12 hours.

Sample	JA (pmol/g)	12-OH-JA (pmol/g)	11-OH-JA (pmol/g)
Col0	186	56	285
Col0 dark 48 hours	70	22	324
Col0 dark→light	82	32	759

Values represent results of a single experiment in which a pool of 30-50 plants grown in magenta boxes on MS media was analyzed.

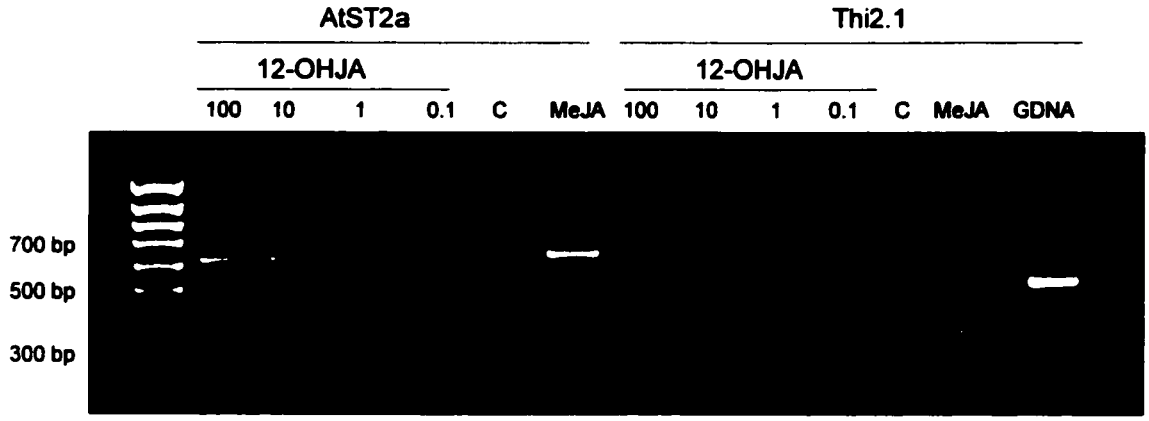


Figure 5.12

RT-PCR of mRNA from *Arabidopsis thaliana* plants treated with 12-OH-JA, Me-JA or water using *thi2.1* specific primers. 15 days old *A. thaliana* plants were treated with indicated concentrations of 12-OH-JA, 100 μM of Me-JA or water (C) for 12 hours. 2.5 μg of total RNA was reverse transcribed and amplified with *AtST2a*- specific primers and *Thi2.1*- specific primers. GDNA indicates amplification from genomic DNA. The amplification products were loaded on ethidium bromide stained agarose gel. The size of DNA fragments in the 100bp ladder are indicated.

with 12-OH-JA. As expected, *Thi2.1* expression is induced when the plants are treated with Me-JA (Epple et al., 1995).

Construction of transgenic *Arabidopsis thaliana* expressing *AtST2a* under the control of *CaMV: 35S* promoter

In order to characterize the biological function of *AtST2a*, its expression was modified in transgenic *A. thaliana*. When transforming *Arabidopsis* with the 35S-*AtST2a* construct, we produced 14 kanamycin-resistant lines, accumulating various amounts of AtST2a. The transgenic lines were analyzed for the segregation ratio of the selectable marker for kanamycin resistance at the T_2 generation (Table 5.4). According to the segregation ratios, line S11 and S16 were predicted to have single insertion of the transgene. The number of inserts present in nine independent transgenic lines was determined by Southern blot (Figure 5.13). Southern blot confirmed insertion of a single copy of the transgene in line S16. The number of insertions in different lines ranged from 1 to 6 copies. The level of AtST2a expression in eight independent transgenic lines was analyzed by Western blot. Plants from all the lines analyzed were found to accumulate various amounts of a protein having the expected molecular mass of AtST2a (41 kDa) (Table 5.4). Protein extracts from wild type Col0 plants did not show accumulation of AtST2a protein under normal growth conditions.

Phenotype of *Arabidopsis thaliana* plants overexpressing *AtST2a*

Seven independent *35S: AtST2a* transgenic lines expressing varying amounts of AtST2a were selected for phenotypic analysis. Germination and growth of the *35S:*

Table 5.4.

Characterization of *35S: AtST2a* transgenic lines.

Line	Kan_R : Kan_S	AtST2a level determined by western blot	Copy number of transgene by southern blot
S1	5:1	++	>5
S3	4:1	+	nd
S5	4:1	+	6
S6	5:1	+	3
S7	6:1	+	>5
S9	20:1	++	>3
S10	9:1	++	3
S11	3.5:1	++	3
S12	2:1	nd	nd
S13	5:1	nd	5-6
S14	6:1	nd	nd
S15	5:1	nd	nd
S16	3:1	++	1
S18	12:1	nd	nd

Segregation ratio of kanamycin resistance, AtST2a protein levels and copy number of the transgene in *AtST2a: 35S* independent lines as determined by Southern blots. S1 through S18 represent independent transgenic 35S: AtST2a lines. nd = not determined.

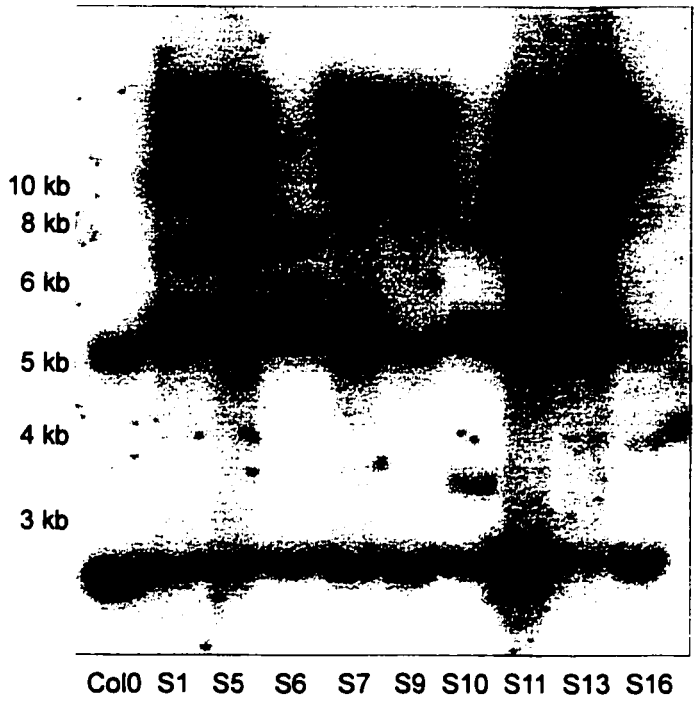


Figure 5.13

Southern blot of *Arabidopsis thaliana 35S: AtST2a* transgenic lines and Col0 plants. S1, S5, S6, S7, S9, S10, S11, S13 and S16 represent independent *35S: AtST2a* lines. 10 μg of genomic DNA from each line was digested with *Eco*R1, run on 1% agarose gel and transferred to a nylon membrane. Hybridization was performed with ^{32}P labeled *AtST2a* cDNA under high stringency conditions.

AtST2a transgenic lines constitutively expressing AtST2a on MS plates and in soil were not different from that of wild-type *A. thaliana*. Seedlings at the cotyledon stage appeared normal and development up to the 8- leaf stage took the same duration of time as in wild type plants (Figure 5.14). Flowers developed normally, were fertile and number of seeds and siliques were not altered. However, all the lines expressing AtST2a to a detectable level on Western blots exhibited a delayed flowering phenotype as compared to wild type plants when grown under long-days (Table 5.5 and Figure 5.15A). The severity of the delay was found to correlate with the level of expression of the transgene and was reproducible from generation to generation (Figure 5.15B). In fact the whole developmental program starting from initiation of flowering is delayed. As observed in Figure 5.15C, the rosette leaves of the wild type plants start to senesce at day 40 after germination while the plants of transgenic line S9 still have green rosette leaves, developing flowers and immature siliques. Several growth parameters were measured after 5 weeks, including the height of the main inflorescence and the number of flowers and siliques (Figure 5.15C). Since the transgenic lines were delayed in flowering time, the number of flowers and siliques were reduced in the transgenic lines as compared with wild type plants of the same age. Flowering time and plant morphology was also compared 85 days after germination under short-day conditions (8 hours light). Under these growth conditions, there was no significant difference in flowering time, between the transgenic lines expressing *AtST2a* and the wild-type plants (data not shown).

. Figure 5.14

Comparison of 20 days old wild type and *35S: AtST2a Arabidopsis* plants grown under long day (16 hours light) conditions. S1, S3, S5, S6, S7, S9 and S10 represent independent *35S: AtST2a* lines. At least 6 plants from each line were examined.

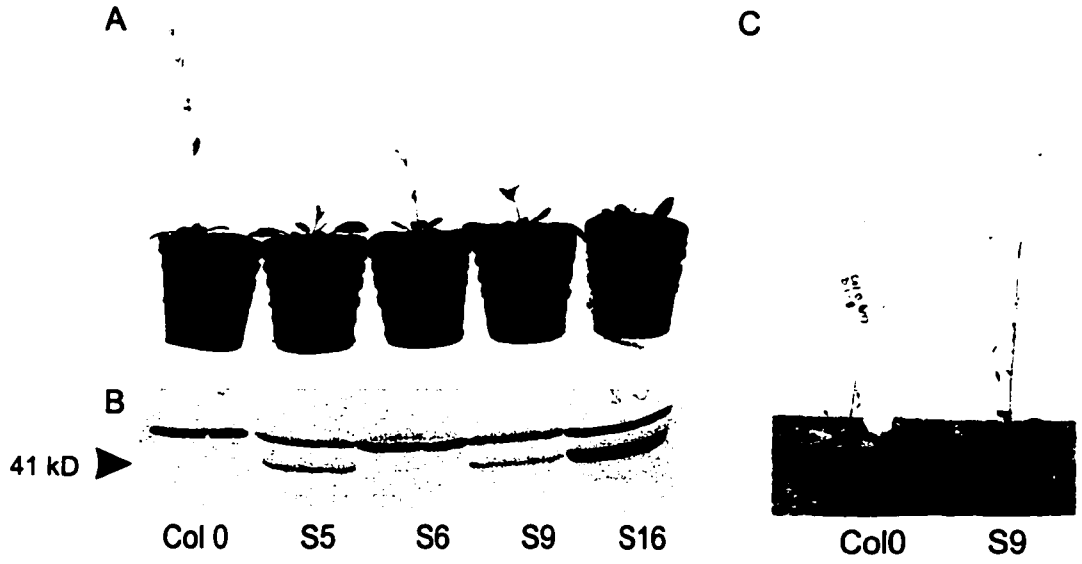
Table 5.5.

Time of flowering of 7 independent *35S: AtST2a* lines as compared to Col0 under long day conditions (16 hours light).

Line	TLN ± SD	FT ± SD
Col0	10.1 ± 0.73	20.6 ± 0.52
S1	11.1 ± 0.4*	24.67 ± 0.52*
S3	10.8 ± 0.4*	26.5 ± 0.55*
S5	11.0 ± 0.63*	24.83 ± 1.17*
S6	11.6 ± 0.56*	26.5 ± 1.38*
S7	11.1 ± 0.4*	24.0 ± 1.41*
S9	11.8 ± 0.4*	27.6 ± 1.37*
S10	11.0 ± 0*	26.0 ± 0.63*

TLN, total leaf number when the first bud appeared; Values are mean ± standard deviation; FT, flowering time is measured as the number of days after germination the first bud appeared. At least 6 plants from each line were analyzed.

* represents values significantly different form wild type ($P<0.05$).



. Figure 5.15

Effect of overexpression of *AtST2a* on flowering time in *Arabidopsis thaliana*. A) Comparison of 26 days old wild type and *35S: AtST2a* transgenic *Arabidopsis* plants grown under long days (16 hours light). S1, S6 , S9 and S16 represent independent *35S: AtST2a* lines. B) Immunoblot of protein extracts from plants corresponding to A. Proteins were extracted by grinding the plants and boiling in sample buffer. Samples were separated on 12 % SDS-PAGE, transferred on nitrocellulose and probed with anti- AtST2a specific antibodies. The arrow indicates the position of the AtST2a specific protein bands. C) Comparison of wild type (Col0) plant with the *35S: AtST2a* line S9, 40 days after germination.

Quantification of jasmonates in wild type and *35S: AtST2a* sense plants

In order to determine the effect of overexpression of *AtST2a* in transgenic plants, the levels of hydroxylated jasmonates and 12-OH-JA sulfate were measured in the transgenic line S9. Table 5.6 shows that an increase in *AtST2a* expression results in higher endogenous level of 12-OH-JA sulfate in the transgenic line S9. Surprisingly, overexpression of AtST2a (line S9) also leads to an increase in endogenous levels of JA and 11-OH-JA (Table 5.6).

Analysis of transgenic *Arabidopsis thaliana* expressing the *AtST2a* in the antisense orientation

To further characterize the function of AtST2a in the control of flowering time, we constructed transgenic *35S:AtST2a* plants expressing the gene in the antisense orientation. When transforming *Arabidopsis* with the 35S- antisense *AtST2a* construct, we produced 17 independent lines, exhibiting resistance to kanamycin. The transgenic lines were analyzed for the segregation ratio of the selectable marker for kanamycin resistance at the T_2 generation (Table 5.7). Based on the segregation ratios (Kan_r: Kan_s::3:1), 5 lines were predicted to have single insertion of the transgene. The number of inserts present in these five independent transgenic lines was analyzed by Southern blot (Figure 5.16A). Except for line 7-18-3, the other lines were found to contain 1-2 inserts. The level of *AtST2a* antisense transcript in the T_3 plants of five independent transgenic lines was analyzed by RT-PCR (Figure 5.16B). With the exception of line 7-18-3 all the lines were found to express the *AtST2a* antisense RNA.

Table 5.6.

Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in 18 days old *Arabidopsis thaliana* (Col0) and *35S:AtST2a* transgenic line S9.

Sample	JA (pmol/g)	12-OH-JA (pmol/g)	11-OH-JA (pmol/g)	12-OH-JA SO_4 (peak area/g)
Col0	186	56	285	211
S9	796	92	5885	2234

Values represent the results of a single experiment in which a pool of 30-50 plants grown on MS media in magenta boxes was analyzed.

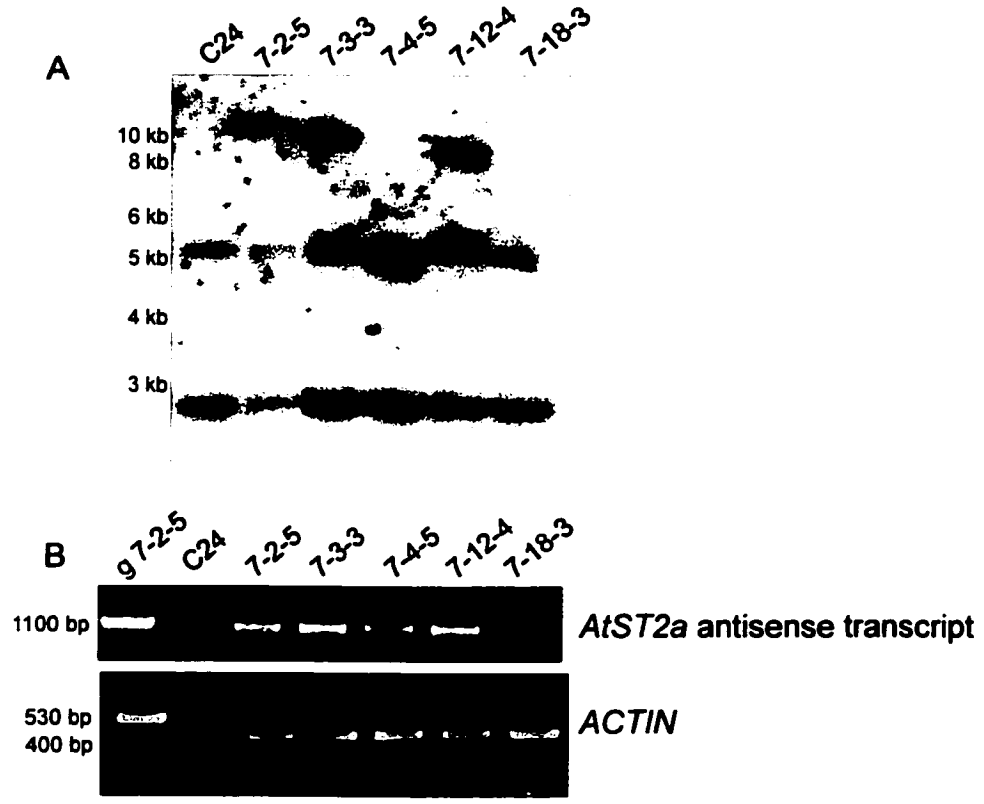


Figure 5.16

Analysis of *Arabidopsis thaliana* transgenic lines expressing *AtST2a* in the antisense orientation. A) Southern blot of *A. thaliana* 35S: antisense *AtST2a* transgenic lines and C24 plants. 7-2-5, 7-3-3, 7-4-5, 7-12-4 and 7-18-3 represent five independent lines. 10 μg of genomic DNA was digested with *Eco*R1, run on 1% agarose gel and transferred to a nylon membrane. Hybridization was performed with ^{32}P labeled *AtST2a* cDNA under high stringency conditions. B) RT-PCR of RNA samples from *A. thaliana 35S*: antisense *AtST2a* transgenic lines and wild type, C24 plants. g7-2-5 represents amplification from the genomic DNA of line 7-2-5. 2.5 μg of total RNA was reverse transcribed and amplified with *AtST2a* 5'-primer and AMV primer (See materials and methods). The products of amplification were loaded on ethidium bromide stained agarose gel. The RT products were also amplified with *ACTIN*- specific primers to confirm that equal amounts of RT product were used in each amplification reaction.

Phenotype of *Arabidopsis thaliana* plants with reduced levels of *AtST2a* transcript

Figure 5.17 shows the phenotype of wild type *Arabidopsis* plants of ecotype C24 as compared to transgenic plants expressing the *AtST2a* in the antisense orientation under the control of the *CaMV: 35S* promoter. In this experiment, plants were grown under conditions that are non inductive for flowering in *A. thaliana (*8-hours light). Under these conditions, wild type plants flower approximately 80-85 days after germination. Several independent transgenic lines exhibited an early flowering phenotype 72 days after germination. The phenotype varied from line to line ranging from 8 to 15 days earlier than the wild type plants. Lines 7-2-5 and 7-3-3 exhibited the most severe phenotype with flowering time 12-15 days earlier than the wild type plants (Figure 5.17 and Table 5.8). Apart from the early flowering phenotype, the growth behavior and the size of the transgenic plants could not be distinguished from the non-transformed control plants. No significant difference in flowering time was observed when the plants were grown under long-days (Table 5.9).

Quantification of jasmonates in the *35S: AtST2a* antisense plants

In order to determine the effect of increased levels of *AtST2a* antisense transcript in the plant, the levels of jasmonates were quantified in the transgenic line 7-2-5. Table 5.10 shows the levels of jasmonates in wild type plants as compared to plants from the transgenic line 7-2-5. The lower endogenous expression of *AtST2a* results in increased accumulation of 12-OH-JA and a lower endogenous level of 12-OH-JA sulfate in 7-2-5 plants. Surprisingly, the antisense plants show a 4 fold decrease in the levels of 11-OH-JA and JA.

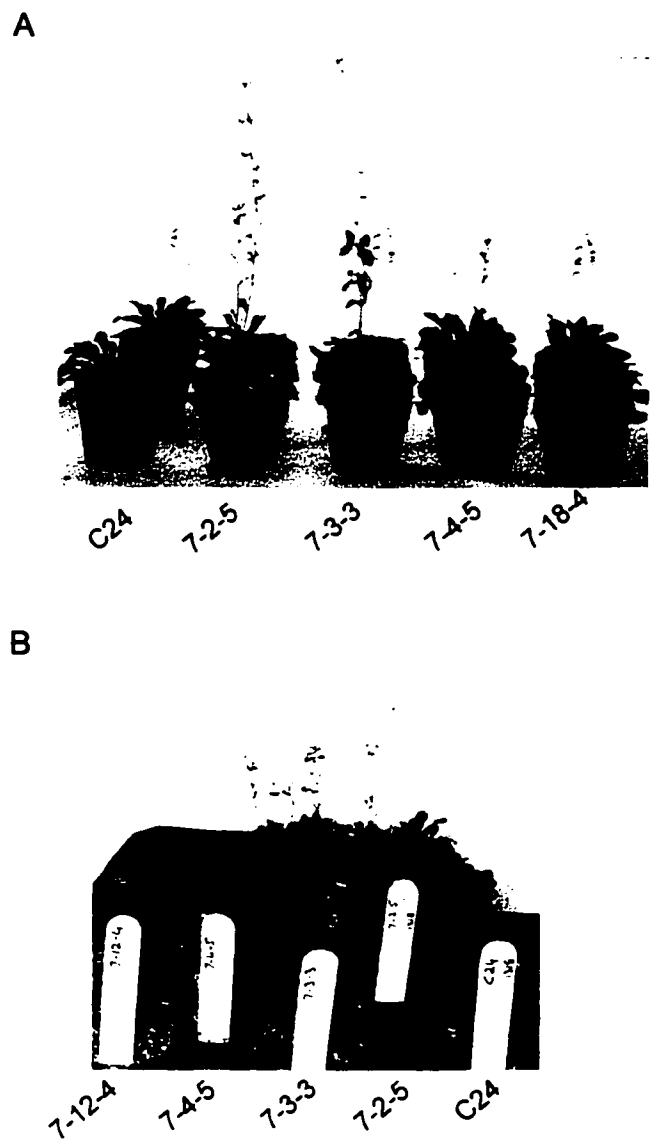


Figure 5.17

Phenotype of *Arabidopsis* plants (C24) expressing *AtST2a* in the antisense orientation grown under short day conditions (8 hours light). A) Comparison of 72 days old wild type and transgenic plants. 7-2-5, 7-3-3, 7-4-5, 7-12-4 and 7-18-4 represent independent lines. B) Photograph of a number of plants from each transgenic line under similar growth conditions as in A.

Table 5.8.

Flowering time of *35S: AtST2a* antisense lines compared to wild type *Arabidopsis thaliana* (C24) grown under short day conditions (8 hours light).

Line	FT ± SD
C24	82 ± 2.64
7-2-5	70.8 ± 9.74*
7-3-3	67.5± 2.58*
7-4-5	73.5± 4.12*
7-12-4	74.25 ± 4.27

Values are mean ± standard deviation FT, flowering time measured as the number of days after sowing until the first bud appeared. At least 4 plants from each line were analyzed.

*Represents values significantly different from wild type ($P<0.05$).

Table 5.9.

Flowering time of *35S: AtST2a* antisense lines compared to wild type *Arabidopsis thaliana* (C24) grown under long day conditions (16 hours light).

Line	TLN ± SD	FT ± SD
C24	13.8 ±3.83	27.4 ± 5.02
7-2-5	11.8 ± 0.44	23.4 ± 2.07
7-3-3	13.4 ± 2.07	27.0± 2.54
7-4-5	13.6 ± 1.14	27.4± 2.40
7-12-4	12.4± 1.51	25.8 ± 2.56

TLN, total leaf number when the first bud appeared; Values are mean ± standard deviation; FT, flowering time is measured as the number of days after sowing until the first bud appeared. At least 6 plants from each line were analyzed.

Table 5.10.

Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in 18 days old *Arabidopsis thaliana* wild type (C24) compared to transgenic line 7-2-5 expressing *AtST2a* in the antisense orientation.

Sample	JA (pmol/g)	12-OH-JA (pmol/g)	11-OH-JA (pmol/g)	12-OH-JA SO_4 (peak area/g)
C24	418	34	405	990
7-2-5	76	239	108	448

Values represent the results of a single experiment in which a pool of 30-50 plants grown on MS media in magenta boxes was analyzed.

Table 5.10.

Quantification of JA, 12-OH-JA, 11-OH-JA and 12-OH-JA sulfate in 18 days old *Arabidopsis thaliana* wild type (C24) compared to transgenic line 7-2-5 expressing *AtST2a* in the antisense orientation.

Sample	JA (pmol/g)	12-OH-JA (pmol/g)	11-OH-JA (pmol/g)	12-OH-JA SO_4 (peak area/g)
C24	418	34	405	990
7-2-5	76	239	108	448

Values represent the results of a single experiment in which a pool of 30-50 plants grown on MS media in magenta boxes was analyzed.

Effect of 12-OH-JA treatment on flowering time of *Arabidopsis thaliana* plants

Since manipulating the levels of 12-OH-JA and 12-OH-JA sulfate in transgenic plants resulted in the alteration of flowering time, the effect of exogenous application of 12-OH-JA to *Arabidopsis* plants was tested. 18 days old *A. thaliana* plants germinated in MS media in magenta boxes were treated with 10 μM 12-OH-JA. The plants were photographed 28 days after germination. Wild type *Arabidopsis* plants grown in magenta boxes flowered between day 28 and 30 when grown under *long-day* conditions. Treatment with 12-OH-JA resulted in plants flowering 2 days earlier than their wild type counterparts (Figure 5.18).

Figure 5.18

Phenotype of *Arabidopsis thaliana* plants treated with 12-OH-JA. *Arabidopsis* plants (Col0) were grown in MS medium in magenta boxes under long day conditions (16 hour light). 18 days old plants were then treated with 10 μM concentration of 12-OH-JA or water. Photographs were taken 28 days after germination.

Discussion

Amino acid sequence alignment of AtST2a and AtST2b indicates that they share 85% amino acid sequence identity and 92% similarity, suggesting that they might have similar substrate specificities. Amino acid sequence alignment of AtST2a and AtST2b with the flavonol 3-ST shows that AtST2a and AtSt2b have identical amino acids at the position corresponding to Leu 96 of the flavonol 3-ST and Tyr 106 of the flavonol 4'-ST (Figure 5.1B). Other amino acids known to interact with the sulfonate acceptor substrate are identical between the two sequences, further supporting the hypothesis that AtST2a and AtST2b might have similar substrate specificities.

The AtST2a and b proteins share 40% sequence identities with the flavonol STs of *Flaveria species*, 45% identities with the *A. thaliana* and *B. napus* 24-epibrassinosteroid STs and approximately 25% identities with mammalian STs. Based on the phylogenetic analysis presented in Chapter 2, *AtST2a* and *AtST2b* form a distinct ST family. When expressed in *E. coli*, the enzyme encoded by *AtST2a* exhibits strict specificity for 12-OH-JA and 11-OH-JA. This high level of substrate specificity is a common feature of plant STs and has been observed with the flavonol and brassinosteroid STs (Varin and Ibrahim, 1989; Rouleau et al., 1999). Recently, a mouse cDNA clone encoding an ST accepting compounds structurally similar to jasmonates has been characterized (Liu et al., 1999). This enzyme, which is predominantly expressed in the kidneys and uterus, accepts eicosanoids (prostaglandins, thomboxanes and leukotreines) as substrates. However, the biological function and the regulation of this enzyme have not been studied.

The kinetic parameters of AtST2a were determined from substrate interaction kinetic experiments. AtST2a exhibited preference for 12-OH-JA over 11-OH-JA with Km values of 10 and 50μM, respectively. These values are probably not reflecting the real affinity of the enzyme for its substrate since the assay mixture contained four stereoisomers as a result of the presence of two chiral carbons at C-3 and C-7 of the cyclopentanone ring. Substrate dependent stereoselectivity has been reported for the rat hydroxysteroid ST and phenol ST and more recently for the *Brassica napus* steroid ST, BnST3 (Banoglu and Duffel, 1997; Rouleau et al., 1999). Though the stereochemistry of naturally occurring 12- and 11-OH-JA has not been established, naturally occurring 12-OH-JA seems to have a *cis* conformation, and it was shown that *cis*-12-OH-JA has a much stronger tuber-inducing activity than the *trans* isomer (Koda, 1992). In addition, Yoshihara et al., (1989) reported that the side chain of purified 12-OH-JA is in the *cis* conformation.

12-OH-JA, also known as tuberonic acid, was initially isolated as a tuber-inducing compound from *Solanum tuberosum* (Yoshihara et al., 1989). To date, the presence of 12-OH-JA has also been reported to occur in *Solanum demissum* (Helder et al., 1993) and in the fungus *Botryodiplodia theobromae* (Miersch et al., 1991). The glucoside of 12-OH-JA (TAG) has also been detected in *Helianthus tuberosus* (Matsura et al., 1993) and *Astragalus complanatus* (Cui et al., 1993). The natural occurrence of 11-OH-JA in *Solanum demissum* was also reported (Helder et al., 1993). However, its tuber-inducing activity was shown to be very low as compared with 12-OH-JA (O. Miersch, unpublished results).

In order to validate the results of the *in vitro* studies, we searched for the presence of this metabolite in *A. thaliana* methanolic extracts using GC-MS. 11- and 12-OH-JA were detected in the two ecotypes that were analyzed (Table 5.2). Their quantification in both ecotypes shows that 11-OH-JA is more abundant than 12-OH-JA. This may be related to the higher Km value for 11-OH-JA exhibited by AtST2a. The fact that the extracts used for GC-MS analyses were from 15 to 18 days old plants indicate that 11- and 12-OH-JA synthesis is taking place in the vegetative tissue prior to the formation of the inflorescence. Preliminary results indicate that 12-OH-JA is also present in barley, tomato and tobacco leaves suggesting that it has a wide distribution in the plant kingdom (O. Miersch, unpublished results).

12-hydroxysulfonyloxyjasmonic acid (12-OH-JA sulfate) has been purified and characterized from *Cistoides tribules* (Zygophyllaceae) but has not been reported to accumulate in *A. thaliana* (Achenbach et al., 1994). In order to confirm that AtST2a catalyzes the sulfonation of 12-OH-JA *in vivo*, LC-MS methods were developed to detect the presence of 12-OH-JA sulfate in *A. thaliana* extracts. Both Col-0 and C24 ecotypes were found to accumulate a compound eluting at the same retention time and giving identical fragmentation patterns (Figure 5.6) as authentic 12-OH-JA sulfate. The presence of 12-OH-JA sulfate in both ecotypes (Table 5.2) confirms that the enzymatic reaction characterized *in vitro* is taking place *in vivo*. The natural occurrence of a sulfate derivative of 11-OH-JA has not been reported in the literature and we do not have as yet any evidence that it accumulates in *A. thaliana*.

AtST2a expression is induced in a dose-dependent manner by 12-OH-JA (Figure 5.9A). 12-OH-JA- stimulated upregulation of *AtST2a* indicates the presence of a feed-

forward regulation mechanism that would control its concentration. Similar feed-forward mechanisms have been shown to regulate the level of the enzymes abscisic acid 8-hydroxylase and gibberellin-2 oxidase involved in the catabolism of abscisic acid and gibberellins, respectively (Windsor and Zeevart, 1997; Thomas et al., 1999). The presence of a regulatory mechanism responding to the endogenous level of 12-OH-JA supports the hypothesis that this metabolite may be regulating an important aspect of plant development.

AtST2a mRNA levels increase rapidly in Me-JA treated plants (Figure 5.7). However, analysis of methanolic extracts of *Arabidopsis* treated with Me-JA reveals that in addition to a rise in JA levels, the levels of 11- and 12-OH-JA also increase considerably. This raises the possibility that the increase in *AtST2a* transcript level in response to Me-JA treatment may be the result of an increase in the levels of the hydroxylated jasmonate derivatives. *AtST2a* expression is also induced by sorbitol, and JA-Ile (Figure 5.9A). It has been demonstrated that treatment of plants or plant tissues with these compounds results in increased endogenous levels of JA (Lehmann et al., 1995, Kramell et al., 1997, Creelman et al., 1992). Again, we cannot exclude the possibility that 12-OH-JA is responsible for the increase in *AtST2a* expression following these treatments since the increase of JA will lead to an increase of 12-OH-JA. In future studies, the expression of *AtST2a* in response to Me-JA and 12-OH-JA should be tested in the *Arabidopsis coi1* mutant. This mutant is insensitive to Me-JA and to coronatine (Feys et al., 1994). Induction of *AtST2a* expression in the *Arabidopsis coi1* mutant following 12-OH-JA treatment will confirm the presence of a 12-OH-JA response pathway distinct from the Me-JA response pathway.

We already have experimental evidence for the existence of two independent pathways. RT-PCR experiments showed that *Thi2.1* expression is induced by Me-JA but not by 12-OH-JA (Figure 5.12). *Thi2.1* encodes a thionin that is specifically induced by wounding, pathogen infection and following treatment with Me-JA (Epple et al., 1995). The lack of induction of *Thi2.1* following 12-OH-JA treatment suggests that Me-JA and 12-OH-JA mediate their response via two independent pathways. In addition, unlike JA, 12-OH-JA does not exhibit inhibitory effects on plant growth, such as promotion of leaf senescence of oat leaves, inhibition of soybean callus growth and inhibition of lettuce seedling growth (Koda, 1992). A number of plant responses that are mediated by JA or Me-JA are accompanied by altered gene expression. For example, *JIP 6* (thionin) and *JIP 23* are known to be specifically induced in response to Me-JA in barley leaves. However, these genes are not induced when barley leaves are treated with 11- and 12-OH-JA (Miersch et al., 1999).

The treatment of *Arabidopsis* plants with the jasmonic acid biosynthetic precursor, 12-oxophytodieonic acid (12-OPDA), leads to increased accumulation of the *AtST2a* transcript (Figure 5.9). It has been shown previously that 12-OPDA can alter the expression of specific genes (Kramell et al., 2000) and was found to exhibit a strong activity in several JA bioassays (Koch et al., 1999; Blechert et al., 1999). Similar inducing activities were exhibited by coronatine and indanone amino acid conjugates (Weiler et al., 1994; Wasternack et al, 1998). These activities are not surprising when we consider the fact that they share structural similarity with 12-oxo-phytodieonic acid. In contrast with 12-OPDA, coronatine and indanone amino acid conjugates cannot be converted to JA *in vivo* suggesting that their effect on *AtST2a* expression is not mediated

via JA or its hydroxylated derivatives. To confirm this hypothesis, JA, 11- and 12-OH-JA should be quantified in plants treated with these compounds.

Another important aspect of *AtST2a* regulation, is its induction in dark-grown plants. The *AtST2a* transcript starts to accumulate 6 hours after the plants are exposed to the dark treatment (Figure 5.11). This indicates that *AtST2a* is expressed for 8-10 hours when the plants are growing under short-day conditions (dark period = 14 to16 hours) as compared to 2 hours when the plants are growing under long-day conditions (dark period = 6-8 hours). This suggests that the level of unconjugated 12-OH-JA would be higher in plants growing under long-day conditions. This hypothesis is supported by the fact that we observe a slight decrease in the levels of JA and 12-OH-JA in dark grown plants (Table 5.3). However, this trend will have to be confirmed using a larger sample set. The decrease in the level of jasmonates may also be due to a decrease in their biosynthesis in the dark. However this aspect of jasmonic acid biosynthesis has not been addressed so far.

In order to determine the biological significance of 11- and 12-OH-JA accumulation in plants, we constructed transgenic *Arabidopsis* overexpressing *AtST2a* in the sense and antisense orientation. Transgenic plants constitutively expressing *AtST2a* exhibit delayed flowering time when grown under inductive photoperiods (Figure 5.15 and Table 5.5). However, no significant difference in flowering time was observed when the plants were grown under non-inductive photoperiods. As expected, the increased expression of *AtST2a* results in higher endogenous level of 12-OH-JA sulfate in the transgenic line S9 (Table 5.6). Surprisingly, the overexpression of *AtST2a* also leads to increased endogenous levels of JA and 11-OH-JA. This result suggests that the plants

respond to the reduction of the 12-OH-JA pool by increasing the synthesis of JA which then leads to increased accumulation of 11-OH-JA. This result suggests that *in vivo*, the sulfonation of 11-OH-JA might not be taking place or is at least less efficient as compared with the sulfonation of 12-OH-JA.

Transgenic plants expressing *AtST2a* in the antisense orientation exhibit early flowering phenotype under short-day conditions. As expected, the transgenic plants show a decrease in the endogenous level of 12-OH-JA sulfate and an increase in 12-OH-JA accumulation as compared to wild type plants. Interestingly, the level of JA is also reduced in the transgenic plants suggesting that the concentration of hydroxylated jasmonates in the plant regulates JA levels.

The results obtained with the sense and antisense transgenic plants suggest that the function of AtST2a is to inactivate the biological activity of hydroxylated jasmonates, which might be acting as signal molecules in the control of flowering time (Figure 5.19). This hypothesis is consistent with the observation that wild type *Arabidopsis* plants treated with 12-OH-JA flower earlier (Figure 5.18). Our results also demonstrate that the sulfonation of hydroxylated jasmonates is photoperiod-dependent suggesting that the efficiency of the inactivation mechanism is optimal in plants growing under short-days (Figure 5.19). This hypothesis is consistent with the fact that transgenic plants expressing *AtST2a* in the antisense orientation exhibit an early flowering phenotype only when grown under short-days.

Prior to this work, the only proposed function of 12-OH-JA was to act as an inducer of tuberization (Yoshihara et al., 1989). It is interesting to note that tuberization and flowering share several common characteristics. Both developmental processes are

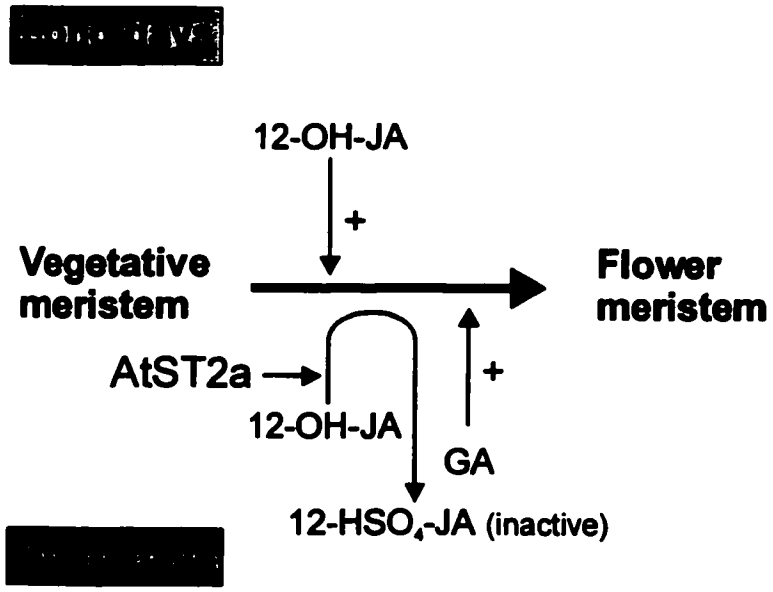


Figure 5.19

Proposed model for the role of AtST2a in the control of flowering time in *Arabidopsis thaliana.*

under the control of photoperiod. For example, reduced levels of PHYB in transgenic antisense Solanum led to strong induced state of tuberization (Jackson et al., 1996) while the *A. thaliana phyB* mutant exhibits an early flowering phenotype (Reed, 1993). Furthermore, tuber and flower induction seems to be controlled by a signal produced in the leaves under inducing conditions that can be translocated through a graft union (Ewing, 1995; Lang, 1977). A direct link between flowering and tuberization was also established by inter species grafting experiments. Grafting leaves from tobacco plants induced to flower onto potato plants grown under conditions that do not favor tuber formation resulted in tuberization of the potato plants, whereas grafted leaves from non-induced tobacco plants had no tuber-inducing properties (Ewing, 1995). Similar results were obtained in grafting experiments between induced-sunflower leaves and Jerusalem artichoke stocks demonstrating that the signal was not restricted to plants belonging to the Solenaceae family (Ewing, 1995). Based on these results, it was proposed that flower induction and tuberization share interchangeable signals. Our results support this hypothesis and indicate that 11- and/or 12-hydroxyjasmonate might be involved in the control of both developmental processes.

Recently genetic studies of *A. thaliana* have begun to identify molecular components that participate in promoting flowering under inductive photoperiods. This pathway has been referred to as the photoperiodic promotion pathway. The pathway involves photoreceptors (such as *PHYA* and *CRY2*) that perceive light quality and day length. When the length of the dark period decrease below a critical point, genes that promote flowering such as; *CO* and *FHA* are activated. This activation ultimately lead to the upregulation of floral meristem identity genes such as LEAFY and finally flowering

is initiated. *CO* encodes a zinc- finger protein (Putterhill et al., 1995) and has been shown to activate expression of *LEAFY* (*LFY*) and *APETELA1* (*AP1*) (Simon et al., 1996), the two genes that directly control the initiation of flowering. (Weigel et al., 1992: Mandel et al., 1992). The fact that the expression of *CO* in the leaves and not at the shoot apex, can promote early flowering (Pineiro et al., 1998), led to the hypothesis that *CO* controls the synthesis in the leaves of a signal (florigen) that is transmitted to the shoot apex to activate floral gene expression. Results presented in this thesis suggest that hydroxylated jasmonates might be the signals generated under the control of the photoperiod promotion pathway. To test this hypothesis, hydroxylated jasmonates should be quantified in *co* mutants or in transgenic plants expressing *CO* constitutively.

Several lines of evidence indicate that gibberellins (GA) fulfill a role similar to hydroxylated jasmonates when the plants are growing under non-inductive photoperiods (Wilson et al., 1992). The presence in the *LEAFY* promoter of independent regulatory elements responding to GAs and to photoperiod support the idea that different signals are integrating at *LEAFY* and are promoting flowering in *A. thaliana*.

A direct implication of jasmonates in regulating flower development was demonstrated in *A. thaliana* plants deficient in JA biosynthesis or perception (McConn and Browse, 1996; Stintzi and Browse, 2000; Sanders et al., 2000). These mutants exhibit abnormal timing of anther dehiscence leading to male sterility. The mutant phenotype of the JA-deficient mutants could be rescued by the addition of JA suggesting that it acts as a signaling molecule that induces and coordinates elongation of the anther filament and opening of the stomium at anthesis leading to the production of viable pollen (Stintzi and Browse, 2000). However, one may argue that the phenotype observed in these mutants is

the result of a deficiency in 11- and/or 12-hydroxyjasmonic acid, since they are believed to be synthesized from JA by a single hydroxylation step (Yoshihara et al., 1996). To better understand the contribution of JA and its derivatives in the control of flowering time and in anther development, the octadecanoids will have to be quantified in the vegetative and reproductive tissues of JA-deficient and JA-insensitive mutants. Furthermore, in order to evaluate the possibility that hydroxylated jasmonic acids might be involved in proper anther development, rescue experiments with 12-hydroxyjasmonic acid should be attempted with the male sterile JA-deficient mutants.

If the hypothesis that hydroxylated jasmonates are involved in proper anther development is true, then the overexpression of AtST2a in transgenic plants should exhibit male sterile phenotype. However such a phenotype was not observed. Our method of selecting. *35S: AtST2a* transgenic lines involved plating seeds of the transformed plants on kanamycin. We may have failed to identify transgenic plants with sufficiently low levels of 12-OH-JA that were sterile. A number of mutants that exhibit altered flowering time have been identified but none of them was found to map in *AtST2a*. It is possible that AtST2b also accepts hydroxylated jasmonates as substrates resulting in genetic redundancy. Determination of the substrate specificity of AtST2b is required to confirm this hypothesis.

CONCLUSIONS AND PERSPECTIVES FOR FUTURE WORK

This thesis presents the sulfotransferase gene family of *A. thaliana* which comprises 18 members. The existence in the *A. thaliana* genome of 18 different sulfotransferases indicates that the sulfonation reaction is involved in a wide variety of metabolic processes in this plant. Several members of the *Arabidopsis* ST gene family are clustered and exhibit high amino acid sequence identity, suggesting that multiple gene duplication events took place. In the context of my doctoral studies, two of the eighteen ST-coding genes were characterized at the biochemical and molecular levels.

We have identified a novel sulfotransferase enzyme AtST2a, that specifically sulfonates 11- and 12-hydroxy jasmonic acid. Although 11- and 12-OH-JA have been reported to occur previously in tuber producing plants, in this study these metabolites were detected for the first time in *Arabidopsis*. The product of the enzymatic reaction with 12-OH-JA was identified as 12-OH-JA sulfate based on HPLC retention time and MS/MS fragmentation patterns. 12-OH-JA sulfate has been purified and characterized from *Cistoides tribules* (Zygophyllaceae) but has not been reported to accumulate in other plant species. The detection of this metabolite in the methanolic extract of two *A. thaliana* ecotypes confirms that the enzymatic reaction characterized *in vitro* is also taking place *in vivo*. Based on the phenotype of transgenic plants, we propose that the function of AtST2a is to inactivate the biological activity of hydroxylated jasmonates. Furthermore, our results suggest that hydroxylated jasmonates might be involved in the control of flowering time in *A. thaliana* in a photoperiod-dependant manner.

The expression pattern of *AtST2a* indicates that the sulfonation of hydroxylated jasmonates is light regulated, and suggests that the inactivation mechanism is effective in

plants growing under short day conditions. This pattern of expression is consistent with the fact that transgenic plants expressing *AtST2a* in the antisense orientation exhibit an early flowering phenotype only when grown under non-inductive photoperiods. Taken together, our results suggest that hydroxylated jasmonic acid acts as a signal promoting the transition from vegetative to reproductive growth when *A. thaliana* is exposed to an inductive photoperiod. 12-OH-JA, also known as tuberonic acid, was initially isolated from *Solanum tuberosum* as a tuber-inducing compound. Based on grafting experiments, it has been proposed that flower induction and tuberization may share interchangeable signals. Our results support this hypothesis and indicate that 12-hydroxyjasmonic acid may be the signal involved in the control of both developmental processes.

It would be interesting to know if the synthesis of hydroxylated jasmonates is also light regulated. In the future, GC-MS methods should be used to determine the levels of 11- and 12-OH-JA during the development of plants growing under long-day and short-day conditions. To confirm the hypothesis that 12-OH-JA is involved in the control of flowering time, the effect of 12-OH-JA treatment on the expression pattern of genes known to regulate flowering time should be studied. In addition, mRNA profiling experiments with *A. thaliana* DNA chips should be performed to identify genes other than *AtST2a* that are regulated by 12-OH-JA.

In order to study the localization of *AtST2a* expression, histochemical analysis of transgenic *A. thaliana* plants expressing *AtST2a* promoter: *GUS* gene fusion should be performed. Furthermore, promoter deletion analysis could be performed to identify *cis* regulatory elements that are involved in the regulation of expression of *AtST2a* in response to by Me-JA, 12-OH-JA and light.

The presence in the *A. thaliana* genome of another sulfotransferase (AtST2b) exhibiting 85% amino acid sequence identity with AtST2a raises the question of the possible role of this enzyme in the control of flowering time. It is therefore important to determine the expression pattern and the substrate specificity of AtST2b.

This thesis also presents the biochemical and molecular characterization of the gene *AtST3a*. AtST3a was found to sulfonate the position 7 of flavonols, flavone as well as their 3 or 4' monosulfate derivatives. *AtST3a* transcript was only detected at the early seedling stage of plant development. This is in contrast with the expression pattern of the flavonol 3-ST from *Flaveria* species, where ST gene expression was detected at all developmental stages with the highest activities found in the first pair of leaves and the terminal buds. The natural occurrence of a ST exhibiting high specificity for flavonoids in *A. thaliana* suggests that sulfated flavonoids may be of more common occurrence in the plant kingdom than once thought. However, we still have to demonstrate that sulfated flavonoids accumulate in *A. thaliana*.

The absence of any phenotypic consequence resulting from antisense *AtST3a* expression in transgenic *A. thaliana* prevented us from reaching a conclusion regarding the biological function of this gene. However, our results suggest that flavonoid sulfation is probably not involved in auxin polar transport. Transgenic plants expressing *AtST3a* in the sense orientation should be constructed to try to define its function *in vivo*.

So far, our approach to characterize the sulfotransferase enzymes from *A. thaliana* relies on the identification of putative substrates using *in vitro* assays with a large number of molecules. This approach is limited by the fact that the natural substrate of the enzyme might be absent from our collection. Furthermore, to validate the results obtained *in vitro*,

we have to demonstrate that the substrate and the sulfated products are present *in vivo*. In order to simplify this approach, a systematic identification of all the sulfated metabolites present in *A. thaliana* should be attempted.

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