# NOTE TO USERS

# The Photoperiod Dependent Sulfonation of 12-Hydroxyjasmonate Establishes a Link Between Jasmonates and the Control of Flowering

Anastasia Levitin

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**ABSTRACT**

The Photoperiod Dependent Sulfonation of 12-Hydroxyjasmonate Establishes a Link Between Jasmonates and the Control of Flowering

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Concordia University, 2004

It recently has been shown that 12-hydroxyjasmonate and its sulfonated derivative occur naturally in *A. thaliana* (Gidda *et al.*, 2003). The enzyme catalyzing the sulfonation of 12-OHJA is encoded by the *AtST2a* gene, one of the 18 sulfotransferase (ST)-coding genes present in the *A. thaliana* genome (Gidda *et al.*, 2003). We demonstrate that 12-OHJA induces floral evocation in *A. thaliana* plants growing under inductive photoperiods and that this inducing activity is abolished by sulfonation under non-inductive photoperiods. We also demonstrate that *CONSTANS*, a putative transcription factor that accelerates flowering in response to long photoperiod, and TERMINAL FLOWER 2, a homolog of *Drosophila* heterochromatin-associated protein 1, which acts on meristem identity genes to repress the initiation of flowering, promote or repress flowering through the direct or indirect regulation of *AtST2a* expression. We, therefore, propose that AtST2a is a member of the photoperiod-dependent flower induction pathway downstream from CO and TFL2.

In *Nicotiana tabaccum* 12-OHJA is found to participate in the determination of flower structures and regulates the expression of *NtPLE36*, a flower organ identity gene.

The *A. thaliana* genome contains a sequence (*AtST2b*) that is closely related to *AtST2a.* The deduced amino acid sequences of the two genes share 85% identity and 92% similarity. Despite this high level of similarity AtST2b did not accept 12-OHJA as substrate. Transgenic plants overexpressing *AtST2b* in sense or antisense orientation, as well as *AtST2b* knock out mutant plants did not show a visible phenotype suggesting that the role of *AtST2b* in *Arabidopsis thaliana* is different from AtST2a.

To My Family

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**TABLE OF CONTENTS**


| | Page |
|---|---|
| ABSTRACT | iii |
| DEDICATION | v |
| ACKNOWLEDGEMENTS | vi |
| TABLE OF CONTENTS | vii |
| LIST OF FIGURES | xii |
| LIST OF TABLES | xvi |
| LIST OF ABBREVIATIONS | xvii |
| CHAPTER 1: LITERATURE REVIEW | |
| Introduction | 1 |
| Sulfotransferases | 1 |
| The importance of PAPS in the regulation of sulfation | 2 |
| Role of the sulfation reaction of sulfotransferases | 3 |
| Classes of sulfotransferases | 4 |
| Membrane-bound mammalian sulfotransferases | 6 |
| Membrane-bound plant sulfotransferases | 6 |
| Cytosolic mammalian sulfotransferases | 8 |
| Cytosolic plant sulfotransferases | 9 |
| Jasmonates | 11 |
| Jasmonic acid response mutants | 14 |
| Jasmonic acid biosynthesis | 15 |
| Octadecanoid pathway | 16 |

Hexadecanoid pathway 21

"Oxylipin signature" 22

Jasmonic acid derivatives 23

Induction of tuberization and flowering 23

Flower initiation 27

Flowering models 27

Flowering time genes 28

The meristem identity genes 31

Flower organ identity genes 32

Photoreceptors 35

Cryptochromes 35

Phytochromes 36

Phytochromes and Flowering Time Genes 38

CHAPTER 2: MATERIALS AND METHODS 41

Materials 41

Cloning of the *AtST2a* promoter 42

Histochemical Analysis of *AtST2a* promoter-*GUS* Fusions 42

Cloning of *AtST2b* 43

DNA and protein sequence analysis 43

Expression of recombinant AtST2a and AtST2b 44

12-hydroxyjasmonate sulfate Preparation of anti-AtST2a 44

antibodies

SDS-Polyacrylamide Gel Electrophoresis 45

Sulfotransferase assay 45

Detection and quantification of jasmonates from *Arabidopsis thaliana* 46

Detection of 12-hydroxysulfonyloxyjasmonic acid by LC MS/MS 48

Northern blot analysis 49

Quantificaton of signal intensity using NIH Image program 50

Plasmid constructs for *AtST2a* and *AtST2b* transformation in *Arabidopsis thaliana* 50

*Agrobacterium tumefaciens* transformation 52

*Arabidopsis thaliana* transformation 52

Southern blot analysis 52

Detection of the AtST2a and AtST2b proteins in transgenic lines 53

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) 54

Screening for *coi1* mutant plants 54

Oligonucleotides used for gene amplification in various PCR and RT-PCR experiments 55

Plant growth conditions 58

CHAPTER 3: BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF 12-HYDROXYJASMONATE SULFOTRANSFERASE FROM *ARABIDOPSIS THALIANA* 59

*AtST2a* transgenic plants 62

Regulation of *AtST2a* expression in response to light 64

Phytochromes and *AtST2a* expression 68

Analysis of various mutants in relation to the initiation of flowering and pattern of *AtST2a* expression 72

Analysis of *AtST2a* expression in *Arabidopsis thaliana* mutants in the photoperiod dependent promotion pathway of flowering 72

AtST2a regulation by 12-hydroxyjasmonate 77

Analysis of *AtST2a* expression in *Arabidopsis thaliana* jasmonate response and biosynthetic mutants 79

Histochemical Analysis of *AtST2a* Promoter Activity 84

Effect of exogenously applied jasmonates on plant development 87

AtST2a in *Nicotiana tabaccum* 89

Complementation of *opr3* with 12-hydroxyjasmonate 98

Discussion 102

*AtST2a* transgenic plants 104

Regulation of *AtST2a* expression in response to light 105

Phytochromes and *AtST2a* expression 108

Analysis of *AtST2a* expression in *Arabidopsis thaliana* mutants in the photoperiod dependent promotion pathway of flowering 109

*AtST2a* regulation by 12-OHJA 114

Analysis of *AtST2a* expression in *Arabidopsis* 114

*thaliana* jasmonate response and biosynthetic mutants

Histochemical Analysis of *AtST2a* Promoter Activity 117

Effect of exogenously applied jasmonates on plant development 119

Model for 12-OHJA/AtST2a participation in floral evocation 122

AtST2a/12-OHJA role in anther development and floral patterning of tobacco 125

AtST2a/12-OHJA role in anther development of *Arabidopsis thaliana* 127

CHAPTER 4: BIOCHEMICAL AND MOLECULAR CHARACTTERIZATION OF *ARABIDOPSIS THALIANA* SULFOTRANSFERASE 2B 129

Biochemical Studies 129

Characterization of the AtST2b gene product 132

Discussion 136

CONCLUSIONS AND PERSPECTIVES FOR FUTURE WORK 138

REFERENCES 142

## LIST OF FIGURES


Page

**Chapter 1**

Figure 1.1 Comparison of the structures of phytodienoic acid and jasmonic acid, which are derived from linolenic acid, with the structure of a prostaglandin derived from arachidonic acid 13

Figure 1.2 α-Linolenic acid is a central molecule for the production of defense-related compounds 17

Figure 1.3 The apical meristem 25

Figure 1.4 Flower development pathways 29

Figure 1.5 ABC model 33

**Chapter 3**

Figure 3.1 Amino acid sequence alignment of AtST2a, AtST2b, and the flavonol 3-ST from *Flaveria chloraefoliata* 60

Figure 3.2 Location of the *AtST2a* and *AtST2b* genes on chromosome V of *Arabidopsis thaliana.* 61

Figure 3.3 *Arabidopsis thaliana AtST2a* transgenic plants and the initiation of flowering 63

Figure 3.4 Light regulation of *AtST2a* expression 65

Figure 3.5 Western blot analysis of AtST2a protein levels from 24 days old *Arabidopsis thaliana* plants following a 48 hours dark treatment 66

Figure 3.6 Western blot analysis of AtST2a protein levels in *Arabidopsis thaliana* plants of different ages. 67

Figure 3.7 Correlation of phytochrome mutants flowering time with the *AtST2a* expression profile in *Arabidopsis thaliana* 69

Figure 3.8 Correlation between flowering time and the AtST2a protein level in photoperiod promotion pathway mutants. 74

Figure 3.9 Kinetic of *AtST2a* mRNA accumulation following treatment of 15 days old *Arabidopsis thaliana* plants with 100 μM 12-hydroxyjasmonate 78

Figure 3.10 RNA gel blot analysis of *AtST2a* expression in various *Arabidopsis thaliana* mutants 80

Figure 3.11 Correlation between flowering time and the AtST2a protein level in the jasmonic acid perception or biosynthesis mutants. 81

Figure 3.12 Hypothetical additional pathways for 12-hydroxy jasmonate biosynthesis 83

Figure 3.13 Histochemical Analysis of *AtST2a* promoter activity in young seedlings 85

Figure 3.14 Histochemical Analysis of *AtST2a* promoter activity in mature plants 86

Figure 3.15 Phenotype of *Arabidopsis thaliana* plants treated with various derivatives of jasmonic acid 88

Figure 3.16 Flower phenotype of the wild type *N.tabaccum* plants and transgenic *AtST2a* tobacco overexpression lines 91

Figure 3.17 Photograph of dissected wild type *N.tabaccum* flower and transgenic *AtST2a* overexpression line 92

Figure 3.18 GC/MS quantification of various jasmonates in wild type *Nicotiana tabaccum* and tobacco *AtST2a* over-expression line 7 93

Figure 3.19 Complementation of *N.tabaccum* plants with 12-hydroxyjasmonate 94

Figure 3.20 RT-PCR analysis of *NtPLE36* expression in *N.tabaccum* wild type flowers and tobacco plants overexpressing *AtST2a* 96

Figure 3.21 Flower phenotype of *Antirrhinum plena* mutant and *N.tabaccum AtST2a* overexpression transgenic plants 99

Figure 3.22 Complementation of the male sterile phenotype of the *opr3* mutant plants with 12-hydroxyjasmonate 101

Figure 3.23 Phylogenetic tree of the *Arabidopsis thaliana* STs 103

Figure 3.24 Quantification of jasmonic acid and 12-hydroxy jasmonate from *Arabidopsis thaliana* 106

Figure 3.25 Proposed location of AtST2a in the photoperiod dependent flower induction pathway 111

Figure 3.26 Proposed model for the role of AtST2a in the control of flowering time in *Arabidopsis thaliana* 123

**Chapter 4**

Figure 4.1 SDS-PAGE of fractions collected during purification of recombinant AtST2a and AtST2b proteins 130

Figure 4.2 Chemical structures of compounds used as substrates for the recombinant AtST2b protein 131

Figure 4.3 *AtST2b* expression in various *Arabidopsis thaliana* organs determined by RT-PCR 133

Figure 4.4 *AtST2b* expression in *Arabidopsis thaliana* phytochrome mutants determined by RT-PCR 134

Figure 4.5 *AtST2b* expression in response to jasmonate treatment 135

## LIST OF TABLES

Table 3.1 Quantification of Northern blot and Western blot signal intensities of Figure 3.7 71

Table 3.2 Quantification of Western blot signal intensities of Figure 3.8 75

Table 3.3 Quantification of *NtPLE36* expression in wild type tobacco plants versus *N.tabaccum* plants overexpressing *AtST2a* 97

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AMV | *Alfalfa* mosaic virus |
| bp | base pairs |
| BR | Brassinosteroid |
| BSA | Bovine serum albumin |
| COI1 | Coronatine insensitive 1 |
| GA | Gibberellin |
| GC | Gas chromatography |
| GUS | β-Glucoronidase |
| HPLC | High performance liquid chromatography |
| IAA | Indole-3-acetic acid |
| JA | Jasmonic acid |
| JIP | Jasmonate inducible protein |
| kDA | kilo Daltons |
| LC | Liquid chromatography |
| JAMe | Methyl jasmonate |
| MS | Mass spectrometry |
| 12-OHJA | 12-hydroxyjasmonic acid |
| 11-OHJA | 11-hydroxyjasmonic acid |
| 12-$HSO_4$-JA | 12-hydroxysulfonyloxyjasmonic acid |
| 12-OHJA-Ile | Isoleucine ester of 12-hydroxyjasmonate |

| | |
|---|---|
| OPDA | Oxo-phytodienoic acid |
| OPR | 12-oxophytodienoate reductase |
| PAGE | Polyacrylamide gel electrophoresis |
| PAPS | 3'-phosphoadenosine 5'-phosphosulfate |
| PCR | Polymerase chain reaction |
| $R_t$ | Retention time |
| RT | Reverse transcription |
| SDS | Sodium dodecyl sulfate |
| TA | Tuberonic acid |
| TAG | Tuberonic acid glucoside |
| VSP | Vegetative storage protein |

# CHAPTER 1

# LITERATURE REVIEW

## Introduction

This thesis presents the results on the characterization of two *Arabidopsis thaliana* sulfotransferase enzymes, AtST2a and AtST2b. The literature review first concentrates on mammalian and plant sulfotransferases as well as on the importance of the universal sulfate donor, PAPS. Since AtST2a accepts as a substrate 12-hydroxyjasmonate (12-OHJA), a derivative of the plant hormone jasmonic acid, part of the review chapter is dedicated to jasmonates, their biosynthesis, and their role in plant development and various stress responses.

In view of the experimental results suggesting the involvement of AtST2a and 12-OHJA in the photoperiodic response and the initiation of flowering, both of these processes are discussed in the last section of the literature review.

## Sulfotransferases

The sulfotransferases (STs) constitute a super family of enzymes catalyzing the sulfonation of a substrate with a sulfuryl group ($-SO_3-$) (Weinshilboum *et al.*, 1997). Many different chemicals are substrates for sulfotransferases and their sulfonation is dependent on the presence of a specific functional group. The most common functional groups that accept

the sulfuryl moiety are $-NH_2$, $-SO_2NH_2$, and -OH (Duffel *et al.*, 2001; Weinshilboum *et al.*, 1997).

Sulfotransferases catalyze the transfer of the sulfonyl group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to some endogenous substances (*e.g.* hormones and neurotransmitters), as well as a number of xenobiotics. When compounds are sulfoconjugated they are more water-soluble than the parent compound and, therefore, are readily transported or excreted (Klaassen and Boles, 1997).

**The Importance of PAPS in the Sulfation Reaction**

PAPS is an obligate co-substrate for sulfonation in higher organisms since the reaction does not proceed in its absence or under conditions that limit PAPS synthesis (Klaassen and Boles, 1997). PAPS is the "activated" form of sulfate. Inorganic sulfate is essential for PAPS synthesis, and there exist several mechanisms, which help to maintain sulfate homeostasis. Among these mechanisms are the sulfoxidation of the sulfur-containing amino acids cysteine and methionine, the transport-mediated renal re-absorption of inorganic sulfate, the degradation of sulfate-containing macromolecules, and through the activity of sulfatases (Klaassen and Boles, 1997).

Rapid biosynthesis of PAPS is required for sulfation. PAPS is synthesized in a two-step, coupled reaction:

$$ATP + SO_4^{2-} \xrightleftharpoons{\text{ATP-sulfurylase}} APS + PPi \quad (1)$$

$$ATP + APS \xrightleftharpoons{\text{APS-kinase}} PAPS + ADP \quad (2)$$

The second reaction takes place in the presence of $Mg^{2+}$.

PAPS is formed in the cytosol where the sulfation of most xenobiotics occurs. However, the sulfation of macromolecules occurs in the lumen of the Golgi apparatus; therefore, their sulfation is dependent not only on the synthesis of PAPS, but also on the transport of PAPS from the cytosol into the lumen of the Golgi (Klaassen and Boles, 1997).

**Role of the Sulfation Reaction Catalyzed by Sulfotransferases**

Sulfation affects many different physiological processes. Sulfotransferases play a prominent role in the elimination of end-products of catabolism. Deactivation or bioactivation of some xenobiotics (substances of exogenous origin) also is accomplished by sulfonation. For example, members of the sulfotransferase multi-gene family catalyze the sulfonation of xenobiotics (detoxication) and bioactivate polycyclic aromatic hydrocarbon carcinogens. In addition, because sulfated hormones are no longer recognized by their receptors, sulfotransferases are in a prime position to regulate bioactive hormone levels and, as a consequence, are believed to play a major role in the development of hormone-responsive tumors in humans (Duanmu *et al.*, 2001). For example, the estrogen sulfotransferase (EST) is the major form of human

cytosolic ST involved in the conjugation of estrogens. Steroid sulfates do not interact with the appropriate hormone receptors, and the presence of the charged sulfate moiety increases the aqueous solubility and excretion of most steroids (Kotov *et al.*, 1999). In addition, sulfotransferases are important factors in the inactivation of catecholamines (a group of hormones such as adrenaline and noradrenaline that are derived from catechol).

Sulfotransferases also can alter the structure and function of macromolecules. Some sea vegetables have been shown to be unique sources of sulfated carbohydrate-like substances called "fucans" that can reduce the body's inflammatory response. The inhibitory effects of fucans on both coagulation and cell proliferation are directly proportional to their level of sulfation (Haroun-Bouhedja *et al.*, 2000).

**Classes of Sulfotransferases**

Sulfotransferases are widely distributed in species ranging from bacteria to humans and share significant sequence homology at both the DNA and the amino acid levels, suggesting that they evolved from a common ancestral gene. The human sulfotransferase gene family now comprises over thirty members. In contrast to the large number of sulfotransferases that have been characterized in mammals, there have been few enzymes studied in bacteria. For instance, the *Rhizobia* sulfotransferases, NodH and NodE, catalyze the sulfonation of secreted

glycolipid root nodulation factors. Since the sulfate group is an important determinant of host specificity, the mutant strains lacking the sulfotransferases involved in the biosynthesis of nodulation factors exhibit a host range distinct from wild type (Dooley, 1998).

Although several sulfated metabolites are known to accumulate in a variety of plant species, plant sulfotransferases are not as well studied as their mammalian homologs. The function of plant-sulfated metabolites is difficult to predict, since their accumulation is often restricted to a limited number of species (Varin *et al.*, 1997b).

Sequencing of the *Arabidopsis thaliana* genome uncovered the existence of 18 gene sequences apparently coding for sulfotransferases and allowed our lab to initiate a functional genomics project with the objective to characterize their biological function. The STs that we characterized so far are specific for various substrates including flavonoids, steroids, glucosinolates and hydroxyjasmonates (Gidda *et al.*, 2003; Varin *et al.*, 1997b).

Sulfotransferases can be divided into two groups according to their intracellular localization. There are membrane-bound sulfotransferases, which are associated with the Golgi apparatus (Weinshilboum, 1994), and there are cytosolic or soluble sulfotransferases, which catalyze the sulfonation reaction in the cytosol. Both the membrane-bound and cytosolic sulfotransferases are widely distributed in plants, animals, and prokaryotes (Falany, 1997b).

**Membrane-Bound Mammalian Sulfotransferases**

These sulfotransferases are primarily localized in the *trans*-Golgi apparatus. The membrane-bound sulfotransferase enzymes transfer sulfate to protein tyrosine residues, to glycoprotein carbohydrates, or to proteoglycans (Bowman and Bertozzi, 1999). For example, protein tyrosine sulfation is a widespread post-translational modification. It is catalyzed by the tyrosyl protein sulfotransferase (TPST), an integral membrane glycoprotein residing in the *trans*-Golgi network (TGN) whose catalytic site is oriented toward the TGN lumen. Accordingly, proteins trafficking through the TGN have been found to become tyrosine-sulfated, including several identified plasma membrane and secretory proteins. As for its physiological role, tyrosine sulfation has been shown to promote protein–protein interaction, be it between (*i*) two secretory proteins, (*ii*) a secretory protein and its cell surface receptor, or (*iii*) two plasma membrane proteins (Beisswanger *et al.*, 1998).

**Membrane-Bound Plant Sulfotransferases**

In contrast with the extensive information on membrane bound mammalian sulfotransferases, higher plant membrane sulfotransferases have not been thoroughly characterized.

Phytosulfokine-α (PSK-α) is a disulfated pentapeptide (Tyr($SO_3H$)-Ile-Tyr($SO_3H$)-Thr-Gln), which was isolated from a conditioned medium (CM) derived from asparagus (*Asparagus officinalis* L.) mesophyll cell

culture (Matsubayashi and Sakagami, 1996). The same growth factor was isolated from both monocot and dicot cell cultures and exhibited mitogenic activity and stimulated colony formation of rice protoplasts. Sulfation of PSK-α by a phytosulfokine-α sulfotransferase is imperative for its mitogenic activity and competitive ability for ligand binding (Matsubayashi and Sakagami, 1999). A homolog of the mammalian tyrosylprotein sulfotransferase (TPST) recently has been characterized from rice, asparagus and carrot tissues (Hanai *et al.*, 2000). It was found to be involved in the sulfation of phytosulfokine-α.

The gallic acid glucoside sulfotransferase is another example of a membrane bound sulfotransferase (Varin *et al.*, 1997a). This enzyme was characterized from microsomal preparations of *Mimosa pudica*. *Mimosa pudica* (Leguminosae) has the ability to perform both nyctinastic (leaf closure at night) and seismonastic (response to touch) movements. It was demonstrated that the leaf movement was a result of cooperation of electrical and chemical signals. In 1916 it was suggested that a substance responsible for the leaf closure activity was released in the transpirational stream following mechanical stimulation of the plant. This hypothesis was based on the observation that an extract from *M. pudica* or from other plants exhibiting nyctinastic movements was able to induce leaf closure when applied to the cut end of *a M. pudica* stem (Ricca, 1916). The actual factor, which could induce leaf-closing activity in *M. pudica*, was determined to be gallic acid 4-O-(β-D-glucopyranosyl-6'-sulfate). This

compound was named periodic leaf movement factor 1 (PLMF-1) (Schildknecht, 1981). The gallic acid glucoside sulfotransferase enzyme catalyzes the last step in the biosynthesis of PLMF-1. The results of the tissue distribution of the gallic acid sulfotransferases suggest that the site of synthesis of PLMF-1 is restricted to plasma membrane preparations from primary (base of the petiole), secondary (the junction between the petiole and the rachillae) and tertiary motor organs (the base of the leaflet) (Varin *et al.*, 1997a).

**Cytosolic Mammalian Sulfotransferases**

The cytosolic sulfotransferases are responsible for the sulfation of small compounds such as drugs, steroids and catecholamines (Falany, 1997a).

The human cytosolic sulfotransferase super family (SULT) comprises five well-characterized members with widely differing substrate specificities. Based on amino acid sequence identity and substrate preferences, the various human SULTs can be subdivided into three families: 1) phenol SULTs (P-PST, SULT1A2, M-PST and EST), 2) hydroxysteroid SULTs (HST) and 3) a single novel SULT of unknown function. SULT1 family members (of which seven members are currently known and characterized) sulfonate primarily phenols (including estrogens and iodothyronines) and catechols (including catecholamines), whereas members of the SULT2 family (of which three are known)

sulfonate primarily steroids, sterols, and other alcohols. The novel SULT has been identified through the human chromosome 22 sequencing project and phylogenetic analysis suggests it may be (distantly) related to the SULT2 family (Coughtrie, 2002).

Recent analysis of expression patterns of cytosolic sulfotransferases in humans showed that SULTs might be a major detoxification enzyme system in fetus and developing human (Coughtrie, 2002). Variation in sulfation capacity was suggested to play a role in determining an individual's response to xenobiotics. In addition, polymorphism in sulfotransferase sequences could lead to various consequences including cancer (Coughtrie, 2002). Recently, the first gene knockout experiments with mammalian SULTs have shown that mice deficient in estrogen sulfotransferase lead to compromised reproductive capacity (Coughtrie, 2002).

**Cytosolic Plant Sulfotransferases**

Plants accumulate various natural products, which are synthesized in response to environmental stimuli and developmental signals. Flavonoids are one group of such compounds recognized as important stress metabolites which are synthesized in response to UV irradiation and microbial attack (Varin *et al.*, 1992).

The genes encoding the 3- and 4'- flavonol sulfotransferases from *Flaveria chloraefolia* were isolated. These two sulfotransferases share

significant sequence similarity with steroid and aryl STs found in animal tissues suggesting an evolutionary link between plant and animal STs (Varin *et al.*, 1992). Structure-function studies using the plant flavonol 3- and 4'-sulfotransferases have revealed internal regions responsible for their substrate and position specificity (Varin *et al.*, 1995).

Analysis of brassinosteroid-deficient mutants showed that plants, like humans, use steroids to regulate their growth and development (Rouleau *et al.*, 1999). Recently a new member of a *Brassica napus* sulfotransferase gene family was found to catalyze the *O*-sulfonation of brassinosteroids and of mammalian estrogenic steroids. The enzyme catalyzed the sulfonation of a hydroxyl group of the brassinosteroid intermediate, 24-epicathasterone. Enzymatic sulfonation of 24-epi-brassinolide was found to abolish its biological activity in the bean second internode bioassay (Rouleau *et al.*, 1999).

In the present thesis, I report the characterization of two *Arabidopsis thaliana* cytosolic sulfotransferases named AtST2a and AtST2b. 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 specificities. The AtST2a and AtST2b proteins share 45% sequence identity with the *A. thaliana* and *B. napus* 24-epibrassinosteroid STs, 40% sequence identity with the flavonol STs of *Flaveria species*, and ~25% sequence identity with the mammalian sulfotransferases (Gidda *et al.*, 2003).

**Jasmonates**

Kinetic studies of the *Arabidopsis thaliana* sulfotransferase AtST2a showed that this enzyme has high affinity for 12-hydroxyjasmonate and to a lesser extent for 11-hydroxyjasmonate. Both substrates are hydroxylated derivatives of jasmonic acid (JA) (Gidda *et al.*, 2003).

Jasmonates (JAs) comprise a group of growth regulators first isolated as growth inhibitors of rice seedlings (Karssen, 1991). Using different methods in a screening program with plants from more than 160 families, jasmonates were found to be widespread in *Angiospermae* (flowering plants), and *Gymnospermae* (palms and conifers). Algae such as *Euglena* or *Chlorella* also contain JA and MeJA (methyl ester of jasmonic acid) (Karssen, 1991).

Derived from α-linolenic acid via the octadecanoid pathway (Schaller, 2001), jasmonates stimulate various effects such as inhibition of shoot, root and callus growth, inhibition of pollen and seed germination, embryogenesis, and flower bud induction. JAs also are involved in processes such as leaf senescence and abscission, tuber induction, stomata closure, chlorophyll degradation and leaf respiration (Karssen, 1991). In addition, jasmonic acid has been proposed to be part of a signal transduction pathway that mediates the induction of plant defense genes in response to insect and pathogen attacks (Doares *et al.*, 1995).

Jasmonates have been identified as plant signaling compounds and are structurally similar to the animal prostaglandins (Fig.1.1).

Prostaglandins are derivatives of fatty acids that are produced in most tissues of the body and mediate varying physiological responses. They are often second messengers within cells in response to other hormones. Prostaglandins are synthesized from arachidonic acid in the cell membrane by the action of phospholipase $A_2$. Because they are lipid soluble, prostaglandins can diffuse easily through cell membranes (Bergey *et al.*, 1996).

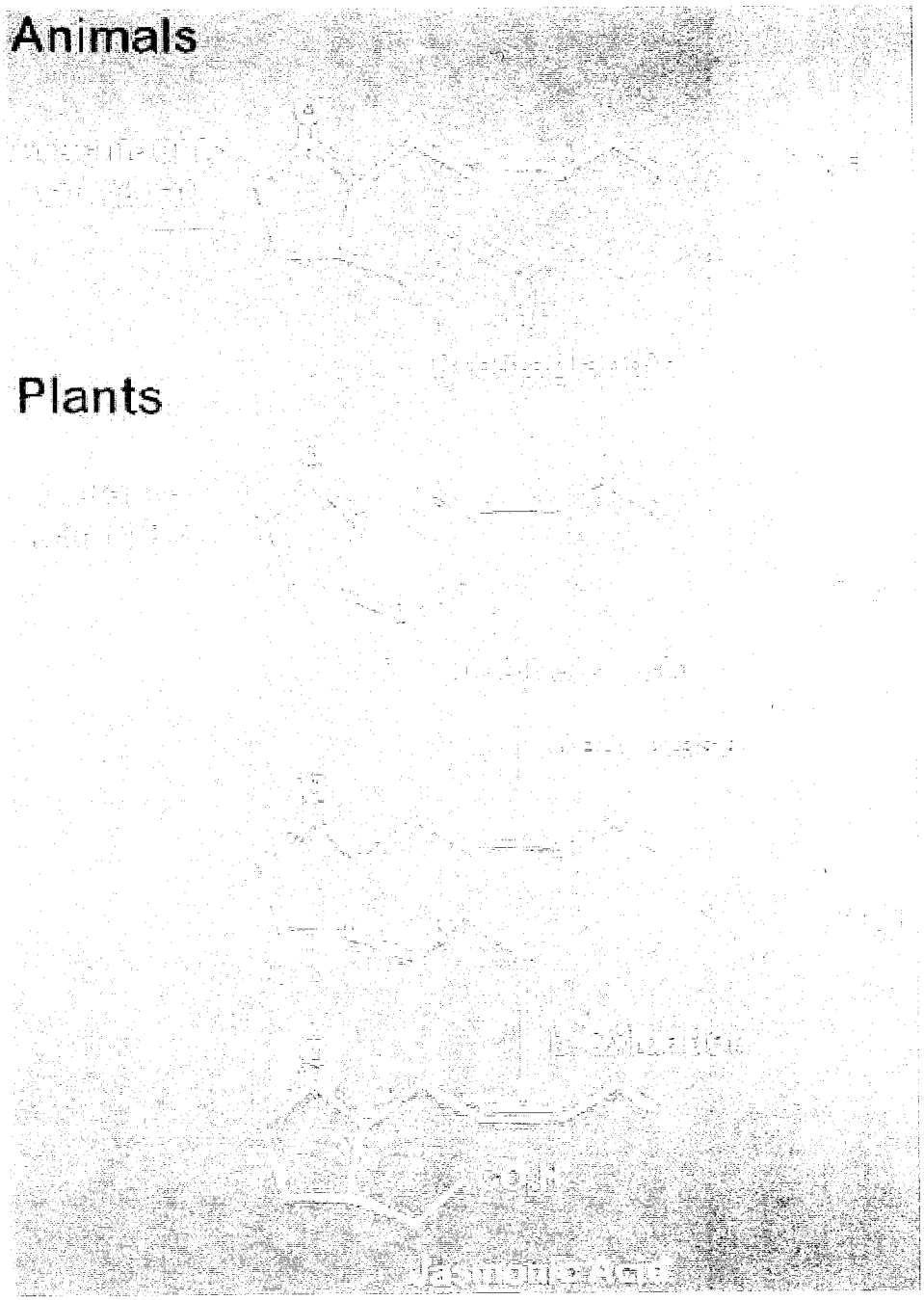



**Figure 1.1 Comparison of the structures of phytodienoic acid and jasmonic acid, derived from linolenic acid, with the structure of a prostaglandin derived from arachidonic acid.**

( Adapted from Bergey *et al.*, 1996)

**Jasmonic Acid Response Mutants**

*Arabidopsis* mutants have provided a wealth of information on the mode of action of jasmonates. For example, two apparently unrelated events: silique formation and defense responses have been linked by the characterization of the male-sterile *coi1* mutant. This mutant was obtained by screening for plants resistant to coronatine, a chlorosis-inducing toxin produced by the phytopathogenic bacterium *Pseudomonas syringae* (Xie *et al.*, 1998). Coronatine is a close structural homolog of the cyclic C18 precursor of jasmonic acid, 12-oxo-phytodienoic acid (OPDA) (Weiler *et al.*, 1994).

*COI1* encodes a protein with 16 leucine-rich repeats and a F-box motif (involved in the ubiquitin conjugation pathway of protein degradation). This protein can recruit regulatory proteins involved in JA response for ubiquitination. COI1 also was found to control the expression of myrosinase binding proteins (MBP), which are present in immature flowers and are localized in several floral organs, including the ovary, ovules, style, anthers and filament (Capella *et al.*, 2001). In contrast with the JA biosynthetic mutants, the male-sterile *coi1* mutant cannot be rescued by the exogenous application of JA defining this mutation as a component of the JA signal transduction pathway.

Screening for constitutive expression of jasmonate-dependent genes led to the discovery of the *cev1* mutant. CEV1 (Constitutive Expression of Vegetative storage protein) encodes a cellulose synthase (CeSA3) that acts

as a negative regulator of jasmonic acid signaling. The *cev1* plants are smaller than wild type, have stunted roots with long root hairs, accumulate anthocyanin (characteristic of a plant treated with JA), have constitutive expression of the defense-related genes, and enhanced resistance to powdery mildew diseases (Ellis and Turner, 2001).

The jasmonic acid (JA)-dependent regulation of the defense response thionin gene *Thi2.1* is a constituent of the signal transduction pathway of *Arabidopsis thaliana*. Several *cet* mutants have been isolated which showed a Constitutive Expression of the Thionin gene. These mutants also showed spontaneous leaf cell necrosis and upregulation of *PR1* gene expression, reactions often associated with systemic acquired resistance (SAR). Four of these *cet* mutants, *cet1, cet2, cet3* and *cet4.1* were crossed with the *fad* triple and *coi1* mutants that are blocked at two steps within the JA-dependent signaling pathway, and with transgenic *NahG* plants that are deficient in salicylic acid (SA) and are unable to activate systemic acquired resistance. Analysis of the various double-mutant lines revealed that the four *CET* genes act within a signaling cascade at or prior to the branch points from which not only JA-dependent signals but also SA-dependent signaling and cell death pathways diverge (Nibbe *et al.*, 2002).

**JA Biosynthesis**

Jasmonic acid is synthesized through at least two pathways, the well-studied octadecanoid pathway from linolenic acid (18:3) and the

novel hexadecanoid pathway from hexadecatrienoic acid (16:3) (Hedden and Phillips, 2000).

**Octadecanoid Pathway**

The first step of this pathway is the release of α-linolenic acid (LA) from membrane lipids located in the chloroplasts (Fig. 1.2). The release of LA might be triggered by local or systemic signals or following wounding. A primary wound signal for the signaling cascade is an 18-amino-acid polypeptide hormone called systemin. The interaction of systemin with its receptor regulates a complex cascade of intracellular events that are all orchestrated to activate a phospholipase $A_2$ to release linolenic acid from membranes (Ryan, 2000). A *fad3 fad7 fad8* triple mutant of *Arabidopsis* is affected in the synthesis of trienoic fatty acids, one of which is linolenic acid, and therefore is deficient in JA production. The triple *fad* mutant plants are male sterile and fertility can be restored by spraying the buds with linoleate or jasmonic acid (McConn and Browse, 1996).

As free LA becomes available, a 13-lipoxygenase (13-LOX) incorporates $O_2$ at carbon 13. The product of this reaction is then converted by the enzyme allene oxide synthase (AOS) to an unstable epoxide. AOS was first purified from flax seeds (Song and Brash, 1991) and was found to be a cytochrome P450 of the CYP74 family (Laudert *et al.*, 1996). Overexpression of flax *AOS* in transgenic *Arabidopsis* and tobacco plants did not alter basal JA levels, but in these transgenic plants

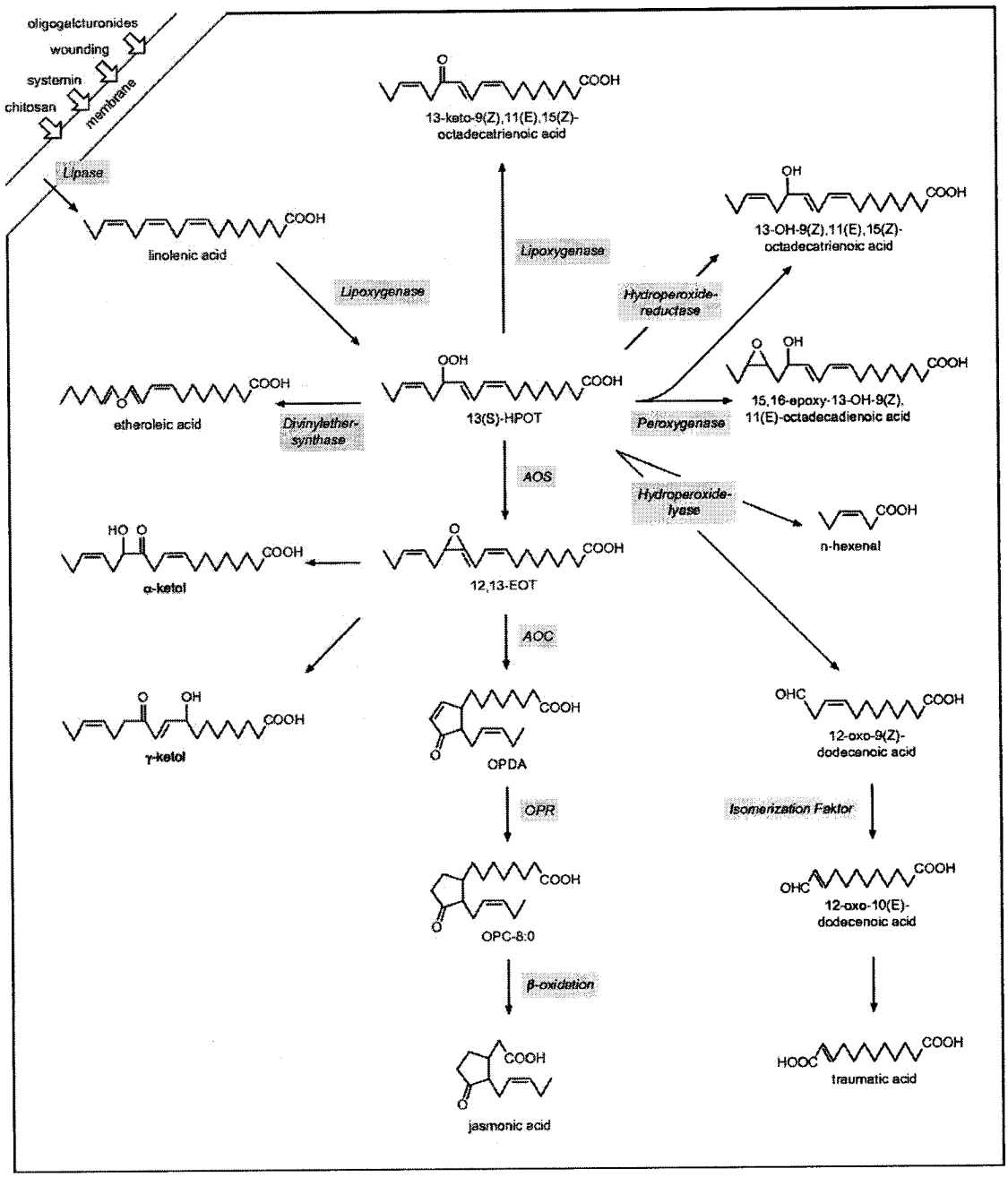



**Figure 1.2 α-Linolenic acid is a central molecule for production of defense-related compounds.**

(Adapted from Schaller, 2001)

peak jasmonate levels were 2-3 folds higher after wounding as compared to the wounded wild type plants. These results suggest that overexpression of *AOS* might be a way of controlling defense dynamics in *Arabidopsis* and tobacco (Laudert *et al.*, 2000). The highly unstable allene oxide can rapidly decay in aqueous solution by chemical hydrolysis to α- and γ-ketols and racemic 12-oxophytodienoic acid or OPDA. In the presence of the allene oxide cyclase (AOC), exclusive formation of the *cis*- (+)-enantiomer (9-*S*, 13-*S*) of OPDA occurs (Hause *et al.*, 2003). Thus, the specificity of the octadecanoid biosynthetic pathway results from AOC, rather than LOX or AOS. The extremely short half-life of allene oxide, the optical purity of natural OPDA, and the absence of α- and γ-ketols in plant tissues *in vivo* (Weiler, 1998) suggest a tight coupling of the AOS and AOC reactions possibly in a synthase-cyclase complex (Schaller, 2001). OPDA then is transported by an unknown mechanism to the peroxisomes where the 10,11-double bond in the cyclopentanone ring is reduced. This reaction is catalyzed by the enzyme 12-oxophytodienoate reductase (OPR3). Molecular cloning of the reductase from *Arabidopsis* revealed a close relationship to Otto Warburg's Old Yellow Enzyme (OYE), a flavoprotein enone reductase (Warburg, 1933), and to morphinone reductase of *Pseudomonas putida*, an enzyme which reduces morphinone to hydromorphone or codeinone to hydrocodone (French and Bruce, 1994). The Old Yellow Enzyme was the first enzyme shown to possess a flavin cofactor (Warburg, 1933). Up to now three OPR isoforms from *Arabidopsis*

have been described (OPR1, OPR2 and OPR3). Sequence similarities to OPR homologs from other organisms were observed over the entire length of the protein sequences. The enzymes of the OPR3-type from *Arabidopsis* and tomato are more closely related to the OYEs than OPR1 and OPR2. The sequence similarities reflect the enzymatic properties of the enzymes. *Le*OPR1 from tomato (Strassner *et al.*, 1999) and OPR1 and OPR2 from *Arabidopsis* preferentially catalyze the reduction of *cis*(-)-OPDA (Schaller *et al.*, 2000), whereas *Le* OPR3 from tomato and OPR3 from *Arabidopsis* prefer *cis*(+)-OPDA, the naturally occurring precursor of JA, as their respective substrate. OPR3 rather than its related genesOPR1 and OPR2 is thus the enzyme involved in JA biosynthesis in *Arabidopsis* and tomato. The functions of OPR1 and OPR2 are not yet understood, but it seems likely that they are not involved in JA biosynthesis. However, looking at the broad substrate specificity of *Le* OPR1 and *Arabidopsis* OPR1 (Strassner *et al.*, 1999) it can be hypothesized that other physiologically similar compounds might be substrates for this reductase (Schaller, 2001).

Mutants homozygous for the disrupted *OPR3* gene are male sterile due to a delay in the release of pollen grains from the anthers and have defective pollen development (Weber, 2002). The mutant phenotype can be rescued (generating fertile plants) by the exogenous application of MeJA.

The last three steps in JA biosynthesis are successive β-oxidation reactions. The compartmentalization of these β-oxidation steps has not

been investigated yet. Presumably, they are localized in the peroxisomes and/or glyoxysomes, since these are the only sites where β-oxidation is known to occur in plants (Gerhardt, 1983). Likewise, the question of whether the transformation of the C18-compound OPDA to the C12-compound JA should be considered a biosynthetic, or rather, a catabolic reaction is still open to debate. It has been reported that OPDA is active *per se* in the induction of benzophenanthridine alkaloid synthesis after elicitation of plant suspension cultures (Blechert, 1995). In this publication, the authors assumed that OPDA and OPC-8:0 are primary signal transducers in the elicitation process, notwithstanding the biological activity of JA and its possible interaction with the same hypothetical receptor. Furthermore, OPDA is 50–100 times more active than JA in eliciting tendril coiling in *Bryonia dioica* (Weiler *et al.*, 1994). Thus, just like in mammalian eicosanoid metabolism where β-oxidation is a characteristic degradation route and usually associated with complete loss of the biological activity, it has been proposed that JA might represent a degradation product of biologically more active C18-compounds, which is then further inactivated in the cell by conjugation (Sembdner, 1988). However, there is strong evidence, based on detailed pharmacological studies, that the natural oxylipin capable of eliciting the synthesis of alkaloids like sanguinarine in *Eschscholtzia* cell cultures is JA (Haider *et al.*, 2000). The availability of *Arabidopsis* plants lacking a functional *OPR3* has allowed the importance of JA to be proven. In the *opr3* mutant, JA

cannot be produced; these plants are male sterile because they produce predominantly unviable pollen and have delayed anther dehiscence. Male sterility in these plants is rescued efficiently by JA but not by OPDA, ruling out an active role for OPDA in pollen development. Thus evidence based on well characterized mutants indicates an exclusive role of JA in male gametophyte development in *Arabidopsis* (Stintzi *et al.*, 2001). On the other hand, the *opr3* plants survived as well as wild-type plants in response to the attack by caged *Bradysia* larvae. Therefore, in the case of defense against this insect, JA production seems unnecessary (Stintzi *et al.*, 2001).

**Hexadecanoid Pathway**

All known jasmonates (and almost all other fatty acid signals in plants) have been shown to derive from unsaturated octadecanoic acids. It is not known whether other unsaturated fatty acids can act as precursors for the biosynthesis of other regulators in plants. In plants, octadecanoid-derived signal compounds are well known, but few hexadecanoid-derived signal molecules have been characterized. A novel 16-carbon cyclopentenoic acid was identified in leaf extracts from *Arabidopsis* and potato. The new compound, a member of the jasmonate family of signals, was named dinor-oxo-phytodienoic acid (dnOPDA). Dinor-oxo-phytodienoic acid was not detected in the *Arabidopsis* mutant *fad5*, which is incapable of synthesizing hexadecatrienoic acid (16:3), suggesting that the metabolite

is derived directly from plastid 16:3 rather than by β-oxidation of the 18-carbon 12-oxo-phytodienoic acid. Treatment of *Arabidopsis* with micromolar levels of dinor-oxo-phytodienoic acid increased the ability of leaf extracts to transform linoleic acid into the α-ketol octadecenoic acid indicating that the compound can regulate part of its own biosynthetic pathway (Weber *et al.*, 1997). It is not yet known whether dnOPDA is further metabolized, but it might be a substrate for one of the OPR enzymes. The presence of dnOPDA in leaf tissue from both *Arabidopsis* and potato hints to a broad distribution of this molecule in the plant kingdom, because these two plants belong to widely separate families of the angiospermae. Over 30 plant families are known to contain 7Z,10Z,13Z-hexadecatrienoic acid, the proposed dnOPDA precursor (Jamieson, 1971).

**The "Oxylipin Signature"**

Oxidative metabolism of polyunsaturated fatty acids gives rise to a group of biologically active compounds, collectively termed oxylipins, which perform a variety of functions in plants. The preferential accumulation of distinct octadecanoids (the C18 compounds, including the precursors of JA, OPDA) or compounds derived from them including jasmonates, led to the suggestion that each plant may have a distinct pattern designated as the "oxylipin signature" (Weber *et al.*, 1997). In general, oxylipins are not pre-formed but rather are synthesized *de novo* in response to mechanical injury, herbivore and pathogen attack, and

other environmental and developmental signals (Howe and Schilmiller, 2002).

**JA derivatives**

Plants can transform jasmonic acid by methylation to its volatile derivative MeJA, which can act as a signal in interplant communication and also move in the intercellular spaces within the plant (Weber, 2002).

A primary step in JA biotransformation is the hydroxylation at C-11 and to a lower extent at C-12 to form 11-OHJA or 12-OHJA derivatives. Although the enzyme(s) responsible for hydroxylation of these compounds still remain(s) to be characterized, cytochrome P450 enzymes would be good candidates.

Another major route of biotransformation of JA is its conjugation with certain amino acids. In the case of barley seedlings or shoots, valine and isoleucine conjugates have been isolated and identified (Parthier, 1991).

A glucoside of 12-OHJA, potassium β-D-glucopyranosyl 12-hydroxyjasmonate was found to play a role in leaf closing of the silk tree, *Albizzia julibrissin* (Ueda, 2001).

**Induction of Tuberization and "Florigen"**

Another glucoside of 12-OHJA also was isolated from the leaves of potato plants and identified as 3-oxo-2 (5'-β-D-glucopyranosyloxy-2-*cis*-pentenyl) cyclopentane-1-acetic acid (Yoshihara, 1989). The aglycone of

the substance was named "tuberonic acid" and was found to stimulate tuberization in potato and Jerusalem artichoke plants (Koda, 1992). Tuberonic acid has four stereoisomers, although the naturally occurring tuberonic acid is found only in the *cis* conformation. The *cis* isomer exhibits much stronger activity than the *trans* isomer. *Cis*-tuberonic acid and its glucoside have no inhibitory effects on plant growth as opposed to their precursor, jasmonic acid (Koda, 1992).

Tuberization in potato plants is controlled by photoperiod and temperature (short days promote and long days inhibit tuberization). It was demonstrated that the tuberization stimulus is formed in the leaves under short days and is transmitted to the underground parts to induce tuberization. This mechanism is similar to the one proposed to initiate flowering where a "florigen" (flowering inducing substance) (Aukerman and Amasino, 1998) is produced in the leaves under the inductive conditions and is translocated to the SAM (Shoot Apical Meristem) of the plant to induce flowering (Fig.1.3) (Chailakhyan, 1936).

Despite the extensive research conducted over the past fifty years, the structure of the hypothetical florigen has not been determined.

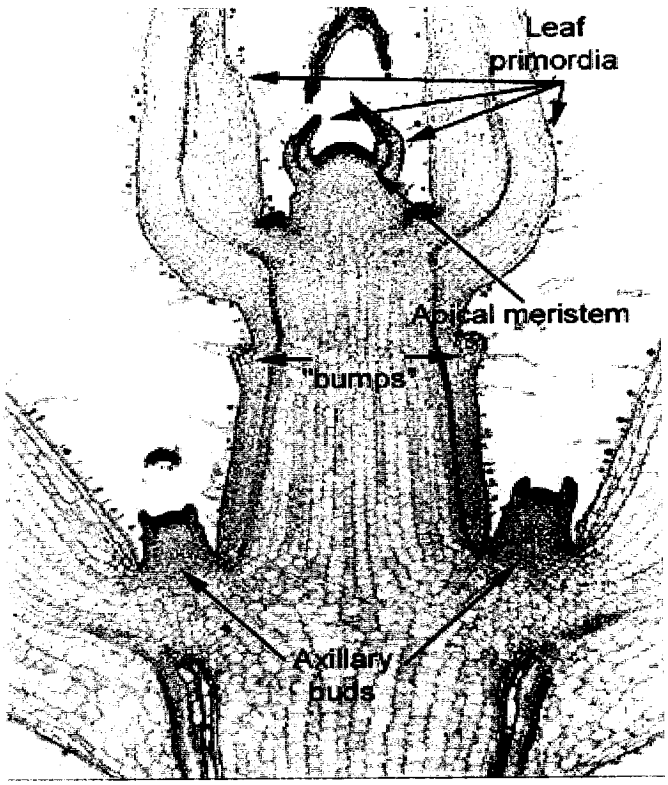



**Figure 1.3 Apical meristem**

( Adapted from Aukerman and Amasino, 1998)

The existence of the florigen was hypothesized from results obtained from grafting experiments where induced shoot was united with a non-induced partner (King, 1973). This grafting union triggered flowering in non-induced plants. Additional grafting experiments between photoperiod-insensitive plants and photoperiod sensitive plants, or between photoperiod-insensitive plants, gave similar results (*i.e.*, floral induction) (Lang, 1977). These data indicate that signal production and translocation are not restricted to responses to photoperiod. Other factors such as vernalization, day length, light intensity, soil moisture, and the developmental stage of the plant control flowering time as well. Recently, an attempt was made to analyze the phloem sap of *Perilla ocymoides* L and *Lupinus albus* L. More than 100 components were detected and the sequence of 16 peptides with masses ranging from 1 to 9 kDa was obtained. Four small proteins were identified that could potentially play a role in the induction of flowering. One of them showed no similarity to any known protein sequences, another showed similarity to Ser/Thr receptor-like protein kinases. The other two proteins were similar to permeases, which might play a role in the transport of signalling molecules. Therefore, kinase-like proteins and permeases may act together to induce flowering directly or may participate in the transport of the flower inducing molecules to the site of action (Hoffmann-Benning *et al.*, 2002).

**Flower Initiation**

Initiation of flowering depends on a number of endogenous and exogenous factors such as the age of the plant, stratification (transient exposure to cold temperatures), light quality, availability of water and nutrients (Koornneef *et al.*, 1998).

Plants like animals pass through certain stages during the course of their lives (Levy and Dean, 1998b). The first phase is called the juvenile or vegetative phase during which the SAM is incompetent to respond to external or internal stimuli that would trigger flowering in a mature meristem. During this phase the SAM contains a group of undifferentiated stem cells that give rise to an indeterminate number of leaves. When the plant has matured and the environmental factors are favorable, the SAM switches from the generation of leaf nodes to the production of flowering nodes giving rise to a determinate number of flowers.

In some plants, the timing of flowering is influenced primarily by environmental factors (long day or short day plants). Other species flower mostly in response to internal stimuli (day neutral plants).

**Flowering Models**

Few models have been proposed for the control of flowering time (Bernier, 1988; Thomas and Vince-Prue, 1997). The first one involves the "florigen" (Levy and Dean, 1998a). This concept was based on the results of grafting experiments, which suggested the existence of a substance

produced in the leaves and translocated to the SAM to promote the switch from the vegetative to the reproductive phase.

Another model is called the multifactorial model which proposes that there are a number of limiting elements that must be present at the apical meristem in the right concentrations and at the right time in order to initiate flowering (Koornneef *et al.*, 1998).

Finally the most recent model proposes that flowering involves the sequential action of three groups of genes (Fig. 1.4) (Blazquez, 2000). The first group comprises genes that sense the endogenous and exogenous factors to prepare for the switch of the meristem (flowering time genes). Secondly, there are genes that make the actual switch of the apical meristem to the reproductive phase (floral meristem identity genes). Finally, there are genes that direct the formation of flower (flower organ identity genes) (Levy and Dean, 1998b). Through the study of mutants, researchers have isolated a number of genes belonging to the three groups.

**Flowering Time Genes**

These genes promote or repress the initiation of flowering. In *Arabidopsis*, there are multiple pathways, which trigger the onset of flowering. Mutations in genes representing each of the pathways cause early or late flowering phenotypes. More that 20 late-flowering genes have been described so far. However, a mutant that remains in the vegetative

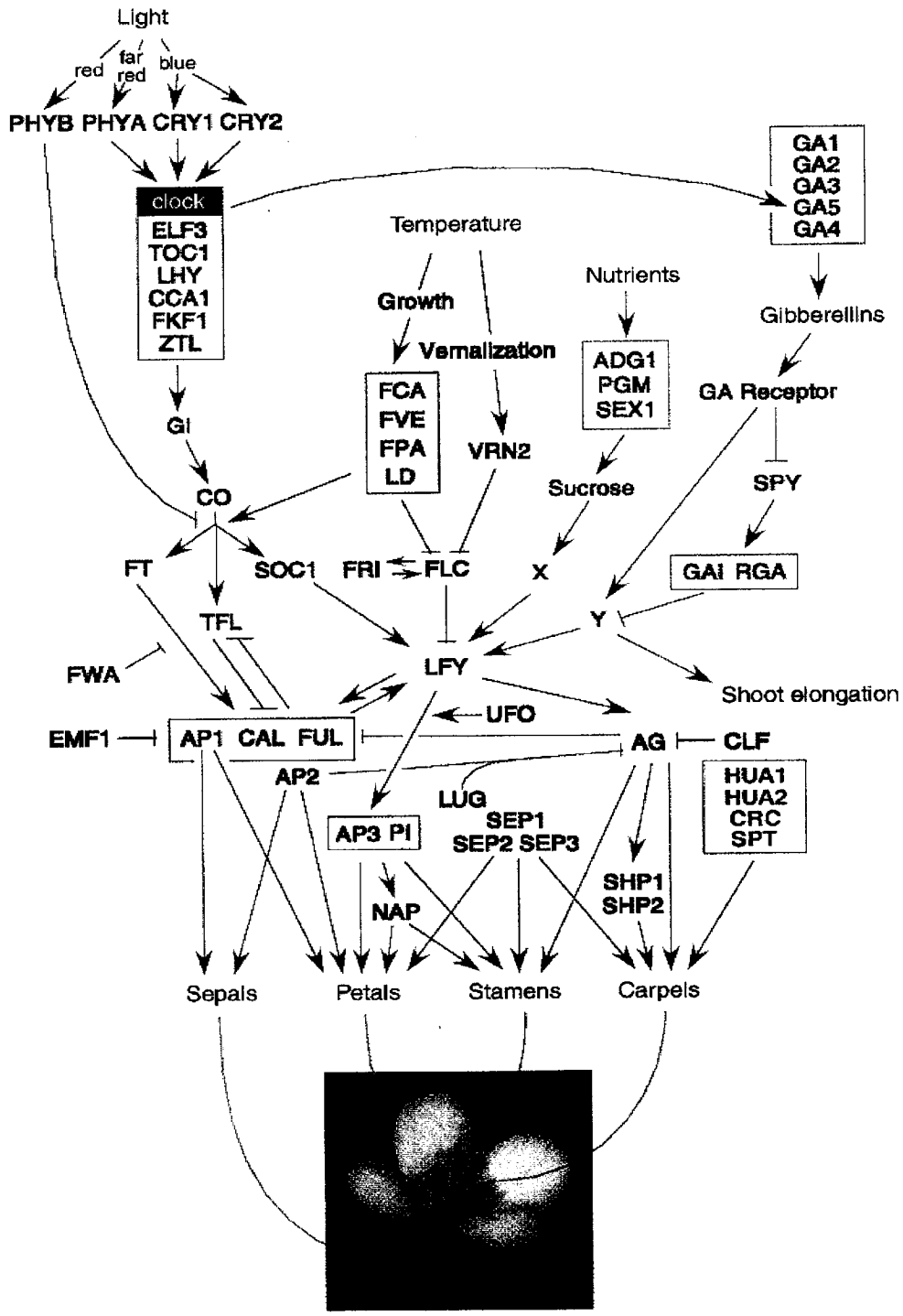



**Figure 1.4 Flower development pathways**

( Adapted from Blazquez, 2000)

phase indefinitely has not been isolated. This suggests that there is redundancy in the action of genes that promote flowering.

There are four pathways that control flowering time in *Arabidopsis*: 1) repression (genes that repress initiation of flowering), 2) autonomous promotion (genes that promote flowering independently of environmental conditions), 3) photoperiodic promotion under long days and under short days (or gibberellin dependent pathway), and 4) vernalization (promotes flowering if the seeds are exposed to cold for a period of time) (Levy and Dean, 1998a).

The photoperiod pathway is linked to the expression of *CONSTANS* (*CO*), which encodes a Zn-finger transcription factor (Putterill *et al.*, 1995). *CO* directly upregulates the transcription of meristem identity genes such as *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)/AGAMOUS LIKE 20 (AGL20)* genes (without *de novo* protein synthesis) to promote floral transition (Lee *et al.*, 2000; Samach *et al.*, 2000). *EARLY FLOWERING 3 (ELF3)* is another component of the photoperiod dependent pathway, which was proposed to serve as a mediator protein that regulates the light input to the circadian clock. Circadian clocks are capable of self-sustained oscillations in the absence of environmental time cues, but in natural conditions they must be set to local time. In *Arabidopsis*, the synchronization (or 'entrainment') of the clock to day-night cycles is mediated by multiple photoreceptors, including cryptochromes and phytochromes (Somers *et al.*, 1998).

ELF3 encodes a novel nuclear protein that is expressed rhythmically and interacts with PHYTOCHROME B (Carre, 2002). ELF3 was identified through a mutation that caused early flowering and abolished responsiveness to photoperiod. The *elf3* mutant exhibited phenotypes usually associated with defective light perception, namely pale leaves and elongated hypocotyl and petioles (Zagotta *et al.*, 1996). ELF3 post-translationally regulates the photoreceptors (*PHYA*, *PHYB*, *CRY1* and *CRY2*) and downstream genes such as GIGANTEA. GIGANTEA is a nuclear protein, which shows no sequence similarity to any known proteins. This protein was proposed to function as a PHYB signaling intermediate in the photoperiodic control of flowering in *Arabidopsis* (Huq *et al.*, 2000).

**The Meristem Identity Genes**

The four pathways described above converge at a group of meristem identity genes. Meristem identity genes include LEAFY (LFY), APETALA1 (AP1), and CAULIFLOWER (CAL), which specify flower meristem identity, as well as TERMINAL FLOWER (*TFL1* and *TFL2*) genes, which maintain inflorescence meristem identity. The *tfl1* and *tfl2* mutants initiate flowering early and terminate the inflorescence with floral structures. However, only *tfl2* mutants are dwarfed and have reduced photoperiod sensitivity and more variable terminal flower structure (Larsson *et al.*, 1998). RNA *in situ* hybridization experiments showed that *TFL2* transcripts accumulated in proliferating cells in the meristematic tissues

of vegetative, inflorescence, and floral organs (Kotake *et al.*, 2003). The *tfl1* and *tfl2* double mutants terminate the inflorescence without development of lateral flowers. The enhanced phenotype of double mutant shows that *TFL2* has a regulatory role more global than that of *TFL1*. *TFL2* function influences developmental processes controlled by the meristem identity gene, *APETALA1*, but not those regulated by *LEAFY* (Larsson *et al.*, 1998).

The *TFL2* gene encodes a homolog of *Drosophila* heterochromatin-associated protein 1. Heterochromatin-associated protein 1 (HP1) of *Drosophila* is involved in the generation and maintenance of an inactive heterochromatin structure that silences gene expression (Eissenberg and Elgin, 2000). TFL2 represses *FLOWERING LOCUS T*, which is involved in regulation of flowering time and several floral homeotic genes that regulate flower structure (Kotake *et al.*, 2003). While *FT* is upregulated in the *tfl2* mutant, *CO* and other floral pathway integrators are not affected (Kotake *et al.*, 2003) suggesting that at least one of the roles of TFL2 is to repress the initiation of flowering by acting on FT levels.

**Flower Organ Identity Genes**

A great deal of attention has focused on the characterization of genes that specify organ identity. The analysis of such genes allowed the "ABC model" of flower development (Fig. 1.5) to be proposed (Bowman and Meyerowitz, 1991). This model suggests that at around the time of organ

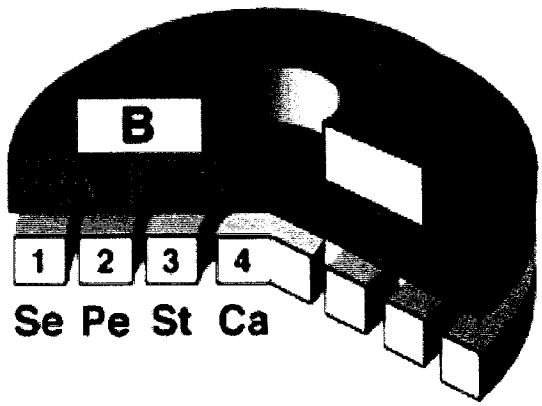



**Figure 1.5 ABC model of Flower Development**

Schematic representation of the ABC model of floral organ determination, showing how the three organ-identity functions (A, B, and C) combinatorially specify the identity of the different types of organs in the four whorls of a wild type flower (Se-sepals, Pe-petals, St-Stamens, Ca-carpel)

(Adapted from Bowman and Meyerowitz, 1991)

initiation, the flower meristem is partitioned into three overlapping fields of gene activity (A, B, and C), each field defining two adjacent whorls. The model further suggests that the A and C functions are mutually antagonistic, such that in a-function mutants, the C domain expands to include all whorls, and similarly in c-function mutants, the A domain expands to include all whorls.

The primary class A gene in *Arabidopsis* is *APETALA2 (AP2)* (Bowman and Meyerowitz, 1991; Meyerowitz *et al.*, 1991). Mutations in *AP2* lead to the development of reproductive or leaf-like organs in the outer whorls (perianth), where sepals and petals would normally form (Keck *et al.*, 2003).

The *Arabidopsis* floral organ identity genes APETALA3 (AP3) and PISTILLATA (PI) encode related DNA-binding proteins of the MADS family. Considerable evidence supports the hypothesis that a heterodimer of AP3 and PI is an essential component of B class activity. All *ap3* and *pi* mutant alleles characterized to date exhibit equivalent phenotypic defects in both whorls 2 and 3. In strong *ap3* and *pi* mutants, petals and stamens are missing and sepals and carpels develop in their place. Weak *ap3* and *pi* mutants exhibit partial conversions of petals to sepals and stamens to carpels (Yang *et al.*, 2003).

An example of a class C gene is AGAMOUS (AG), which is required for determinacy in addition to stamen and carpel development. As a consequence, *ag* mutant flowers are indeterminate and continue to

elaborate new whorls of organs, giving rise to the "flower within a flower" phenotype. In *ag* mutant flowers, the 3rd whorl stamens are replaced by petals, and the 4th whorl carpels are replaced by a new flower (Gustafson-Brown *et al.*, 1994).

**Photoreceptors**

Light is perceived by plants via several photoreceptors. The photoreceptors modulate plant development by detecting the changes in light quality, intensity, and duration and by transducing light signals to photo-responsive nuclear genes through various pathways (Bagnall *et al.*, 1995).

There are three major classes of photoreceptors: the red(R)/far-red (FR) light absorbing phytochromes (600-750 nm), the blue (B)/UV-A absorbing cryptochromes and phototropins (320-500 nm), and the UV-B sensing UV-B receptors (282-320 nm) (Wang, 2002). Among these photoreceptors, phytochromes and cryptochromes are known to regulate flowering time (Lin, 2000a).

**Cryptochromes**

A cryptochrome was first identified in *Arabidopsis thaliana*. Several photomorphogenic mutants were isolated, including *hy4 (elongated hypocotyls 4)*, which had impaired blue-light-dependent inhibition of hypocotyls elongation, resulting in long hypocotyls when grown in blue

light. *HY4* (renamed CRYPTOCHROME 1) amino acid sequence was found to be 30% identical to the microbial DNA-repairing enzyme DNA photolyase (Sancar, 1994). DNA photolyases are flavoenzymes that catalyze a blue-UV-A-light-dependent DNA-repair reaction through an electron-transfer mechanism (Sancar, 1994). *CRY2* was isolated by screening a cDNA library using *CRY1* as a probe. In contrast to *CRY1*, which is expressed constitutively, CRY2 accumulation is down regulated in blue light. However, no change of *CRY2* mRNA could be detected suggesting that blue light triggers degradation of the CRY2 protein (Lin, 2000b).

**Phytochromes**

Phytochromes are protein kinases that most probably convey the light signals to other molecules through light-dependent enzymatic activity. Phytochromes also may relay signals by changing their conformations. A phytochrome consists of a backbone, which is covalently attached to a chromophore (linear tetrapyrrole). A phytochrome backbone contains two structural domains: a photosensory (chromophore-binding) domain, which is involved in light absorption and photoreversibility, and a regulatory domain important for dimerization and downstream signaling. Phytochromes interconvert between the Far Red light (FR) - absorbing Pfr (physiologically active form) and the Red light (R)-absorbing phytochrome (Pr) form (inactive).

In *Arabidopsis* there are five phytochromes (termed *PHYA-PHYE*) encoded by five distinct members of the phytochrome gene family. PHYA is a type I phytochrome, and PHYB-PHYE are all type II phytochromes. PHYB and PHYD polypeptides are about 80% identical and are somewhat more related to PHYE than they are to either PHYA or PHYC (about 50% identity). The PHYB, PHYD and PHYE polypeptides are the most recently evolved members of the phytochrome family. Homologs of PHYA, PHYB and other phytochrome genes are present in most, if not all, higher plants (Wang, 2002).

The *phyA* mutants show a fairly normal shade avoidance response, but exhibit a late flowering phenotype, and are impaired in the perception of daylength. The *phyB*-deficient plants have a constitutive elongated-petiole and an early-flowering phenotype. The monogenic *phyD* mutant plants have no obvious phenotypic abnormality, whereas plants impaired in both the *PHYB* and the *PHYD* genes display significantly longer hypocotyls under either red or white light and flower earlier than the *phyB* monogenic mutants. Moreover, the triple mutant *phyABD* still develops elongated rosette internodes and exhibits accelerated flowering responses. The *phyE* mutants show no phenotypic alteration unless it is in the *phyB* mutant background and the *phyBE* double mutants flower much earlier than the *phyB* monogenic mutants (Devlin *et al.*, 1998). These studies show that PHYB, PHYD and PHYE control shade avoidance responses and inhibit flowering in a conditional redundant manner. No mutation for

PHYC has been reported yet, but over-expression studies suggest a role in primary leaf expansion (Qin *et al.*, 1997). The *phyA* mutants flower later than wild type plants under long days, whereas *phyE* mutants flower slightly early. Plants having null mutants for both *phyA* and *phyE* flower at the same time as *phyE* single mutant plants, indicating that the *phyE* mutation is epistatic to *phyA* with respect to flowering time (Whitelam, 2001).

**Phytochromes and Flowering Time Genes**

Like *phyB* mutants, both the *gigantea (gi)* and the *early flowering 3 (elf3)* mutants display elongated hypocotyls in red light. However, the *gi* mutants are late flowering, which is in contrast with the early flowering phenotype of the *phyB* and *elf3* mutants. This clearly suggests that ELF3 and GI play different roles or use different mechanisms in controlling hypocotyl elongation and flowering responses. Both ELF3 and GI are nuclear proteins and are most likely involved in regulating the expression of flowering-time genes (Huq *et al.*, 2000; Liu *et al.*, 2001). *PhyA* mutants flower late but *phyB* mutants flower early. These findings suggest that the observed alterations in flowering time of the *phyA* and *phyB* mutants are unlikely to be the direct consequence of a malfunction of the circadian clock. Instead, these photoreceptors may directly affect the floral initiation process, but the signal transduction of photoreceptors may be gated (rather than executed) by the circadian clock. This notion gained support

from a recent genetic study, which showed that ELF3 requires PHYB function in early morphogenesis but not for the regulation of flowering time, suggesting that ELF3 and PHYB control flowering via independent signal transduction pathways (Liu *et al.*, 2001).

The notion of phytochrome interconversion between the active Pfr and inactive Pr forms imposes an apparent difficulty in explaining how PHYA may mediate an FR response in light-grown adult plants in which the PHYA abundance is significantly lower than that in etiolated seedlings. However, it has been reported recently that the active form of PHYA may be neither Pfr nor Pr. Instead a short-lived intermediate generated during photoconversion from Pfr to Pr may be the active form. This hypothesis explains not only some well studied FR-dependent high-irradiance responses, such as FR inhibition of hypocotyl elongation, but also the PHYA-mediated FR promotion of flowering (Shinomura *et al.*, 2000). It has been found that the coincidence of light perception by CRY2 and PHYA with the peak circadian expression of the *CO* gene is critical for the induction of the expression of the flowering-time gene *FT* and photoperiodic flowering (Yanovsky and Kay, 2002).

The red and far-red light–absorbing phytochrome photoreceptors are light-regulated Ser/Thr-specific protein kinases that regulate diverse photo-morphogenic processes in plants. It recently was discovered that phytochromes functionally interact with the catalytic subunit of a Ser/Thr-specific protein phosphatase 2A designated FyPP. The interactions were

influenced by phosphorylation status and spectral conformation of the phytochromes. FyPP was expressed predominantly in floral organs. Transgenic *Arabidopsis* plants with overexpressed or suppressed FyPP levels exhibited delayed or accelerated flowering, respectively, indicating that FyPP modulates phytochrome-mediated light signals in the timing of flowering (Kim *et al.*, 2002).

# CHAPTER 2

# MATERIALS AND METHODS

## Materials

12-OH-JA, 12-$HSO_4$-JA, and jasmonic acid 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, C24, WS, Col-Gl, L*er* were obtained from Lehle seeds, USA. The *coi1, cev1* and *opr3* mutant plants were provided by Dr. J. Turner, School of Biological Sciences, University of East Anglia, Norwich, United Kingdom. Mutants such as *constans (co)* (seed stock number CS175), *early flowering (elf3)* (CS3787), *gigantea (gi)* (CS51), *flowering locus T (ft)* (CS56), *terminal flower 1 (tfl1)* (CS6235), *terminal flower 2 (tfl2)* (CS3796), *cryptochrome (cry2)* (CS3732), *phytochrome B (phyB)* strong allele (CS6211), and weak allele (CS6212), *phytochrome A* strong allele (CS6219), and weak allele (CS6221), *phytochrome A phytochrome B* double mutant *(phyAB)* (CS6224), *AtST2a* knockout T-DNA insertion line (SALK_053325) and *AtST2b* knockout line (SALK_009093) were obtained from the *Arabidopsis* Biological Resource Center. All reagents were of analytical or molecular biology grade.

**Cloning of the *AtST2a* promoter**

The sequence of a 4 kb DNA intergenic region upstream from the start codon of *AtST2a* was retrieved from Genbank (MOJ9 clone, accession number: AB010697.1 GI: 2828182, from nucleotides 54992 to 58972).

Oligonucleotide 5'-CCTCTAGAGTAGACTTCATTCGCTGTGGATT-3' was designed to introduce an *Xba*1 site at the 5'- end of the 4kb promoter region, and another primer 5'-GCGGATCCGCTCTTCATGCTTGAGGTAGC-3' was designed to introduce a *Bam*HI site at the 3' end of the *AtST2a* promoter region. The promoter region was amplified by the Polymerase Chain Reaction with Ex-Taq DNA polymerase (TaKaRa), the above primers, and *Arabidopsis thaliana* (Col-0) genomic DNA.

The amplified product was digested with *Xba*1 and *Bam*HI restriction endonucleases and ligated into the corresponding sites of the binary vector pBI101 (Clontech) upstream from the *β-glucoronidase (GUS)* reporter gene. The clones containing the *AtST2a* promoter region were isolated by restriction enzyme analysis and confirmed by sequencing. All enzymes used for cloning were from New England Biolabs and were used under the conditions recommended by the manufacturer.

**Histochemical analysis of *AtST2a* promoter-*GUS* fusions**

Plants harboring *AtST2a* promoter-*GUS* fusions were grown in soil or in magenta boxes. The GUS staining was performed according to standard procedures (Campisi *et al.*, 1999).

**Cloning of *AtST2b***

The cDNA sequence of *AtST2b* (accession number NM_120782) was retrieved from Genbank.

Oligonucleotide 5'-CATAGGATCCATGGCGATCCCAAGTTTCT-3' was designed to introduce a *Bam*H1 site at the 5'- end of the gene, and another primer 5'-CGCGGGATCCACTAAAGTGTGGACGTTAC-3' was designed to introduce a *Kpn*1 site at the 3' end of the gene. *AtST2b* was amplified by the Polymerase Chain Reaction with Ex-Taq DNA polymerase (TaKaRa) using the above primers and *Arabidopsis thaliana* (Col-0) genomic DNA.

The amplified product was digested with *Bam*HI and *Kpn*1 restriction endonucleases and ligated into the *Bam*H1/*Kpn*1 sites of the bacterial expression plasmid, pQE30 (Qiagen). Clones containing *AtST2b* were determined by PCR, restriction endonuclease digestion analysis and by sequencing. All enzymes used for cloning were from New England Biolabs and were used under the conditions recommended by the manufacturer.

**DNA and protein sequence analysis**

DNA and protein sequence alignments were performed using the Multialin Interface program (http://prodes.toulouse.inra.fr/multalin /multalin.html) as well as the ClustalW program (http://dot.imgen.bcm.tmc.edu:/multialign/multi-align.html), and the

similarity/identity values determined from the pairwise comparisons of *AtST2a* and *AtST2b* genes.

**Expression of recombinant AtST2a and AtST2b**

A culture of *E. coli*, strain XL1-blue harboring *AtST2a* or *AtST2b* ($O.D_{600}$ = 0.7) was induced with 1mM isopropylthio-β-D-galactopyranoside for 10 hours at 22°C. Bacterial cells were collected by centrifugation, resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 0.3M NaCl and 14 mM 2-mercaptoethanol. The cells were lysed by sonication and the recombinant proteins recovered in the soluble fraction by centrifugation at 12 000 x g for 15 min at 4°C. 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 and the proteins were eluted with the same buffer containing 0.5 M imidazole. The Ni-agarose purified protein was desalted on a PD-10 (Pharmacia Biotech) column pre-equilibrated in 25 mM bis-Tris, pH 6.5. Protein concentration was estimated using the Bradford Reagent (Bio Rad) and bovine serum albumin (BSA) as reference protein.

**Preparation of anti-AtST2a antibodies**

Anti-AtST2a polyclonal antibodies were raised in rabbits using PAP-agarose purified recombinant enzymes from *E.coli.* Purified protein (100

μg) in Freund's complete adjuvant was injected subcutaneously into a rabbit. 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 (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 AtST2b 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- or AtSt2b-sulfated

reaction product was extracted with 1-butanol saturated with water and an aliquot was counted for radioactivity in scintillation fluid.

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

Fresh plant material (1g) was homogenized with 10 ml methanol and 100 ng of ($^2H_6$) JA, 12-($^2H_3$)OAc-JA and 11-($^2H_3$)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 (500 mg; Machery-Nagel). The flow-through containing JA and acetylated forms 11-OHJA and 12-OHJA 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-OHJA 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 100µl 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-hydroxysulfonyloxyjasmonic acid 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 an 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 collision energy of 30 eV (collision gas: argon, collision pressure: 1.8 x $10^{-3}$ Torr). All mass spectra are averaged and background subtracted. 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, 1999).

**Northern blot analysis**

*A. thaliana* plants were pulverized in liquid nitrogen and extracted in buffer/phenol/chloroform as described (Cashmore, 1982) to isolate total RNA. Total RNA (10 μg) was used for agarose gel electrophoresis after denaturing with glyoxal and DMSO (McMaster and Carmichael, 1977). Northern blot analysis of total RNA was achieved under high stringency conditions according to standard procedures (Sambrook *et al.*, 2001) using the [$^{32}$P]- labeled coding region of *AtST2a* as a probe.

**Quantificaton using NIH Image program**

The quantifications were performed using the NIH Image software, version 1.63 (RSB-NIMH-NIH http://rsb.info.nih.gov/nih-image/). When quantifying *AtST2a* signal intensity in Northern blot, we subtracted the background signal intensity from either *AtST2a* or *ACTIN* signal intensities.

The anti-AtST2a antibodies cross react with a ~43 kDa protein migrating above the predicted molecular weight of AtST2a. This cross reacting protein was found to be constitutively expressed in all samples tested so far and was used as a reference to quantify AtST2a accumulation. Therefore, when AtST2a signal intensity was quantified from Western blot, the background signal intensity was subtracted from the bands representing either AtST2a protein or the loading control.

Subsequently, the calculated intensities of AtST2a were divided by the corresponding intensities of *ACTIN* in Northern blot or the loading control in Western blot, which gave us the normalized intensities. The relative intensity values were obtained by dividing the intensity value of each of the treated sample by the intensity level of the untreated or control sample.

**Plasmid constructs for *AtST2a* and *AtST2b* transformation in *Arabidopsis thaliana***

The cassette of the pBI526 construct (Dalta *et al.*, 1993) was removed by digestion with *Hind*III *and Eco*RI, and re-ligated into the

corresponding sites of the binary vector pBI101 (Clontech). The resulting vector called pRD526 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 upstream of the *AtST2b* coding sequence using oligonucleotide 5'-CCTCTAGAGTGCCCCATTTCTACTCTTGT TCTGA-3'. A *Bam*H1 site also was introduced downstream the *AtST2b* coding sequence using oligonucleotide 5'-GCGGATCCGAGAAACTTGG GATCGCCATTGGAA-3'. The PCR product obtained using the above primers was digested with *Bam*HI, and ligated into the corresponding site of the pRD526 vector. The constructs were tested by PCR for the orientation of the inserted *AtST2b* sequence. The vectors which carried the *AtST2b* insertion in the correct orientation were called AtST2b sense strains. In such constructs, the translation initiation codon of *AtST2b* was 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 vector which carried the *AtST2b* sequence in the reverse orientation was called the antisense construct. The orientation of the *AtST2b* insert in the pRD526 vector was verified by sequencing using the AMV oligonucleotide 5'-AATTTTCTTTCAAATACTTCCACCATG-3' (binds to the region correspon-ding to the translational enhancer and located upstream of the multiple cloning sites) and the 3' and 5' *AtST2b* oligonucleotides.

### *Agrobacterium tumefaciens* transformation

The pRD526-*AtST2b* sense and antisense constructs, and the pBI 101 *AtST2a* promoter-*GUS* construct were transformed into *Agrobacterium tumefaciens* GV3101 pmp90 strain using the freeze-thaw method (An *et al.*, 1998).

### *Arabidopsis thaliana* transformation

*A. thaliana* plants of ecotype Columbia-0 were transformed with the *Agrobacterium* strain containing the *AtST2a* promoter-*GUS* fusion as well as *AtST2b* in the sense and antisense orientations by the vacuum infiltration method as described previously (Benchtold *et al.*, 1993).

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 medium 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 that were sensitive.

### 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 homogenized in extraction buffer (3% CTAB, 1.4 M NaCl, 52 mM β-mercaptoethanol, 20 mM EDTA and 100 mM 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 for 20 min with 95% ethanol, containing 10 mM ammonium acetate. 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* coding sequence was used as a probe. The blot was hybridized under stringent conditions according to standard procedures (Sambrook *et al.*, 2001).

**Detection of the AtST2a and AtST2b proteins in transgenic lines**

For the analysis of *AtST2a* and *AtST2b* expression in independent transgenic lines, 16 day old $T_2$ plants were pooled, ground in liquid nitrogen, and the powder was boiled in 2X SDS sample buffer to extract total protein. To confirm the integrity of the proteins and equal loading of each sample, protein extracts were also separated by 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 and AtST2b were immunodetected using anti-AtST2a polyclonal antibodies (dilution 1:1000)

and goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase (dilution 1:3000; BioRad).

**Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)**

Total RNA (2.5 μg) was treated with 20 U of DNase I (Roche Molecular Biochemicals) in 50 μl of 0.1 M sodium acetate, 5 mM $MgSO_4$, 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. The cDNA was synthesized using Moloney Murine Leukemia virus reverse transcriptase (New England Biolabs) in a 25 μl reaction volume as recommended by the manufacturer. RT reaction product (1μl) was used for PCR with Ex Taq DNA polymerase (TaKaRa Biochemicals).

**Screening for *coi1* mutant plants**

*Coi1* mutant plants were pulverized in liquid nitrogen and incubated in CTAB buffer (Tris (100mM), NaCl (1.4 M), EDTA (20 mM), CTAB (2% w/v), β-mercaptoethanol (0.2% v/v) at 65°C for 40 minutes with intermittent vortexing. The DNA was extracted with phenol/chloroform/isoamyl alcohol (50:48:2) and washed with ethanol. The DNA was used as a template to amplify *COI1* by PCR using the oligonucleotides 5'-GGTTCTCTTTAGTCTTTAC-3' and 5'-CAGACAACTATT TCGTTACC-3'. The resulting PCR product was digested with the *Xcm*1 restriction endonuclease. Since the mutation in the *COI1* gene in *coi1*

mutant plants results in loss of the *Xcm*1 site, the PCR product of *coi1* mutant samples could not be digested with the *Xcm*1 endonuclease and gave a 1.5 kbp fragment as opposed to the PCR product derived from wild type *Arabidopsis* samples which showed 2 bands of 1 kbp and 500 bp.

**Oligonucleotides used for amplifications in various PCR and RT-PCR experiments**

Oligonucleotides were designed using the OLIGO software (Molecular Biology Insights Inc.), and the ABI Prism Premier Express Software 2.0. The sequence of the primers used in different experiments are presented below.

**Primers for PCR analysis of *A. thaliana* samples**

- Primers used to amplify the *ACTIN* gene of *A. thaliana*

(Genebank Acc. No. U39449)

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

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

- Primers used to amplify *AtST2a* in RT-PCR experiments

(Genebank Acc. No. T43254)

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

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

- Primers used to amplify the *AtST2b* sense and antisense transcript from *A. thaliana* plants

(AMV) 5'- AATTTTCTTTCAAATACTTCCACCATG-3', AtST2b 3' *Bam*HI, and AtST2b 5' *Bam*HI.

- Primers used to select for homozygous *AtST2a* knockout plants

(salk_053325 LP) 5'-GAACAATTTGTGATTTCTCATGCCA-3'

(salk_053325 RP) 5'-CGGAAAGGAGAAGTGAGTGATTGG-3'

- Primers used to select for homozygous *AtST2b* knockout plants

(salk_009093 LP) 5'-CGGTGACATGATGTTGACATTGA-3'

(salk_009093 RP) 5'-TACTGATCGGATTTGGCCCGT-3'

- Primers used to select for *coi1* mutants

(Coi1_mRNA-5') 5'-GGTAAGTGGCTTCATGAGCTT-3'

(Coi1_mRNA-3') 5'-CGTTTGGACTGTACTGTCCGAT-3'

**Primers used for RT-PCR analysis of *N.tabaccum* samples**

- Primers used to amplify the *SQUAMOSA15* gene of *N.tabaccum*

(Genebank Acc. No. U63162)

(NtSqua15-5') 5'-CATACACAGAGAGACGTTTGCT-3'

(NtSqua15-3') 5'-GTTGTAGGAGGAAAGATGTTGAAGT-3'

- Primers used to amplify the *NtPLE36* gene of *N.tabaccum*

(Genebank Acc. No. U63163)

(NtPle36-5') 5'-GCTGATTCCACCAGTCAA-3'

(NtPle36-3') 5'-GCAGATTTACAGGCAGGA-3'

- Primers used to amplify the *NtSQUA4* gene of *N.tabaccum*

(Genebank Acc. No. U63160)

(NtSqua4-5') 5'- GTACTCATATGCTGAGAGGCAGCTT-3'

(NtSqua4-3') 5'-CTGATCCTTCAACTTCTCCGTTGT-3'

- Primers used to amplify the *NtDEF* gene of *N.tabaccum*

(Genebank Acc. No. X96428)

(NtDEF-5') 5'-CACGACCAAGCAGTTGTTCGATCT-3'

(NTDEF-3') 5'-GAAGATGATGATTTGGCTGTTGTTT-3'

- Primers used to amplify the *NAG* gene of *N.tabaccum*

(Genebank Acc. No. Q43585)

(NAG-5') 5'-GGTGAAAGCAACAATTGAGAGGTA-3'

(NAG-3') 5'-GGTTGTTGGTTTGCAAACCAT-3'

- Primers used to amplify the *NtGLO* gene of *N.tabaccum*

(Genebank Acc. No. X67959)

(NtGLO-5') 5'-CTTCGTTGGTTGATATTTTGGATCA-3'

(NtGLO-3') 5'-CTGAACTCGGAAGGCAAAAGG-3'

- Primers used to amplify the *NtS6* gene of *N.tabaccum*

(Genebank Acc. No. X68050)

(NtS6-5') 5'-CCGCGGCACTCCTTGTTT-3'

(NtS6-3') 5'-GGGTGTCACAAGCCTCTGATCT-3'

**Plant growth conditions**

*A. thaliana* plants were grown in soil in a growth chamber with 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 then were 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 then were spread on agar-solidified medium containing Murashige and Skoog salts, B5 vitamins, 1% sucrose, 0.5 g/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

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

The *AtST2a* gene is located on chromosome V and can be retrieved from the Genbank database under the accession number AB010697 from nucleotides 53936 to 55015 of clone MOJ9.

The *AtST2a* open reading frame codes for a protein of 359 amino acids, which corresponds to a molecular mass of 41.3 kDa. The AtST2a protein sequence contains all the amino acids characteristic of the PAPS binding site and the catalytic site (Fig. 3.1) (Marsolais, 2000). AtST2a is 85% identical and 92% similar to AtST2b at the amino acid level. The two sequences are located 2 287 bp apart on chromosome V (Fig. 3.2). The results of biochemical studies showed that AtST2a exhibits preference for 12-hydroxyjasmonate (12-OHJA), over 11-hydroxyjasmonate (11-OHJA) with $K_m$ values of 10 μM and 50 μM, respectively (Gidda, 2001).

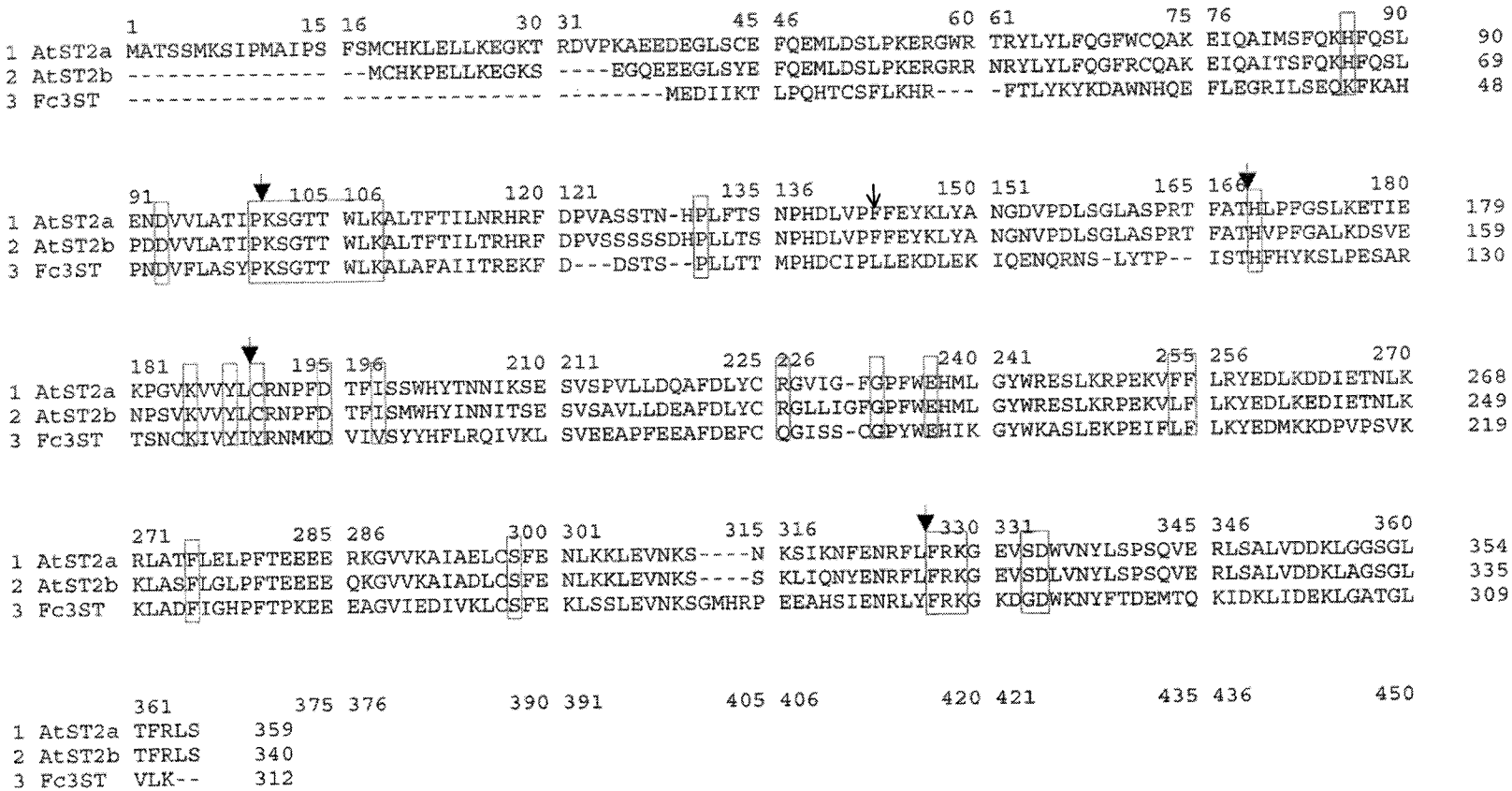



**Figure 3.1 Amino acid sequence alignment of AtST2a, AtST2b, and the flavonol 3-ST from *Flaveria chloraefoliata* (accession number M84135).** Deduced amino acid sequences were aligned with the CLUSTALW 1.8 program. Black arrows indicate amino acid residues that are involved in catalysis in the flavonol 3-ST. The open arrow identifies the amino acid involved in determining the substrate specificity of the flavonol 3-ST (Marsolais *et al.*, 2000).

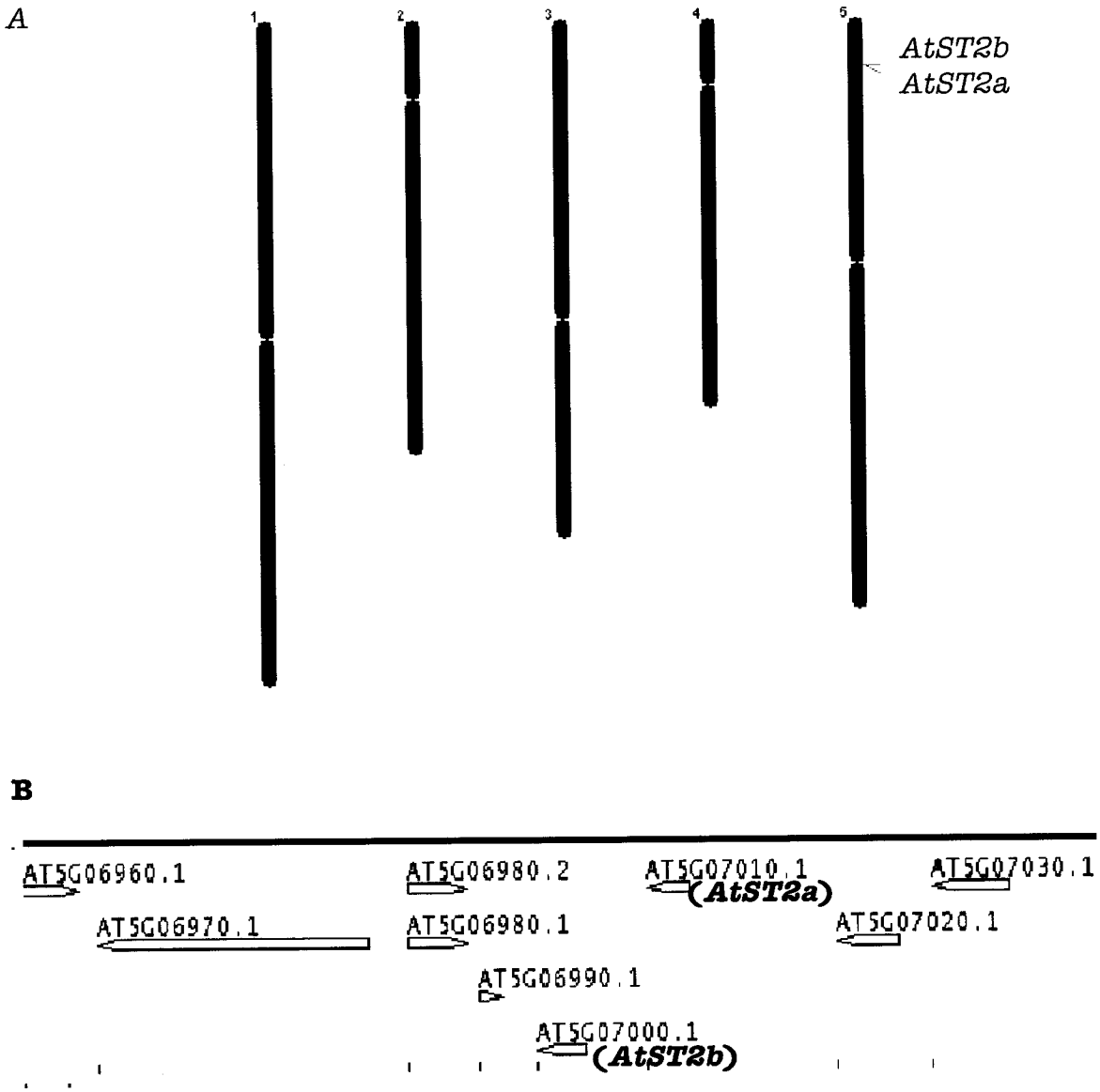



**Figure 3.2 Location of the *AtST2a* and *AtST2b* genes on chromosome V of *Arabidopsis thaliana*.**

(A) Graphical representation of *A.thaliana's* chromosomes

(B) Enlarged view of a segment of chromosome V containing *AtST2a* and *AtST2b*

### *AtST2a* transgenic plants

In order to determine the biological function of AtST2a and 12-OHJA, we constructed transgenic *Arabidopsis thaliana* plants with altered levels of the AtST2a protein (Gidda, 2001; Gidda *et al.*, 2003). Transgenic plants overexpressing *AtST2a* grown under long day conditions flowered later than wild type plants grown under the same conditions (Fig. 3.3); however, flowering time under short days was not altered. The results suggested that elevated levels of AtST2a cause a delay in the initiation of flowering. Since the biochemical function of AtST2a is to modify 12-OHJA by sulfonation, the delay in flowering can be attributed to either the reduced level of 12-OHJA or the increased level of 12-hydroxysulfonyloxy jasmonic acid (12-$HSO_4$-JA), which might act as a flowering inhibitor.

Transgenic plants overexpressing *AtST2a* in the antisense orientation also exhibited alteration in the time of flower initiation. These plants flowered earlier than their wild type counterparts. However, the early flowering phenotype was only observed under the short day (SD) conditions (Gidda, 2001).

The alteration of floral evocation time observed in transgenic plants showed dependence on light growth conditions suggesting that *AtST2a* is regulated in response to photoperiod.

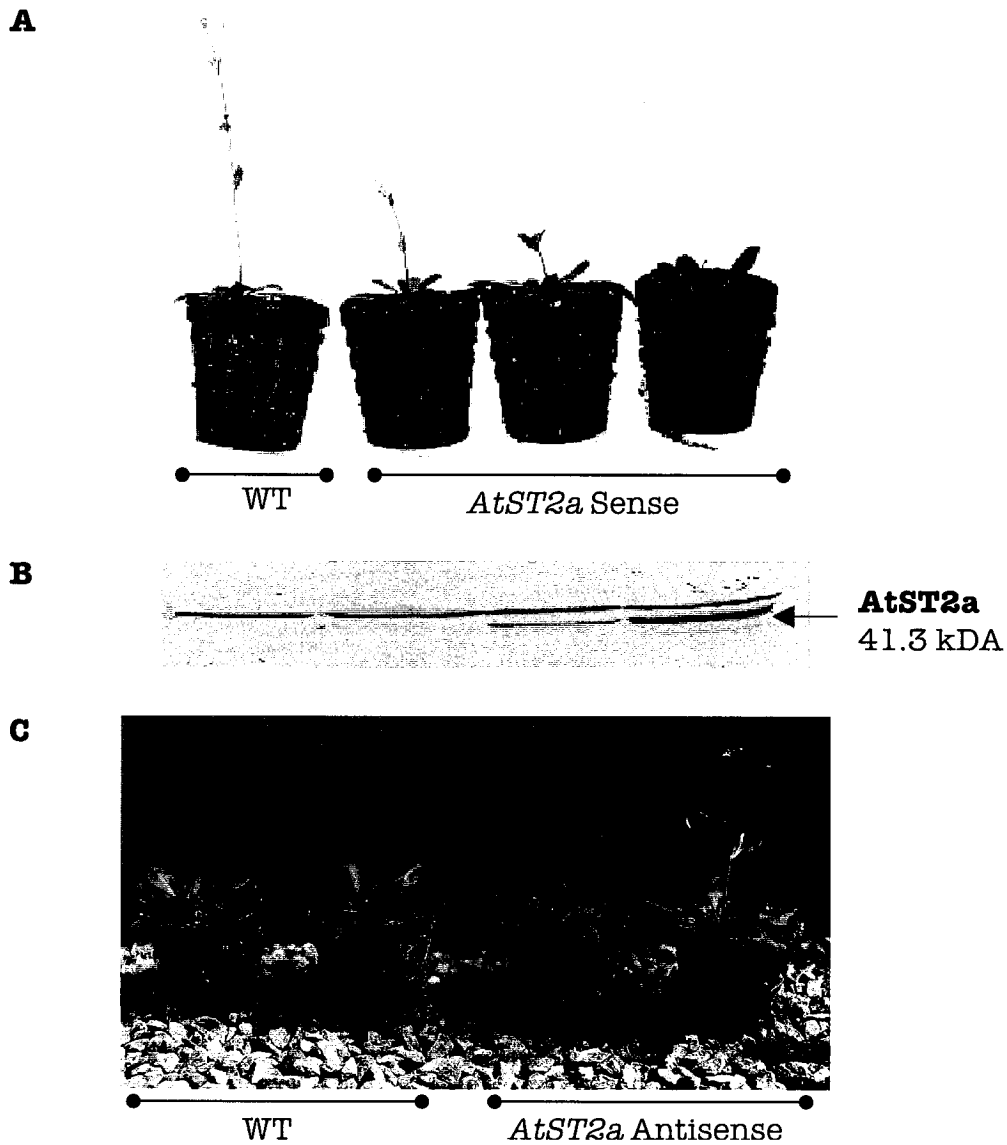



**Figure 3.3 *Arabidopsis thaliana AtST2a* transgenic plants and the initiation of flowering**

(A) Transgenic plants overexpressing *AtST2a* in the sense orientation grown under long day conditions; (B) Western blot analysis of the transgenic plants shown in (A); (C) Transgenic plants overexpressing *AtST2a* in the antisense orientation grown under short day conditions.

**Regulation of *AtST2a* expression in response to light**

In view of the photoperiod-dependent flowering initiation of the transgenic plants, we analyzed *AtST2a* expression in response to light. Analysis of various RNA samples by Northern blots using *AtST2a* as a probe revealed no detectable *AtST2a* transcript in samples from plants grown under light in long days (16 hours of light and 8 hours of dark) (Fig. 3.4). However, *AtST2a* mRNA could be detected in plants grown in the dark for more than 8 hours. In samples originating from plants grown in the dark for 48 hours and subsequently placed under the light, the *AtST2a* mRNA transcript disappeared after 4 hours (Fig. 3.4) (Gidda, 2001). However, at the protein level AtST2a never completely disappeared and a low signal was present even after 24 hours of light exposure (Fig. 3.5). The presence of basal level of AtST2a protein in long day grown plants might result from its inherent stability or *de novo* synthesis. Taken together, the results suggest that AtST2a accumulates to a significant level only under short day conditions, where the dark period is more than 8 hours.

Western blot analysis of plants grown for different numbers of days under long day conditions (16 hours of light) or dark treated for 24 hours shows that AtST2a protein levels do not change with age (Fig. 3.6). These results suggest that *AtST2a* expression is independent of the stage of development of the plant.

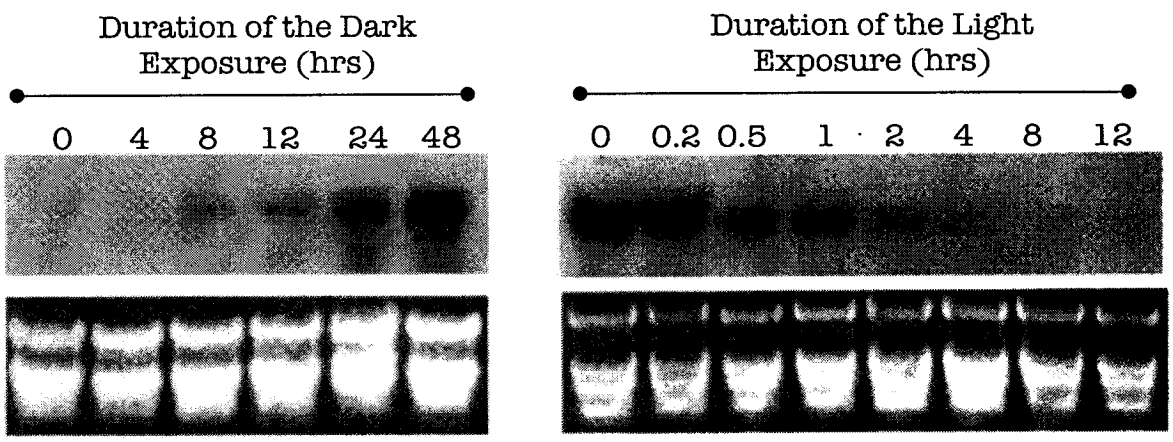



**Figure 3.4 Light regulation of *AtST2a* expression.**

(A) Northern blot of total RNA isolated from 15 day old *Arabidopsis* plants kept in the dark for the indicated time periods. (B) *Arabidopsis* plants kept in the dark for 48 hours were brought back into the light and total RNA extracted at the indicated time intervals. Total RNA samples from rosette leaves (10 μg) were denatured with glyoxal, resolved on a 1% agarose gel, transferred to a Zeta-probe nylon membrane and hybridized with an *AtST2a* probe. The lower panels show the amount of RNA loaded as revealed by ethidium bromide staining of the rRNA bands.

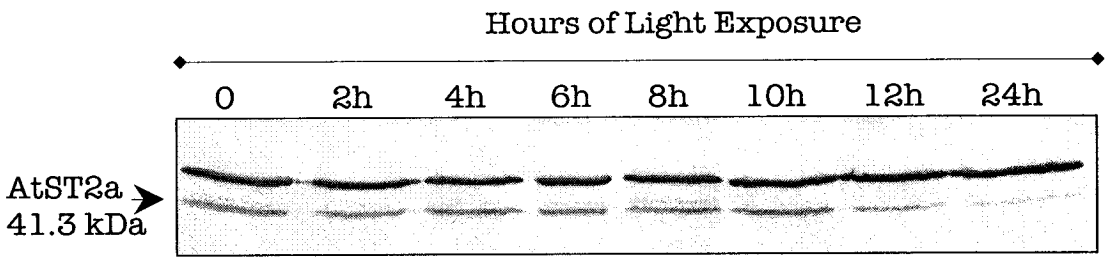



**Figure 3.5 Western blot analysis of AtST2a protein levels from 24 day old *Arabidopsis thaliana* plants following a 48 hour dark treatment.**

*Arabidopsis* plants kept in the dark for 48 hours were brought back to the light and the proteins were extracted at the indicated time intervals. Proteins were extracted by grinding the plants and boiling the extract in sample buffer. Samples were separated on 12% SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with anti-AtST2a specific antibodies.

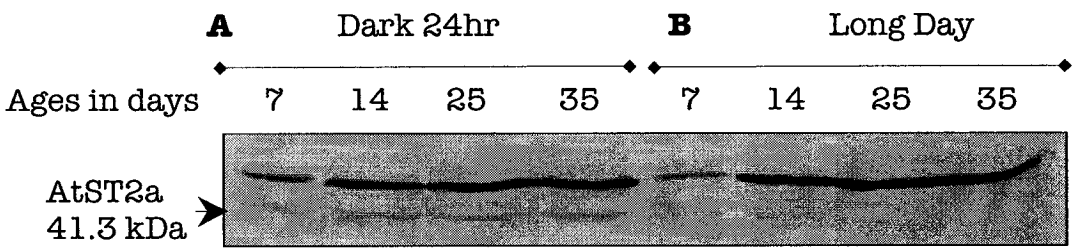



**Figure 3.6 Western blot analysis of AtST2a protein levels in *Arabidopsis thaliana* plants of different ages.**

*Arabidopsis* plants were grown under long day conditions (16 hours of light). (A) Samples from plants treated for 24 hours in the dark. (B) Samples from plants grown under 16 hours light/ 8 hours dark conditions collected at the indicated days after germination. All the samples were collected at the same time of the day for consistency.

**Phytochromes and *AtST2a* expression**

Plants monitor changes in the ambient light environment using different sensory photoreceptors such as: the phototropins, the cryptochromes and the phytochromes. Light-induced signal transduction starts with the perception of light by these photoreceptors, which in turn regulate light-modulated gene expression (Gyula *et al.*, 2003). The effect of light on *AtST2a* expression raised a question as to the involvement of photoreceptors in its expression. To test this hypothesis, we analyzed the pattern of *AtST2a* expression in photoreceptor mutants, such as *phyA*, *phyB*, and *phyAB* phytochrome mutants and in the cryptochrome mutant (*cry2*). Northern and Western blot analyses of *phyA*, *phyB* and *phyAB* mutants showed that *AtST2a* expression and AtST2a accumulation is upregulated in the dark in all three mutants (Fig. 3.7), as compared to wild type plants (Landsberg *erecta* ecotype). However, under long day conditions, *AtST2a* was strongly expressed only in *phyB* and *phyAB* double mutants. These results suggest that PHYA and PHYB downregulate *AtST2a* expression in the dark, but under long day conditions only PHYB seems to repress *AtST2a* expression. Interestingly, only *phyB* and *phyAB* mutants exhibit an early flowering phenotype. However, transgenic plants overexpressing *PHYB* also flower early indicating a more complex mechanism for phytochrome B participation in the regulation of flowering time (Bagnall *et al.*, 1995).

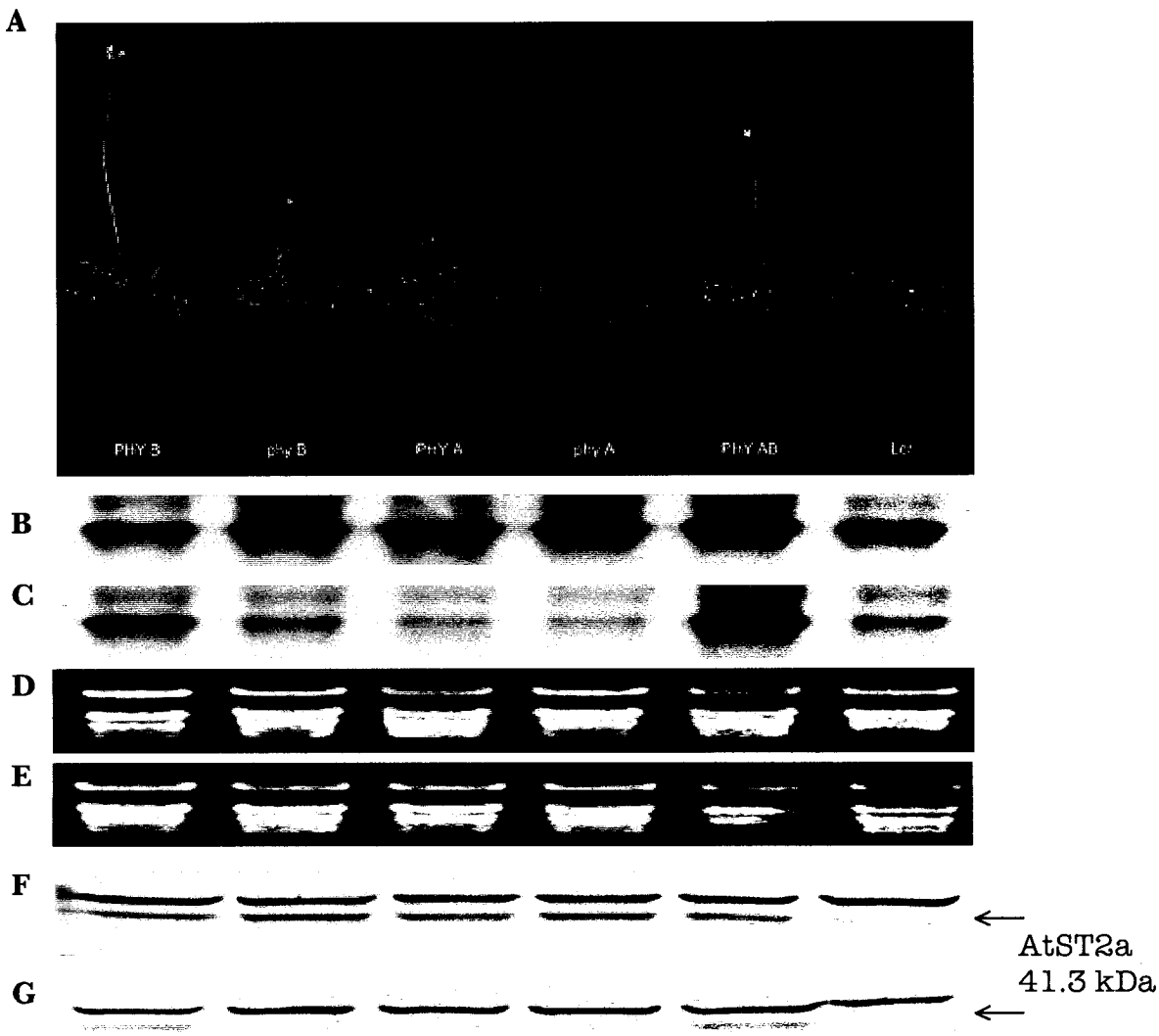



**Figure 3.7 Correlation of phytochrome mutant flowering time with *AtST2a* expression in *Arabidopsis thaliana*.**

The legend is presented on page 70, and the signal intensities are quantified in table 3.1.

**Figure 3.7 Correlation between flowering time of *Arabidopsis thaliana* phytochrome mutants and *AtST2a* expression profile.**

A) Comparison of 20 day old phytochrome mutants and wild type *Arabidopsis* plants grown under long days (16 hours of light). *PhyB*, *PhyA* and *PhyAB* represent a phytochrome B mutant, a phytochrome A mutant and a phytochrome AB double mutant. *PHYB*, *PHYA*, *PHYAB* represents strong mutant alleles of the respective phytochromes, *phyB* and *phyA* represent weak mutant alleles.

B and C) Northern blot analyses of RNA samples from plants shown in A and grown for 24 hours in the dark (B) or grown under long day conditions (C).

D and E) The amount of RNA loaded for Northern blot analysis as revealed by ethidium bromide staining of the rRNA bands.

F and G) Western blot analyses of protein extracts from the plants shown in A and grown for 24 hours in the dark (F) or grown in the light (G).

**Table 3.1**

**Quantification of Northern Blot and Western Blot signal intensities of Figure 3.7**

| Samples* | Northern Blot (Fold Increase) | Western Blot (Fold Increase) |
|---|---|---|
| *PHYB* Dark | 2.5 | 6.3 |
| *phyB* Dark | 7.2 | 9.5 |
| *PHYA* Dark | 6.5 | 5.4 |
| *phyA* dark | 6.8 | 10.4 |
| *PHYAB* Dark | 6.4 | 4.6 |
| Wild Type Dark | 1.0 | 1.0 |
| *PHYB* Light | 7.2 | 3.3 |
| *phyB* Light | 2.1 | 1.4 |
| *PHYA* Light | 1.1 | 1.2 |
| *phyA* Light | 1.0 | 1.3 |
| *PHYAB* Light | 12.5 | 3.1 |
| Wild Type Light | 1.0 | 1.0 |

* ecotype Landsberg *erecta*

The signal intensities of Figure 3.7 were quantified using the NIH Image program (version 1.63). The quantification was done according to the protocol described in the Materials and Methods section.

The cryptochrome mutant (*cry2*) showed no apparent change in *AtST2a* expression (data not shown) suggesting that *ATST2a* expression is regulated by red/far-red light through the action of phytochromes A and B and not by blue light through the action of CRY2.

**Analysis of various mutants in relation to the initiation of flowering and pattern of *AtST2a* expression.**

Assuming that 12-OHJA and AtST2a are implicated in the regulation of floral initiation as seen from the analysis of *AtST2a* sense and antisense transgenic plants, AtST2a protein levels should correlate with the onset of flowering (*i.e.* elevated AtST2a levels should be observed in late flowering mutants and *vice versa*). We tested this hypothesis in various photoperiod dependent flowering time mutants.

**Analysis of *AtST2a* expression in *Arabidopsis thaliana* mutants in the photoperiod dependent promotion pathway of flowering.**

In view of *AtST2a* regulation by light and its participation in flower initiation, AtST2a protein levels have been analyzed in various photoperiod dependent flowering time mutants such as *constans*, *terminal flower 2*, *early flowering 3*, *gigantea*, and *flowering locus T*.

The *A. thaliana* gene *CONSTANS* encodes a putative transcription factor that promotes flowering in response to LD conditions. The onset of

flowering is promoted by long photoperiods, and the *constans (co)* mutant flowers later than wild type under these conditions (Putterill *et al.*, 1995). We tested the impact of a *CONSTANS* loss of function mutation on the accumulation of the AtST2a protein. RT-PCR and Western blot analyses of *co* mutants revealed constitutive expression of *AtST2a* under light and dark growth conditions (Fig. 3.8). As expected *co* plants were found to flower 16±1 days later than the wild type plants (Fig. 3.8 and Table 3.2). These results suggest that in wild type plants, *CONSTANS* represses *AtST2a* expression in the light.

We also examined the expression of *AtST2a* in the *terminal flower 2* mutant *(tfl2)*, a repressor of euchromatic genes involved in meristem and floral identity (*FLOWERING LOCUS T, PISTILLATA, APETALA 3, AGAMOUS* and *SEPALLATA 3*). The *TFL2* gene encodes a protein with homology to heterochromatin protein 1 (HP1) of animals and Swi6 of fission yeast (Kotake *et al.*, 2003). RT-PCR and Western blot analyses results showed that the *tfl2* mutant does not express *AtST2a* following dark or JA treatments as opposed to wild type plants (Fig. 3.8 and Table 3.2). The *tfl2* mutant plants flowered 8±1.5 days earlier than the wild type plants under long day condition. These results suggest that in wild type *Arabidopsis* plants TFL2 upregulates *AtST2a* expression.

In contrast, *AtST2a* gene expression was not altered in the meristem identity *flowering locus T (ft)* mutant (data not shown). FT is located downstream from TFL2 in the photoperiod dependent pathway.

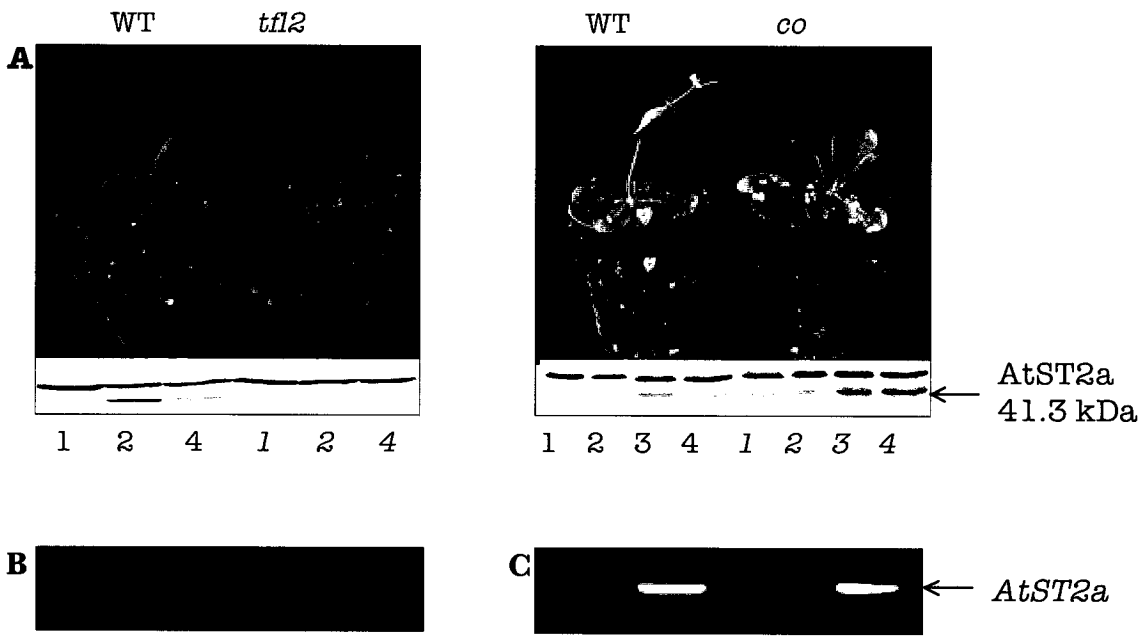



**Figure 3.8 Correlation between flowering time and the AtST2a protein level in the photoperiod promotion pathway mutants.**

(A) Wild type (WT) and two mutant plants grown under long days (16 hours light). The lower panels represent Western blot analysis of the plant samples (1: untreated, 2: 12OHJA 100 μM 24 hrs, 3: MeJA 100 μM 24 hrs, 4: Dark 24 hrs)

B and C) RT-PCR of *AtST2a* transcript in total RNA samples from 14 day old *tfl2* and wild type plants (L*er*) shown in B and 14 day old *co* and wild type plants (Col-0) shown in C grown under LD and collected after 8 hours of light or after 24 hour dark treatment.

**Table 3.2**

**Quantification of Western blot signal intensities of Figure 3.8**

| Sample | Relative Intensity | Fold Increase |
|---|---|---|
| WT* Control | 12.9 | 1.0 |
| WT 12-OHJA | 42.8 | 3.5 |
| WT Dark | 23.6 | 1.9 |
| *tfl2* Control | 12.8 | 1.0 |
| *tfl2* 12-OHJA | 12.3 | 1.0 |
| *tfl2* Dark | 12.5 | 1.0 |
| | | |
| WT** Control | 11.7 | 1.0 |
| WT 12-OHJA | 14.4 | 1.3 |
| WT MeJA | 32.7 | 2.8 |
| WT Dark | 21.9 | 1.9 |
| *co* Control | 29.2 | 2.5 |
| *co* 12-OHJA | 24.7 | 2.1 |
| *co* MeJA | 42.9 | 3.7 |
| *co* Dark | 55.9 | 4.8 |

* ecotype Columbia-0

** ecotype Landsberg *erecta*

The signal intensities of Figure 3.8 were quantified using the NIH Image program (version 1.63). The quantification was done according to the protocol described in the Materials and Methods section.

In addition, *FT* is a target gene of CONSTANS, and is expressed at a similar time of day as *CONSTANS* (Suarez-Lopez *et al.*, 2001). Analysis of the early flowering mutants *gigantea (gi)* which encodes a nuclear protein involved in phytochrome signalling, *early flowering (elf-3)* which encodes a mediator protein between the light input and the circadian clock, as well as the *cry2* mutant, which encodes a photoreceptor cryptochrome, showed no apparent change in *AtST2a* expression (data not shown).

The analysis of flowering time mutants demonstrated a strong correlation between the level of expression of *AtST2a* and flowering time (*i.e.*, *AtST2a* levels were directly proportional to the delay in flowering). Similar effects were observed in *AtST2a* sense and antisense transgenic lines. Plants overexpressing *AtST2a* in the sense orientation flowered late under long day conditions, whereas antisense plants flowered earlier than the wild type when grown under short day conditions. The level of *AtST2a* expression and the time required for floral evocation are correlated suggesting that 12-OHJA plays a role in the control of flowering time.

These results also suggested that AtST2a acts in the photoperiod dependent flower induction pathway downstream of CO. In view of the involvement of TFL2 in *AtST2a* regulation, we propose the existence of an additional branch in the photoperiod dependent pathway where TFL2 upregulates *AtST2a*, and CONSTANS downregulates *AtST2a* expression. TFL2 and CONSTANS are known to act on *FLOWERING LOCUS T*, a floral pathway integrator found to regulate the switch of the apical meristem

from vegetative to reproductive state (Araki, 2001) suggesting a possible participation of AtST2a and 12-OHJA on *FT* expression. It also is possible that AtST2a and 12-OHJA participate in floral evocation via an additional pathway independent from FT.

**AtST2a regulation by 12-OHJA**

*AtST2a* expression was found to be upregulated by its substrate through a feed-forward mechanism. *AtST2a* expression was induced in *Arabidopsis* plants treated with 12-OHJA or MeJA (volatile derivative of JA) at concentrations as low as 10 μM. In addition, the accumulation of *AtST2a* mRNA was observed in plants treated with 100 μM 12-OHJA or MeJA 30 minutes following the treatment and reaching a maximum of expression after approximately 2 hours (Gidda, 2001) (Fig. 3.9). To show that a functional JA response is required for the light/dark control of *AtST2a* expression, we compared AtST2a accumulation in wild type and JA perception mutants *cet1*, *coi1*, and *cev1*, as well as JA biosynthesis mutant *opr3*.

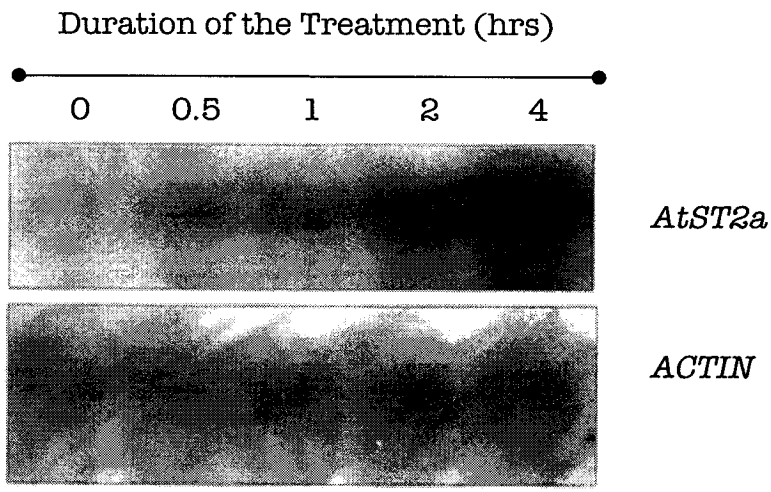



**Figure 3.9 Kinetic of *AtST2a* mRNA accumulation during treatment of 15 days old *Arabidopsis thaliana* plants with 100 μM 12-OHJA.**

(Adapted from Gidda *et al.*, 2003).

The plants were germinated on MS medium in magenta boxes and total RNA was extracted at the indicated time period after treatment. Total RNA (10μg) was resolved on 1% agarose gel, transferred to a nylon membrane and hybridized with $^{32}P$ labeled *AtST2a* cDNA as probe. The same nylon membrane was probed with *ACTIN* cDNA to confirm equal RNA loading.

**Analysis of *AtST2a* expression in *Arabidopsis thaliana* jasmonate response and biosynthetic mutants.**

The *cet1* (Constitutive Expression of Thionin) mutant expresses consitutively the JA defense response pathway (Nibbe *et al.*, 2002). Flowering time and the pattern of *AtST2a* expression are not altered in a *cet1* mutant (Fig. 3.10). The wild type expression pattern of *AtST2a* observed in the *cet1* mutant suggests that AtST2a is not part of the JA defense response pathway. In addition, there is experimental evidence for the existence of a MeJA response pathway that is distinct from the 12-OHJA response pathway. For instance, it was shown that the expression of *Thi2.1,* a defense response gene, induced by MeJA, does not respond to exogenous application of 12-OHJA (data not shown).

The *coronatine-insensitive 1 Arabidopsis* mutant (*coi1)* has a defect in the wound-inducible JA response pathway (coronatine is a structural analogue of jasmonic acid). Using a microarray analysis of RNA samples from *coi1* and wild type plants, it became possible to sort the JA indicible genes into *COI1* -dependent and *COI1* -independent classes (Reymond *et al.*, 2000).

Northern and Western blot analyses showed that *AtST2a* is not expressed in *coi1* mutant plants (Fig. 3.10). In addition, *AtST2a* expression could not be induced following the application of jasmonates or by dark treatment in this mutant (Fig. 3.11). These results indicate that *AtST2a* is a COI1-dependent gene. *Coi1* mutants flowered 7±1 days earlier

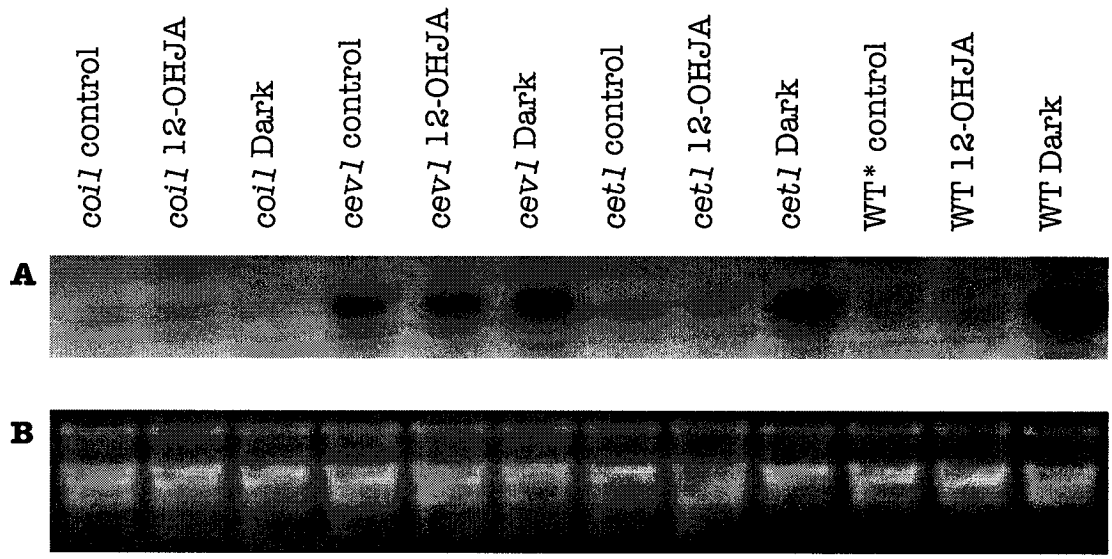
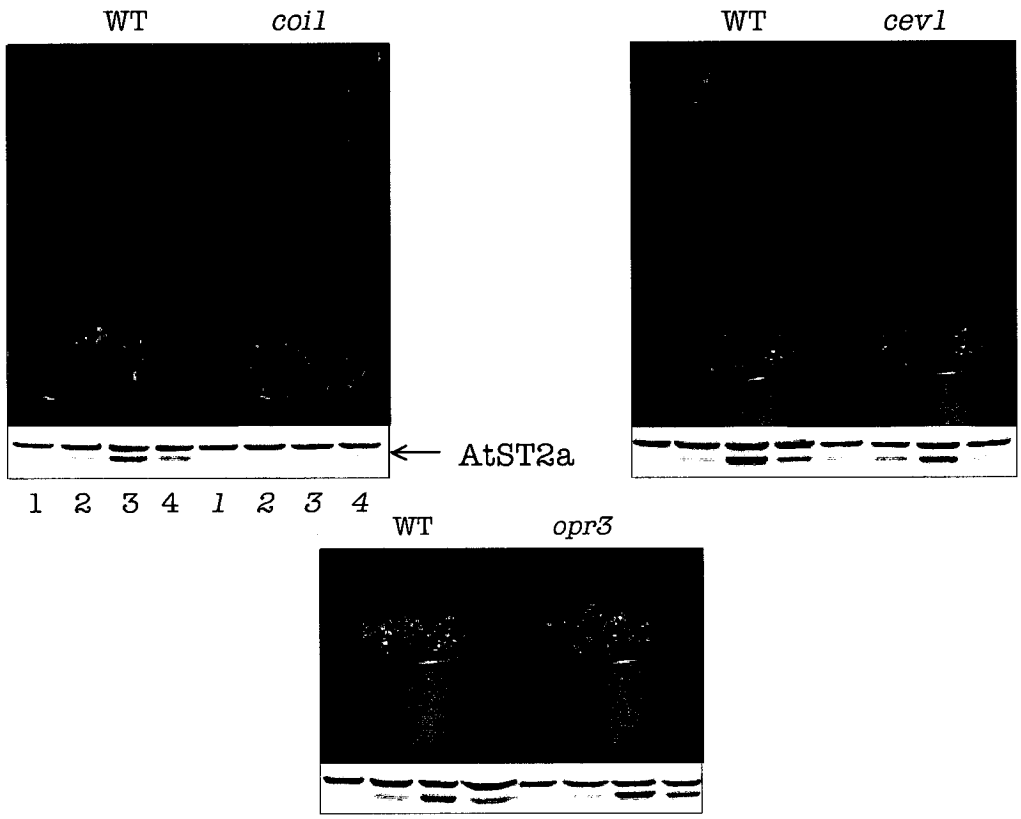



**Figure 3.10 RNA gel blot analysis of *AtST2a* expression in various *Arabidopsis thaliana* mutants**

* ecotype Columbia-0

(A) 14 day old plants were treated with 100 μM 12-OHJA (12-OHJA) for 24 hours or kept in the dark for the same amount of time. Total RNA (10μg) was hybridized with $^{35}P$ labeled *AtST2a* cDNA as a probe. (B) Ethidium bromide staining was used to confirm equal loading.




| Sample | Relative Intensity | Fold Increase | Sample | Relative Intensity | Fold Increase |
|---|---|---|---|---|---|
| WT Control | 41.1 | 1.1 | *cev1* Control | 115 | 3.0 |
| WT 12-OHJA | 76.2 | 2.0 | *cev1* 12-OHJA | 125 | 3.3 |
| WT MeJA | 152.3 | 4.0 | *cev 1* MeJA | 150.4 | 3.9 |
| WT Dark | 114.3 | 3.0 | *cev1* Dark | 110.3 | 2.9 |
| *coi1* Control | 41.7 | 1.1 | *opr3* Control | 40.9 | 1.1 |
| *coi1* 12-OHJA | 38.1 | 1.0 | *opr3* 12-OHJA | 70.7 | 1.9 |
| *coi1* MeJA | 38.9 | 1.0 | *opr3* MeJA | 153.1 | 4.0 |
| *coi1* Dark | 38.6 | 1.0 | *opr3* Dark | 119.3 | 3.1 |

**Figure 3.11 Correlation between flowering time and AtST2a protein level in the JA perception or biosynthesis mutants.**

Wild type (WT) and various mutant plants were grown under long days (16 hours light). The lower panels represent western blot analysis of the plant samples (1: untreated, 2: 12-OHJA 100 μM 24 hrs, 3: MeJA 100 μM 24 hrs, 4: Dark 24 hrs)

than wild type plants (Col-0 ecotype) when grown either under long day or short day conditions (Fig. 3.11). Again, a strict correlation was observed between flowering time and *AtST2a* expression.

The *cev1* mutant was shown to accumulate high endogenous levels of JA and OPDA and to express constitutively JA- and OPDA-responsive genes such as *VSP* and *Thi2.1* (Ellis and Turner, 2001). *Cev1* plants flowered 6±1.5 days later (Fig. 3.11) than wild type *Arabidopsis* plants (Col-0 ecotype). Northern and Western blot analyses revealed that AtST2awas expressed at elevated levels under both inductive and non-inductive conditions (Fig. 3.10 and 3.11).

Mutants homozygous for the disrupted *OPR3* gene of the JA biosynthetic pathway flowered at the same time as the wild type plants (Wassilewskaja ecotype) (Fig. 3.11). Northern and Western blots (Fig. 3.10 and 3.11) showed that *AtST2a* expression in this mutant is similar to that observed in wild type plants. GC/MS quantification analysis of jasmonates in *opr3* mutant plants showed trace amounts of jasmonic acid, but normal endogenous levels of 12-OHJA (C. Wasternack, personal communication). The trace amount of JA is consistent with the disruption of the JA biosynthetic pathway. However, it does not explain the high level of 12-OHJA since the later is proposed to be derived from JA by hydroxylation on carbon 12. The normal 12-OHJA levels suggest that there is an additional pathway for 12-OHJA biosynthesis in *Arabidopsis thaliana* (Fig. 3.12).

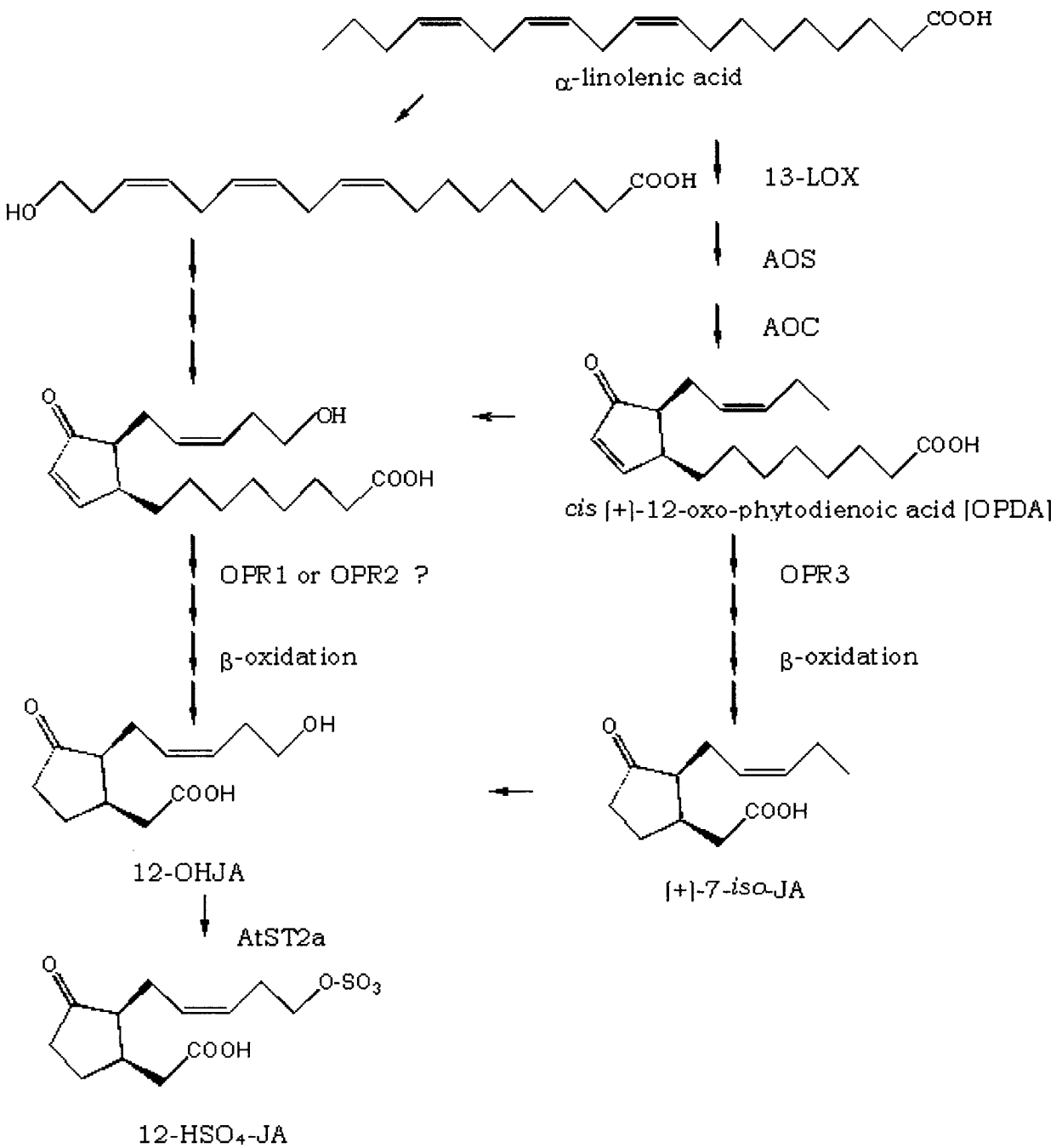



**Figure 3.12 Hypothetical additional pathways for 12-OHJA biosynthesis**

**Histochemical Analysis of *AtST2a* Promoter Activity**

In view of the involvement of AtST2a and 12-OHJA in the initiation of flowering, we were interested in identifying the tissue(s) in which the *AtST2a* promoter is active. To study the localization of *AtST2a* expression, wild type *Arabidopsis* plants (Col-0) were transformed with a plasmid construct containing the bacterial *β-glucuronidase* gene (commonly referred to as the *GUS* reporter gene) under the control of the *AtST2a* promoter (4 kb intergenic region upstream from the start codon of *AtST2a*).

Histochemical analysis of transgenic lines homozygous for a single insertion of the *AtST2a* promoter-*GUS* fusion revealed staining in the cotyledons, roots and the hypocotyls of young seedlings (2 to 14 day old plants) (Fig. 3.13). A uniform staining of the young plantlets was observed as compared to the localized staining pattern of more mature plants (Fig. 3.14). Older plants showed staining at the floral primordia of the reproductive apical meristem. Staining also was localized in different flower organs such as the style and the anther, silique and floral abscission zones. *AtST2a* promoter activity also was observed at the base of trichomes on the adaxial surface of mature leaves, at the tips of emerging leaves and at the root tips as well as at lateral root primordia suggesting a role for AtST2a and 12-OHJA in plant developmental processes other than flowering.

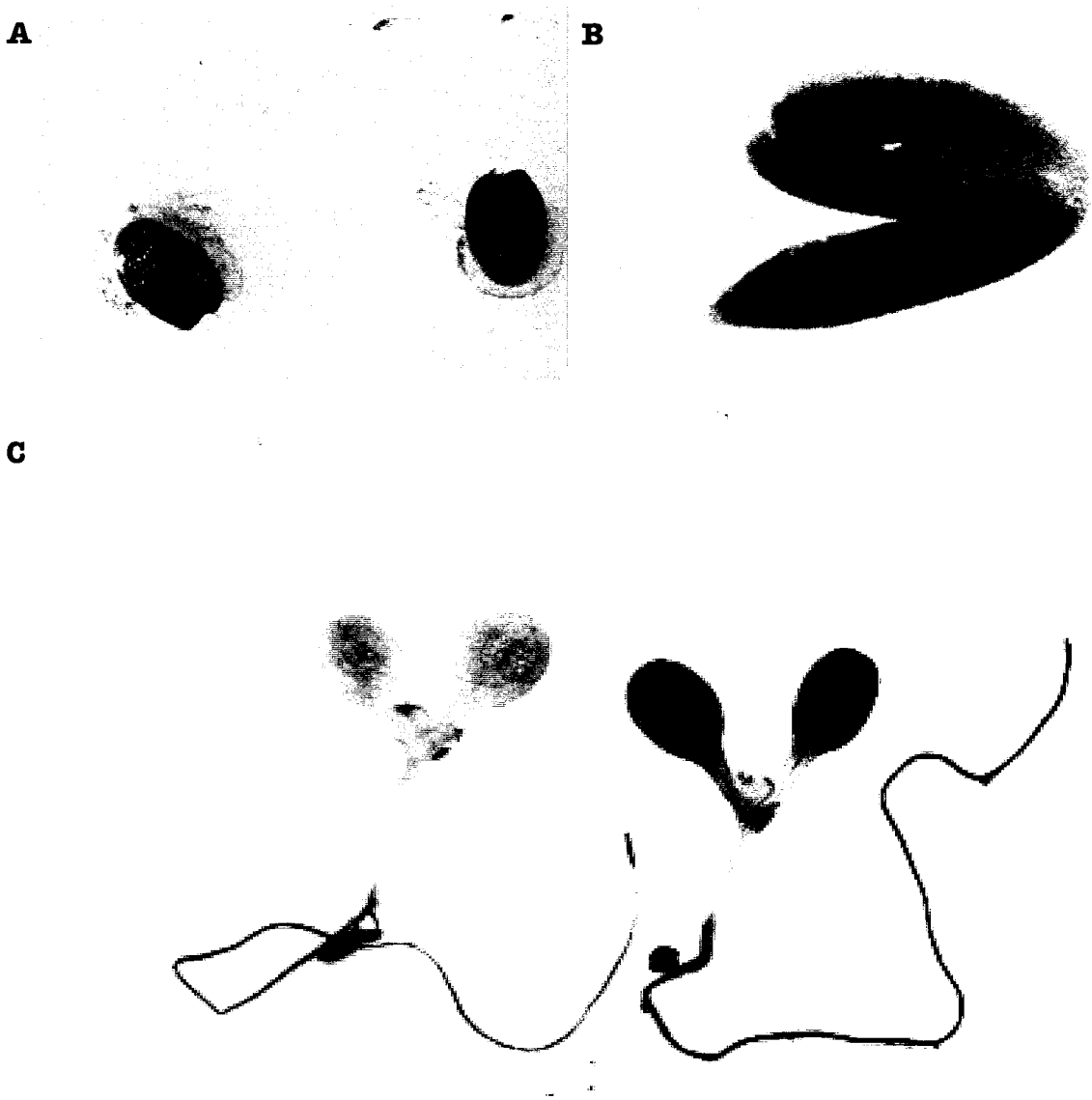



**Figure 3.13 Histochemical analysis of *AtST2a* promoter activity in young seedlings.**

$T_3$ generation of transgenic plants homozygous for a single insertion of *AtST2a* promoter - *GUS* fusion.

A) Seeds. B) Dark treated 2 day-old seedling; C) Long-day grown (left) and 24 hours dark treated (right) 12 day old seedlings.

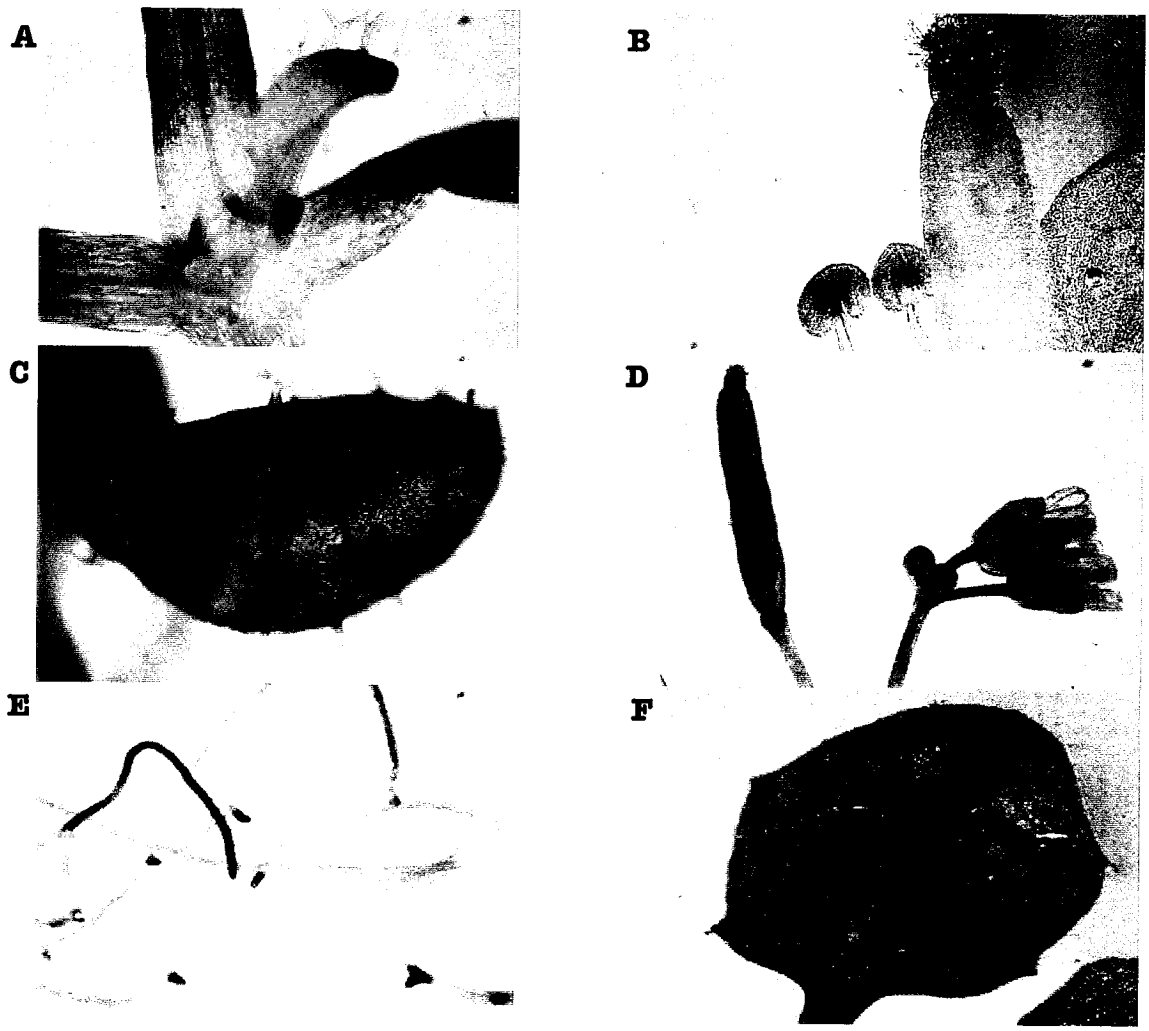



**Figure 3.14 Histochemical analysis of *AtST2a* promoter activity in mature plants.**

$T_3$ generation of transgenic plants homozygous for a single insertion of the *AtST2a* promoter - *GUS* fusion. A) Apical meristem of 26 day old light grown plant; B) Close up of the anther and carpel of a light grown mature flower; C) Trichomes on the leaf of a 14 day-old dark treated (24 hours) seedling; D) Silique and flower staining of a 30 day old mature plant; E) Root system of 26 day old light grown plant; F) Young leaf from a light grown plant.

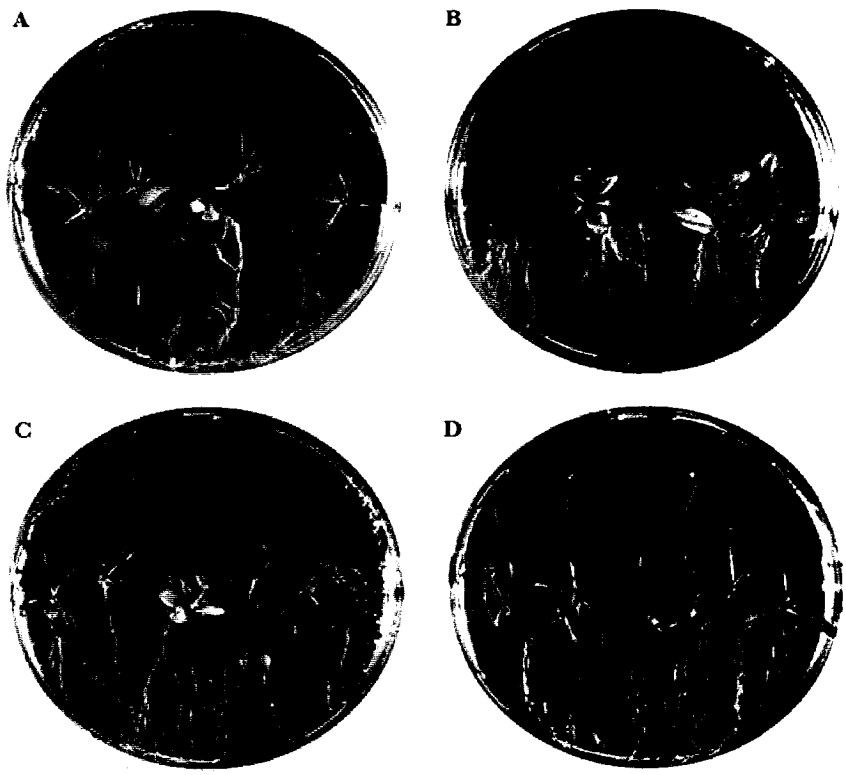
It is worth noticing that the staining was observed in light grown plants (confirming the Western blot data outlined in Fig. 3.5). However, dark exposure or jasmonate treatment (data not shown) visibly enhanced the promoter activity confirming the results of *AtST2a* regulation by photoperiod as well as by jasmonate treatment. These results correlate with the results of Western blot analyse of plants of different ages (Fig. 3.6) where AtST2a protein was present at all the developmental stages of *Arabidopsis.*

*AtST2a* promoter activity also was observed in mature seeds but not in young seeds suggesting a possible role of AtST2a and 12-OHJA in seed germination or seed dormancy.

**Effect of exogenously applied 12-OHJA, 12-$HSO_4$-JA and MeJA on plant development**

We analyzed the effect of 12-OHJA on plant development as compared to MeJA. In the same experiment, we investigated how sulfonation affects 12-OHJA activity.

Addition of 20 μM 12-OHJA or MeJA to the germination medium interfered with normal plant growth and development (Fig. 3.15). Although, wild type plants grown on 12-OHJA flowered at the same time as plants grown on medium without any addition, the 12-OHJA grown plants




E

| | TREATMENT | Root Length (cm) ±SD | Stem Length (cm) ±SD | Total Leaf Number ±SD | Flowering Time ±SD |
|---|---|---|---|---|---|
| A | Control | 12.9±3.9 (n=22) | 3.5±0.5 (n=5) | 7.6±1 (n=5) | 19.4±1.5(n=5) |
| B | MeJA | 4.3±1.5 (n=29) | 1.1±1 (n=5) | 6.5±1.3(n=5) | 23.2±1.8(n=5) |
| C | 12-OHJA | 10.2±3.4 (n=21) | 1.1±0.6 (n=6) | 7.8±1(n=6) | 19±0.6(n=6) |
| D | 12-$HSO_4$-JA | 12.9±2.7 (n=34) | 5.7±3 (n=6) | 7.2±0.8(n=6) | 18.2±1.2(n=6) |

**Figure 3.15 Phenotype of *Arabidopsis thaliana* plants treated with various derivatives of jasmonic acid**

A) *Arabidopsis* plants (Ws ecotype) were grown for 26 days under long day conditions (16 hours light) in MS medium. B), C) and D) 26 days old wild type plants grown under long day conditions (16 hours light) in MS media containing 20 μM MeJA, 20 μM 12-OHJA or 20 μM 12-$HSO_4$JA, respectively. (E) Quantification of flowering time, stem and root lengths, and the total leaf number in wild type plants treated with jasmonate derivatives.

displayed shorter petioles and slower stem elongation than untreated plants. In contrast, MeJA treated plants exhibited a delayed flowering phenotype, root growth inhibition and early senescence. Plants grown on 12-$HSO_4$-JA-containing medium had the same number of leaves, equivalent flowering time, and similar root and stem lengths as compared with untreated plants (Fig. 3.15), suggesting that the sulfonation of 12-OHJA abolishes its activity.

**AtST2a in *Nicotiana tabaccum***

To date, the presence of 12-OHJA has been reported in *Solanum tuberosum* (Koda *et al.*, 1988), *Solanum demissum* (Helder *et al.*, 1993) and Jerusalem artichoke plants where this compound was shown to have tuber-inducing activity. In *Arabidopsis thaliana* 12-OHJA was shown to play a role in the initiation of flowering in response to photoperiod. It was, therefore, interesting to test if other properties of 12-OHJA will be observed in a non-tuberizing day-neutral plant such as *Nicotiana tabaccum*. GC/MS analysis of wild type *N.tabaccum* samples (SNN ecotype) showed the presence of 12-OHJA and 12-$HSO_4$-JA suggesting the existence of an *AtST2a* functional homolog in this plant (O. Miersch, unpublished results).

*N.tabaccum* plants overexpressing *AtST2a* (*35S:AtST2a*) show no alteration in flowering time probably because initiation of flowering in a day neutral plant is insensitive to photoperiod. However, these plants

exhibit aberrant flower development and male sterility. In the transgenic flowers, the point of junction between sepals and petals is much higher than in wild type. Furthermore, stamens have petal-like structures with dark shriveled anthers, are partially attached to the petals, and are shorter than the wild type stamens so the stigmas remain unpollinated (Fig. 3.16-3.17).

GC/MS analysis of jasmonates in transgenic tobacco plants expressing *AtST2a* in the sense orientation showed that they contain less 12-OHJA as compared to wild type plants. In addition, wild type tobacco plants contain high 12-OHJA levels in stamens, while only minute levels are observed in the same organs of *35S:AtST2a* transgenic plants (Fig 3.18). To determine if the low level of 12-OHJA was the cause of sterility and aberrant flowers in the transgenic tobacco plants, we applied 12-OHJA to the developing tobacco flower buds every day for 10 days before the opening of the first flower bud. Figure 3.19 shows that flowers treated with 12-OHJA exhibit a wild type development. The complementation result suggests that 12-OHJA plays an important role in anther development and in the determination of proper flower structures.

The aberrant flower phenotype observed in *N.tabaccum* transgenic plants suggested that *AtST2a* overexpression might have interfered with the expression of organ identity gene(s) responsible for petal/stamen development. According to the ABC model for floral patterning, flower

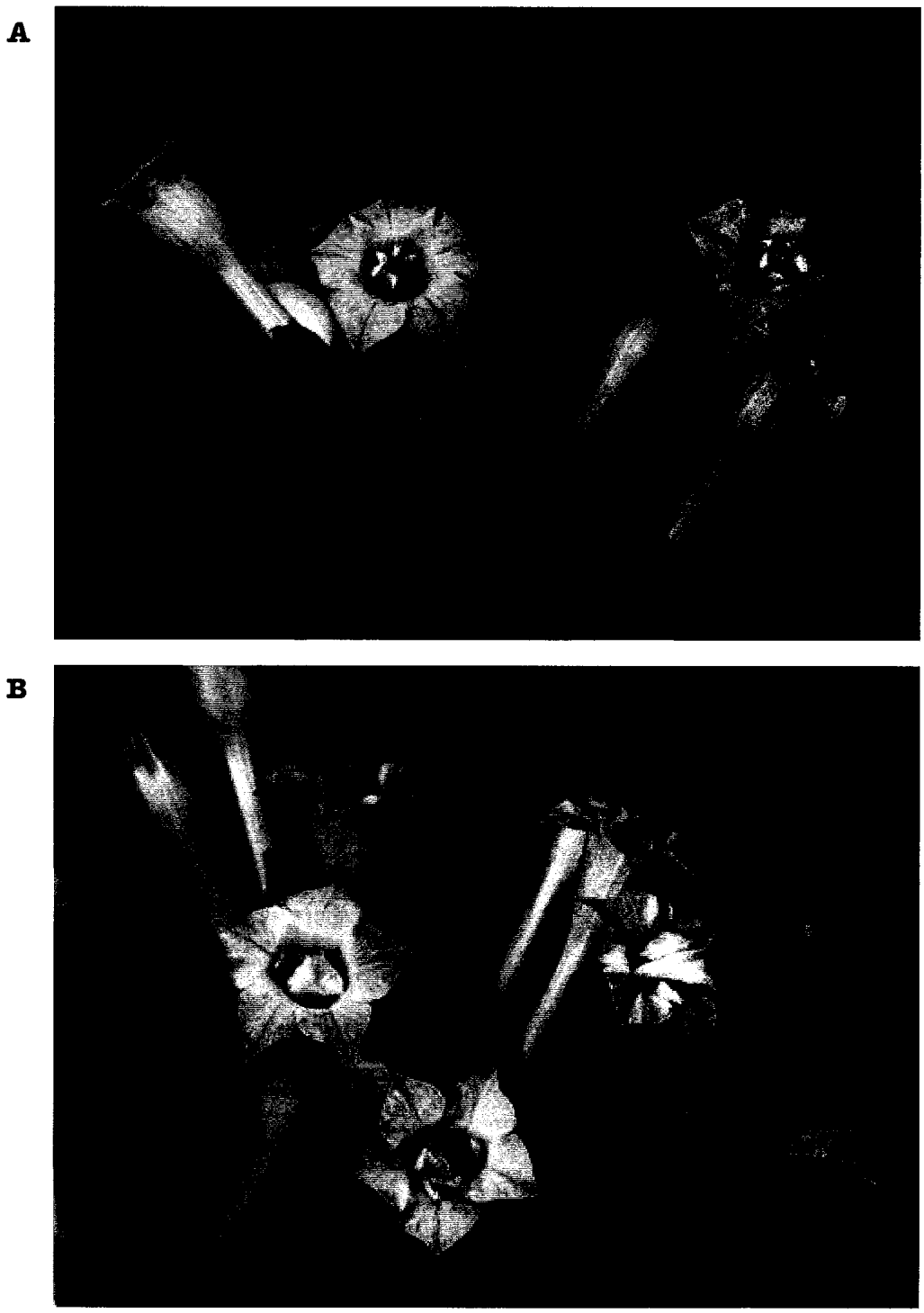



**Figure 3.16 Flower phenotype of the wild type *N.tabaccum* plants (A) and transgenic *AtST2a* tobacco overexpression lines (B)**

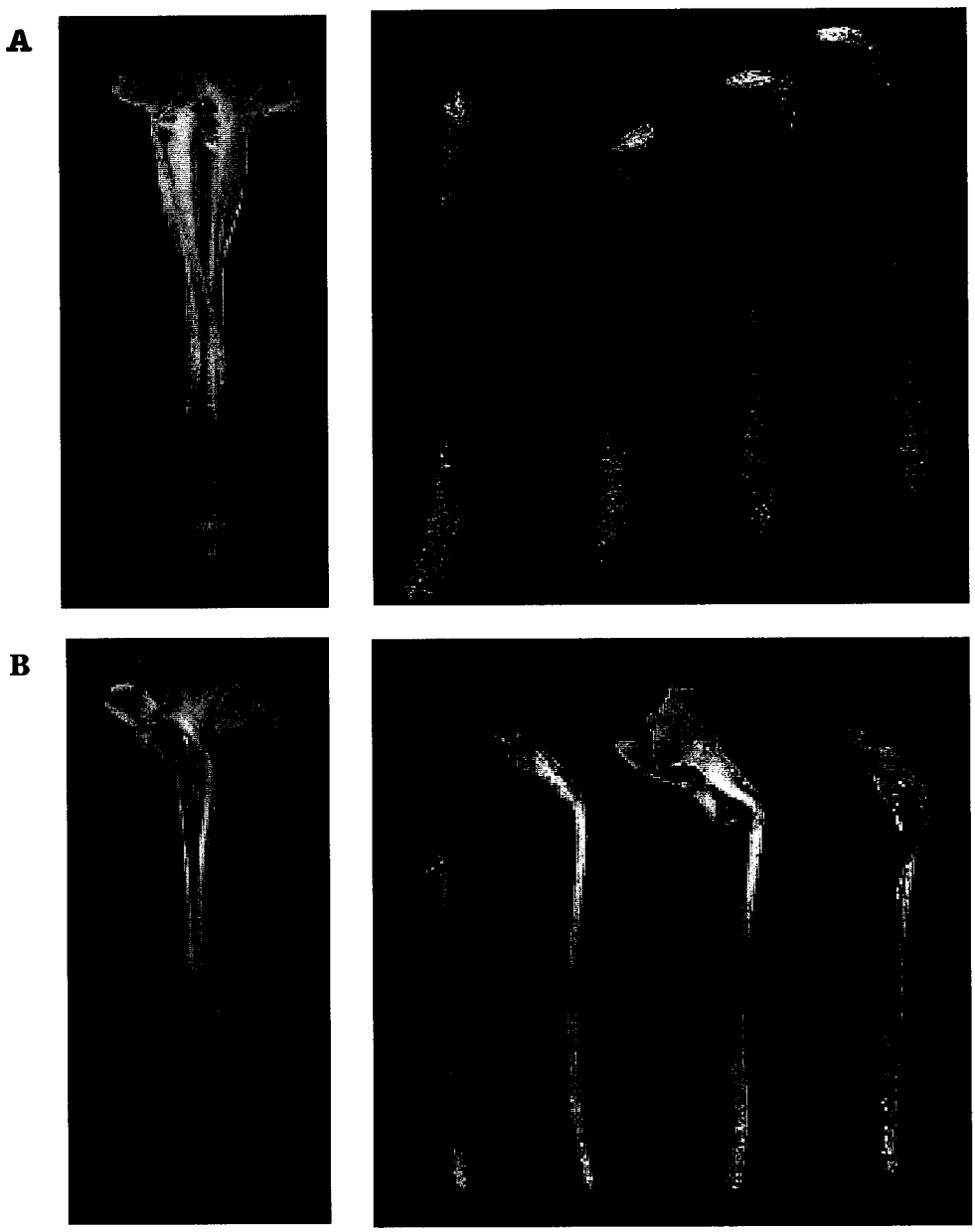



**Figure 3.17 Photograph of dissected wild type *N.tabaccum* flower (A) and transgenic *AtST2a* overexpression line (B)**

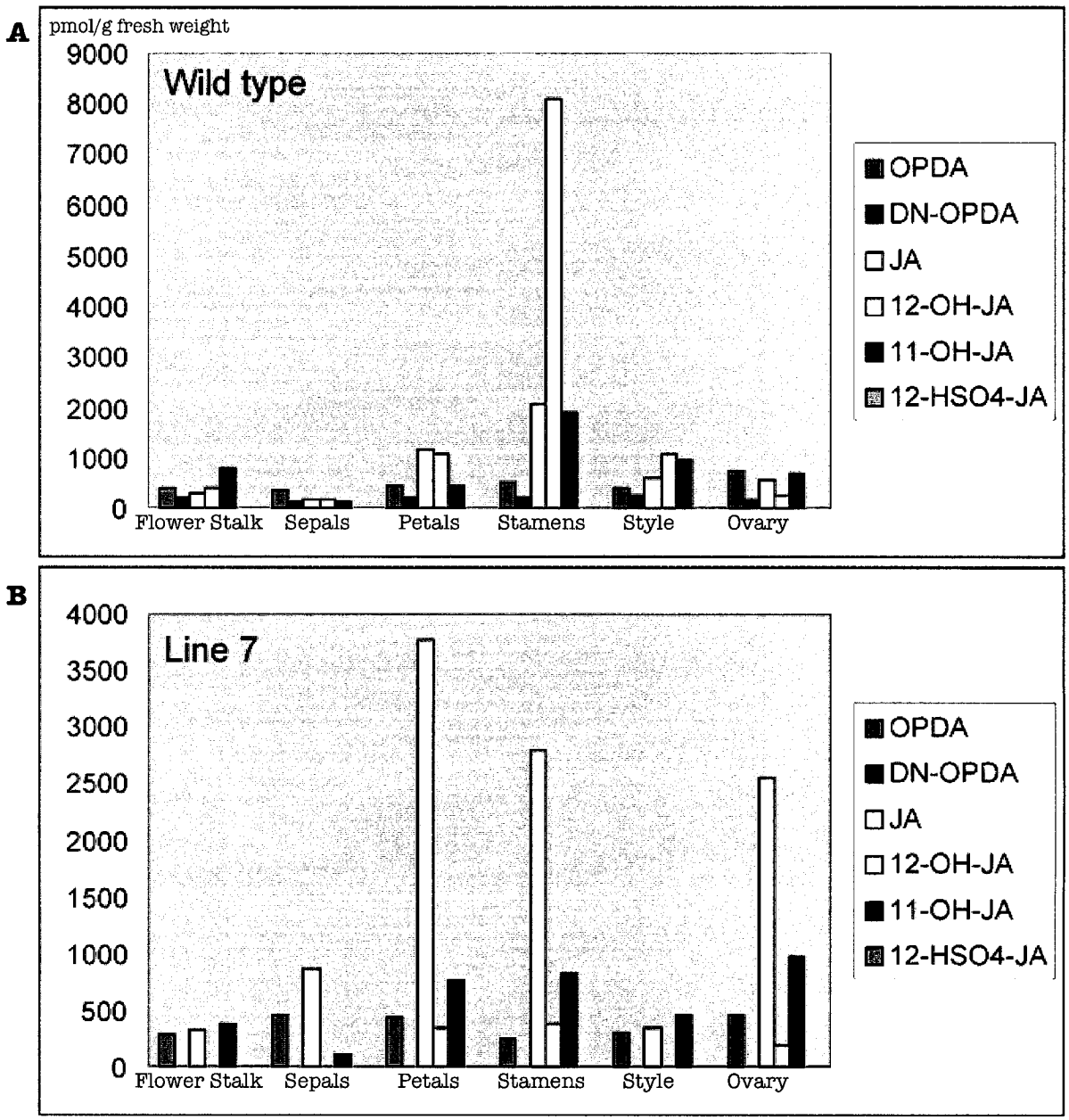
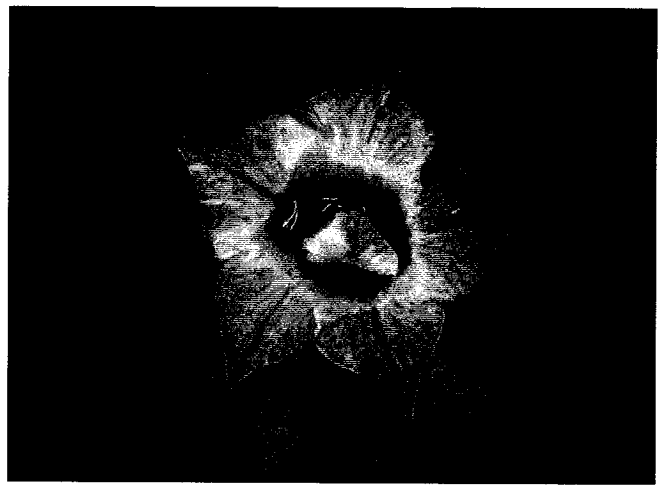



**Figure 3.18 GC/MS quantification of various jasmonates in wild type *Nicotiana tabaccum* (A) and tobacco *AtST2a* overexpression line 7 (B).**

A



B

**Figure 3.19 Complementation of transgenic *N.tabaccum 35S:AtST2a* plants with 12-OHJA.**

*N.tabaccum* plants were grown in soil under greenhouse conditions. Control plants were sprayed with water containing 0.1% Tween 20 (A). A 0.1M solution of 12-OHJA in 0.1% Tween 20 was applied to plants daily for a period of 10 days starting with the appearance of the first flower buds (B).

organ identity genes can be divided into 3 groups. Group A genes are responsible for sepal and petal production, class B genes participate in petal and stamen formation and class C genes take part in stamen and carpel development. The model further suggests that the A and C functions are mutually antagonistic, such that in a-loss of function mutants, the C domain expands to include all whorls, and similarly in c-loss of function mutants, the A domain expands to include all whorls. Therefore, it can be proposed that the presence of 12-OHJA in the stamens might down regulate expression of one or few class A genes in whorl 3, or might be required for the expression of the C function in this whorl. We have analyzed the pattern of expression of all known *N.tabaccum* flower organ identity genes such as *GLOBOSA, AGAMOUS, DEFICIENS, PLENA,* and *SQUAMOSA*. Preliminary RT-PCR results showed that the abnormal organ development was not due to a defect in the spatial expression of the tobacco *SQUAMOSA* gene (an ortholog of the *Arabidopsis* class A organ identity gene *APETALA1),* of the orthologs of class B organ identity genes (*GLOBOSA* and *DEFFICIENS),* nor of the ortholog of the class C gene *AGAMOUS* (data not shown). However, the expression of the homolog of the *Antirrhinum PLENA* gene called *NtPLE36* was found to be upregulated mostly in stem and sepals of the transgenic tobacco plants (Fig. 3.20 and Table 3.3). The *Antirrhinum* class C MADS-box gene *PLENA (PLE)* is a homolog of the *AGAMOUS Arabidopsis* gene. The class C genes of

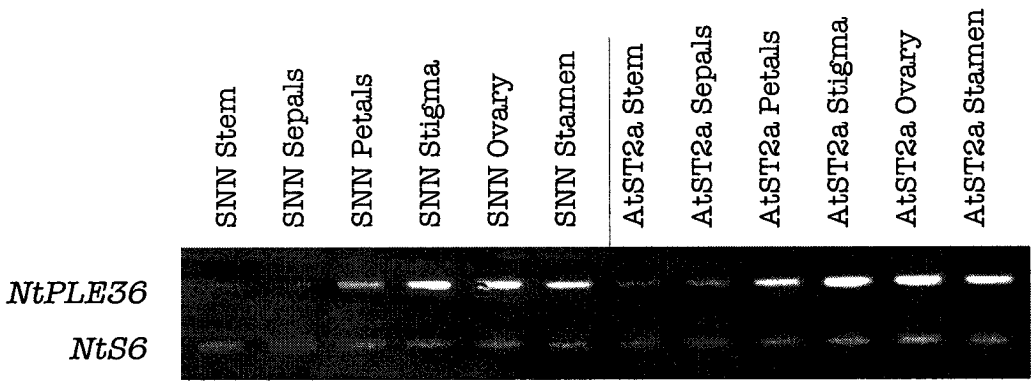



**Figure 3.20 RT-PCR analysis of *NtPLE36* expression in *N.tabaccum* wild type flowers (cultivar SNN) and tobacco plants overexpressing *AtST2a*.**

The cDNA products were amplified with *NtPLE36* specific primers as well as with *NtS6*-specific primers (encoding ribosomal protein S6) to confirm that equal amounts of cDNA were used in each amplification reaction.

**Table 3.3**

**Quantification of *NtPLE36* expression in wild type tobacco plants versus *N.tabaccum* plants overexpressing *AtST2a***

| Samples* | Fold Increase |
|---|---|
| *35S:AtST2a* ** /wild type Stem | 3.5 |
| *35S:AtST2a* /wild type Sepal | 2.0 |
| *35S:AtST2a* /wild type Petal | 2.0 |
| *35S:AtST2a* /wild type Stigma | 1.2 |
| *35S:AtST2a* /wild type Ovary | 1.0 |
| *35S:AtST2a* /wild type Stamen | 1.1 |

* ecotype SNN

** transgenic plants overexpressing *AtST2a*

The RT-PCR products of Figure 3.20 were used for quantification.

The signal intensities were quantified using the NIH Image program (version 1.63). The quantification was done according to the protocol described in the Materials and Methods section.

*Antirrhinum* [*PLENA* (*PLE*)] and *Arabidopsis* [*AGAMOUS* (*AG*)] are expressed in the third and fourth whorls of the flower. Mutations in *PLENA* and *AGAMOUS* give rise to the same conversion of reproductive organs to perianth organs (Davies *et al.*, 1999). These preliminary results suggest that in tobacco the partial conversion of stamens into petals results from the upregulation of *NtPLE36* as opposed to the similar phenotype resulting from a loss of function mutation of PLENA in *Antirrhinum* (Figure 3.21).

It is interesting to note that the tobacco genome contains two class C genes. The first one called *NAG* is a homolog of *AGAMOUS* from *Arabidopsis thaliana* and the second one is a homolog of *PLENA* from *Antirrhinum.* The presence of two class C genes in tobacco suggests that the function of *NtPLE36* might be different in specifying stamen and carpel identity as compared to other organisms.

**Complementation of *opr3* with 12-OHJA**

Jasmonic acid is known to play a role in male fertility. The *Arabidopsis thaliana opr3* plants accumulating trace amount of jasmonic acid are male sterile due to a delay in the release of pollen grains from the anthers and a defect in pollen development (Weber, 2002). The *opr3* male-sterile phenotype can be complemented by the addition of the volatile derivative of jasmonic acid, MeJA (Stintzi and Browse, 2000). On the other

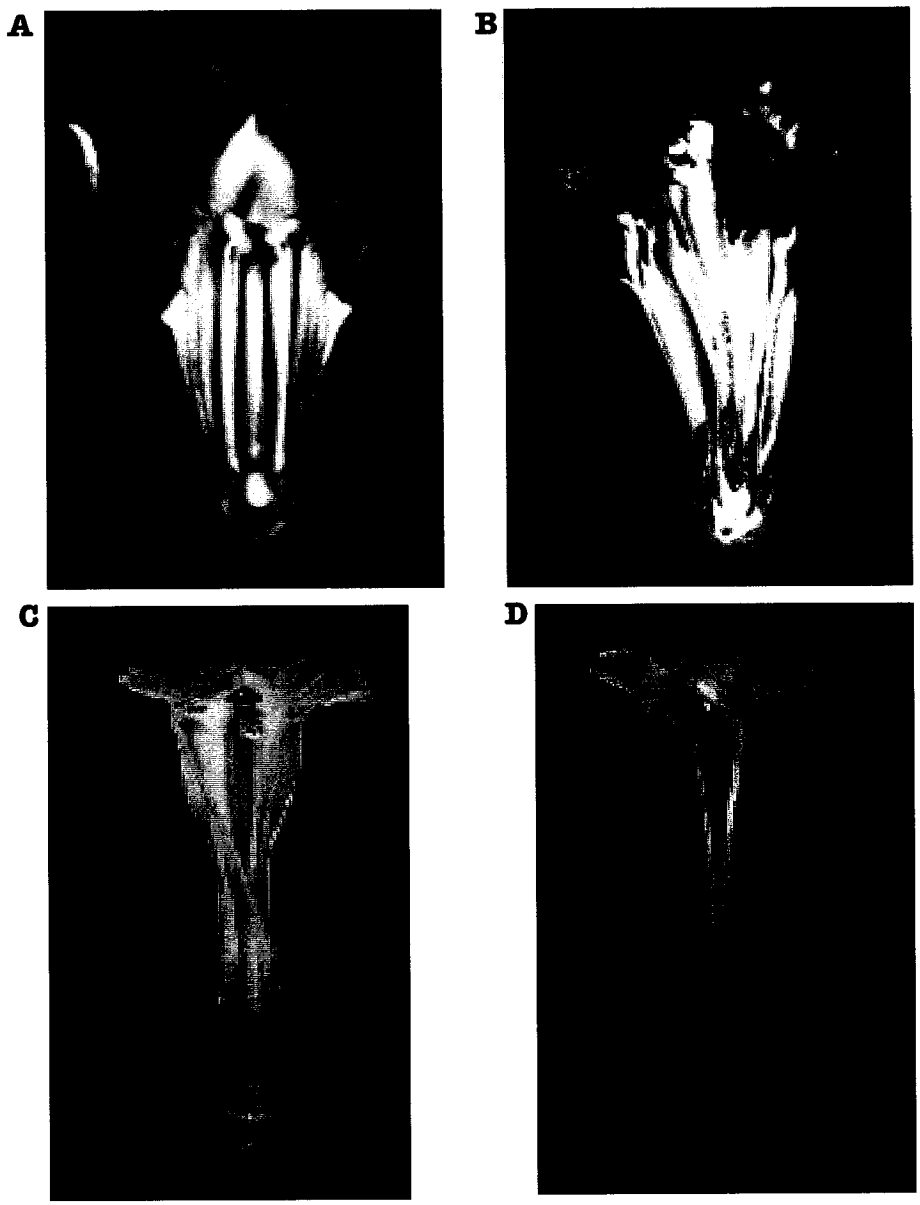



**Figure 3.21 Flower phenotype of *Antirrhinum plena* mutant and *N.tabaccum 35S:AtST2a* transgenic plants.**

Photographs of *Antirrhinum* wild type flower (A) and *plena* mutant flower (B). *N.tabaccum* wild type flower (C), and flower phenotype of *AtST2a* overexpression transgenic plant (D).

hand, transgenic *N.tabaccum* plants overexpressing *AtST2a* contain less 12-OHJA as compared to wild type plants and are also male sterile. Therefore, we wanted to test if 12-OHJA alone is sufficient to rescue the male-sterile phenotype of *opr3* mutant plants. In addition, the histochemical analysis of *AtST2a* promoter-*GUS* fusion plants revealed *AtST2a* activity in the anthers, the organs defective in *opr3* mutants and tobacco *AtST2a* sense transgenic plants.

Male-sterile *opr3* plants were treated with 450 μM 12-OHJA or MeJA. Silique formation was observed in plants following the treatment with 12-OHJA and MeJA (Fig. 3.22). However, in plants treated with 12-OHJA, silique elongation began later as compared to plants treated with MeJA. In addition, siliques derived from the 12-OHJA-treated *opr3* plants were approximately half the size (0.5 cm±0.1, n=16) of MeJA-treated siliques (1 cm±0.2, n=15), but contained a similar number of seeds. The 12-OHJA- treated *opr3* plants contained 7.6±0.7 seeds/pod, n=15,whereas MeJA-treated siliques contained 8.2±0.7 seeds/pod, n=15. The delay in silique development observed in 12-OHJA treated plants can be attributed to lower levels of 12-OHJA absorption by the plant as compared to the volatile MeJA. These results are consistent with the *AtST2a* promoter activity observed in the anther abscission zone of *AtST2a-GUS* transgenic plants.

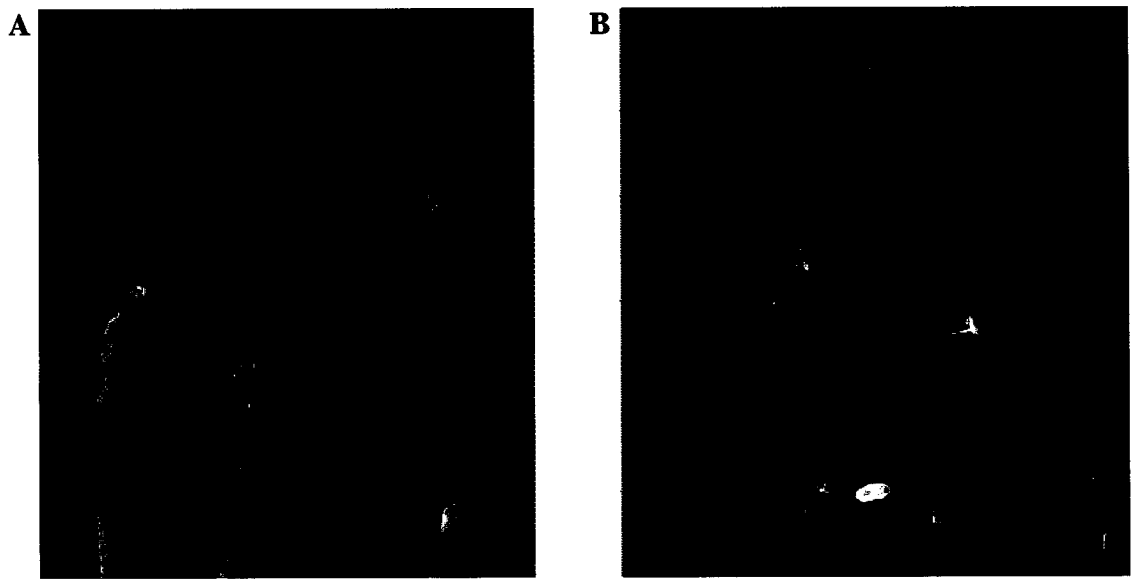



**Figure 3.22 Complementation of the male sterile phenotype of *opr3* mutant plants with 12-OHJA.**

*Arabidopsis thaliana opr3* plants were grown in soil under long day conditions (16 hours light). A solution containing 450 μM of 12-OHJA or MeJA in 0.1% Tween 20 was sprayed on plants during flowering. Control plants were sprayed with water containing 0.1% Tween 20.

**Discussion**

The *Arabidopsis thaliana* genome contains 18 sulfotransferase coding genes. Recently, we initiated a functional genomic project with the objective of characterizing their biological function.

In this thesis we describe the results of our study of two *Arabidopsis thaliana* sulfotransferases, AtST2a and AtST2b. The two genes lie in tandem on chromosome V and might be the result of a duplication event. The amino acid alignment of the deduced proteins indicates that AtST2a and AtST2b share 85% amino acid identity and 92% similarity. AtST2a and AtST2b constitute a separate group among the *Arabidopsis* sulfotransferases with the closest relative being *AtST1*, which encodes an epi-brassinosteroid sulfotransferase (Fig. 3.23). The amino acid sequence of AtST1 is 42% identical and 58% similar with AtST2a. The position of the *AtST2a* and *AtST2b* genes on the chromosome and their high degree of similarity suggest that the gene duplication occurred relatively recently (Eichler and Sankoff, 2003). However, the differences in their sequences suggest that they might have distinct substrate specificities. AtST2a was shown to accept 12-OHJA and to a lesser degree 11-OHJA, whereas the substrate for AtST2b is still unknown (see chapter 4).

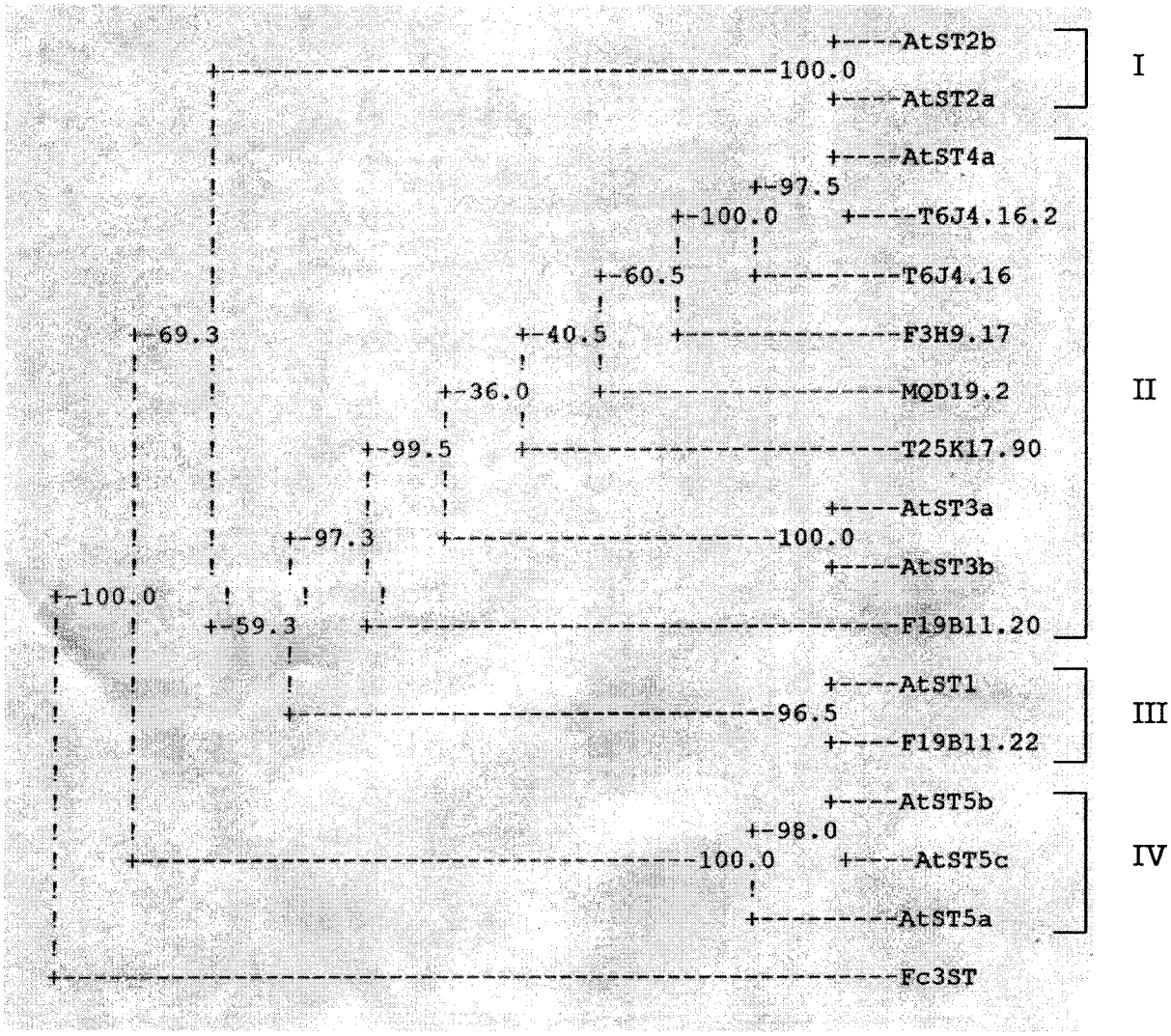



**Figure 3.23 Phylogenetic tree of the *Arabidopsis thaliana* sulfotransferases.** Numbers indicate bootstrap values of branches from 100 replicates. I, II, III, and IV designate the proposed subfamilies defined by a minimal level of 45% amino acid identity among members. The parsimony tree has been generated from an alignment of full length STs with the ClustalW 1.5 program using PILEUP of GCG and PROTPARs of PHYLIP (Felsenstein, 1993; Gidda, 2001).

The variations in the amino acid sequences of these two proteins might also explain their difference in solubility. Recombinant AtST2a could be easily purified from *E.coli* lysates, whereas only a very low yield of recombinant AtST2b could be obtained. The low yield was probably due to protein aggregation and might explain the lack of activity of the recombinant AtST2b enzyme.

### *AtST2a* transgenic plants

In order to determine the biological function of AtST2a and of 12-OHJA, we developed transgenic *Arabidopsis thaliana* plants with altered levels of the AtST2a protein (Gidda, 2001; Gidda *et al.*, 2003). Transgenic plants overexpressing *AtST2a*, grown under long day conditions flowered later than wild type plants grown under the same conditions. However, flowering time under short days was not altered. The results suggest that AtST2a plays a negative role in the initiation of flowering. Since the biochemical function of AtST2a is to sulfonate 12-OHJA, the delay in flowering can be attributed to either reduced levels of 12-OHJA or increased levels of 12-$HSO_4$JA, which might act as a flowering inhibitor. The significant increase of 12-OHJA observed at the onset of flowering supports the former hypothesis. Quantification of JA and 12-OHJA during development of wild type *Arabidopsis* (Col-0) plants exposed for 25 days to LD, revealed a continuing decrease in JA levels, whereas 12-OHJA levels decreased between day 5 and day 10, followed by a 4-fold increase between

day 10 and day 20 (Fig. 3.24)(Gidda *et al.*, 2003). Furthermore, several well documented examples show that enzymatic sulfonation abolishes the biological activity of such molecules as steroids in plants and animals, and thyroid hormones and catecholamine neurotransmitters in mammals (Rouleau *et al.*, 1999; Strott, 1996; Visser *et al.*, 1984).

Transgenic plants overexpressing *AtST2a* in the antisense orientation also exhibited alteration in the time of flower initiation. These plants flowered earlier than their wild type counterparts. However, the early flowering phenotype was only observed under short day (SD) conditions (Gidda, 2001).

**Regulation of *AtST2a* expression in response to light**

The alteration of the time of floral initiation observed in transgenic plants showed dependence on photoperiod suggesting that *AtST2a* expression is regulated by light/dark. Northern and Western blot analyses showed that *AtST2a* expression is induced by prolonged exposure to dark conditions (more than 8 hours) suggesting that AtST2a accumulates only when the days are sufficiently short. The AtST2a protein is also present at a low basal level in long day grown plants.

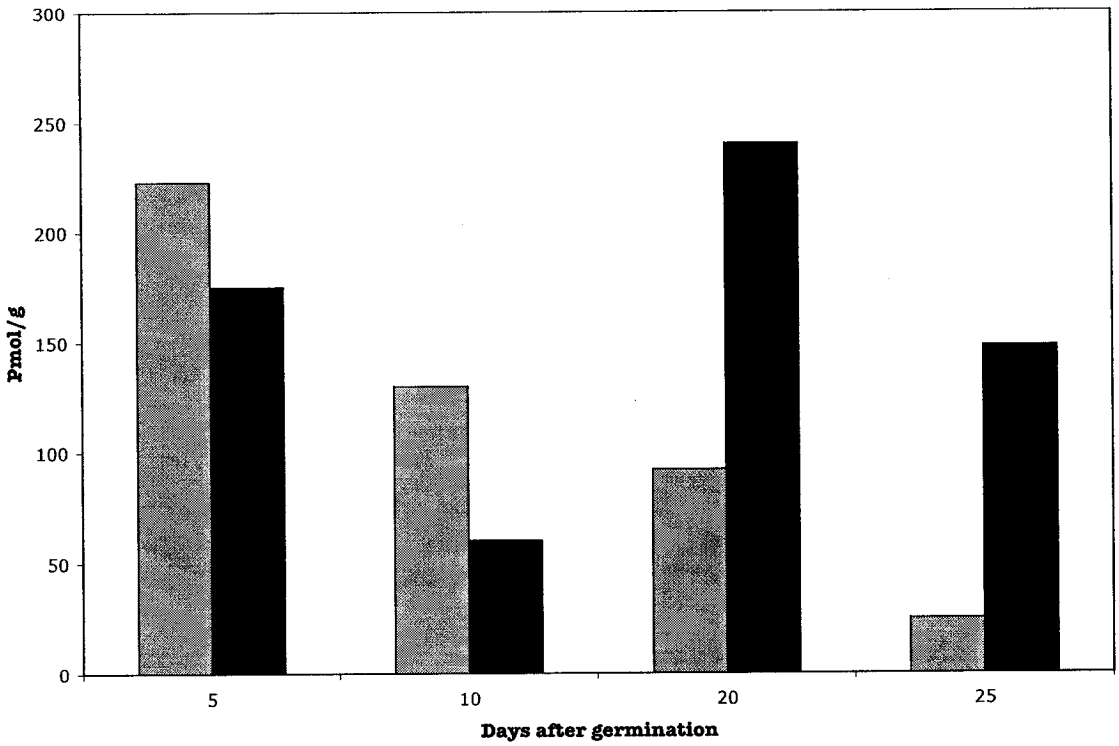



**Figure 3.24 Quantification of JA and 12-OHJA from *Arabidopsis thaliana* (Col-0)**

Jasmonates were extracted from plants growing under LD and JA (open bars) and 12-OHJA (black bars) were quantified by GC/MS. To minimize the effects of individual variability, pools consisting of 30-50 plants were used for metabolite extraction and internal standards were used to compensate for differences in extraction efficiencies.

(Adapted from Gidda *et al.*, 2003)

The results obtained from the study of the transgenic plants suggest the involvement of AtST2a and 12-OHJA in the control of flowering time. Initiation of flowering requires sensing of the environment by the photoreceptors and evaluating endogenous growth conditions of the plant (such as age, nutrient content, *etc.*). The most recent flowering model attempts to explain how exogenous and endogenous factors influence the timing of floral evocation. This model consists of a number of pathways each of which is activated in response to a particular condition. Two of these pathways mediate signals from the environment. The photoperiod pathway promotes flowering in response to inductive photoperiods, while the vernalization pathway allows flowering to occur after a prolonged period of cold temperatures. Two other pathways monitor the developmental state of the plant. The repression pathway prevents flowering until the plant reaches a certain age, while the autonomous pathway antagonizes the repression pathway and promotes flowering as the plant develops. Finally, the giberellin pathway plays a major role in promoting flowering under non-inductive photoperiods. We propose that AtST2a and 12-OHJA are part of the photoperiod pathway and that the photoperiod dependant regulation of *AtST2a* expression is a mechanism to modulate the flower inducing activity of 12-OHJA in response to photoperiod.

**Phytochromes and *AtST2a* expression**

The analysis of *AtST2a* expression in *phyA* and *phyB* phytochrome mutants revealed that it is downregulated in the dark by phytochromes A and B, but it is only repressed by PHYB in the light indicating the involvement of the phytochromes in the regulation of *AtST2a* expression.

The analysis of transgenic plants as well as flowering time mutants demonstrated a strong correlation between the level of expression of *AtST2a* and flowering time (*i.e.*, *AtST2a* levels were directly proportional to the delay in flowering). However, the *phyB* mutants accumulate elevated levels of AtST2a, but exhibit an early flowering phenotype. The function of PHYB in floral inhibition is apparently more complex since transgenic *Arabidopsis* plants overexpressing *phyB* also flower earlier than wild type plants (Bagnall *et al.*, 1995). The early flowering phenotype of the *phyB* mutant is the opposite of what we should expect according to our model where high AtST2a levels are proposed to inhibit flowering. A plausible explanation for this observation might be that the absence of PHYB might trigger another pathway independent of 12-OHJA that promotes flowering.

The photoperiod-dependent alterations of flowering time of *AtST2a* sense and antisense transgenic plants are very similar to those reported for the phytochrome-associated protein phosphatase 2A (FyPP) sense and antisense transgenic plants (Kim *et al.*, 2002). FyPP was shown to interact directly with phytochromes A and B and to act as a negative regulator in

the photoperiodic control of flowering. The phenotypic similarities between *AtST2a* and *FyPP* sense and antisense plants suggest that AtST2a also plays a negative role in the photoperiod control of flowering.

**Analysis of *AtST2a* expression in *Arabidopsis thaliana* mutants in the photoperiod dependent promotion pathway of flowering**

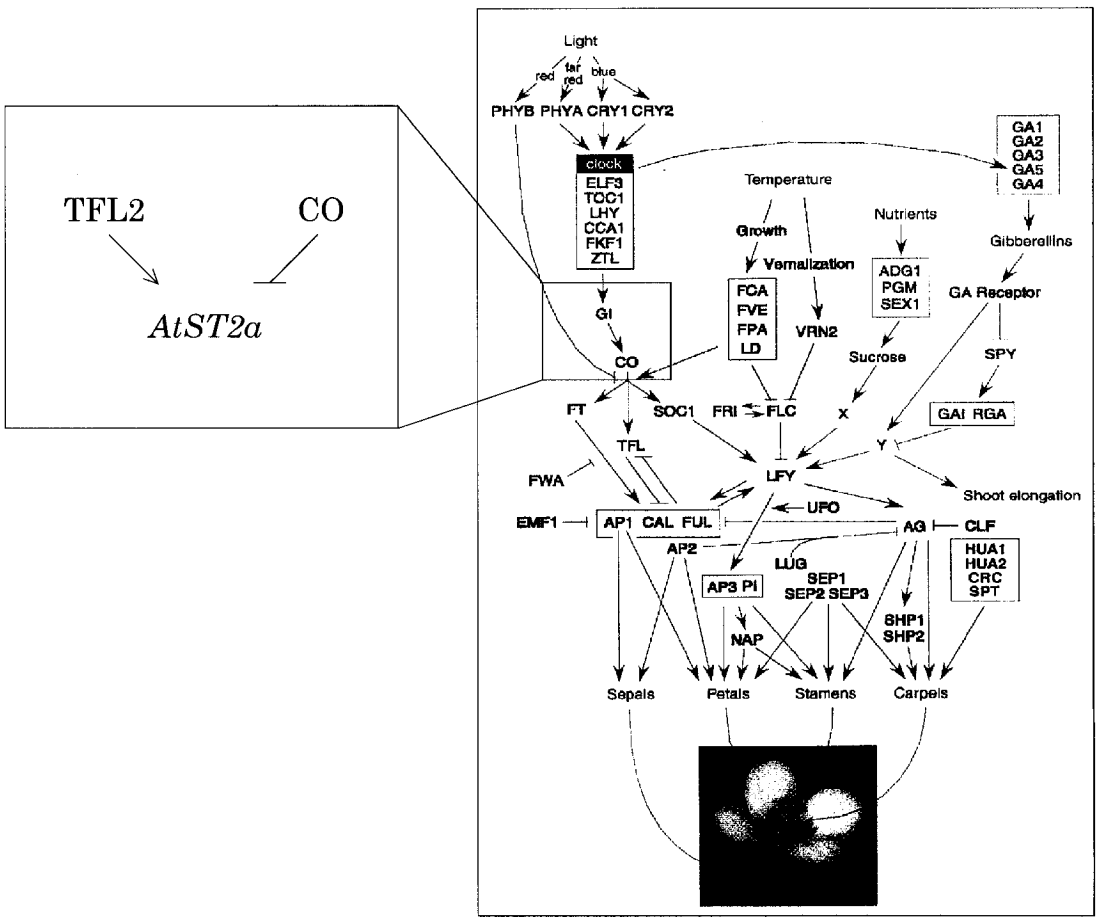
Through the analysis of various mutants we tried to correlate AtST2a levels with the onset of flowering and found that AtST2a levels are directly proportional to the delay in flowering.

The analysis of *Arabidopsis* plants carrying mutations at various loci of the flower induction pathways allowed us to position *AtST2a* and 12-OHJA in the photoperiod dependent flower induction pathway downstream of CONSTANS. The floral inducer, CONSTANS was found to repress *AtST2a* expression in wild type plants, whereas the floral repressor TERMINAL FLOWER 2 (TFL2) was found to upregulate *AtST2a* expression in *Arabidopsis*.

*CONSTANS (CO)* encodes a transcription factor, which is expressed in leaves, yet it can activate the expression of three genes, *LEAFY, FLOWERING LOCUS T* and *APETALA1* that are expressed at the reproductive meristem, and that control the initiation of flower development. Therefore, CONSTANS could be involved in the regulation of a systemic signal previously defined as the florigen (Yu and Ma, 2001).

*TFL2*, on the other hand, was shown to be expressed in proliferating cells of the meristem and young leaves, but is absent from mature leaves (Kotake *et al.*, 2003). A similar pattern of expression is observed for *AtST2a* suggesting that TFL2 upregulates *AtST2a* expression to avoid precocious flowering. Expression of *TFL2* and *AtST2a* in young leaves, but not in mature leaves correlates with the currently accepted model that the "florigen" is produced in the leaves and is translocated to the apical meristem. Taken together, our results suggest that 12-OHJA might induce floral evocation and that CONSTANS and TFL2 control the level of this metabolite by regulating *AtST2a* expression (Fig. 3.25). It should be interesting to see if AtST2a is downstream of FT or SOC1, two genes that are regulated positively by CONSTANS.

Prior to this work, the only proposed function of 12-OHJA was to act as an inducer of tuberization (Yoshihara *et al.*, 1989). A glucoside of 12-OHJA was first isolated from *Solanum tuberosum* leaf extracts and identified as 3-oxo-2 (5'-(-D-glucopyranosyloxy-2-cis-pentenyl) cyclopentane-1-acetic acid (Yoshihara *et al.*, 1989). The aglycone of this compound, 12-OHJA was named "tuberonic acid" and has been reported to occur only in tuber-producing plant species (Koda, 1997) and in the fungus *Botryodiplodia theobromae* (Miersch *et al.*, 1991).




(Blazquez, 2000)

**Figure 3.25 Proposed location of AtST2a in the photoperiod dependent flower induction pathway.**

It was demonstrated that 12-OHJA could act as a tuberization stimulus, which is produced in the leaves and is translocated to the underground parts of tuber producing plants. Since *Arabidopsis* is not a tuber producing plant, 12-OHJA might play other role(s) in plant development. It is important to emphasize that, in both environmentally responsive and autonomous plants, the signals that regulate the transition to flowering originate outside the SAM; in both cases, they probably originate in the leaves (Bernier *et al.*, 1993; Lang *et al.*, 1977). Through the grafting of the aerial parts from plants exposed to flower inducing photoperiods onto plants that had not been exposed to inducive stimulation, it was found that a signal of measurable mobility moved from the leaves to the SAM to cause the transition to flowering. These experiments showed that the floral stimulus had the properties of a mobile growth regulator, but a chemically defined substance with florigenic activity has never been isolated (Yu and Ma, 2001).

Despite the difference in the outcome of these developmental processes, tuberization and flowering share several characteristics. Both developmental processes are under the control of photoperiod. For instance, reduced levels of PHYB in transgenic antisense *Solanum tuberosum* led to strong induced state of tuberization, while the *A. thaliana phyB* mutant was shown to exhibit an early flowering phenotype (Reed *et al.*, 1993). Tuberizaton and flowering also seem to share an interchangeable signal(s) produced in the leaves under the proper

photoperiod (Ewing, 1995). The increases in 12-OHJA levels at the onset of flowering in *Arabidopsis* (Fig. 3.24), and at the onset of tuberization in potato (Yoshihara *et al.*, 1989), further support this hypothesis. Furthermore, the recent report of the control of photoperiod-regulated tuberization in potato by the overexpression of the *Arabidopsis* flowering-time gene *CONSTANS* provides strong evidence for a conserved photoperiodic functional module involved in the control of tuberization in potato and flowering in *A. thaliana* (Martinez-Garcia *et al.*, 2002).

### *AtST2a* regulation by 12-OHJA

*AtST2a* expression is induced in a dose-dependent manner by 12-OHJA (Gidda *et al.*, 2003). Upregulation of *AtST2a* stimulated by 12-OHJA indicates the presence of a feed-forward regulation mechanism that would control its level. Similar feed-forward mechanisms have been shown to regulate the level of the enzymes abscisic acid 8-hydroxylase and GA-2 oxidase involved in the catabolism of abscisic acid and gibberellins, respectively (Thomas *et al.*, 1999; Windsor *et al.*, 1997). The presence of a regulatory mechanism responding to the endogenous level of 12-OHJA supports the hypothesis that this metabolite acts as a signal molecule regulating important aspects of plant development.

### Analysis of *AtST2a* expression in *Arabidopsis thaliana* jasmonate response and biosynthetic mutants

It has been shown previously that in addition to 12-OHJA, MeJA and JA can induce *AtST2a* expression. We wanted to determine if a functional JA-response pathway is required for *AtST2a* expression. We looked at *AtST2a* expression in the JA perception mutants *cet1*, *coi1*, and *cev1*, and in the JA deficient mutant *opr3*.

CORONATINE INSENSITIVE 1 (COI1) encodes a protein with 16 leucine-rich repeats and a F-box motif, which is part of the $SCF^{COI1}$ complex. During JA response, signals pass via the $SCF^{COI1}$ complex to target proteins for degradation. $SCF^{COI1}$, an E3 ubiquitin ligase, is probably

involved in the ubiquitination of regulatory proteins. Possible targets for ubiquitination include transcriptional repressors (Siberil *et al.*, 2001; Turner *et al.*, 2002) and other regulators (Farmer *et al.*, 2003). COI1 regulates the expression of some JA response genes (called COI1-dependent), whereas the genes that are not regulated through the action of COI1 are called COI1-independent genes (Reymond *et al.*, 2000). In *coi1* mutants *AtST2a* expression is not detectable suggesting that in wild type *Arabidopsis thaliana AtST2a* gene expression involves the removal of a repressor by the action of the $SCF^{COI1}$ complex.

CEV1 (Constitutive Expression of Vegetative storage protein) encodes a cellulose synthase (CeSA3) that acts as a negative regulator of jasmonic acid signaling. *Cev1* plants were shown to accumulate high endogenous levels of JA and OPDA and to express constitutively JA- and OPDA responsive genes such as *VSP* and *Thi2.1* (Ellis and Turner, 2001). Northern and Western blot analyses revealed a constitutive accumulation of the AtST2a protein taking place under inductive and non-inductive conditions. Since *AtST2a* also is regulated by 12-OHJA, the constitutive expression of *AtST2a* could be explained by elevated endogenous levels of 12-OHJA in the *cev1* mutant.

Despite the high 12-OHJA levels observed in *cev1* extracts (Table 3.2), this mutant flowers late. Our results suggest that the absence of *cev1* might trigger the action of other signaling components controlled by the photoperiod promotion pathway. In addition, in wild type plants AtST2a

might control 12-OHJA levels in specific tissues involved in floral evocation.

12-oxophytodienoate reductase (OPR3) is an enzyme responsible for the reduction of OPDA leading to the production of jasmonic acid. Therefore, when *OPR3* is mutated, JA biosynthesis does not take place. Consequently, GC/MS metabolite quantification of *opr3* mutants showed trace amounts of JA. However, normal levels of 12-OHJA could be detected in *opr3* extracts (O. Miersch, personal communication). 12-OHJA is proposed to originate from the hydroxylation of jasmonic acid and should not, therefore, be produced in the *opr3* mutants.

According to the existing model, JA is synthesized from linolenic acid through a series of enzymatic reactions (Fig. 1.2). An important component of this pathway is the OPR3 enzyme. It has been shown that OPR3 is the only enzyme in *Arabidopsis thaliana* that can convert OPDA in the JA biosynthetic pathway. The presence of 12-OHJA in the *opr3* mutant suggests the existence of an additional pathway acting independently from JA. The first step of this hypothetical pathway could be an early hydroxylation at position C18 of linolenic acid (Fig. 3.12). There are a number of lipid hydroxylases that could catalyze this reaction. For instance, the C18 fatty acid derivatives of the structurally similar oleic acid 9,10-epoxystearic acid and 9,10-dihydroxystearic acid can be hydroxylated on the terminal methyl by microsomes of yeast cells expressing the *CYP94A1* gene from *Vicia sativa* (Pinot *et al.*, 1993). The

hydroxylated linolenic acid could then undergo a number of reactions catalyzed by enzymes similar to those involved in JA biosynthesis.

It is interesting to note that the *Arabidopsis thaliana* genome has two homologs of *OPR3 (OPR1* and *OPR2).* One of those is a good candidate for the reduction of a hydroxylated derivative of OPDA. It has been shown that OPR1 and OPR2 are capable of converting (9S,13S)-12-oxophytodienoic acid (OPDA) although with greatly reduced efficiency compared to OPR3 (Schaller *et al.*, 2000). It is interesting to note that similar early and late oxidation pathways were recently demonstrated for brassinosteroid biosynthesis (Fujioka *et al.*, 2003).

**Histochemical Analysis of *AtST2a* Promoter Activity**

Histochemical analysis of *AtST2a* expression shed light on possible additional roles of AtST2a and 12-OHJA in *Arabidopsis* development. Based on our Western blot data, we have evidence that the AtST2a protein is produced at similar levels throughout the life of the plant. The histochemical analyse of the *AtST2a* promoter activity support these data and show that the localization of expression shifts as the plant matures. For instance, *AtST2a* promoter activity is observed in the leaves of young seedlings, whereas, old plants do not show staining in the leaves, but in the flowers. In addition, the staining of younger plants is more uniform as compared to the more localized expression observed in older plants. The

expression of *AtST2a* in specific tissues or organs suggests that it might be involved in several developmental processes including flower initiation.

The *AtST2a* promoter was shown to be active in emerging leaves, tips of growing leaves, bases of trichomes, root apical meristem and emerging lateral roots. It is interesting to note that all of these tissues are sites of cell division. In addition, the staining observed at the plant apical meristem and at floral primordia supports the hypothesis that AtST2a is involved in the control of floral evocation.

*AtST2a* also was shown to be expressed at the seed stage. The gene was expressed only in mature seeds and not in newly produced seed-containing siliques suggesting that AtST2a/12-OHJA might play a role in dormancy by preventing precocious germination. It is interesting to note that mutants lacking *phytochrome B* show reduced seed germination suggesting that PHYB plays a role in seed germination (Bentsink *et al.*, 2002). Preliminary data on the germination of *AtST2a* knockout lines show that only 12% of the mutant seeds germinate as compared to 90% germination for the parental line. These plants probably accumulate higher levels of 12-OHJA as a result of the absence of AtST2a. Since JAs have been known to inhibit seed germination (Preston *et al.*, 2002), a similar role can be attributed to its hydroxylated derivative, 12-OHJA. The reduced germination of *AtST2a* knockout seeds is in line with the 12-OHJA levels found to accumulate in wild type tomato seeds and seedlings during development. The GC/MS quantification of jasmonates showed that as

tomato seeds germinate - 12-OHJA levels decrease pointing out to a direct correlation between 12-OHJA levels and seed germination (H. Maucher, unpublished results). Therefore, *AtST2a* expression might be required to inactivate 12-OHJA to allow the seeds to germinate. It would be interesting to quantify 12-OHJA levels in the seeds of *AtST2a* knockout plants. Furthermore, the effect on seed germination of adding 12-OHJA to germination medium should be evaluated.

A light induced stimulation of seed germination was suggested to depend on R/FR ratio experienced by the mother plant and, therefore, during seed maturation to affect the subsequent germination behavior of mature seeds. In fact, *phyB* mutants were shown to be affected in seed germination linking PHYB to the germination process (Bentsink *et al.*, 2002). In view of the photoperiodic and PHYB involvement in the regulation of *AtST2a* expression we can hypothesize that PHYB might regulate seed germination through the regulation of *AtST2a* expression and the inactivation of 12-OHJA.

**Effect of exogenously applied 12-OHJA, 12-$HSO_4$-JA and MeJA on plant development**

To understand the function of 12-OHJA in *Arabidopsis thaliana*, we compared the effect on growth of this compound with MeJA, which is another derivative of jasmonic acid. Plants grown on 12-OHJA supplemented media exhibited shorter internodes and petioles as

compared to untreated plants. This phenotype is similar to the one observed in plants lacking the brassinosteroid (BR) and gibberellin (GA) phytohormones. In addition, indole-3-acetic acid (IAA) also has been proposed to be an important determinant in phytochrome-mediated stem growth suppression (Chory *et al.*, 1996). A mutation in *PHYB* was shown to result in increased internode growth in part by blocking the ability of the phytochrome to decrease epidermal IAA levels (Behringe *et al.*, 1992). In addition, as seen from the expression analyses of *phyB* mutants, these plants accumulate AtST2a possibly resulting in reduced 12-OHJA levels. Therefore, 12-OHJA endogenous levels might have an effect on internode elongation by positively or negatively regulating the response or accumulation of various hormones (BR, GA or IAA) under the control of phytochrome B.

MeJA exhibited different effects on plant growth as compared to 12-OHJA such as: delay in flowering, promotion of leaf senescence, and root growth inhibition (Staswick *et al.*, 1992) suggesting a difference in the mode of action of these two metabolites. For instance, a number of plant responses that are mediated by JA or MeJA are accompanied by altered gene expression. For example, *JIP6* (thionin) and *JIP23* are known to be specifically induced in response to MeJA in barley leaves. However, these genes are not induced when barley leaves are treated with 12-OHJA (Miersch *et al.*, 1999).

Treatment with MeJA and/or JA leads to 20- and 4-fold increases of the endogenous levels of 12-OHJA and 12-$HSO_4$-JA, respectively (Gidda, 2001). However, RT-PCR experiments have shown that *Thi2.1* expression is induced by MeJA, but not by 12-OHJA. The *thi2.1* gene encodes a thionin that is specifically induced by wounding, pathogen infection and following treatment with MeJA (Epple *et al.*, 1995). Therefore, the difference in the effect induced by 12-OHJA and MeJA as well as the lack of *Thi2.1* induction following 12-OHJA treatment suggests that MeJA and 12-OHJA mediate their responses via two independent pathways, and have different functions during *Arabidopsis thaliana* development.

It was shown previously that the sulfonation reaction can abolish the biological activity of molecules including hormones, neurotransmitters and xenobiotics (Hobkirk and Glasier, 1993; Rouleau *et al.*, 1999; Strott, 1996; Visser, 1994). We tested if the sulfonation of 12-OHJA abolishes its biological activity. Plants grown on 12-$HSO_4$-JA had a phenotype similar to the one observed with plants grown without any supplement suggesting that the sulfonation turns off the biological activity of this metabolite on plant development. These results further support the proposed role of AtST2a in the inactivation of the biological activity of 12-OHJA. However, we cannot exclude the possibility that the addition of a sulfate group prevents the uptake of 12-OHJA by the plant cells.

**Model for 12-OHJA/AtST2a participation in floral evocation**

In the model presented in Figure 3.26, we propose that 12-OHJA is the signal or is part of a more complex mixture of floral inducers collectively called "florigen". The role of AtST2a is to negatively regulate the initiation of flowering by modulating the levels of 12-OHJA under noninductive conditions. CONSTANS and TFL2 in their turn control 12-OHJA levels by regulating *AtST2a* expression. CONSTANS acts as negative regulator of *AtST2a* expression, whereas, TFL2 acts as a positive regulator. The low level of *AtST2a* expression in the presence of light and the slow kinetics of *AtST2a* induction after the plants are transferred to dark is in line with this prediction. This pattern of expression would allow *AtST2a* to be expressed significantly only when the dark period is sufficiently long (SD) and explains why the overexpression of *AtST2a* antisense RNA is effective only under these conditions. The results are also in line with the finding that the delayed flowering phenotype associated with *AtST2a* overexpression is not observed under SD. Under these growth conditions, *AtST2a* is already expressed at a level that is probably sufficient to control 12-OHJA levels below a threshold concentration required to induce floral evocation. The basal level of *AtST2a* expression under inductive photoperiods might be required to control 12-OHJA levels to prevent precocious flowering. In view of the fact that the flower-inducing compound might be generated in the leaves and then translocated to the apical meristem to induce a switch from vegetative to reproductive

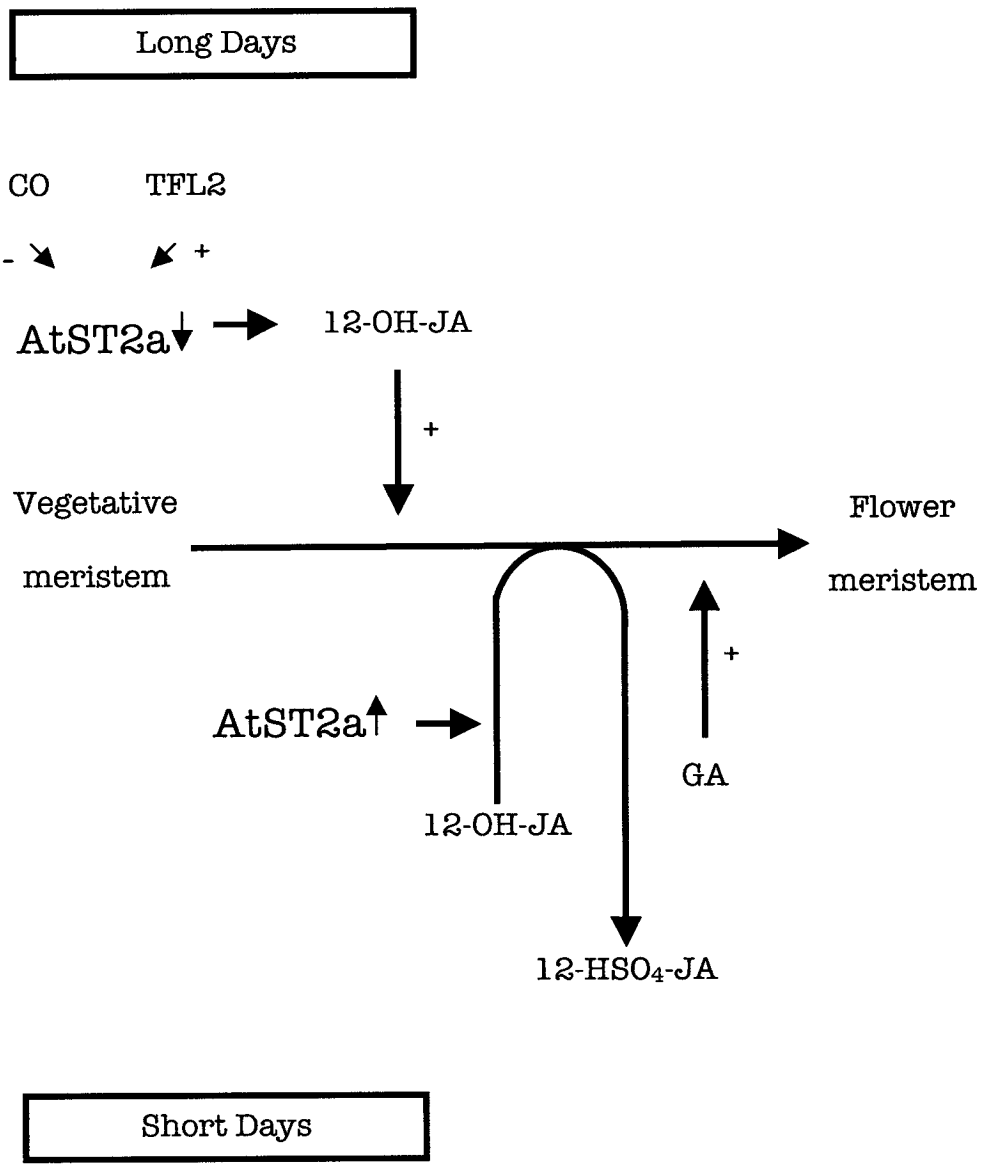



**Figure 3.26 Proposed model for the role of AtST2a in the control of flowering time in *Arabidopsis thaliana***

growth, it is possible that the role of AtST2a in the leaves is to control the active 12-OHJA levels. The histochemical analysis of *AtST2a* promoter activity showed an extensive staining of cotyledons in dark-grown seedlings. No staining was observed in the leaves of flowering *Arabidopsis* plants suggesting that 12-OHJA levels are not controlled in the leaves after flowering takes place.

The results obtained with *coi1* and *cev1* as well as those observed with *co* and *tfl2* indicate a strong correlation between flowering time and the levels of expression of *AtST2a*, which suggest a direct relationship between jasmonates, the photoperiod promotion pathway and the control of flowering time.

**AtST2a/12-OHJA role in anther development and floral patterning of tobacco**

The presence of 12-OHJA and 12-$HSO_4$-JA in tobacco plants suggests the existence of an AtST2a homolog in these plants (NtST2). When *AtST2a* was overexpressed in *N.tabaccum* plants, no change in flowering time was observed since floral initiation in this species is independent of photoperiod. However, these plants exhibit aberrant flower development. In such flowers, the point of junction between sepals and petals is much higher than in wild type flowers.

According to the ABC model for floral patterning around the time of organ initiation, the flower meristem is partitioned into three overlapping fields of gene activity, each field defining two adjacent whorls (*i.e.,* class A includes sepals and petals, class B-petals and stamens, class C-stamens and carpels) (Bowman and Meyerowitz, 1991; Meyerowitz, 1994). The model further suggests that the A and C functions are mutually antagonistic, such that in a-loss of function mutants, the C domain expands to include all whorls, and similarly in c-loss of function mutants, the A domain expands to include all whorls. The tobacco transgenic flowers contained petalloid stamens suggesting that either the class C gene(s) was (were) down regulated, or the class A gene(s) was (were) overexpressed. Therefore, the results obtained with the transgenic tobacco plants suggest that in wild type tobacco 12-OHJA and the AtST2a homolog NtST2 affect the expression of flower organ identity gene(s).

In support of this hypothesis, a homolog of the *Antirrhinum* gene called *PLENA* was found to be upregulated in the transgenic flower parts. In *Antirrhinum, PLENA* is a class C gene and a homolog of *AGAMOUS* from *Arabidopsis*. In addition, a phenotype similar to the one observed in tobacco transgenic plants overexpressing *AtST2a* also is seen in *Antirrhinum* plants carrying a mutation in the *PLENA* gene. This result suggests that in tobacco a homolog of AtST2a and PLENA participates in the flower development pathway. Although, in tobacco, the function of PLENA seems to be the opposite of what is observed in *Antirrhinum*. It is interesting to note that the tobacco genome contains two class C genes. The first one called *NAG* is a homolog of *AGAMOUS* from *Arabidopsis thaliana* and the second one is a homolog of *PLENA* from *Antirrhinum*. The presence of two class C genes in tobacco suggests that the function of *NtPLE36* might be different in specifying stamen and carpel identity as compared to other organisms.

In order to confirm that the phenotype of the transgenic plants is caused by changes in AtST2a activity, we quantified jasmonates in transgenic tobacco as well as in wild type plants. GC/MS results showed a considerable decrease of 12-OHJA levels in the stamens of the *AtST2a* overexpression lines. It can, therefore, be proposed that in the wild type *N.tabaccum* plants, 12-OHJA controls the expression of "petal gene(s)" outside of its/their field.

The aberrant flower structure of the transgenic *N.tabaccum* plants overexpressing *AtST2a* can be explained by the decrease in 12-OHJA levels, specifically in the stamens. The complementation of the aberrant flower phenotype after the exogenous application of 12-OHJA to the growing floral buds suggests that 12-OHJA is sufficient to determine proper anther development in the transgenic plants. Furthermore, *AtST2a* overexpression in tobacco resulted in shorter anther filaments and as a consequence the flowers were male sterile. When the tobacco transgenic plants were complemented with 12-OHJA the fertility was restored suggesting a link between the lowered levels of 12-OHJA and the defect in anther development.

**AtST2a/12-OHJA role in anther development of *Arabidopsis thaliana***

*Arabidopsis thaliana* mutants homozygous for the disrupted *OPR3* gene are also male sterile. The anther filaments on *opr3* plants do not elongate before anthesis (the time of a flower's opening), and anther locules do not dehisce at anthesis, so that mutant stigmas typically remain unpollinated (Stintzi and Browse, 2000). The male-sterile phenotype of *opr3* can be complemented by the exogenous application of MeJA to the flower buds (Stintzi and Browse, 2000). Previously it was shown that only JA, not OPDA, could render *opr3* plants fertile. The complementation also takes place following the application of 12-OHJA (Fig. 3.22) supporting the proposed role of 12-OHJA in anther development. It is interesting to note

that overexpression of *AtST2a* in *Arabidopsis thaliana* did not produce male-sterile plants. These plants have reduced 12-OHJA levels, but are still male fertile. However, since our method of screening for transgenic plants involves seed collection rather than tissue culture, it is possible that transgenic plants with sufficiently low levels of 12-OHJA cannot be isolated because of their male-sterile phenotype.

Inconclusion, 12-OHJA was found to play a similar role in anther development in both *Arabidopsis thaliana* and *Nicotiana tabaccum*. However, 12-OHJA had a distinct role in floral evocation of photoperiod dependent *Arabidopsis thaliana* as compared to a role in floral patterning of the day neutral *Nicotiana tabaccum*.

# CHAPTER 4

# BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *ARABIDOPSIS THALIANA* SULFOTRANSFERASE 2B.

*AtST2b* is localized on chromosome V and its sequence can be retrieved from the Genbank database under the accession number AB010697 from nucleotides 50627 to 51649 of the clone MOJ9. The *AtST2b* open reading frame codes for a protein of 347 amino acids corresponding to a molecular mass of 39.6 kDa. AtST2b is 85% identical and 92% similar to AtST2a at the amino acid level. The ORFs of two genes are located 2 287 bp apart on chromosome V.

## Biochemical Studies

In order to characterize the biochemical function of AtST2b, we expressed the recombinant protein in *E.coli.* The histidine-tagged recombinant AtST2b enzyme was recovered from *E.coli* lysates at relatively low levels when compared with AtST2a. This low yield is probably due to protein aggregation (Fig. 4.1). The purified soluble enzyme was used to test a variety of sulfonate acceptor molecules. In view of the high level of similarity between the *AtST2a* and *AtST2b* sequences, we expected that the latter might accept 12-OHJA or a substrate structurally similar to 12-OHJA (Fig. 4.2). The compounds tested such as the α-ketol of

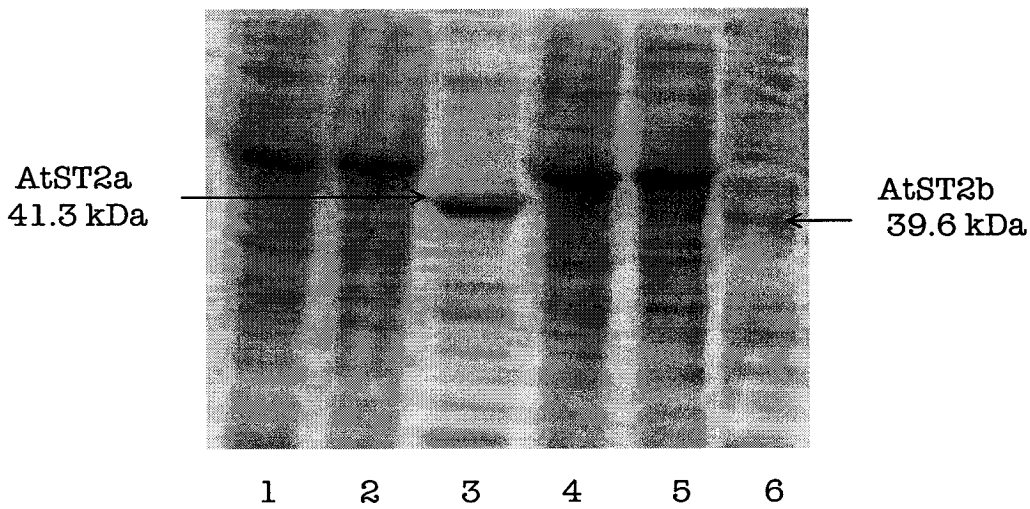
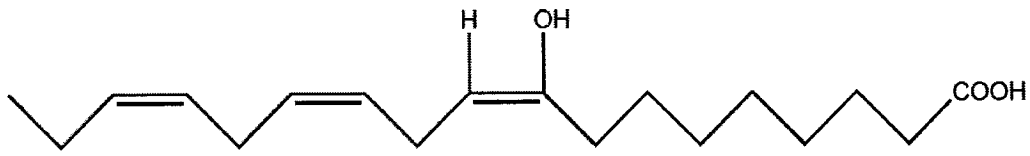
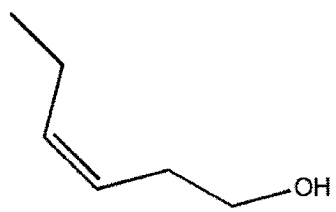



**Figure 4.1 SDS-PAGE of fractions collected during purification of recombinant AtST2a (Lanes 1-3) and AtST2b proteins (Lanes 4-6).**

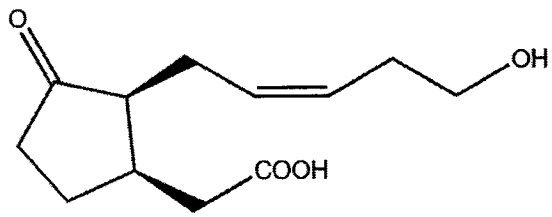
Lanes 1 and 4 Crude *E.coli* extracts; Lanes 2 and 5, Ni-agarose purified extract; Lanes 3 and 6 PAP-agarose purified extract. Proteins were visualized by Coomassie staining.



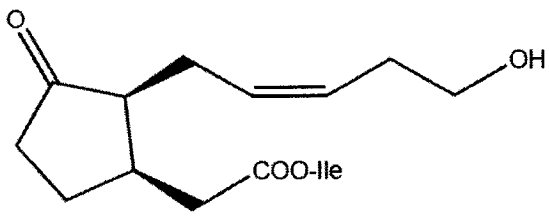

α-ketol of linolenic acid



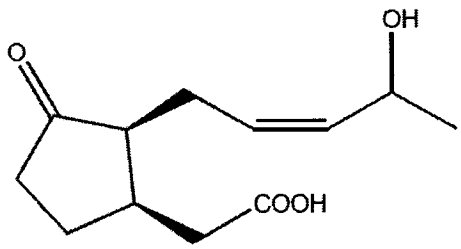

*cis*-3-hexen-1-ol




12-OHJA




12-OHJA-Ile




11-OHJA

**Figure 4.2 Chemical structures of compounds used as substrates for the recombinant AtST2b protein**

linolenic acid, *cis*-3-hexen-1-ol, 12-OHJA, 11-OHJA and 12-OHJA-Ile were not accepted by AtST2b. However, there is also a possibility that the purified enzyme was catalytically inactive.

**Characterization of the AtST2b gene product**

The pattern of *AtST2b* expression is different form the one of *AtST2a*. RT-PCR results demonstrate that *AtST2b* is constitutively expressed in all tissues of the plant (Fig. 4.3). *AtST2b* expression does not fluctuate in response to light or dark treatments. *AtST2b* is regulated independently of phytochromes A and B since *AtST2b* expression is not altered in the *phyA, phyB or phyAB* phytochrome mutants (Fig. 4.4). The results of RT-PCR experiments also reveal that *AtST2b* expression is not regulated by 12-OHJA or MeJA (Figure 4.5).

Finally, transgenic plants overexpressing *AtST2b* in the sense and antisense orientation as well as *AtST2b* knockout mutants show no visible phenotypic alterations (data not shown).

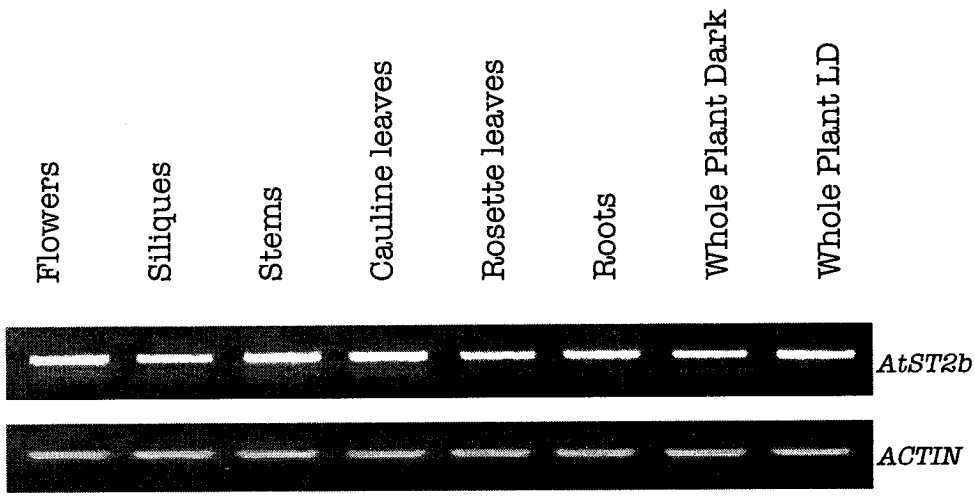



**Figure 4.3 *AtST2b* expression in various *Arabidopsis thaliana* organs as determined by RT-PCR**

Plants were grown for 14 days under long day conditions (16 hours of light) and various plant organs were collected. The cDNA products were amplified with *AtST2b* specific primers as well as with *ACTIN*-specific primers to confirm that equal amounts of cDNA were used in each amplification reaction.

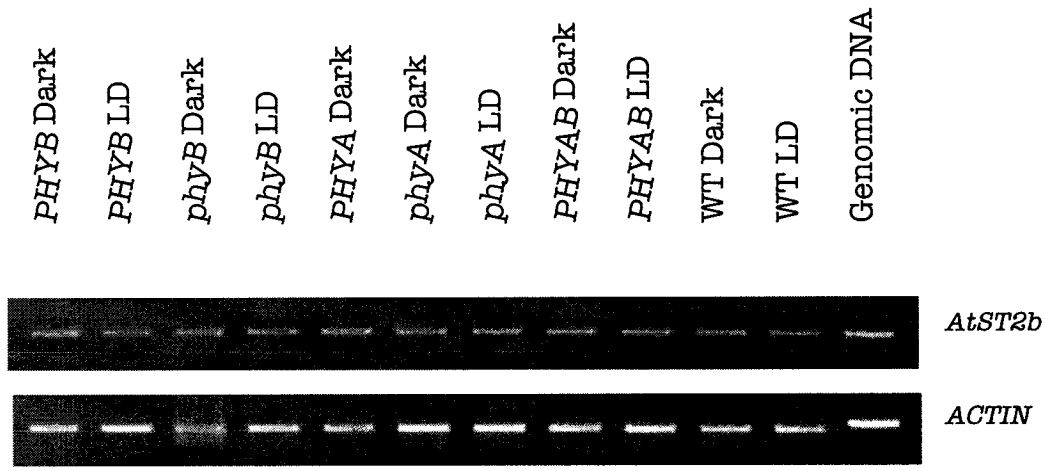



**Figure 4.4 AtST2b expression in *Arabidopsis thaliana* phytochrome mutants as determined by RT-PCR.**

Phytochrome mutants and wild type *Arabidopsis* plants were grown for 14 days under long day conditions (16 hours of light). *PhyB*, *PhyA* and *PhyAB* represent phytochrome B, phytochrome A and double phytochrome AB mutants. *PHYB*, *PHYA*, *PHYAB* stand for strong alleles of the respective phytochromes, while *phyB* and *phyA* represent weak alleles.

The cDNA products were amplified with *AtST2b* specific primers as well as with *ACTIN*-specific primers to confirm that equal amounts of cDNA were used in each amplification reaction.

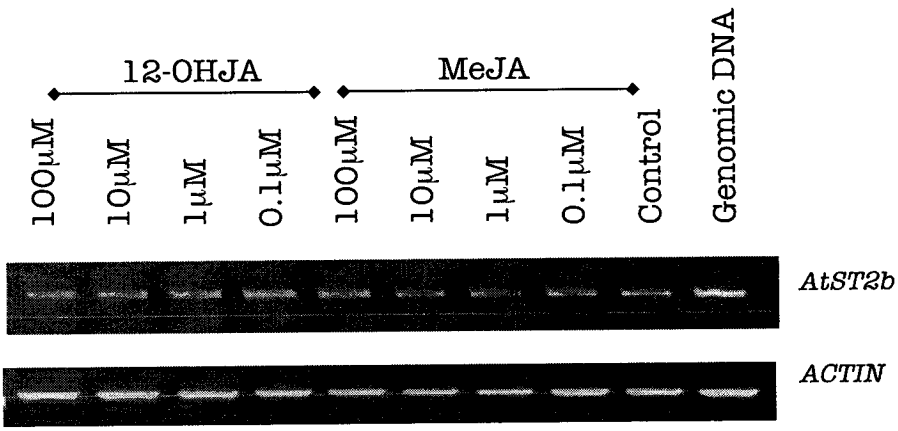



**Figure 4.5 *AtST2b* expression in response to jasmonate treatment**

Wild type *Arabidopsis thaliana* plants were grown for 14 days under long day conditions (16 hours of light). The plants were treated with various concentrations of 12-OHJA and MeJA dissolved in 0.1% methanol for a period of 24 hours. The control plants were treated for 24 hours with a 0.1% methanol solution. The cDNA products were amplified with *AtST2b* specific primers as well as with *ACTIN*-specific primers to confirm that equal amounts of cDNA were used in each amplification reaction.

**Discussion**

*AtST2a* and *AtST2b* share a high degree of amino acid sequence similarity. According to the sequence alignment, the two proteins share the same amino acid at the position responsible for substrate specificity suggesting that they sulfonate the same or a similar substrate. We tested a number of substrates that are structurally similar to JA or intermediates in the JA biosynthetic pathway (12-OHJA, 11-OHJA, the isoleucine derivative of 12-OHJA (12-OHJA-Ile), the α-ketol of linolenic acid (a signal compound produced in response to stress (Suzuki *et al.*, 2003)), and *cis*-3-hexen-1-ol (a green leaf volatile compound derived from the octadecanoid pathway (Kessler and Baldwin, 2001) None of the tested compounds were accepted by the AtST2b enzyme. Additional putative sulfate acceptor compounds should be tested including phenolic acids, brassinosteroids, desulfoglucosinolates, salicylic acid, gibberellic acid, phenylpropanoids, flavones, flavonols, and coumarins. The absence of activity might be due to the poor solubility of the recombinant enzyme. Other expression systems will have to be tested to address this question.

The results of RT-PCR experiments revealed that *AtST2b* expression is not affected by treatment with 12-OHJA or MeJA. *AtST2b* is constitutively expressed under various light conditions and in various plant tissues. *AtST2b* knockout mutants as well as transgenic plants overexpressing *AtST2b* in sense and antisense orientation showed no visible growth phenotype and no alteration of flowering time.

The constitutive expression of *AtST2b* under the tested conditions suggests that either the gene product is present constitutively, or that the expression was tested under conditions that upregulate *AtST2b*. To address this question more growth conditions should be tested including temperature regime as well as various nutrient contents in the growth media.

**PERSPECTIVES FOR FUTURE WORK**

Quantification of JA and 12-OHJA during development of wild type *Arabidopsis* (Col-0) plants exposed for 25 days to LD, revealed a constant decrease in JA levels, whereas 12-OHJA levels decreased between day 5 and day 10, followed by a 4-fold increase between day 10 and day 20. In the model presented in Figure 3.26, we propose that 12-OHJA is the signal or is part of a more complex mixture of floral inducers that are produced in the leaves and translocated to the apical meristem. Therefore, additional quantification of jasmonates in different organs of *Arabidopsis thaliana* during various developmental stages should be done to confirm the relationship between 12-OHJA and the control of flowering time. The quantification analysis also will allow us to determine where 12-OHJA is synthesized and if a transport mechanism is responsible for its translocation to the apical meristem.

I also demonstrated that the accumulation of the AtST2a protein is repressed by CONSTANS, a putative transcription factor that accelerates flowering in response to long photoperiod. *AtST2a* expression also was upregulated by TFL2, a homolog of *Drosophila* heterochromatin-associated protein 1, which acts on meristem identity genes to repress the initiation of flowering. These results suggest that CONSTANS and TFL2 promote or repress flowering through the direct or indirect regulation of *AtST2a* expression, therefore, controlling 12-OHJA levels. CONSTANS and TFL2 also regulate expression of one of the floral regulators, *FLOWERING LOCUS*

*T*. The pattern of *FT* expression in response to 12-OHJA applications should be tested, to determine if CONSTANS and TFL2 regulate *FT* through the action of 12-OHJA or if 12-OHJA acts via an additional pathway.

In the *coi1* jasmonate response mutant *AtST2a* expression could not be induced by application of jasmonates or by dark treatments. The absence of induction by dark in this mutant suggests a link between jasmonate response and the developmental response to light. Furthermore, the absence of induction of jasmonate response genes in the *tfl2* mutant defines a new link between photoperiod promotion pathway and jasmonate response. Therefore, a thorough analysis of gene expression in response to MeJA should be conducted in *tfl2* mutant. Emphasis should be placed on genes that are upregulated during the wound response and following pathogen infection. Finally, we have a preliminary indication, using Affymetrix DNA chips, that there is a lot of overlap between JA- and 12-OHJA-inducible genes. Further experiments should be conducted to demonstrate that JA and 12-OHJA mediate their responses via separate pathways.

The first step in JA biotransformation is the hydroxylation at C-11 and to a lower extent at C-12 to form 11-OHJA or 12-OHJA derivatives. Although the enzyme(s) responsible for hydroxylation of JA still remain(s) to be characterized, cytochrome P450 enzymes would be good candidates. It would be interesting to isolate the hydroxylase catalyzing the production of 12-OHJA from JA. This should be done first by

phenotypic analysis of all available cytochrome P450/hydroxylase mutants.

The analysis of *AtST2a* expression in *phyA* and *phyB* phytochrome mutants revealed that it is downregulated in the dark by phytochromes A and B, but it is only repressed by PHYB under long day conditions. The *phyB* mutants accumulate elevated levels of *AtST2a*, but exhibit an early flowering phenotype. The function of PHYB in floral inhibition is apparently more complex since transgenic *Arabidopsis* plants overexpressing *phyB* also flower earlier than wild type plants. Therefore, a more thorough study of PHYB involvement in *AtST2a* regulation should be done including the analysis of *35S:phyB* transgenic plants.

Also, 12-OHJA was found to participate in the determination of *Nicotiana tabaccum* flower structures as suggested by the results of the complementation experiments of the male-sterile phenotype of tobacco transgenic plants overexpressing *AtST2a*. *N.tabaccum* plants overexpressing *AtST2a* develop aberrant flowers. These flowers have additional petal-like structures on stamens suggesting that the effect is mediated by the upregulation of class A gene(s) or downregulation of class C flower organ identity gene(s). The expression analysis of all the known tobacco flower organ identity genes revealed the upregulation of *NtPLE36*, a homolog of *Antirrhinum PLENA* and *Arabidopsis thaliana AGAMOUS*. Alteration of expression of *NtPLE36* suggests a possible involvement of 12-OHJA in expression of this gene in wild type *N.tabaccum*. In order to

confirm that the misexpression of *NtPLE36* is responsible for the phenotype observed in *35S:AtST2a* transgenic tobacco plants, we should attempt to replicate the *35S:AtST2a* phenotype by overexpressing *NtPLE36* in tobacco.

I also attempted to characterize the biochemical function of *AtST2b*, a gene sharing a high level of sequence identity with *AtST2a*. However, we did not succeed in finding the natural substrate of this enzyme. A library of compounds available in our laboratory should be tested as potential substrates for this enzyme. Alternatively, we can isolate metabolites from *Arabidopsis thaliana* to try to identify the endogenous substrate of AtST2b.

*AtST2b* was found to be constitutively expressed in all tissues of the plant. Furthermore, transgenic plants overexpressing *AtST2b* in the sense or antisense orientation, as well as *AtST2b* knock out mutants revealed no visible phenotype suggesting a different function for this enzyme as compared to AtST2a. More growth conditions should be tested to try to detect a phenotype associated with disruption of the *AtST2b* gene in *Arabidopsis thaliana.*

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