INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

Molecular and Biochemical Characterization of
Steroid Sulfotransferases from *Brassica napus* and *Arabidopsis thaliana*

Frédéric Marsolais

A Thesis

in

The Department

of

Biology

Presented in Partial Fullfilment of the Requirements
for the Degree of Doctor of Philosophy at
Concordia University
Montreal, Quebec, Canada

November 2000

© Frédéric Marsolais, 2000

National Library of Canada

Acquisitions and Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada

Acquisitions et services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-59210-3

Canada

ABSTRACT

Molecular and Biochemical Characterization of Steroid Sulfotransferases from *Brassica napus* and *Arabidopsis thaliana*

Frédéric Marsolais, Ph. D.

Concordia University, 2000

We describe the characterization of four genes designated *BNST1* to *-4* encoding steroid sulfotransferases from *Brassica napus*. Recombinant BNST3 catalyzes the sulfonation of brassinosteroids and of mammalian estrogenic steroids. The enzyme is stereospecific for 24-epibrassinosteroids, with a substrate preference for 24-epicathasterone, a metabolic precursor of 24-epibrassinolide. The bean second internode bioassay was used to demonstrate that enzymatic sulfonation of 24-epibrassinolide abolishes its biological activity. This mechanism of hormone inactivation is similar to the modulation of estrogen biological activity observed in mammals. The sulfonation of an intermediate in the biosynthesis of 24-epibrassinolide may prevent the formation of biologically active end products. *BNST* genes are inducible by salicylic acid, a signal molecule in the defense response, suggesting that plants respond to pathogen infection by modulating steroid-dependent growth and developmental processes.

BNST genes along with alcohol dehydrogenase (*ADH*) and xyloglucan endotransglycosylases (*XET*) genes are also inducible by ethanol. *BNST4* displays the fastest response among *BNST* genes, with peak mRNA levels within one hour after treatment. Plants respond to low oxygen stress by a switch to ethanolic fermentation and the induction of anaerobic proteins, including ADH and XET. BNST proteins are induced under low oxygen stress, suggesting that endogenous ethanol may act as a chemical signal regulating gene expression.

The localization of *BNST2* and *-3* expression was studied in transgenic *A. thaliana* expressing promoter fusions with the ß-glucoronidase reporter gene. Expression was observed at the apex of leaf organs, including cotyledons, rosette and cauline leaves. Expression was also observed in the transition zone of seedlings. The tissue distribution of expression is compatible with a function of the sulfotransferases in brassinosteroid inactivation. We tested this hypothesis in transgenic *A. thaliana* expressing *BNST3* under the control of the constitutive CaMV35S promoter. No effect of the transgene was observed on plant growth and development, and the response of transgenic lines to exogenous 24-epiteasterone or 24-epibrassinolide was similar to that of the wild-type. Alternative experimental approaches will be required in order to determine the function of the sulfotransferases. The results of preliminary experiments performed in order to purify endogenous substrates of the BNST enzymes are discussed.

à mon frère,

Jean-Christophe

ACKNOWLEDGMENTS

I would like to express my gratitude to my thesis supervisor, Dr. Luc Varin, for giving me the opportunity to work in his laboratory and for sharing his passion for plant biology. I am grateful to the Natural Sciences and Engineering Research Council (NSERC), for supporting me with a postgraduate scholarship, and to Concordia University, for a J. W. McConnell Memorial fellowship and an external award holder scholarship.

I thank the members of my committee, Drs. Paul Joyce and Patrick Gulick, for their support and patience. I appreciate the collaboration of Dr. Van Luu-The as external examiner, and of Dr. Joanne Turnbull as external-to-program examiner. I am much obliged to Graduate Program Directors at the Department of Biology, Drs. Narinder Kapoor, Luc-Alain Giraldeau and Paul Albert, for their warm reception and encouragement, and their help with administrative procedures.

I would like to thank Drs. Bernd Schneider, Jean Rivoal and Francis Durst, who offered scientific advice during my studies. I thank undergraduate students who worked in the laboratory under my supervision, Anna-Maria Schinas, Samer Hossein and Yosabeth Paredes. I also thank Tonia De Bellis, Dr. Narinder Kapoor and Dr. Paul Widden, who helped me with microscopy and microphotography. Special thanks to Satinder Gidda, Annie Rousseau, and other members of Dr. Luc Varin's laboratory, for their friendly and warm company during several years.

I would like to express my deep gratitude to my family, who encouraged me through the most difficult times. Finally, I thank Cinta Hernández Sebastià, who generously offered her scientific advice and help during my work. She was a constant

source of inspiration, as a talented woman taking her place in science. I would like to express my love and admiration, which goes back to our meeting during the conference of the Phytochemical Society of North America at Concordia University.

CONTRIBUTION OF AUTHORS

In chapter 2, my contribution was the detailed characterization of the substrate specificity for steroid substrates and kinetic analysis of BNST3. Martine Richard performed the isolation of the *Brassica napus* genomic clones containing the *BNST* genes. Ludovic Nicolle did the restriction mapping and sequencing of the clones, the subcloning of *BNST3* into a bacterial expression vector, and the preliminary analysis of recombinant BNST3 expression in *Escherichia coli*. Michèle Rouleau performed the Northern and Western Blot experiments, and the initial characterization of the enzymatic activity of BNST3 with estrogenic steroids. Brunhilde Voigt and Günter Adam synthesized the brassinosteroids used in the experimental study. In the chapter, I wrote the sections of the experimental procedures and results describing my work. I also had a significant contribution to the writing of the introduction and the discussion, and to the revision of the manuscript.

In chapter 3, my contribution was the sequencing of the *BNST4* cDNA clone, the subcloning of *BNST4* into a bacterial expression vector, and the characterization of the substrate specificity and kinetic analysis of BNST4. I also performed the studies on the expression of the BNST genes and proteins using Western blot and RT-PCR. Annie Rousseau isolated the *BNST4* clone, and performed the Northern blot experiments using the *BNST4* and *XET* probes. I was responsible for writing chapter 3.

In chapter 4, my contribution was the study of the salicylate induction of the *BNST* genes by RT-PCR, the preparation of DNA constructs and *Agrobacterium tumefaciens* strains used for plant transformation, and the characterization of transgenic *Arabidopsis thaliana* plants expressing promoter-GUS fusions by histochemical staining.

Cinta Hernández Sebastià collaborated with the GUS histochemistry and microphotography. Samer Hossein, an undergraduate student doing a research project under my supervision, did the Southern blot experiments. I was responsible for writing chapter 4.

In chapter 5, my contribution was the preparation of the DNA construct and *A. tumefaciens* strain used for plant transformation, the characterization of the phenotypes of the *A. thaliana* transgenic plants expressing *BNST3*, and of their response to exogenous 24-epibrassinosteroids. Cinta Hernández Sebastià provided advice for the experimental design of the bioassays, and performed the statistical analysis of the results. Samer Hossein did the Western and Southern blot experiments. I was responsible for writing chapter 5.

In annex I, Anna-Maria Schinas performed the subcloning of the human steroid sulfotransferase cDNAs in a bacterial expression vector, and participated to the characterization of the enzymatic activity of recombinant enzymes with brassinosteroids, as part of an undergraduate research project under my supervision. Yosabeth Paredes performed the subcloning of *ATST1* in a bacterial expression vector, and the analysis of the substrate specificity and kinetic analysis of ATST1. She also did this work as part of an undergraduate research project under my supervision.

TABLE OF CONTENTS

	Page
List of Figures	xiv
List of Tables	xvii
List of Abbreviations	xviii
Introduction	1
Chapter 1: Review of Literature	1
Introduction	3
Sulfotransferases	3
Structural and functional conservation between plant and animal soluble sulfotransferases	4
Plant soluble sulfotransferases	5
Mammalian soluble sulfotransferases	7
Function of mammalian steroid sulfotransferases	8
Steroids in plants	12
Brassinosteroids	12
Physiological effects of brassinosteroids	13
Cell elongation	14
Root inhibition	15
Vascular differentiation	15
Light control of development	16
Expression of stress-regulated genes	16
Brassinosteroid biosynthesis	17

From cycloartenol to sterol precursors 19

From campesterol to brassinolide 21

Biosynthetic origin of 24-epibrassinosteroids 23

Regulation and site of brassinosteroid biosynthesis 24

Metabolism of brassinosteroids 26

Brassinosteroid signal transduction 29

Estrogens and androgens 31

Modification of hormone levels in plants 33

Plant response to stress 37

Defense response against pathogen infection 38

Response to ethanol 42

Response to low oxygen stress 44

Chapter 2: Inactivation of brassinosteroid biological activity by a salicylate-inducible steroid sulfotransferase from *Brassica napus* 55

Summary 57

Introduction 58

Experimental procedures 60

Isolation of the *BNST* genes 60

Expression of recombinant BNST3 in *Escherichia coli* 60

Expression of *BNST* genes and of *ATST1* in plants 61

Enzymatic assays 61

Bean second internode test 62

Synthesis of brassinosteroids 62

Synthesis of 6-deoxo-24-epicathasterone 62

Results 63

Isolation of a family of *Brassica napus* sulfotransferase genes 63

The expression of *BNST* genes is inducible by salicylic acid 63

BNST3 is a steroid sulfotransferase 64

Sulfonation of 24-epibrassinolide abolishes its biological activity 66

Discussion 67

Acknowledgments 70

Chapter 3: Molecular and biochemical characterization of an ethanol-inducible steroid sulfotransferase from *Brassica napus* 76

Summary 78

Introduction 79

Experimental procedures 82

Plant growth and induction 82

Isolation of the *BNST4* cDNA 82

Expression of recombinant BNST4 in *Escherichia coli* 82

Enzyme assays 83

Expression of *BNST* genes in *Brassica napus* seedlings 84

Results 85

Discussion 89

Acknowledgments 93

Chapter 4: Regulation of steroid sulfotransferase genes from *Brassica napus* in response to salicylic acid and during development 103

Summary 105

Introduction 106

Experimental procedures 109

Plant growth and induction 109

Expression of *BNST* genes in *Brassica napus* seedlings 109

Expression of *BNST* promoter-GUS fusions in transgenic *Arabidopsis thaliana* 110

Histochemical GUS assays 112

Results 112

Salicylate induction of *BNST* genes in *Brassica napus* seedlings 112

Localization of expression in transgenic *Arabidopsis thaliana* expressing *BNST2* or *BNST3* promoter-GUS fusions 113

Discussion 115

Acknowledgments 118

Chapter 5: Expression of the *BNST3* steroid sulfotransferase gene in transgenic *Arabidopsis thaliana* and purification of an endogenous substrate from *Brassica napus* 125

Summary 127

Introduction 128

Experimental procedures 129

Preparation of transgenic *Arabidopsis thaliana* plants expressing *BNST3* 129

Characterization of *Arabidopsis thaliana* transgenic lines expressing *BNST3* 130

Bioassays with 24-epibrassinosteroids 131

Experimental design and statistical analyses 131

Expression of recombinant sulfotransferases in *Escherichia coli* 132

Preparation of *Brassica napus* extracts and enzyme assays 133

Purification of sulfated products by HPLC 133

Results 134

Discussion 137

Acknowledgments 142

Conclusion 149

References 157

Annex I 182

LIST OF FIGURES

Page

Chapter 1

Figure 1. Sterol precursors of brassinosteroids. 50

Figure 2. Pathway of brassinosteroid biosynthesis. 51

Figure 3. Pathway of sterol biosynthesis. 52

Figure 4. Biosynthesis of 24-epicampesterol. 53

Figure 5. Metabolism of 24-epicastasterone in tomato and serradella cell cultures. 54

Chapter 2

Figure 1. Chemical structure of the steroids used in this study. 72

Figure 2. Comparison of the deduced amino acid sequences of *B. napus* sulfotransferase genes and *A. thaliana ATST1*. 73

Figure 3. A) Northern analysis of *BNST* gene expression in *B. napus* seedlings treated with 10 mM salicylic acid. B) same blot as in A hybridized with an actin probe. C) Western analysis of sulfotransferase gene expression in *B. napus* and *A. thaliana*. 74

Figure 4. Biological activity of 10^{-10} mol epibrassinolide (A) and 10^{-10}mol epibrassinolide sulfate (B) in the bean second internode bioassay. 75

Chapter 3

Figure 1. Chemical structure of the steroids used in this study. 95

Figure 2. Time-course of *BNST* expression in *B. napus* seedlings treated with 2% ethanol. A) Northern analysis of *BNST* gene expression. B) same blot hybridized with *A. thaliana TCH4*. C) same blot hybridized with an actin probe. D) Western analysis of BNST expression. 96

Figure 3. Western analysis of BNST induction by treatment with ethanol in different tissues of the seedling. 97

Figure 4. Western analysis of BNST induction in response to treatment with different alcohols. 98

Figure 5. Western analysis of BNST induction by treatment with phenobarbital. 99

Figure 6. Western analysis of the dose of ethanol required for BNST induction. 100

Figure 7. Western analysis of BNST induction during hypoxia and anoxia. 101

Figure 8. RT-PCR analysis of *ADH* and *BNST* induction by treatment with ethanol. 102

Chapter 4

Figure 1. RT-PCR analysis of *BNST* expression in *Brassica napus* seedlings treated with salicylic acid. 119

Figure 2. Western analysis of BNST expression in *Brassica napus* seedlings treated with salicylic acid. 120

Figure 3. Southern analysis of independent transgenic lines of *Arabidopsis thaliana* expressing *BNST2* or *BNST3* promoter-GUS fusions. 121

Figure 4. Histochemical localization of *BNST3* promoter-GUS expression in transgenic *Arabidopsis thaliana*. 122

Figure 5. Histochemical localization of *BNST2* promoter-GUS expression in transgenic *Arabidopsis thaliana*. 123

Figure 6. Function of the BNST steroid sulfotransferases in brassinosteroid metabolism. 124

Chapter 5

Figure 1. Southern blot of independent transgenic lines of *Arabidopsis thaliana* expressing *BNST3*. 144

Figure 2. Western blot of independent transgenic lines of *Arabidopsis thaliana* expressing *BNST3*. 145

Figure 3. Wild-type *Arabidopsis thaliana* and transgenic lines expressing *BNST3*. 146

Figure 4. Response of wild-type *Arabidopsis thaliana* and transgenic lines expressing *BNST3* to treatment with exogenous 24-epibrassinolide. A) Hypocotyl elongation. B) Root elongation. 147

Figure 5. Root elongation of wild-type *Arabidopsis thaliana* and transgenic lines expressing *BNST3* in response to treatment with exogenous A) 24-epicathasterone; B) 24-epiteasterone. 148

Annex I

Figure 1. Phylogenetic reconstruction of plant soluble sulfotransferases. 184

LIST OF TABLES

Page

Chapter 2

Table 1. Substrate specificity of recombinant BNST3. 71

Table 2. Kinetic parameters of recombinant BNST3 for the preferred brassinosteroid substrates. 71

Chapter 3

Table 1. Kinetic parameters of recombinant BNST4 for the preferred brassinosteroid substrates. 94

Table 2. BNST4 inhibition by mammalian sex steroids. 94

Chapter 5

Table 1. Retention times of sulfated products in HPLC 143

Annex I

Table 1. Relative activity of recombinant ATST1 with steroid substrates. 182

Table 2. Kinetic parameters of recombinant ATST1 for steroid substrates. 182

Table 3. Enzymatic activity of recombinant human hydroxysteroid sulfotransferase with brassinosteroids. 183

LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ANP	Anaerobic protein
ARE	Anaerobic response element
CTAB	Hexadecyltrimethyl ammonium bromide
EDTA	Ethylene diamine tetraacetate
EREBP	Ethylene response element binding protein
EST	Estrogen sulfotransferase
GST	Glutathione *S*-transferase
GUS	ß-Glucuronidase
HSST	Hydroxysteroid sulfotransferase
MES	2-[*N*-Morpholino]ethane sulfonic acid
PAGE	Polyacrylamide gel electrophoresis
PAPS	3'-Phosphoadenosine 5'-phosphosulfate
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PDC	Pyruvate decarboxylase
PR	Pathogenesis related
PST	Phenol sulfotransferase
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
XET	Xyloglucan endotransglycosylase
X-Gluc	5-Bromo-4-chloro-3-indolyl-ß-D-glucuronide

INTRODUCTION

In mammals, sulfotransferases play an important role in steroid hormone metabolism (Strott, 1996). According to the molecular classification of soluble sulfotransferases, the steroid sulfotransferases are classified in two groups, the estrogen (*SULT1B*) and hydroxysteroid sulfotransferases (*SULT2*) (Weinshilboum et al., 1997). The steroid sulfotransferases may have two different types of function. Steroid sulfonation constitutes a mechanism of inactivation, whereby expression of the sulfotransferases in specific tissues prevents hormone action (Roy, 1992, Strott, 1996). In addition, sulfonation of steroid hormone precursors in the adrenal gland is required for transport to the site of action, where they are further metabolized and bioactivated (Hobkirk, 1993).

In plants, brassinosteroids play an important role in regulating growth and development. Their status as hormones has been widely accepted, since the characterization of *Arabidopsis thaliana* mutants which are deficient in their biosynthesis or response (Li et al., 1996, Szekeres et al., 1996, Li and Chory, 1997). The field of research on brassinosteroid hormone metabolism and conjugation is still in its infancy. Polar and non-glycosidic metabolites of brassinosteroids purified from mung bean and rice have been proposed to be sulfate esters, based on their chromatographic behavior and susceptibility to solvolysis, but their structure has not been identified (Yokota et al., 1991, 1992). Several acyl and glucosyl conjugated brassinosteroids have been characterized (Adam and Schneider, 1999). However, the biological function of these metabolites is unknown.

This thesis presents the results of the biochemical and molecular characterization of four genes designated *BNST1* to *-4* encoding steroid sulfotransferases from *Brassica napus*. The general objective of this thesis was to characterize the biochemical and biological functions of the *BNST* genes. The substrate specificities of recombinant BNST3 and BNST4 were characterized using mammalian-specific steroids and brassinosteroids, and the biological activity of a brassinosteroid sulfate conjugate was determined. The study of *BNST* expression in response to salicylic acid and ethanol allowed a link with specific physiological responses resulting from pathogen infection or low oxygen stress to be established. Furthermore, the tissue localization of expression during normal growth and development was determined in transgenic *A. thaliana* expressing a ß-glucoronidase reporter gene under the control of *BNST* promoters. The main hypothesis that sulfonation leads to brassinosteroid inactivation was tested in transgenic *A. thaliana* expressing the *BNST3* gene in sense orientation. Finally, the possibility that the physiological substrate of the BNST enzymes is different from those identified *in vitro* was evaluated.

CHAPTER 1

REVIEW OF LITERATURE

INTRODUCTION

This review of the literature covers the main subjects related to the experimental results presented in this thesis. The characterization of plant soluble sulfotransferases is first described, with particular emphasis on enzymes characterized at the molecular level. The function of mammalian steroid sulfotransferases is also discussed. Our knowledge of steroids in plants is presented, especially that of the brassinosteroid hormones. The mechanisms of plant hormone inactivation, and experimental approaches used to manipulate hormone levels *in vivo* are outlined. Physiological adaptation of plants to stress conditions requires changes in hormone action. This aspect is discussed in relation to the plant defense response against pathogen infection, and the responses to ethanol and low oxygen stress caused by flooding.

SULFOTRANSFERASES

Sulfotransferases catalyze the transfer of the sulfonate group (SO_3) of an activated nucleotide donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to the appropriate hydroxyl or amino group of acceptor substrates. Sulfotransferases may be classified in two groups according to their localization (Huxtable, 1986). The membrane-bound sulfotransferases, localized in the Golgi apparatus, catalyze the sulfonation of glycosaminoglycans, glycolipids, and tyrosines in proteins and peptides (Bowman and Bertozzi, 1999, Kehoe and Bertozzi, 2000). Until recently, members of this group had been characterized from animal tissues only. However, a sulfotransferase activity involved in the biosynthesis of the peptide hormone phytosulfokine has recently been

characterized from plant cell cultures (Hanai et al., 2000). The members of the second group are often described in the literature as the cytosolic sulfotransferases. This terminology is misleading and should be replaced by "soluble sulfotransferases", since it does not reflect accurately the subcellular distribution of the protein. The soluble sulfotransferases accept as substrates small organic molecules such as flavonoids, steroids, neurotransmitters as well as xenobiotics with diversified structures. They have been characterized from bacteria, plants and animals (Varin et al., 1997, Weinshilboum et al., 1997), and can be subdivided in two subgroups according to their biological function. The first subgroup is represented by enzymes involved in the detoxication of xenobiotics and/or endogenous compounds, which are generally characterized by their broad substrate specificities (Mulder and Jakoby, 1990). Members of the second subgroup are involved in important metabolic processes such as steroid transport or inactivation, and they exhibit high specificity for their substrates (Strott, 1996).

Structural and functional conservation between plant and animal soluble sulfotransferases

The evolutionary link between soluble sulfotransferases from different phyla is obvious when we consider their common structural domains and the similarity in the chemical structure of their substrates. Structural similarities shared between members from different phyla represent a distinctive feature of soluble sulfotransfersases, as compared with other families of enzymes involved in conjugation reactions of plant secondary metabolism. For instance, mammalian and plant soluble sulfotransfersases generally share 25 to 30 % amino acid sequence identity. Soluble sulfotransferases have similar lengths and hydropathy profiles, and contain four regions of conserved amino

acid residues (Varin et al., 1992). Based on their conserved features, they should have similar three-dimensional structures. The function in cosubstrate binding and/or catalysis of amino acid residues present in all soluble sulfotransferases has been characterized (for a review, see Marsolais et al., 2000). In addition, the plant and mammalian soluble sulfotransferases use similar residues and protein segments to form their acceptor substrate binding sites.

Plant soluble sulfotransferases

At the beginning of the 1990s, biochemical research on plant sulfotransferases was focused on enzymes involved in the biosynthesis of choline sulfate and glucosinolates (for a review see Varin et al., 1997). Understanding the biosynthesis of these sulfated metabolites may lead to biotechnological applications, since choline sulfate is involved in resistance to salt stress in *Limonium* species, while glucosinolates are responsible for the distinctive flavor of plants from the Brassicaceae family. Research on the sequential pathway of flavonol polysulfation in *Flaveria* resulted in the isolation of the first plant cDNA clones coding for sulfotransferases (Varin et al., 1992). The flavonol 3- and 4'-sulfotransferases from *Flaveria chloraefolia* catalyze the sulfonation of flavonol aglycones and flavonol 3-sulfates, respectively. The cloning of soluble sulfotransferases from plant origin allowed the main features of the general structure of these enzymes to be established. Despite extensive knowledge of the biochemistry of flavonol sulfate biosynthesis, the understanding of its biological function is limited. The results of studies on the distribution of flavonol sulfates, and on the regulation of expression of flavonol sulfotransferases may provide clues about their function. The accumulation of flavonol sulfates is developmentally regulated in *F. bidentis*, being more abundant at the apex and

in the first pair of extended leaves (Hannoufa et al., 1991). In cell culture, flavonol sulfotransferase expression is induced by the synthetic auxin 2,4 D (Ananvoranich et al., 1994). Furthermore, flavonol aglycones are known to inhibit polar auxin transport by binding to the naphthylphthalamic acid receptor, while sulfate conjugates have an antagonistic effect (Jacobs and Rubery, 1988, Faulkner and Rubery, 1992). Flavonol sulfate biosynthesis may therefore allow auxin efflux from tissues where it is produced in large amounts (Ananvoranich et al., 1994).

RaR047 was the first cDNA clone encoding a sulfotransferase to be characterized from *A. thaliana* (Lacomme and Roby, 1997). This work was particularly important since *A. thaliana* is the model species in plant molecular biology. The results of the *A. thaliana* genome project, which is essentially completed, led to the identification of 13 sulfotransferase-coding genes in this plant. In order to simplify the nomenclature of *A. thaliana* sulfotransferase-coding genes, *RaR047* was renamed *ATST1* (Marsolais et al., 2000). *ATST1* was isolated from a cDNA library constructed from cell cultures of *A. thaliana* inoculated with an avirulent strain of *Xanthomonas campestris*. *ATST1* was found to encode a protein having 302 amino acids with an overall identity of 52% with the previously characterized flavonol sulfotransferases from *Flaveria* species. No attempt was made in the original report to demonstrate the sulfotransferase activity or to define the substrate specificity of ATST1.

Studies on the developmental regulation of *ATST1* expression showed that it is expressed in the aerial parts of young seedlings. This pattern of expression is not unique to *ATST1* and was also observed for the flavonol sulfotransferase genes in *F. bidentis* (Hannoufa et al., 1991). The expression of *ATST1* was induced in response to salicylate

and pathogen infection. Furthermore, the expression of *ATST1* was induced preferentially in response to avirulent pathogens as compared with virulent strains. This pattern of expression is similar to that of previously characterized Pathogenesis-Related (PR) genes (Ward et al., 1991), and suggests that *ATST1* might play a role in the plant defense response to pathogen infection.

Mammalian soluble sulfotransferases

During the 1970's, biochemical characterization of mammalian soluble sulfotransferases revealed the presence of three families of enzymes, the phenol (PST), estrogen (EST) and hydroxysteroid sulfotransferases (HSST), which were classified according to their substrate specificities. PSTs were considered to be involved in the sulfonation of phenolic xenobiotics, while ESTs and HSSTs were shown to catalyze the sulfonation of the phenolic hydroxyl of estrogens and alcoholic hydroxyls of hydroxysteroids, respectively. The partial overlap in substrate specificities observed between these groups of enzymes has slowed down considerably the progress in animal sulfotransferase research during the 1970's and 80's. In the last ten years, molecular cloning of cDNAs coding for soluble sulfotransferases in mammals has allowed the development of a molecular classification of sulfotransferases based solely on the comparison of their amino acid sequences (Weinshilboum et al., 1997). Mammalian soluble sulfotransferases are now classified within two families, the PSTs and HSSTs, with the PSTs being divided in two sub-families, the PSTs and ESTs. The availability of cDNA clones has allowed the characterization of the kinetic properties of recombinant sulfotransferases expressed in bacterial or mammalian cells, so that we have a much clearer view of the biochemical function of these enzymes.

Mammalian sulfotransferases are very important enzymes for the detoxication of drugs and xenobiotics (Mulder and Jakoby, 1990). The transfer of sulfonate increases the aqueous solubility of hydrophobic molecules and facilitates their excretion. In comparison with other Phase II detoxication enzymes such as the UDP-glucuronosyltransferases, sulfotransferases are characterized by their high affinity for the acceptor substrates, so that they represent the first line of defense against foreign compounds in the organism. In addition to the detoxication function, mammalian STs are involved in the metabolic pathways of steroid and thyroid hormones, as well as catecholamine neurotransmitters (Strott, 1996, Visser et al., 1994, Darras et al., 1999, Eisenhofer et al., 1999).

Function of mammalian steroid sulfotransferases

Mammalian HSSTs have a broad substrate specificity. The recombinant human HSST catalyzes the transfer of sulfonate to position 3 of hydroxysteroids and estrogens, although testosterone is sulfated at position 17 with a comparable affinity (Falany, 1997). This enzyme also accepts cholesterol as substrate, as well as a large variety of free and amino acid-conjugated bile acids. There is a lot of controversy regarding the specificity of ESTs *in vivo*. Although the recombinant human EST catalyzes the transfer of sulfonate to dehydroepiandrosterone, pregnenolone and phenolic xenobiotics, estrone and 17ß-estradiol are converted with a thousand-fold more affinity, with K_ms in the nanomolar range (Falany, 1997). Similar kinetic parameters were determined for the guinea pig recombinant EST (Tomizuka et al., 1994). The low K_m values suggest that ESTs can compete efficiently with the estrogen receptor for hormone binding *in vivo*. In addition, the nuclear localization reported for EST in guinea pig adrenal cells suggests that this

enzyme is responsible for the inactivation of estrogens at their site of action (Whitnall et al., 1993). Similar results were obtained on the subcellular localization of EST in rat liver (Mancini et al., 1992).

The function of HSSTs and ESTs has been best characterized in rats and humans (Runge-Morris, 1997, Falany, 1997). In the adult rat liver, the expression of steroid sulfotransferases is regulated according to age and sex, and is consistent with a function in sex steroid hormone inactivation. The expression of one isoform of HSST is high in female. In the adult male, on the contrary, a pattern of high EST expression is observed. The male pattern of HSST and EST expression is also influenced by age, and is correlated with shifts in androgen sensitivity, that are dependent on the levels of the androgen receptor (Chatterjee et al., 1994). The first phase, which occurs before puberty, is characterized by a relative insensitivity to androgens, with a high expression of HSST and a low EST expression. With the onset of puberty, this pattern is reversed. During ageing, there is a constant increase in HSST expression, accompanied by a decrease in EST expression. The cDNA encoding the female-predominant HSST was originally isolated as a marker of senescence in the male rat liver and designated SMP-2 (senescence marker protein-2) (Song et al., 1990).

The rat liver HSST gene is the object of complex regulation. A predominant factor governing its expression is the sex-specific pattern of growth hormone secretion by the pituitary (Labrie et al., 1994). The HSST gene is also negatively regulated by androgens and positively regulated by estrogens (Labrie et al., 1994). The impact of androgen sulfonation by HSST on the expression of the androgen responsive genes has been studied in cell co-transfection assays. In this system, it was demonstrated that androgen

sulfonation results in a decrease in the transcription of androgen responsive genes (Chan et al., 1998).

The physiogical consequences of deregulated liver EST and HSST expression have been studied. In rat liver, the balance of active androgens and estrogens has a critical impact on glucose homeostasis. The rat HSST promoter contains a perfect insulin-responsive element, suggesting that this gene may be positively regulated by insulin (Runge-Morris, 1997). Alterations in liver steroid sulfotransferase expression have been studied extensively in rodent models of chemically and genetically-induced diabetes (Runge-Morris, 1997). In both models, there is a masculinization of steroid sulfotransferase expression in females, leading to a high expression of EST and low expression of HSST. In chemically-induced diabetes, a model for insulino-dependent diabetes, this trend is reversed to normal by insulin treatment (Runge-Morris and Vento, 1995).

In humans, expression of EST in the endometrium is positively regulated by progesterone (Hobkirk, 1993, Falany and Falany, 1996). Thus, up-regulation of EST during the luteal phase of the menstrual cycle decreases the proliferative stimulus of 17ß-estradiol on the endometrium. EST is also highly expressed in testis, where it probably protects this tissue from estrogen action (Song et al., 1995). The function of EST in estrogen inactivation has been demonstrated in breast cancer cell transfection assays (Falany and Falany, 1997, Qian et al., 1998). Expression of EST reduced the expression of an estrogen-responsive reporter gene, as well as estrogen-induced cell proliferation, suggesting that EST expression in normal breast epithelium is important for modulating the levels of biologically active estrogens. Another type of evidence pointing to a critical

role of EST in estrogen inactivation comes from the recent discovery that the estrogenic effects of PCBs may be mediated through the inhibition of ESTs (Kester et al., 2000). These recent results help to explain the effects of so-called "environmental estrogens" having a very low affinity for the estrogen receptors.

In addition to its role in hormone inactivation, steroid sulfonation allows the transport of steroid hormones from their site of biosynthesis to their site of action. Sulfated precursors can be further metabolized and bioactivated through the removal of sulfate catalyzed by steroid sulfatases. A distinguishing feature of humans as compared with other mammalian species is the presence of a high concentration of dehydroepiandrosterone sulfate in the serum. Dehydroepiandrosterone sulfate is synthesized by HSST in the adrenal gland, and may serve as a precursor for the biosynthesis of biologically active steroid hormones. In pregnant women, the high levels of estrogen sulfates present in the serum are derived from dehydroepiandrosterone sulfate that is secreted from the fetal adrenal and transformed in the placenta (Hobkirk, 1985).

In summary, collective evidence supports the hypothesis that ESTs and HSSTs are involved in estrogen and androgen inactivation, respectively. In the few examples that have been well characterized, the steroid sultransferases act to prevent hormone action in specific tissues. This mechanism is generally coupled with a state of hormone insensitivity, determined by a low level of the corresponding steroid receptor. Up-regulation of steroid sulfotransferases by steroid hormones other than their substrates represents a simple mechanism to prevent antagonistic hormone activity, such as in the induction of HSST by estrogens in female rat liver, or the induction of EST by progesterone in human endometrium. Finally, diseases or exposure to environmental

pollutants may lead to an aberrant regulation of steroid sulfotransferase expression or activity, having potential physiological consequences.

STEROIDS IN PLANTS

Brassinosteroids

The discovery of brassinosteroids originated with the finding that *Brassica napus* pollen extracts could stimulate the growth of the bean's second internode, and that this activity was distinct from those of other plant hormones such as gibberellins. In the 1970's, a program was initiated by the United States Department of Agriculture to identify the active agent from rape pollen. This effort culminated in 1978 when 10 mg of brassinolide was purified from 40 kg of bee-collected pollen, and its structure elucidated (Grove et al., 1979, Mandava et al., 1988). Brassinolide, the most biologically active brassinosteroid, is a C28 steroid with an unusual lactone ring B. Today, more than forty naturally occurring brassinoteroids have been identified from a wide range of species (Fujioka, 1999). They are considered to be ubiquitous in the plant kingdom, and have been detected in all aerial parts of plants. The biological activities of brassinosteroids have been studied in detail (Sasse, 1999).

Despite these discoveries, the importance of brassinosteroid hormones was not recognized by the plant research community until about four years ago. At that time, spectacular results were obtained with mutants of *A. thaliana* affected in brassinosteroid biosynthesis. The *det2* (*de-etiolated2*) and *cpd* (*constitutive morphogenesis and dwarfism*) mutants were originally isolated by screening for alterations in the regulation of morphogenesis by light. These mutants display similar phenotypes (Chory et al., 1991, Szekeres et al., 1996). Under dark conditions, they show an aberrant expression of light-

regulated genes and are de-etiolated. However, the mutations have pleiotrophic effects, including reduced male fertility, reduced apical dominance, and extreme dwarfism due to reduced cell size. The brassinosteroid-deficient mutants exhibit a delay in leaf and chloroplast senescence and flowering, suggesting that brassinosteroids are involved in overall maturation (Li and Chory, 1996). In the last few years, knowledge of the physiological actions, and of the genetic targets of brassinosteroid regulation has expanded considerably. The availability of brassinosteroid-deficient mutants has allowed for a better understanding of brassinosteroid biosynthesis, and of its regulation. The molecular genetic approach has also led to the characterization of a protein required for the brassinosteroid response and signal transduction.

Physiological effects of brassinosteroids

In early brassinosteroid research, biological activity was studied using a large number of bioassays (Mandava, 1988). Brassinosteroids show complex interactions with other phytohormones and environmental conditions. In general, their effects are synergistic with auxins, additive with gibberellins, and can be inhibited by abscisic acid. Sasse recently hypothesized that brassinosteroids "might be involved in the control of the overall form of the plant and its modification by environmental signals" (Sasse, 1999). Among other effects, brassinosteroids increase cell elongation, inhibit root growth, stimulate leaf unrolling and pollen tube growth, influence the differentiation of the vascular system and stimulate ethylene production. In recent years, molecular approaches have yielded a much clearer picture of the physiological functions of brassinosteroids. In this section the main aspects of this subject will be reviewed.

Cell elongation. One of the primary functions of brassinosteroids is the promotion of cell elongation, as confirmed by the phenotypes of brassinosteroid-deficient mutants. Brassinosteroid-induced elongation involves changes in microtubule organization and modification of the cell wall (Mayumi and Shibaoka, 1995, 1996, Tominaga et al., 1994). The effect of brassinosteroids on cell elongation is mediated by increasing the expression of specific genes involved in this process. The first identified gene target of brassinosteroid regulation was *BRU1* (*BRassinosteroid Upregulated 1*) of soybean (Zurek and Clouse, 1994). This gene encodes a xyloglucan endotransglycosylase (XET) (Oh et al., 1998), an enzyme involved in cell wall rearrangements during cell expansion. The *TCH4* gene of *A. thaliana* and *LeXET* gene of tomato are other XET genes regulated by brassinosteroids, although they are also regulated by auxin as well as environmental conditions, such as touch, darkness and heat and cold shock (Xu et al., 1995, Catala et al., 1997). Genes encoding expansins and ß-tubulin are also positively regulated by brassinosteroids (Clouse, 1997, Muñoz et al., 1998).

In addition to gene regulation, brassinosteroids may directly stimulate enzyme activities involved in cell expansion. The *det3* mutant, like *det2*, is de-etiolated in the dark. Unlike *det2* however, *det3* has a reduced sensitivity to brassinosteroids (Szekeres et al., 1996). The *DET3* gene encodes the C-subunit of V-ATPase, an enzyme localized in the cell and vacuolar membranes (Schumacher et al., 2000). The C-subunit is responsible for the formation of an active holoenzyme. V-ATPase activity is required for brassinosteroid-dependent cell elongation, which is consistent with earlier physiological studies demonstrating that brassinosteroids stimulate the activity of membrane-bound ATPases (Cerana et al., 1983, 1984, Katsumi, 1985).

Root inhibition. The effects of brassinosteroids on root growth are variable, depending on light and other growth conditions, but are generally inhibitory at concentrations of 0.1 to 1 μM (Roddick and Guan, 1991, Sasse, 1999). This phenomenon is well known for other growth-promoting hormones such as auxins and gibberellins. The inhibitory effect of brassinosteroids on root growth has been well characterized in *A. thaliana* seedlings (Clouse et al., 1993), and this property has been used to isolate brassinosteroid-insensitive mutants (Clouse et al., 1996, Li and Chory, 1997).

Vascular differentiation. Brassinosteroids are required for xylem differentiation. Application of brassinosteroids to mesophyll cell cultures of *Zinnia elegans* promotes tracheary element formation. In addition, inhibition of gibberellin and brassinosteroid biosynthesis by the inhibitor uniconazole blocks the differentiation of tracheids. Application of brassinosteroids, but not of gibberellins, allows the differentiation of tracheids in uniconazole-treated cells (Iwasaki and Shibaoka, 1991). Brassinosteroids are required more specifically for the third and last stage of tracheary element differentiation in *Z. elegans*. During this stage, genes involved in lignin production are induced, such as the phenylalanine ammonia-lyase and cinnamate 4-hydroxylase genes, and secondary growth predominates. Treatment with uniconazole blocks the induction of genes specific to the third stage of differentiation. Rescue with brassinosteroids allowed the induction of those genes as well as lignification (Yamamoto et al., 1997). The role of brassinosteroids in xylem differentiation is supported by observations made on the brassinosteroid-deficient mutant *cpd* (Szekeres et al., 1996). In this mutant, the balance of phloem and xylem tissues is modified, with a predominance of phloem over xylem.

Light control of development. Brassinosteroids are involved in the control of development by light. Brassinosteroid-deficient and brassinosteroid-insensitive mutants of *A. thaliana* are de-etiolated in the dark and express light-regulated genes. A similar phenotype is observed in the brassinosteroid-deficient *dumpy* and brassinosteroid-insensitive *curly* mutants of tomato (Koka et al., 2000). In contrast, the de-etiolation phenotype is not observed in pea mutants (Nomura et al., 1997). Interestingly, plant physiologists noted long ago that brassinosteroids have no effect on etiolated plants (Mandava, 1988), suggesting that the brassinosteroid response was saturated under these conditions.

Brassinosteroids act downstream of phytochromes in regulating the etiolation response of *A. thaliana* (Chory and Li, 1997). The *BAS1* gene of *A. thaliana* has been recently isolated by screening for intergenic suppressors of the phenotype of the *phyB-4* mutant using activation tagging (Neff et al. 1999). Overexpression of *BAS1* rescues the hyper-elongated phenotype of the *phyB-4* mutant. *BAS1* encodes a C26-hydroxylase involved in brassinosteroid inactivation. These results provide further evidence that brassinosteroids are involved in the control of development by light. However, the mechanism by which this control is achieved still remains unclear. Chory et al. (1996) predicted that "light negatively regulates brassinosteroid synthesis or responsiveness in the hypocotyl, while simultaneously promoting brassinosteroid synthesis or responsiveness in leaf cells".

Expression of stress-regulated genes. The brassinosteroid-deficient *cpd* mutant of *A. thaliana* expresses very low levels of PR (Pathogenesis-Related) genes (Szekeres et al., 1996). In transgenic plants overexpressing *CPD*, these genes were significantly

induced. In addition, transcript levels of stress-related genes, such as chalcone synthase, alcohol dehydrogenase, and lipooxygenase were increased in the *cpd* mutant. These results raised the possibility that brassinosteroids act as positive regulators of defense-related genes, and as negative regulators of stress-related genes. The effects of the *cpd* mutation on stress-related and defense-related gene expression were hypothesized to be mediated by jasmonic acid, because of the induction of lipooxygenase. In plants overexpressing *CPD*, the increase in PR gene expression may be due to the production of reactive oxygen species by increased levels of CPD, a cytochrome P450-dependent monooxygenase. No further reports were made on these hypotheses.

Brassinosteroid biosynthesis

The brassinosteroid biosynthetic pathway is much more complex than that of mammalian steroid hormones, which are all derived from the simple precursors cholesterol and pregnenolone. Brassinosteroids are classified as C27, C28 and C29 steroids, depending on the number of carbons present in their chemical structure (Figure 1). The variable number of carbons comes from variations in the structure of the side-chain. Brassinosteroids are derived from seven different sterol precursors, depending on the structure of the side-chain present in the final end product (Yokota, 1997) (Figure 1). The C27 norbrassinosteroids, having no substituent at position 24, are derived from cholesterol. Within the C28 brassinosteroids, dolicholide, having a C24-methylene group, is synthesized from 24-methylenecholesterol. 24-Epibrassinolide, having a 24ß-methyl group is derived from 24-epicampesterol (in the stereochemistry of the steroid side chain, groups behind the plane are in the ß position). Brassinolide, having a 24α-methyl group, is derived from campesterol. Within the C29 brassinosteroids, 28-homobrassinolide,

having an ethyl group at position 24, is synthesized from sitosterol, and 28-homodolicholide, having a 24-ethylidene group, is synthesized from isofucosterol. Finally, 25-methyldolicholide, having 24-methylene and 25-methyl groups, is synthesized from 25-methyl-24-methylenecholesterol.

The biological significance of the structural diversity in the side-chain of brassinosteroids is poorly understood. Brassinosteroids derived from campesterol are the most common and have been detected in all plants examined. However, most species accumulate at least two types of brassinosteroids having different side-chain structures. The capacity to accumulate brassinosteroids with diversified side-chain structures appears to be dependent on the tissue where they are synthesized. In shoots of *Phaseolus vulgaris*, only the campesterol-related brassinosteroids could be detected, whereas in seeds, brassinosteroids are derived from at least five different sterol precursors (Yokota, 1999).

A pathway for the biosynthesis of brassinolide from campesterol has been proposed from studies on the metabolism of radiolabelled intermediates in *Catharantus roseus* cell culture (Yokota, 1997). However, recent results demonstrated the occurrence of an alternative biosynthetic pathway, which involves a late C6 oxidation step (Figure 2). In this pathway, 6-deoxocastasterone is oxidized at C6 to form castasterone, the immediate precursor of brassinolide. The early and late C6 oxidation pathways are probably operational in most higher plants (Sakurai, 1999, Fujioka, 1999).

The characterization of brassinosteroid-deficient mutants has allowed a better understanding of brassinosteroid biosynthesis. The phenotypes of these mutants can

generally be rescued with exogenous brassinosteroids. By testing metabolic intermediates in the pathway, the specific step that is affected can sometimes be identified. These experiments have been coupled to the analysis of endogenous brassinosteroids in the mutants, sometimes after feeding of precursors labelled with stable isotopes. Accumulation of specific intermediates is also informative about the blockage in biosynthesis. For reasons of clarity, the biosynthetic pathway will be presented in two sections. The first section describes the biosynthesis of the sterol precursors. The second one describes the biosynthesis of brassinosteroids from sterol precursors.

From cycloartenol to sterol precursors. Brassinosteroids are terpenoid molecules, synthesized from mevalonic acid and squalene. The first committed step in sterol biosynthesis is the C24-methylation of cycloartenol. A number of brassinosteroid biosynthetic mutants have been isolated which are defective in the steps leading from cycloartenol to campesterol, the precursor to the most common C28 brassinosteroids. The *dwf1/dim1* (*diminuto1*), *dwf5* and *dwf7* mutants, like other brassinosteroid-deficient mutants, are dwarf and de-etiolated in the dark, and can be rescued by brassinosteroids. *DWF7* encodes a Δ^7-sterol C5 desaturase involved in the formation of the double bond between carbons 5 and 6, during the conversion of episterol to 5-dehydroepisterol (Choe et al., 1999b) (Figure 3). *DWF5* encodes a sterol Δ^7-reductase involved in the following step, the conversion of 5-dehydroepisterol to 24-methylenecholesterol (Choe et al., 2000). The DWF5 protein shares significant sequence homology with mammalian sterol Δ^7-reductases. However, the phenotype of the *dwf5* mutant was not rescued by the expression of the human gene.

dwf1 was the first described T-DNA tagged mutant in plants (Feldmann, 1989). A second allele, *dim1* was subsequently characterized (Takahashi et al., 1995). However, the function of the gene was unclear until it was proposed to encode a FAD-dependent oxido-reductase (Mushegian and Koonin, 1995). *DWF1* encodes a sterol $\Delta^{24(28)}$isomerase/C24 reductase which catalyzes the reduction of the 24-methylene group during the conversion of 24-methylenecholesterol to campesterol (Figure 3) (Klahre et al., 1998, Choe et al., 1999a). The enzyme is also active in the reduction of isofucosterol to sitosterol, and the reaction involves a $\Delta^{24,25}$ sterol intermediate (Klahre et al., 1998). A deficiency of the C24 reductase in the *lkb* mutant of pea also leads to a dwarf phenotype (Nomura et al., 1999).

Very recently, two genes involved in earlier steps of sterol biosynthesis have been characterized. The *SMT1* gene of *A. thaliana* encodes a sterol C24-methyltransferase, involved in the conversion of cycloartenol to 24-methylenecycloartenol (Figure 3) (Diener et al., 2000). SMT1 also catalyzes, although with less efficiency, the C28-methylation of 24-methylenelophenol to form a 24-ethylidene sterol, leading to the production of isofucosterol and sitosterol. The *FACKEL* gene encodes a C14-sterol reductase which catalyzes the conversion of a $\Delta^{8,14}$ sterol to 4α-methylfecosterol (Figure 3) (Jang et al., 2000, Schrik et al., 2000). In contrast with brassinosteroid biosynthetic mutants, *smt1* and *fackel* mutants cannot be rescued by exogenous brassinosteroids, although the *fackel* mutant has reduced brassinosteroid levels (Jang et al., 2000). The *smt1* and *fackel* mutants have multiple defects, including aberrant cell division and expansion, and improper organization of the embryo. These results suggest that sterol

molecules, which may be produced downstream from the C14-reductase step, but before the Δ^7 C5 desaturase step, act as signals during plant development.

From campesterol to brassinolide. The brassinosteroid biosynthetic pathway involves the addition and epimerization of several hydroxyl groups that are absolutely required for biological activity (Figure 2). After hydroxylation steps of the steroid side-chain at positions 22 and 23, the 3ß-hydroxyl group is epimerized to the 3α position during the conversion of teasterone to typhasterol. Typhasterol is then transformed via 2α-hydroxylation resulting in the formation of castasterone, the immediate precursor of brassinolide. The stereochemistry of the hydroxyls on ring A is equally important for biological activity (Mandava, 1988).

The study of brassinosteroid-deficient mutants has allowed the characterization of four steps in the pathway. The *DET2* gene was isolated by positional cloning and complementation of the *det2* phenotype (Li et al., 1996). The predicted DET2 protein was found to be approximately 40% identical with mammalian steroid 5α-reductases. The DET2 enzyme was initially hypothesized to convert campesterol into campestanol (Figure 2). Later on, the biochemical function of DET2 was characterized. The recombinant plant steroid 5α-reductase was found to accept androgens such as testosterone, epitestosterone and androsterone as well as progesterone and 17α-hydroxyprogesterone (Li et al., 1997). The enzyme did not accept compounds having a 3ß-hydroxyl group, suggesting that the endogenous substrate is a 3-oxo $\Delta^{4,5}$-campesterol. The kinetic parameters determined for the plant enzyme with mammalian steroids were highly similar to those of the human steroid 5α-reductases. Similarly, the human enzymes were found to be capable to act on the plant sterol substrate, since their

expression in the *det2* mutant rescued the dwarf phenotype. These results revealed a high degree of functional conservation between plant and animal steroid 5α-reductases.

The *det2* mutant accumulates 3-dehydro-$\Delta^{4,5}$-campestanol, and the DET2 enzyme is involved in the conversion of this intermediate to 3-dehydrocampestanol (Figure 2) (Fujioka et al., 1997, Noguchi et al., 1999a). The levels of campestanol in the *det2* mutant are about 10% of those in the wild-type (Fujioka et al., 1997). Brassinosteroids are reduced, but are not absent in the *det2* mutant, like in all other biosynthetic mutants examined so far (Sakurai, 1999). This suggests a certain level of genetic redundancy in the brassinosteroid biosynthetic pathway. The *A. thaliana* database contains at least one homologue of *DET2*, which has not yet been characterized (Li and Chory, 1999).

The *DWF4* gene of *A. thaliana* encodes a cytochrome P450 hydroxylase (CYP90A2) that catalyzes the 22-hydroxylation of campestanol to cathasterone (Figure 2) (Choe et al., 1998). The results of feeding experiments suggest that DWF4 is also enzymatically active with 6-deoxocampestanol. The *CPD* gene (*CYP90A1*) was found to encode a protein showing significant homology with mammalian cytochrome P450 steroid hydroxylases (Szekeres et al., 1996). Rescue of the *cpd* phenotype by application of various intermediates in brassinosteroid biosynthesis suggested that CPD could convert cathasterone to teasterone by inserting a hydroxyl group at position 23 (Figure 2). The *cpd* mutant phenotype is also rescued by application of 6-deoxoteasterone, suggesting that CPD also participates in the late C6 oxidation pathway (Clouse and Feldmann, 1999).

The tomato *DWARF* gene encodes a cytochrome P450 (CYP88) involved in the conversion of 6-deoxocastasterone to castasterone, the last step before the formation of

brassinolide (Figure 2) (Bishop et al., 1999). The Dwarf enzyme catalyzes two reactions, as suggested by the identification of the intermediate 6α-hydroxycastasterone. The first step involves the 6-hydroxylation of 6-deoxocastasterone, followed by oxidation to produce the 6-keto group of castasterone. In tomato, only the late C6 oxidation pathway was found to operate in brassinosteroid biosynthesis.

Biosynthetic origin of 24-epibrassinosteroids. 24-Epibrassinosteroids are generally considered as minor brassinosteroids, as compared with those derived from campesterol. However, their content is variable, and they sometimes are the most abundant, depending on the plant species and tissue analyzed (Fujioka, 1999). Within species of the Brassicaceae family, 24-epibrassinolide has been detected in seeds of *A. thaliana* (Schmidt et al., 1997). Knowledge of 24-epibrassinosteroid biosynthesis is limited as compared with that of their 24-epimers. The biochemical analysis of 24-epibrassinosteroids in brassinosteroid biosynthetic mutants has not been performed so far, and the activity of individual biosynthetic enzymes has not been tested with these metabolites. However, the enzymes of the brassinosteroid biosynthetic pathway, like most enzymes of the sterol biosynthetic pathway, are probably not stereospecific for the steroid side chain, since end products with a large structural diversity in this part of the molecule accumulate in plants. The late and early C6 oxidation pathways both seem to operate in 24-epibrassinosteroid biosynthesis, as suggested by the co-occurence of 24-epicastasterone and 6-deoxo-24-epicastasterone in serradella (*Ornithopus sativus)* (Schmidt et al., 1993).

24-Epibrassinosteroids are derived from a distinct sterol precursor, 24-epicampesterol, as compared with their 24-epimers which are derived from campesterol.

Catharanthus roseus cell cultures fed with labelled mevanolate synthesize a mixture of campesterol and 24-epicampesterol in a 4 to 1 ratio (Suzuki et al., 1995a). In the sterol biosynthetic pathway, 24-epicampesterol is synthesized from cyclolaudenol and cyclosadol, whereas campesterol, as well as the other sterol precursors of brassinosteroids, with the exception of cholesterol, are derived from 24-methylene cycloartenol (Benveniste, 1986, Yokota, 1997). Cyclolaudenol, cyclosadol and 24-methylene cycloartanol are synthesized by distinct C24-methyltransferases acting on cycloartenol (Figure 4) (Nes et al., 1991, Guo et al., 1996). The C24-methyltransferases responsible for the biosynthesis of cyclolaudenol and cyclosadol have yet to be characterized.

The side-chain structures present in the 24-epibrassinosteroid intermediates imply that DWF1, which catalyzes the $\Delta^{24,28}$ isomerization and $\Delta^{24,25}$ reduction of sterols, is not active in the biosynthesis of 24-epicampesterol. The biosynthetic capacity to synthesize 24α-substituted sterols such as campesterol through the stereospecific reduction of a $\Delta^{24,25}$ sterol represents a phylogenetic marker separating plants from fungi (Nes, 1990). This pathway allows the generation of sterols with diversified side-chain structures, in comparison with the pathway leading to 24ß-methyl sterols. In the mutants *lkb* of pea and *dwf1* of *A.thaliana*, the biosynthesis of campesterol and sitosterol is blocked specifically, and these mutants accumulate 24-methylenecholesterol and isofucosterol (Noguchi et al., 1999, Klahre et al., 1999).

Regulation and site of brassinosteroid biosynthesis. The *DET2* gene is expressed constitutively in all parts of the plant (Li and Chory, 1997). In comparison, the side chain hydroxylation steps catalyzed by DWF4 and CPD seem to be the major targets of

regulation of the brassinosteroid biosynthetic pathway. 22-Hydroxylation catalyzed by DWF4 appears to be the rate-limiting step. Several lines of evidence support this hypothesis. *DWF4* is expressed at very low levels in *A. thaliana*, as compared with other biosynthetic genes (Choe et al., 1998). In addition, brassinosteroid-deficient mutants affected in steps before 22-hydroxylation can be rescued by synthetic analogs bearing a 22-hydroxyl group (Choe et al., 2000). For example, while the *dwf5* mutant cannot be rescued by campesterol, the application of 22-hydroxycampesterol rescues the mutant phenotype, suggesting that 22-hydroxylation is rate-limiting in the pathway. Finally, the endogenous levels of cathasterone are 500-fold lower as compared with those of 6-oxocampestanol (Sakurai, 1999).

The step of 23-hydroxylation catalyzed by CPD is under negative feedback regulation at the transcriptional level by end products of the biosynthetic pathway (Mathur et al., 1998). This type of feedback regulation has been well characterized for enzymes of the gibberellin biosynthetic pathway, the GA 20-oxidase and 3ß-hydroxylase (Hedden and Kamyia, 1997). Histochemical staining of *A. thaliana* transgenic plants expressing *CPD* promoter-*GUS* fusions revealed that the gene is expressed in leaves and cotyledons, but not in actively elongating cells of the roots and hypocotyls, the target tissues of brassinosteroid action (Mathur et al., 1998). In contrast, the *DWARF* gene of tomato, involved in a late step in biosynthesis, was reported to be expressed in elongating tissues, suggesting that brassinosteroid precursors are transported and activated in their target tissues (Lenton, 1998).

Metabolism of brassinosteroids

Sulfate conjugates of brassinosteroids have not been characterized from plants. However, feeding experiments with ^{3}H-castasterone in mung bean explants showed that two types of conjugates were formed (Yokota et al., 1991). A minor part were glucosides, but most conjugates were polar and non-glycosidic, since the aglycone could not be released by enzymatic hydrolysis. In a similar experiment with ^{3}H-brassinolide, a reverse pattern of conjugate formation was observed. The major part consisted of a glycoside, 23-*O*-ß-D-glucopyranosylbrassinolide (Suzuki et al., 1993). During feeding experiments with rice seedlings, non-glycosidic water-soluble metabolites of ^{3}H-castasterone and ^{3}H-brassinolide were the major conjugates formed (Yokota et al., 1992). Based on their chromatographic behavior and susceptibility to solvolysis, it was proposed that they might be sulfate esters. The results of experiments with mung bean suggest that, in some species, brassinolide is transformed predominantly into glucosyl conjugates, whereas intermediates are metabolized into polar conjugates. In order to characterize brassinosteroid sulfate conjugates, new methods will be needed for their purification that are compatible with the retention of a labile sulfate group. In particular, the methods presently used for derivatization before mass spectroscopy are not suitable for the analysis of sulfated metabolites (Takatsuto and Yokota, 1999).

Several glucosyl and 3-acyl conjugates of brassinosteroids have been identified from various plant species (Adam and Schneider, 1999). Brassinosteroid metabolism has been studied by feeding labelled precursors to whole plants and excised tissues. Cell cultures have proven to be highly useful for this type of study, since metabolism by bacteria or fungi can be ruled out in these experiments. Metabolic sequences have been

worked out in tomato and serradella cell cultures (Adam and Schneider, 1999). From the results of these studies a few general principles can be deduced. Epimerization at position 3 is a common event in the metabolism of 24-epicastasterone (Figure 5). This reaction is also known to occur in rice, tobacco and pea, and results in biological inactivation (Suzuki et al., 1995b, Kim, 1991). The epimerization proceeds via an oxidation/reduction mechanism (Hai et al., 1996). The opposite reaction occurs during the conversion of teasterone to typhasterol (Suzuki et al. 1994, Griffiths et al. 1995) and of 24-epiteasterone to 24-epityphasterol (Kolbe et al., 1998). Epimerization of brassinosteroids to the 3ß configuration can be followed by three types of reactions, but is an absolute prerequisite of glucosyl or acyl conjugations of the hydroxyl group at this position (Kolbe et al., 1998). 24-Epiteasterone and teasterone are directly metabolized into 3-glucosyl conjugates in tomato and lily cell cultures, respectively (Kolbe et al., 1997, 1998, Soeno et al., 2000).

In cell cultures of serradella, 3, 24-diepicastasterone and 3, 24-diepibrassinolide are further metabolized by side-chain degradation, resulting in the production of pregnane metabolites (Figure 5) (Kolbe et al., 1994, 1995). Pregnanes are known to occur in plants as precursors of cardenolides (Nes and McKean, 1997, Deepak et al., 1989). The side-chain degradation involves the formation of an intermediate bearing a 20-hydroxyl group. This mechanism of side-chain degradation also occurs in the metabolism of 20-hydroxyecdysone in crustaceans (Lachaise and Lafont, 1984), as well as in the formation of pregnenolone from cholesterol in mammals (Sugano et al., 1996). In mammals, this reaction is catalyzed by the cholesterol side chain cleavage P450 ($P450_{scc}$), and involves

successive hydroxylations at positions 22 and 20, followed by cleavage of the bond between C20 and C22.

In addition to their metabolism by conjugation of a 3ß-hydroxyl group or side-chain degradation, brassinosteroids can undergo 25- or 26-hydroxylation followed by the formation of glucosyl esters (Figure 5). 25-Hydroxy-3, 24-diepibrassinolide has been identified as a metabolite of 24-epibrassinolide in serradella cell culture (Kolbe et al., 1996). In tomato cell culture, 24-epicastasterone and 24-epibrassinolide are hydroxylated at position 25 or 26, without prior inversion of configuration at position 3 (Schneider et al., 1994, Hai et al., 1995, 1996). These reactions of side-chain hydroxylation are followed by glucosylation of the new hydroxyl group. Hydroxylation is rate-limiting in the formation of the glucoside products (Hai et al., 1996).

Application of brassinolide or 24-epibrassinolide to tomato cell cultures resulted in an increase of the 25- and 26-hydroxylase activities, that was dependent on protein synthesis, suggesting an effect on gene expression (Winter et al., 1997). In contrast, enzyme activity was not increased in response to typical plant cytochrome P450 inducers such as ethanol, $MnCl_2$ and phenobarbital. The 25- and 26-hydroxylases are induced by their substrates, a mechanism of regulation that provides a tight control of the levels of biologically active brassinosteroids. Substrate-dependent induction of catabolic enzymes, also referred to as "feedforward regulation", has been demonstrated for GA 2-oxidase (Thomas et al., 1999), and ABA 8'-hydroxylase (Windsor and Zeevart, 1997), and seems to be a common mechanism for the regulation of plant hormone levels.

The recent characterization of *BAS1* from *A. thaliana*, encoding a cytochrome P450 (CYP72B1) which catalyzes the 26-hydroxylation of brassinolide, has demonstrated

the function of 26-hydroxylation in hormone inactivation (Neff et al., 1999). Overexpression of *BAS1* in transgenic *A. thaliana* and tobacco resulted in a dwarf phenotype similar to that of biosynthetic mutants. These transgenic plants showed reduced levels of 6-deoxocastasterone, castasterone and brassinolide, but accumulated 26-hydroxybrassinolide. The *BAS1* gene is expressed constitutively in rosette leaves and hypocotyls. Regulation in response to brassinosteroids has not been reported.

Brassinosteroid signal transduction

Screening for brassinosteroid-insensitive mutants of *A. thaliana* in several laboratories resulted in the isolation of more than 25 alleles of the same locus (Clouse and Feldmann, 1999). The *Brassinosteroid-Insensitive 1* gene (*BRI1*) encodes a leucine-rich repeat receptor protein kinase (Li and Chory, 1997), that may act as a brassinosteroid receptor. BRI1 has an extracellular domain containing the leucine-rich repeat, a membrane-spanning domain, and an intracellular kinase domain. As compared with other plant homologs, BRI1 contains a unique 70 amino acid island within the leucine-rich repeat, that may function as a ligand binding domain. However, brassinosteroid binding to the extracellular domain of BRI1 has not been reported (Schumacher and Chory, 2000). Preliminary data on *BRI1* expression indicated that the gene is expressed at constant levels in all tissues (Li and Chory, 1997). These results suggest that the brassinosteroid response is not modulated at the level of hormone sensitivity. *bri1* mutants, as well as the brassinosteroid-insensitive mutant *lka* of pea, accumulate high levels of brassinosteroids, as compared with wild-type plants (Noguchi et al., 1999b, Nomura et al., 1999). Therefore, BRI1 is required for the proper regulation of

brassinosteroid biosynthesis, and probably participates in the feedback regulation of *CPD*.

A very elegant approach was used to test the function of BRI1 in the brassinosteroid response. The extracellular and transmembrane domains of BRI1 were fused to the cytoplasmic serine/threonine kinase domain of XA21, a rice disease resistance receptor (He et al., 2000). Rice cells expressing this chimeric receptor activate the plant defense response against pathogen infection when supplied with exogenous brassinosteroids. This experimental system will be useful to test putative ligands for the plant orphan leucine-rich repeat receptor kinases.

The discovery of BRI1 has important evolutionary implications for the comparison of the mode of action of steroid hormones across phyla. The steroid receptors of insects and mammals are part of the superfamily of nuclear receptors (Thummel, 1995, Beato et al., 1995). One of the main conclusions drawn from the results of the *A. thaliana* genome project is the absence of genes coding for nuclear receptors. Although steroid hormones have been conserved in evolution to regulate growth, development and metabolic processes, their mode of action in plants and animals appears totally different.

Future progress in understanding brassinosteroid signal transduction will involve the identification of protein subtrates of the BRI kinase, which may include transcription factors required for the transcription of brassinosteroid-regulated genes, and proteins involved in the activation of the V-ATPase. New mutant screens, designed to isolate intergenic suppressors of the phenotypes of brassinosteroid-deficient mutants, or mutants that are resistant to inhibitors of brassinosteroid biosynthesis, may allow the identification of new components involved in signal transduction (Wang and Chory, 2000).

Estrogens and androgens

Extensive studies have been performed to detect the presence and evaluate the physiological effects of mammalian sex steroids in plants. However, very few plant physiologists are convinced that they constitute true classes of phytohormones. Their presence has been inferred from numerous phytochemical studies (Geuns, 1978). The results of these studies are considered questionable, because of the ambiguities associated with the methods that were used (Leighton Jones and Roddick, 1988). The failure to reproduce the results of some studies has sometimes been attributed to differences in growth conditions, or in the physiological condition of the material used for extraction. The presence of estradiol in flowering plants and seeds of dwarf *P. vulgaris* was detected by GC-MS (Young et al., 1977, 1978). In addition, androstenedione, dehydroepiandrosterone and testosterone were identified in the pollen of *Pinus nigra* by a combination of analytic TLC, UV spectrophotometry and fluorometry (Saden-Krehula et al., 1979, 1983).

Additional evidence supporting the presence of mammalian sex steroids in plants comes from the finding that they possess enzymes required for steroid interconversions and biosynthesis. *P. vulgaris* seedlings are able to synthesize estradiol from exogenous ^{14}C-mevalonic acid, and are able to convert exogenous estrone-sulfate and estrone into estradiol (Young et al., 1977). Interconversions of androgens have also been characterized in cell cultures of tobacco and *Dioscorea deltoidea*, and in *Cannabis sativa* plants (Geuns, 1978, Leighton-Jones and Roddick, 1988).

These results need to be interpreted carefully, considering the high degree of functional conservation between mammalian and plant steroid biosynthetic enzymes.

Nevertheless, two of the enzymes involved, the aromatase and 17ß-hydroxysteroid dehydrogenase, have yet to be characterized from plants. The functional conservation between mammalian and plant enzymes is not limited to the steroid 5α-reductase (Li et al., 1997). A Δ^5-3ß-hydroxysteroid dehydrogenase involved in the conversion of pregnenolone to progesterone was recently purified from *Digitalis lanata* (Finsterbusch et al., 1999). Progesterone is present in *Digitalis* species as a metabolic precursor to cardenolines (Nes and McKean, 1977, Deepak et al., 1989). The sequence of peptide fragments obtained from the purified Δ^5-3ß-hydroxysteroid dehydrogenase shows significant homology to hydroxysteroid dehydrogenases from bacteria and mammals (Finsterbusch et al., 1999). Since the enzymatic activity was detected in plant species devoid of cardenolides, the Δ^5-3ß-hydroxysteroid dehydrogenase was proposed also to be involved in the conversion of teasterone to 3-dehydroteasterone during brassinosteroid biosynthesis (Finsterbusch et al., 1999, Fujioka and Sakurai, 1997).

Since sex steroids are involved in the development of mammalian reproductive organs, plant physiologists studied the effects of these hormones on the sex determination of flowers. Löve and Löve (1945) conducted the most complete study. They observed that application of estrogens or androgens to the stem of *Melandrium dioecum* had a feminizing or masculinizing effect on the sex of flowers, respectively. Similar effects were later observed with other species. However, these results could not be reproduced with *Cucumis sativa, Cucurbita pepo* and *Spinacia oleracea* in a more recent study (Leighton Jones and Roddick, 1988).

A clue to the role of mammalian sex steroids in the sex determination of flowers may come from the study of the maize *TS2* (*Tasselseed2*) gene. A loss of function

mutation in this gene leads to the development of female instead of male tassel florets, because the female primordia fail to abort in the developing flower (Lebel-Hardenack and Grant, 1997). *TS2* has been cloned by transposon tagging (Delong et al., 1993). The predicted protein shows significant homology with the Δ^5-3β-hydroxysteroid dehydrogenase from *D. lanata* and bacterial hydroxysteroid dehydrogenases (Delong et al., 1993, Finsterbusch et al., 1999). The TS2 enzyme may be involved in the biosynthesis of a steroidal compound responsible for the abortion of female tissue in the floral meristem.

Another plant enzyme that may be involved in steroid metabolism is the product of the *ROT3* gene (*ROTUNDIFOLIA3)* of *A. thaliana* (Kim et al., 1998). The *ROT3* gene specifically regulates the length of leaves (Kim et al., 1998, 1999). *ROT3* encodes a cytochrome P450-dependent monooxygenase (CYP90C1). The closest relatives of ROT3 are CPD (cathasterone 23-hydroxylase), DWF4 (campestanol 22-hydroxylase) and DWARF (6-deoxocastasterone 6-hydroxylase/oxidase). It is clear that the field of plant steroid research is ripe for important discoveries, perhaps of new classes of steroid hormones. Progress in this field depends on the use of multiple approaches, combining the tools of molecular genetics, biochemistry and phytochemistry. It would be interesting to reassess the presence of estrogens and androgens in plants using state-of-the-art spectroscopic techniques.

MODIFICATION OF HORMONE LEVELS IN PLANTS

The modification of hormone levels in transgenic plants can provide a useful functional test for a gene involved in hormone biosynthesis or catabolism. The presentation of this subject will be limited to the classes of hormones where both

biosynthetic and catabolic genes have been tested. Pathogenic bacteria such as *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* modify the levels of auxin and cytokinin in host plants. This is achieved by insertion of genes into the host chromosomes. As a rule, the bacterial genes specify metabolic pathways that are different from those of the plants. This section will review the results of experiments performed with genes of plant origin.

Attempts to modulate hormone levels by sense or antisense expression of biosynthetic genes in transgenic plants have been sometimes of limited success. The levels of active hormones are often under tight and dynamic control. Therefore, metabolic compensation may counteract the effects of the transgene. In addition, hormones are sometimes synthesized by several parallel pathways. This redundancy allows biosynthesis to remain functional when one pathway is blocked. Within individual pathways, there are often multiple genes involved in a single step, making the isolation of knockout mutants difficult.

Overexpression and antisense expression of the brassinosteroid biosynthetic gene *DET2* has been reported to modifiy plant stature (Chory and Li, 1997, Li and Chory, 1999), although no experimental data were presented. In contrast, transgenic plants expressing high levels of *CPD* or *DWARF* transcript did not show growth enhancement, suggesting that enhanced biosynthetic enzyme activity may be compensated for by an increase in the activity of catabolic enzymes (Szekeres et al., 1996, Bishop et al., 1999). As mentioned earlier, overexpression of the *BAS1* gene in *A. thaliana* and tobacco led to a dwarf phenotype through a reduction of the levels of biologically active brassinosteroids (Neff et al., 1999). These results suggest that modifying the levels of

enzymes involved in hormone inactivation is a more efficient approach to manipulate hormone levels, as compared with similar experiments using biosynthetic enzymes.

Attempts to manipulate auxin levels were made by overexpression and antisense expression of nitrilase genes (*NIT1* and *NIT2*), involved in the formation of indole 3-acetic acid from indole 3-acetonitrile. Sense expression of *NIT2* in transgenic tobacco and *A. thaliana* had no effect on plant development, unless exogenous indole 3-acetonitrile was supplied, indicating that substrate availability was limiting in these experiments (Schmidt et al., 1996, Grsic et al., 1998). *A. thaliana* transgenic plants expressing antisense *NIT2* accumulated reduced levels of total indole 3-acetic acid, in free and conjugated forms (Grsic et al., 1998). However, the levels of free indole 3-acetic acid remained unchanged. In plants, most indole 3-acetic acid is found in the form of amino acid conjugates, and several genes coding for hydrolases of indole 3-acetic acid-amino acid conjugates have been isolated from *A. thaliana*. Overexpression of a gene encoding an indole 3-acetic acid-alanine hydrolase (*IAR3*) had no effect on plant morphology (Davies et al., 1999), however, the transgenic plants were more sensitive to root inhibition by the corresponding amino acid conjugate. A reverse effect was observed in the *iar3* mutant.

Overexpression of the *GA1* gene of *A. thaliana*, encoding *ent*-kaurene synthase A, which catalyzes the first committed step of gibberellin biosynthesis, did not result in a modification of plant growth (Sun and Kamiya, 1994). In this experiment, the lack of effect of the transgene could be due the limiting levels of *ent*-kaurene synthase B, since metabolic channeling probably occurs between the two synthases. Alternatively, the lack of effect could be explained by a down-regulation of later steps in biosynthesis. The last

steps of gibberellin biosynthesis, catalyzed by GA 20-oxidase and 3ß-hydroxylase, are under negative feedback regulation by biologically active gibberellins (Hedden and Kamiya, 1997). In *A. thaliana*, GA 20-oxidase is encoded by at least three genes, *At20ox1*, *At20ox2* and *At20ox3*, having different developmental expression (Phillips et al., 1995). Overexpression of GA 20-oxidase in transgenic *A. thaliana* resulted in longer hypocotyls, early flowering, longer stems and reduced seed dormancy, mediated by an increase in the content of GA_4, the main biologically active gibberellin in this species (Huang et al., 1998, Coles et al., 1999). Antisense expression of *At20ox1* resulted in a reduction of GA_4 levels and a semi-dwarf phenotype (Coles et al., 1999).

Other attempts of modifying gibberellin levels *in vivo* involved the expression in transgenic *A. thaliana* of a pumpkin cDNA encoding a GA20-oxidase which is involved in the formation of biologically inactive tricarboxylic acid gibberellins (Lange et al., 1994, Xu et al., 1999). Transgenic plants were only slightly dwarf, with a moderate reduction in GA_4 levels. The effects of the transgene were partially compensated by increases in the expression of the biosynthetic enzymes GA20-oxidase and 3ß-hydroxylase (Xu et al., 1999).

The enzyme GA2-oxidase, involved in gibberellin inactivation, is a very attractive target to manipulate gibberellin levels *in vivo*, and may help circumvent the difficulties associated with metabolic compensation (Thomas et al., 1999, Hedden and Phillips, 2000). In *A. thaliana*, GA 2-oxidases are encoded by at least three genes, *AtGA2ox1*, *AtGA2ox2* and *AtGA2ox3*, and in contrast to the GA 20-oxidase genes, *AtGA2ox1* and *AtGA2ox2* have a similar tissue distribution of expression (Thomas et al., 1999). GA2-oxidases catalyze the 2ß-hydroxylation/oxidation of gibberellic acids. In *A. thaliana*,

expression of GA2-oxidase is stimulated by biologically active gibberellins (Thomas et al., 1999). In the *slender* mutant of pea, a mutation in the GA 2-oxidase gene is responsible for the hyper-elongated phenotype, and increased content of the biologically active GA_1 (Lester et al., 1999, Martin et al., 1999). Overexpression of GA2-oxidase in transgenic *A. thaliana* has been reported to result in an extreme dwarf phenotype, although experimental data have not been presented (Hedden et al., 1999, Hedden and Phillips, 2000). So far, successful manipulation of hormone levels using plant catabolic enzymes has only been achieved with hydroxylases involved in hormone inactivation. The use of enzymes involved in hormone conjugation has yet to be reported.

PLANT RESPONSE TO STRESS

Plants respond to various stresses by physiological and metabolic adaptations, which involve changes in gene expression. The metabolic pathways of hormones are often affected, as well as hormone response and sensitivity. These aspects will be briefly discussed in relation with the plant defense response against pathogen infection, and the responses to ethanol and low oxygen stress.

Defense reponse against pathogen infection

The plant defense response to pathogen infection involves many different components. A very interesting aspect of this response is the development of an induced resistance in some plant-pathogen interactions (Ryals et al., 1996). The plant responds to an initial infection by a localized hypersensitive response, characterized by the production of reactive oxygen species such as H_2O_2 (Lamb and Dixon, 1997), and the development of lesions, through programmed cell death (Richberg et al., 1998), which limit the spread of the infection. Subsequently, the plant develops a systemic acquired

resistance to further infection by various pathogens. This response involves the systemic induction of PR genes, reinforcement of the cell wall and production of phytoalexins (Hammond-Kosack and Jones, 1996). Systemic acquired resistance is strictly dependent on interactions between specific plant disease resistance genes and corresponding avirulence genes from pathogens (Holt et al., 2000). In contrast, plant interactions with virulent pathogens do not lead to the establishment of systemic acquired resistance.

Systemic acquired resistance to various pathogens involves common signal transduction events, including the binding of a pathogen-derived signal to a receptor, an increase in intracellular calcium, the activation of protein kinase cascades, the generation of an oxidative burst, and the induction of defense genes (Scheel, 1998, McDowell and Dangl, 2000). In *A. thaliana*, the induction of defense genes is dependent on two distinct pathways, involving either salicylic acid (Shah and Klessig, 1999), or jasmonic acid and ethylene (Pieterse and van Loon, 1999). The increase in endogenous salicylic acid content is triggered by H_2O_2 produced during the hypersensitive response (Chamnongpol et al., 1998). Induction of the *PR-1*, *PR-2* and *PR-5* genes is dependent on salicylic acid (Ward et al., 1991), while induction of *PR-3* and *PR-4* is dependent on jasmonic acid (Thomma et al., 1998). Some PR genes encode enzymes, such as *PR-2* and *PR-3*, encoding ß-1-3-glucanases and chitinases respectively. The function of others such as *PR-1* and *PR-10* is completely unknown. In general, the function of PR genes has not been a major focus of research, but they have rather been used as molecular markers of the response to pathogen infection. The kinetics of induction of PR genes is rather slow for a molecular response, with peak levels of mRNAs at approximately 12 hours (Ward et al., 1991). However PR induction is sustained, and the time-frame of induction is relevant

to a physiological response to infection. Induction of PR genes is dependent on protein synthesis, suggesting that it constitutes a secondary response to salicylic acid.

Further research on salicylate-inducible gene expression has allowed the identification of genes that are induced earlier than PR-genes, within the first 30 minutes following salicylic acid treatment (Horvath et al., 1998). Genes encoding glutathione *S*-transferases (GSTs) are induced early in response to both H_2O_2 and salicylic acid (Chen et al., 1996, Xiang et al., 1996, Chen and Singh, 1999). The results of recent experiments using functional genomic approaches suggest that many more than the classical PR genes, in fact up to several hundred genes in *A. thaliana*, have altered levels of expression during pathogen infection. It has also become apparent that there is no universal mechanism of gene up-regulation during the defense response, and that the mode of transcriptional regulation may vary depending on the individual gene target.

Several plant genes inducible by salicylic acid, such as *GST* genes, contain an *activation-sequence-1*-related (*as-1*) element in their promoters (Ulmasov et al., 1995, Chen et al., 1996, Xiang et al., 1996, Chen and Singh, 1999). The *as-1* element is present in the CaMV35S promoter, and related elements are found in the promoters of other pathogen genes expressed in plants, such as the nopaline synthase and octopine synthase genes of *A. tumefaciens*. The *as-1* element is a target for transcriptional factors of the basic leucine zipper protein family, such as tobacco TGA2.2 (Niggeweg et al., 2000). In the absence of salicylic acid, the TGA1-related factor exists in the form of an inactive complex present in the cytosol. Treatment with salicylic acid results in the release from an inhibitory component and translocation to the nucleus, where the factor binds the *as-1* element (Jupin and Chua, 1996, Strange et al., 1997). Furthermore, an *as-1*-related

element present in the *PR-1* promoter is required for salicylate induction (Lebel et al., 1998). TGA factors have recently been demonstrated to interact with the NPR1 protein (Zhang et al., 1999, Zhao et al., 2000, Després et al., 2000). The *NPR1* gene is required for the positive regulation of *PR-1* by salicylate in *A. thaliana*, and for resistance to a broad range of pathogens (Cao et al., 1994, Cao and Dong, 1998). The NPR1 protein contains an ankyrin-repeat domain, and shares significant homology with mammalian IkB (Cao et al., 1997, Ryals et al., 1997). NPR-1 is translocated to the nucleus after salicylate treatment, where it probably acts as a transcriptional co-activator (Cao et al., 1997). The *as-1 cis*-element is not specific to salicylic acid, but confers inducibility to other molecules such as auxin and jasmonic acid (Horvath et al., 1998).

Another example of transcriptional regulation of PR-genes involves the ethylene response element binding protein (EREBP) family of transcriptional factors. EREBPs are induced by salicylic acid and ethylene (Horvath et al., 1998, Gu et al., 2000). They confer ethylene regulation to several PR gene promoters (Ohme-Takagi and Shinshi, 1995). In tomato, Pti4, a member of the EREBP family, interacts with Pto kinase, the product of a resistance gene involved in the interaction with *Pseudomonas syringae* pv. *tomato* (Zhou et al., 1997). Pto phosphorylates Pti4, and this enhances binding to a GCC *cis*-element, present in the promoter of PR genes (Gu et al., 2000). These results suggest a direct link between a disease resistance protein and the induction of PR genes.

Plants respond to pathogen infection by the *de novo* synthesis of secondary metabolites called phytoalexins having antimicrobial activity. In several species, increased biosynthesis of sesquiterpenoid phytoalexins during pathogen infection is accompanied by a reduction in sterol biosynthesis, since the two pathways share common

precursors. Reduced levels of sterol precursors may have consequences on the biosynthesis of brassinosteroid hormones. Suppression of phytosterol biosynthesis in response to fungal elicitors has been demonstrated in parsley, potato, tobacco, and *Tabernaemontana divaricata* (Haudenschild and Hartmann, 1995, Brindle et al., 1988, Vogeli and Chappell, 1988, van der Heijden et al., 1989). There is also a coordinated reduction of expression, at the steady-state level of mRNA, of the genes coding for squalene synthase and Δ24-sterol C-methyltransferase, in tobacco and soybean respectively, in response to fungal elicitors (Devarenne et al., 1998, Shi et al., 1996). Squalene synthase acts at an important branch point where a common precursor, farnesyl pyrophosphate, is partitioned between the sterol biosynthetic pathway and the pathways leading to other terpenoids (Newman and Chappell, 1999).

In potato, infection with *Phytophthora infestans* leads to the suppression of squalene synthase and up-regulation of the sesquiterpene cyclase vestipiradiene cyclase (Yoshioka et al., 1999). In addition, two isoforms of HMG-CoA reductase are induced, while a third isoform is suppressed (Choi et al., 1992). HMG-CoA reductase catalyzes the formation of mevalonate, a common precursor to sesquiterpenes and sterols. The results obtained on the regulation of HMG-CoA reductases suggest that the sterol and sesquiterpene phytoalexin pathways may involve distinct pools of intermediates. In *Stevia rebaudiana*, the diterpene steviol glycosides and gibberellin pathways, which share common intermediates, are also spatially and temporally separated (Richman et al., 1999).

During the plant defense response to pathogens, energy normally used for growth has to be reallocated to the establishment of chemical and physical barriers against

infection. Processes of cell elongation and cell division stimulated by auxin, gibberellins and brassinosteroids are incompatible with the reinforcement of the cell wall occurring during the defense response. One of the ways to regulate the activity of these hormones may be to reduce their biosynthesis. Alternatively, hormone response or sensitivity may be down-regulated. An interesting illustration comes from the recent characterization of a MAP kinase cascade involved in oxidative stress signaling in *A. thaliana* (Kovtun et al., 2000). Activation of the MAP kinase kinase ANP1 by H_2O_2 results in the induction of a GST gene, but suppresses the expression of auxin-responsive genes.

Response to ethanol

Ethanol treatment of plants result in the induction of several cytochrome P450s involved in herbicide detoxication (Reichhart et al., 1979). Some of these enzymes are also inducible by other chemicals, such as $MnCl_2$, phenobarbital, or the herbicide safener 1,8-naphthalic anhydride (Reichhart et al., 1979, Zimmerlin and Durst, 1992). In mammalian liver, phenobarbital is the typical inducer of cytochrome P450s involved in detoxication, whereas ethanol is a specific inducer of the cytochrome P450 CYP2E1. In a number of subsequent studies using plant tissues, ethanol has been used alone or in combination with other chemicals to increase the activity of cytochrome P450s involved in glucosinolate biosynthesis or herbicide metabolism (for example, see Frear et al., 1993, Moreland et al., 1995, Du and Halkier, 1996). In maize, ethanol treatment resulted in an increase of the steady-state mRNA levels for members of several cytochrome P450 families (Potter et al., 1995). Since ethanol also induces a *GST* gene, it was proposed that ethanol is perceived as a general chemical stress, leading to the establishment of various detoxication mechanisms (Potter et al., 1995).

Several authors in the field of herbicide detoxication in plants support the view that plants have evolved enzyme systems to detoxify xenobiotic molecules from bacterial, fungal or animal origin (Coleman et al., 1997, Kreuz et al., 1996). However, this view is debatable, and plant detoxication enzymes can certainly not be expected to have appeared under the pressure of agrochemicals, which are of very recent use in natural history. Alternatively, plant detoxication enzymes may be required to detoxify endogenous metabolites produced under stress conditions. In mammalian liver, enzymes of detoxication with broad substrate specificities are expected to have appeared under the pressure of plant secondary metabolites derived from nutrition. Zimmerlin and Durst (1992) have hypothesized that the capacity of plant enzymes to accept xenobiotics may reflect their structural similarities with the physiological substrates. These authors have demonstrated that the physiological substrate of the wheat cytochrome P450 responsible for the metabolism of the herbicide diclofop is lauric acid (Zimmerlin and Durst, 1992). However, no other physiological substrate of a plant enzyme involved in herbicide metabolism has been identified so far.

Detoxication enzymes of mammalian liver are classified in two groups. The phase I enzymes, represented by the cytochrome P450s, catalyze hydroxylation reactions. The phase II enzymes, which include the sulfotransferases, the UDP-glucoronosyltransferases and the glutathione transferases, catalyze conjugation reactions, leading to excretion of the metabolites. In plants, herbicide detoxication involves both phase I and phase II enzymes. However, plants do not have excretion systems. Glutathione and glucoside conjugates of herbicides are targeted to the vacuole, where they are stored (Coleman et al., 1997, Kreuz et al., 1996). Among the few plant endogenous metabolites that are

known to be stored in the vacuole as glutathione conjugates are the anthocyanin pigments (Marrs et al., 1995, Alfenito et al., 1998). The glutathione conjugates are transported into the vacuole by a non-specific ATP-binding cassette transporter protein (Lu et al., 1997). Flavonol sulfates and sulfated xenobiotics appear to be transported into the vacuole by another related non-specific transporter (Klein et al., 1997). The simple storage in plants of toxic xenobiotics as conjugates may have important consequences for human toxicity, since the parent compounds may be easily released after ingestion.

Response to low oxygen stress

Plants produce endogenous ethanol under low oxygen stress. Therefore, ethanol accumulation under these conditions may have an impact on gene regulation. Low oxygen stress in the form of hypoxia or anoxia has been studied in plants as an adaptation to flooding, an ecologically and agronomically important phenomenon (Crawford and Braendle, 1996). Limited oxygen availability results in a switch from aerobic metabolism to ethanolic fermentation (Drew, 1997). This simple pathway involves the conversion of pyruvate to acetaldehyde, catalyzed by pyruvate decarboxylase, followed by the conversion of acetaldehyde to ethanol, catalyzed by alcohol dehydrogenase.

In maize, low oxygen stress results in the induction of anaerobic proteins (Sachs et al., 1996). These proteins start to accumulate 90 minutes after the onset of low oxygen stress. Most anaerobic proteins are enzymes involved in glycolysis or ethanolic fermentation, with the exception of a *XET* gene. Anaerobic induction is dependent on increases in transcription, as well as translational regulation. At the onset of anoxia, protein synthesis is inhibited, and polysomes dissociate (Bailey-Serres and Freeling,

1990). In addition, the translation initiation factor eIF-4A is phophorylated (Webster et al., 1991). Subsequently, mRNAs coding for anaerobic proteins are translated selectively (Fennoy and Bailey-Serres, 1995), and the 5'- and 3'-untranslated regions of the *Adh1* transcript are important for this translational regulation (Bailey-Serres and Dawe, 1996).

An anaerobic response element (ARE) has been identified in the promoters of several genes encoding anaerobic proteins (Olive et al., 1991). In *A. thaliana*, the transcriptional factor AtMYB2 binds the GT-motif present within the ARE of *ADH1* (Hoeren et al., 1998). The *AtMYB2* gene is induced by hypoxia in roots, and is involved in the up-regulation of *ADH1* under these conditions. AtMYB2 is involved in other stress responses, since it is also induced by abscisic acid, salt stress and dehydration, and regulates a dehydration-responsive gene (Urao et al., 1993, Abe et al., 1997).

The early events occurring during low oxygen stress may have consequences for the perception and signal transduction occurring in the molecular response. In maize roots, low oxygen stress blocks mitochondrial respiration, and lactate fermentation takes place during the first 20 minutes (Davies, 1980). This is accompanied by a drop in intracellular pH (Roberts et al., 1984a, 1984b). At low pH, lactate dehydrogenase is inhibited, and metabolism then switches to ethanolic fermentation (Davies et al., 1974). The switch is probably not determined by cytoplasmic acidification, but rather by an increase in pyruvate concentration (Tagede et al., 1999). Pyruvate decarboxylase has a higher *Km* for pyruvate than that of pyruvate dehydrogenase.

Loss of function mutations of maize *Adh1* and *A. thaliana ADH1* have no effect on growth under normal conditions, but result in impaired survival under low oxygen

stress (Freeling and Bennett, 1985, Jacobs et al., 1988, Dolferus et al., 1990). In *A. thaliana*, *ADH1* is assumed to be present as a single copy gene (Chang and Meyerowitz, 1986). *adh1* null mutants have no detectable alcohol dehydrogenase activity in callus cultures, where *ADH1* is normally expressed (Jacobs et al., 1988, Dolferus et al., 1990). However, from a recent inspection of the *A. thaliana* genome database, the *ADH* gene family appears fairly complex. We cannot exclude that other ADH isozymes, distinct from the stress-inducible ADH1, may be required during normal development.

Advances towards the understanding of low oxygen stress were made through the comparison of flooding-tolerant and intolerant species, and the discovery that maize roots treated with hypoxia are more tolerant to anoxic shock (Ricard et al., 1994, Drew, 1997). In general, enhanced survival depends on sustained levels of enzymes involved in glycolysis and ethanolic fermentation in order to generate ATP. In intolerant species, anoxic toxicity results from uncontrolled cytoplasmic acidosis, which is probably dependent on the hydrolysis and depletion of nucleotide triphosphates. However, the return to aerobic conditions after a period of anoxia constitutes a major cause of injury. During post-anoxic shock, oxygen reacts with reduced iron accumulated under anoxia, producing reactive oxygen species. Furthermore, H_2O_2 reacts with ethanol resulting in the production of toxic levels of acetaldehyde (Crawford and Braendle, 1996). In the anoxia-tolerant *Iris pseudocarus*, superoxide dismutase is induced under low oxygen stress, affording a protection against oxidative stress upon return to aerobic conditions (Monk et al., 1987). Antioxidants such as ascorbic acid and glutathione are probably also involved (Crawford and Braendle, 1996).

How plant cells perceive low oxygen stress and the signal transduction mechanism involved in the induction of anaerobic proteins is completely unknown (Drew et al., 1997). Perception of cytoplasmic acidosis or an oxygen-sensing mechanism have been considered. Inhibition of respiration in transgenic tobacco leaves expressing a bacterial pyruvate decarboxylase led to ethanol accumulation, but under those conditions, the *ADH* gene was not induced, suggesting that induction of genes encoding anaerobic proteins is not mediated by a product of ethanolic fermentation (Bucher et al., 1994). However, accumulation of ethanol under low oxygen stress may still have an impact on the regulation of plant ethanol-inducible genes. In support of this hypothesis, it has been demonstrated that low oxygen stress results in the induction of an ethanol-inducible fungal promoter expressed in transgenic plants (Salter et al., 1998).

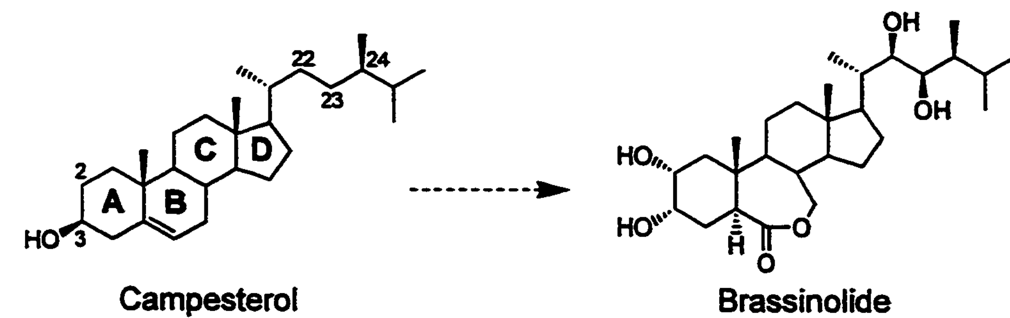
In addition to changes in primary metabolism, low oxygen stress also results in complex physiological adaptations. Maize roots exposed to hypoxia develop aerenchyma which improves the oxygenation of the tissue (Jackson, 1985). In comparison, formation of aerenchyma in roots is a constitutive developmental program in rice (Jackson et al., 1985). The development of aerenchyma is under the control of ethylene, and hypoxia results in an increase in the levels of ethylene biosynthetic enzymes, and ethylene content (Atwell et al., 1988, He et al., 1994, 1996). A *XET* gene, which is inducible by ethylene in hypoxic maize roots, may be involved in cell wall rearrangements during the formation of aerenchyma (Saab and Sachs, 1996). Formation of aerenchyma also requires hydrolytic enzymes, and the increase in cellulase activity in roots under low oxygen stress is also mediated by ethylene (He et al., 1994).

In contrast with other cereal crops, rice has the capacity to germinate anaerobically. In most species, anoxia has an inhibitory effect on α-amylase required for starch breakdown, but rice seeds retain the ability to respond to gibberellic acid, which is required for α-amylase induction (Perata et al., 1993). In addition, submerged deepwater rice has a spectacular capacity for internode elongation. This response allows the plant to emerge in order to reach light and oxygen. Internode elongation of deepwater rice under submergence is stimulated by gibberellic acid (Sauter and Kende, 1992, Sauter et al., 1993). However, the response is not achieved through an increase in gibberellin biosynthesis. As in maize roots, hypoxia results in an increase in ethylene biosynthesis. Increased ethylene renders the tissue more responsive to gibberellic acid, through a decrease in endogenous abscisic acid (Raskin and Kende, 1984a, 1984b, Hoffman-Benning and Kende, 1992). The *Os-EXP4* gene from deepwater rice, which encodes an expansin involved in cell elongation, is induced by submergence and treatment with gibberellin (Cho and Kende, 1997). Induction of *Os-EXP4* mRNA under submergence occurs before the enhanced elongation of the stem. Two rice *XET* genes, *OsXTR1* and *OsXTR3*, are expressed in the zones of internode elongation, and are induced by gibberellins and brassinosteroids (Uozo et al., 2000). However, regulation of these genes under hypoxia was not investigated.

Ethanolic fermentation probably occurs in plants under conditions other than low oxygen stress (Tagede et al., 1999). Under aerobic conditions, ethanolic fermentation occurs in tissues where oxygen availability is limited, such as in roots (Tagede et al., 1998a) and germinating seeds (Raymond et al., 1985). In pollen of maize and tobacco, alcohol dehydrogenase is expressed at high levels (Freeling and Bennett, 1985, Bucher et

al., 1995, Tagede et al., 1997). In tobacco pollen, a specific isoenzyme of pyruvate decarboxylase is also highly expressed (Bucher et al., 1995, Tagede et al., 1997). Pollen grown *in vitro* has a high rate of ethanolic fermentation, without the arrest of mitochondrial respiration. In analogy with yeast, ethanolic fermentation in pollen is regulated by the carbon source rather than oxygen availability. It has been hypothesized that the occurrence of ethanolic fermentation in pollen is related to the large energetic demand of this tissue.

The pathway of ethanolic fermentation may be involved during various stresses. *ADH1* of *A. thaliana* is induced in response to abscisic acid, low temperature and dehydration (Dolferus et al., 1994, De Bruxelles et al., 1996). In potato, an alcohol dehydrogenase gene is induced in response to fungal elicitors, as well as salicylic acid and anoxia (Matton et al., 1990). In addition, various environmental conditions, including dehydration, SO_2 fumigation, ozone exposure and low temperature result in significant accumulation of acetaldehyde and ethanol (Kimmerer and Kozlowski, 1982). It is possible that under stress conditions where mitochondrial respiration may be inhibited, plants use the simple pathway of ethanolic fermentation in order to derive energy. Expression of a bacterial pyruvate decarboxylase gene in transgenic potato plants resulted in the development of spontaneous lesions on the leaves similar to those observed during the hypersensitive response, the constitutive expression of defense-related genes, and enhanced resistance to a fungal pathogen (Tagede et al., 1998b). The transgenic plants also had altered sugar metabolism. This phenotype may be caused by a temperature-dependent accumulation of toxic acetaldehyde.



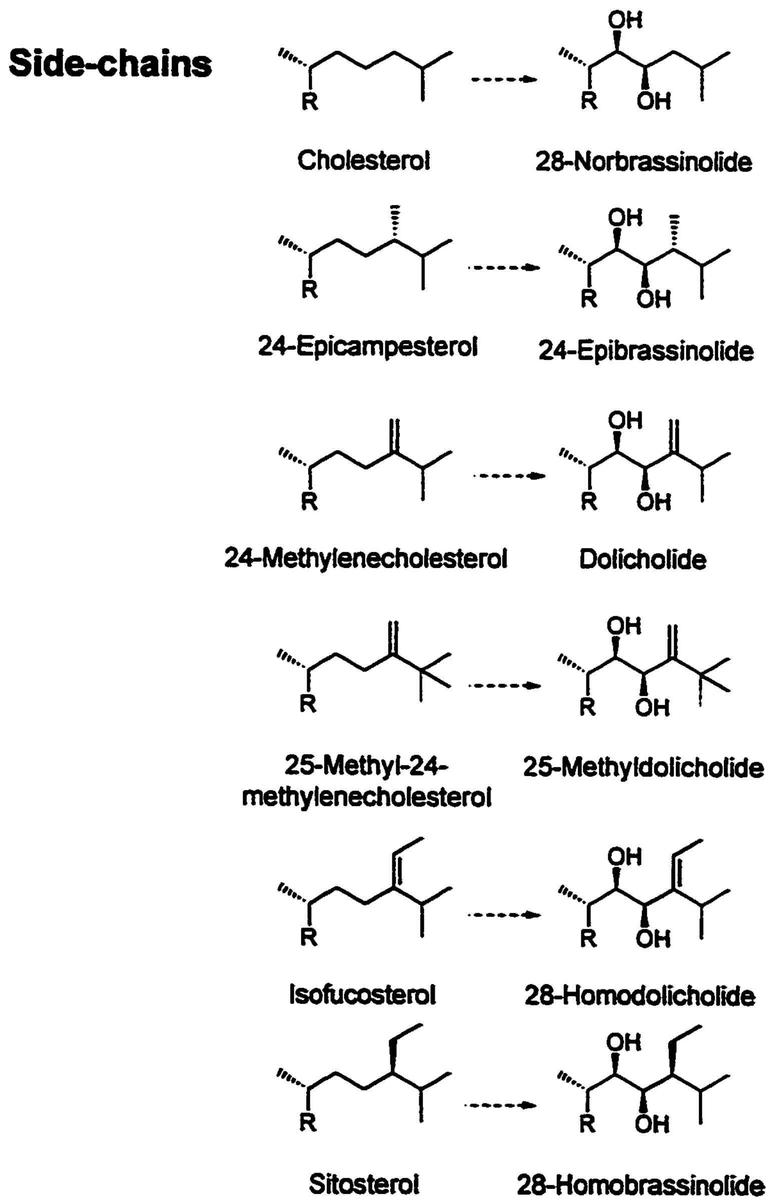


Figure 1. Sterol precursors of brassinosteroids.

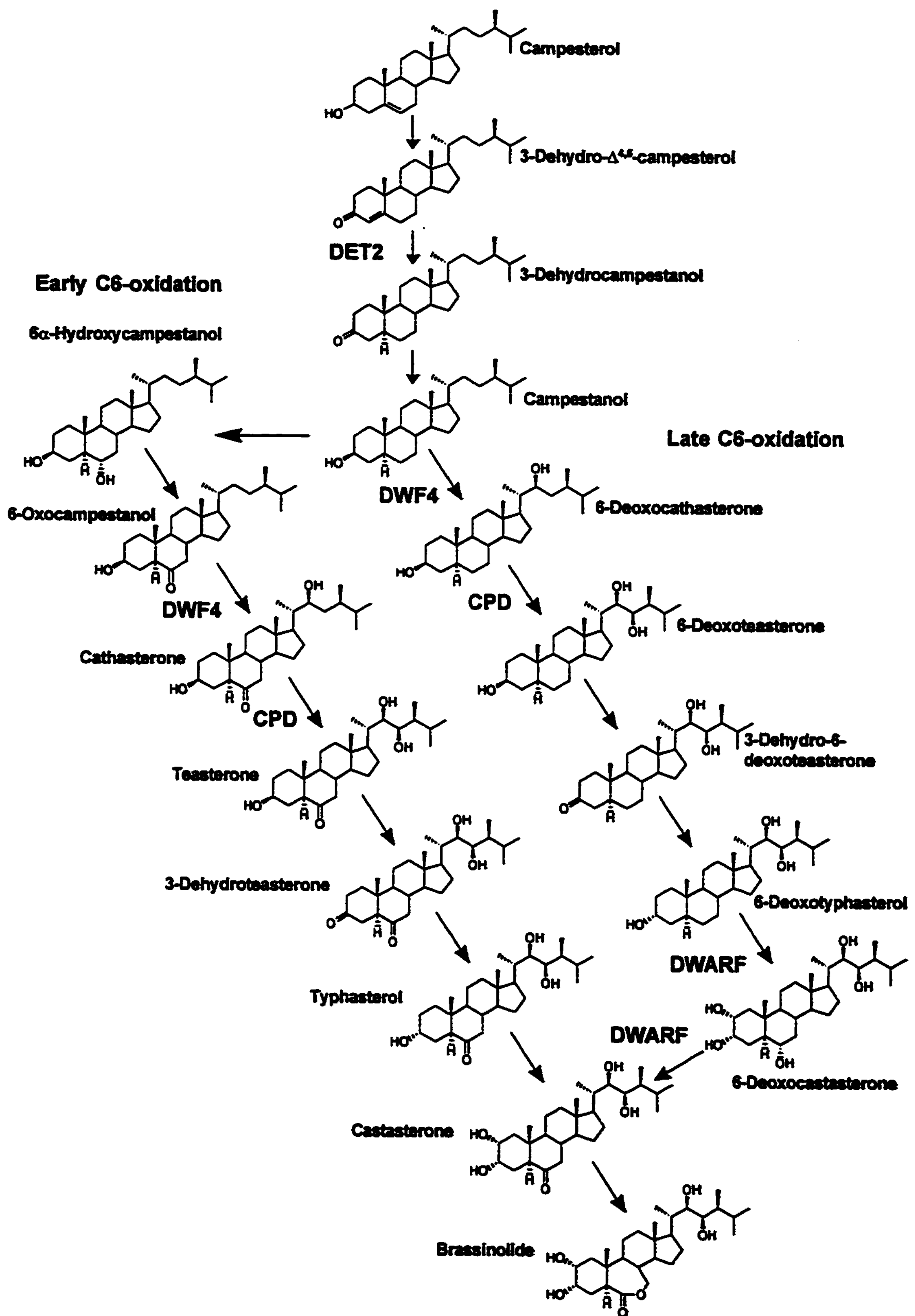


Figure 2. Pathway of brassinosteroid biosynthesis.

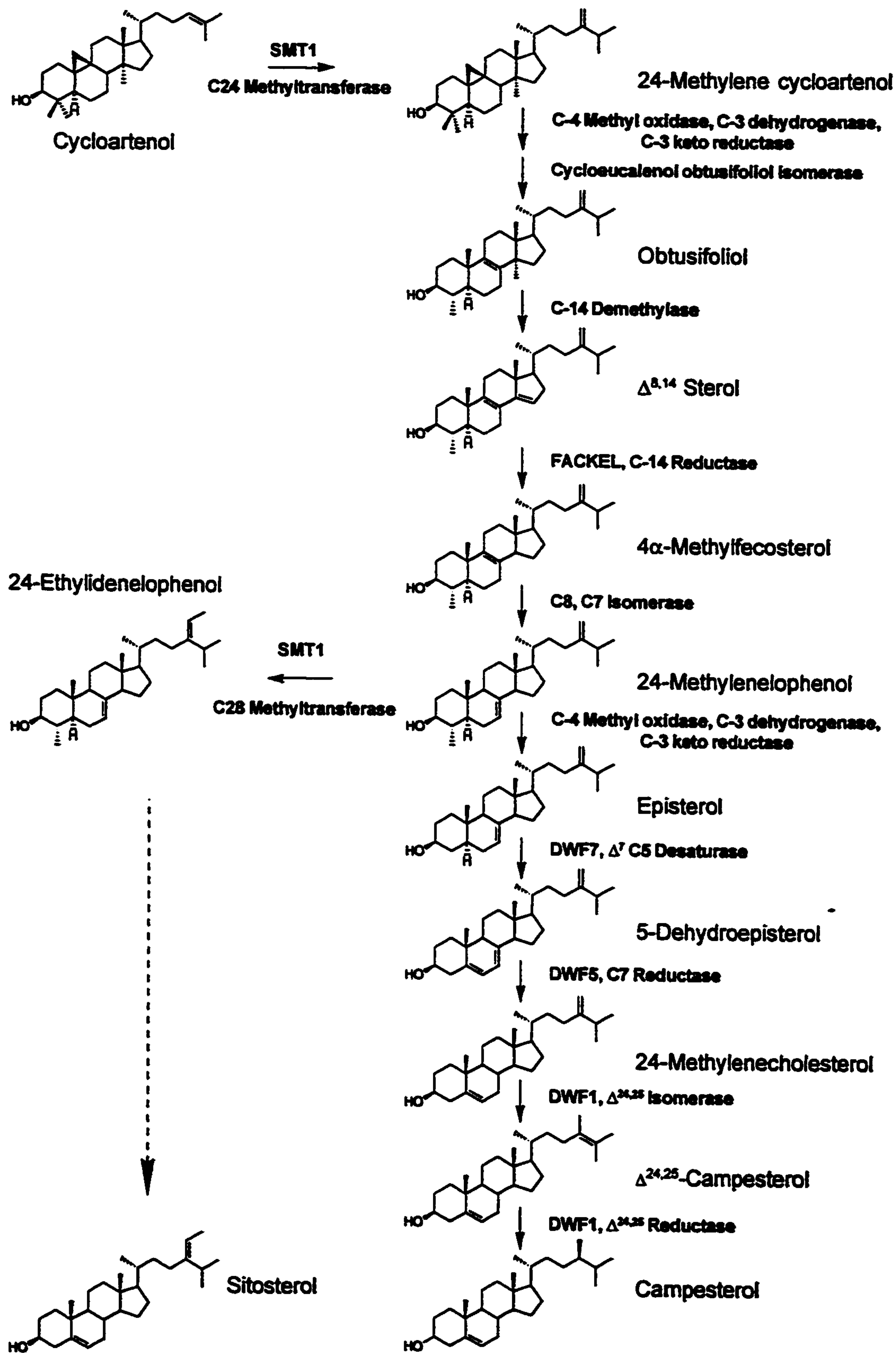
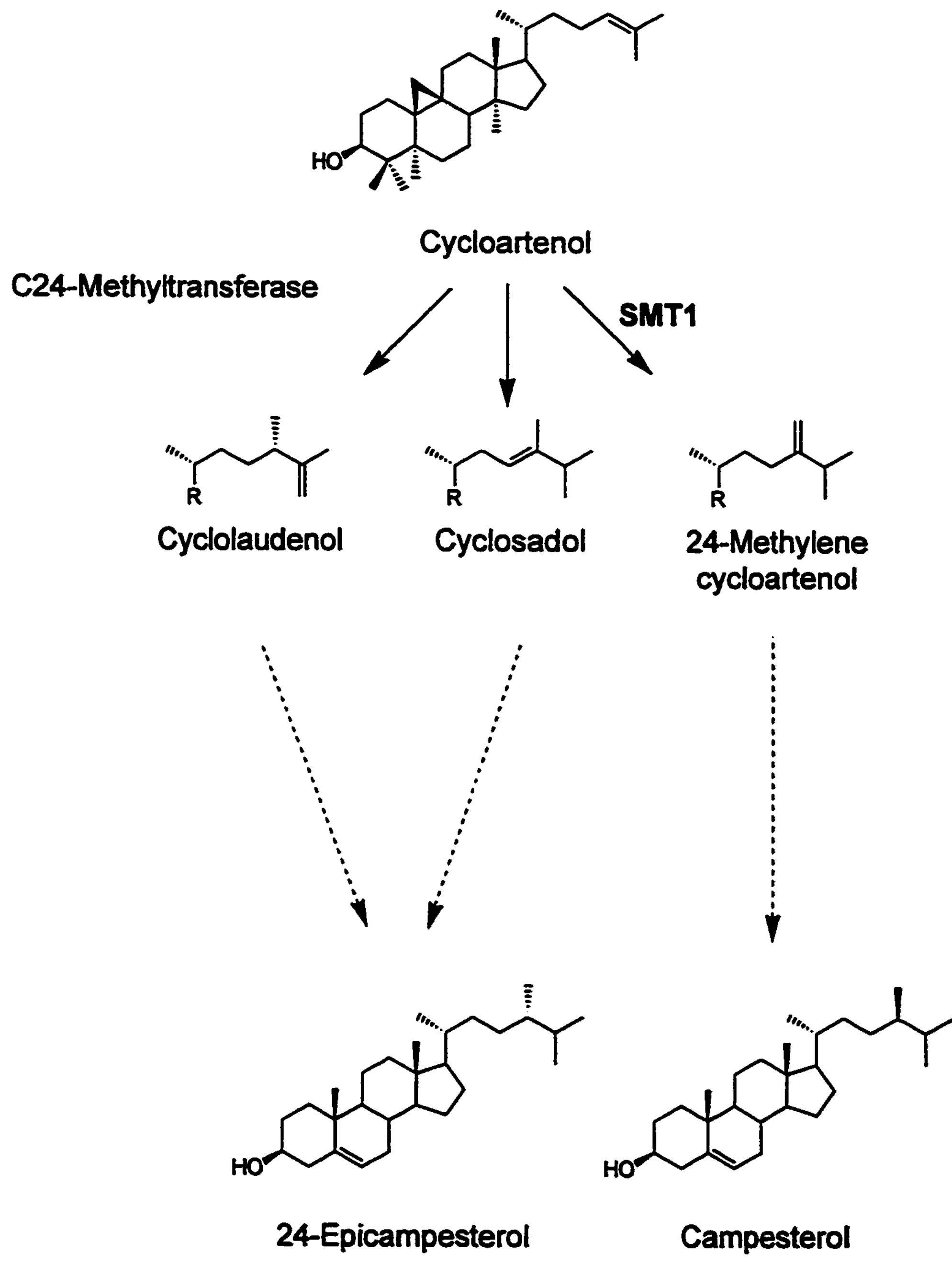
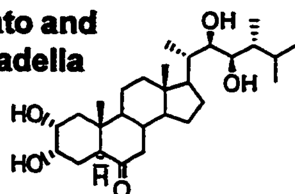


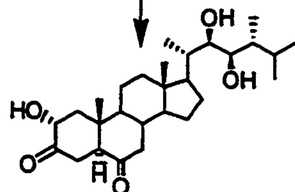
Figure 3. Pathway of sterol biosynthesis.


Cycloartenol
C24-Methyltransferase
SMT1
Cyclolaudenol
Cyclosadol
24-Methylene
cycloartenol
24-Epicampesterol
Campesterol
HO
R

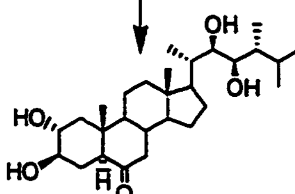
Tomato and Serradella



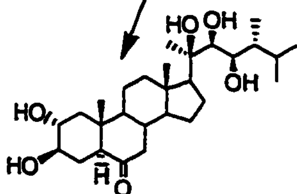
24-Epicastasterone



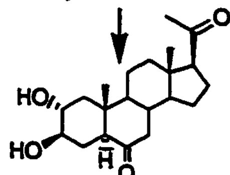
3-Dehydro-24-epicastasterone



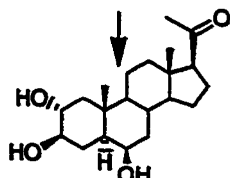
3, 24-Diepicastasterone



(20*R*)-Hydroxy-3, 24-Diepicastasterone

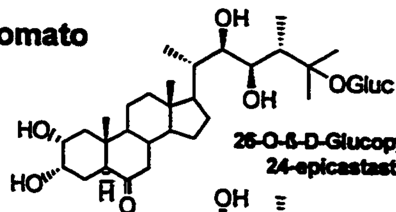


2α, 3ß-Dihydroxy-5α-pregnane-6, 20-dione

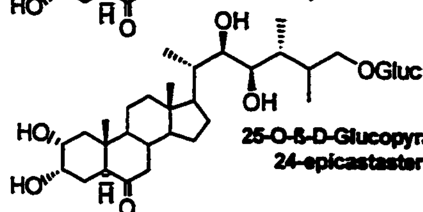


2α, 3ß, 6ß-Trihydroxy-5α-pregnane-6, 20-dione

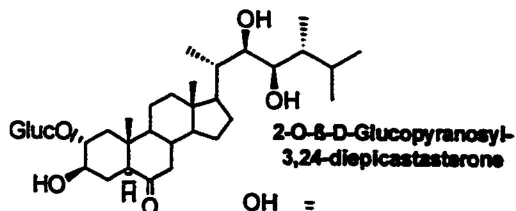
Tomato



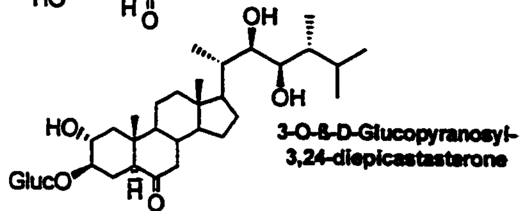
26-O-ß-D-Glucopyranosyl-24-epicastasterone



25-O-ß-D-Glucopyranosyl-24-epicastasterone

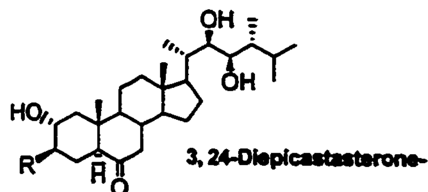


2-O-ß-D-Glucopyranosyl-3,24-diepicastasterone

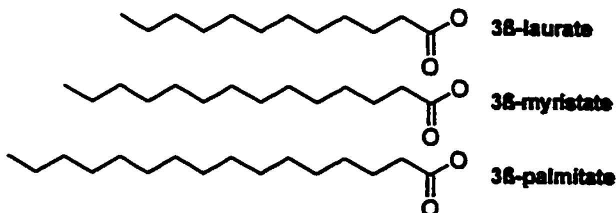


3-O-ß-D-Glucopyranosyl-3,24-diepicastasterone

Serradella



3, 24-Diepicastasterone-



CHAPTER 2

Inactivation of Brassinosteroid Biological Activity by a Salicylate-inducible Steroid Sulfotransferase from *Brassica napus*

In the last part of chapter 1, we have seen how physiological and metabolic adaptations of plants to various stress often involve a modulation of hormone action. In this chapter, we present the first evidence that like in mammals, steroid sulfotransferases exist in plants. The steroid sulfotransferase genes isolated from *Brassica napus* are inducible by salicylic acid, a chemical signal in the defense response against pathogen infection. In mammals, sulfonation of estrogens and androgens results in a loss of biological activity (Roy, 1992, Strott, 1996). Enzymatic sulfonation of the plant steroid hormone 24-epibrassinolide abolishes its biological activity in the bean second internode bioassay. The effect of sulfotransferase induction during pathogen infection may be a reduction in the levels of 24-epibrassinolide, preventing the stimulation of cell elongation. The formation of a physical barrier against pathogen infection by the reinforcement of the cell wall is incompatible with this process. To our knowledge, this is the first time that an enzyme involved in the modulation of hormone biological activity has been linked to physiological changes associated with the plant defense response.

Inactivation of brassinosteroid biological activity by a salicylate-inducible steroid sulfotransferase from *Brassica napus*

Michèle Rouleau[1], Frédéric Marsolais[2], Martine Richard[1], Ludovic Nicolle[1], Brunhilde Voigt[3], Günter Adam[3], and Luc Varin[2,4]

[1]Département de biologie, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4

[2]Department of Biology, Concordia University, 1455 de Maisonneuve W., Montreal, Quebec, Canada H3G 1M8

[3]Department of Natural Products, Institute of Plant Biochemistry, Weinberg 3, PO Box 100432, D-06018 Halle, Germany

[4]Author for correspondence: Tel.: (514)-848-3396; Fax: (514)-848-2881;

E-mail: varinl@clone.concordia.ca

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF000305-AF000307).

Part of this chapter was presented at the conference of the Phytochemical Society of North America, Evolution of Metabolic Pathways, Concordia University, Montreal, Quebec, Canada, July 10-13, 1999.

This chapter was published in the *Journal of Biological Chemistry* (1999) **274**, 20925-20930.

SUMMARY

Recent discoveries from brassinosteroid-deficient mutants led to the recognition that plants, like animals, use steroids to regulate their growth and development. We describe the characterization of one member of a *Brassica napus* sulfotransferase gene family coding for an enzyme that catalyzes the *O*-sulfonation of brassinosteroids and of mammalian estrogenic steroids. The enzyme is specific for the hydroxyl group at position 22 of brassinosteroids with a preference for 24-epicathasterone, an intermediate in the biosynthesis of 24-epibrassinolide. Enzymatic sulfonation of 24-epibrassinolide abolishes its biological activity in the bean second internode bioassay. This mechanism of hormone inactivation by sulfonation is similar to the modulation of estrogen biological activity observed in mammals. Furthermore, the expression of the *B. napus* steroid sulfotransferase genes was found to be induced by salicylic acid, a signal molecule in the plant defense response. This pattern of expression suggests that, in addition to an increased synthesis of proteins having antimicrobial properties, plants respond to pathogen infection by modulating steroid-dependent growth and developmental processes.

INTRODUCTION

Many developmental and physiological processes in organisms ranging from fungi to humans are regulated by a small number of steroid hormones. However, until recently the plant kingdom was almost completely excluded from the field of steroid endocrinology. The recent demonstration that the *Arabidopsis de-etiolated2* (*det2*) and the *constitutive photomorphogenesis and dwarfism* (*cpd*) mutants are defective in the synthesis of brassinosteroids focused attention toward the physiological function of steroids in plants (Li et al., 1996, Szekeres et al., 1996). Since these initial discoveries, several other mutants impaired in brassinosteroid synthesis or perception have been characterized at the molecular level (Kauschmann et al., 1996, Li and Chory, 1997, Choe et al., 1998).

Brassinosteroids have been shown to elicit a broad spectrum of responses including the promotion of cell elongation and cell division, inhibition of de-etiolation in the dark, repression of light-regulated genes in the dark and repression of stress-regulated genes (Szekeres et al., 1996, Cutler et al., 1991, Clouse and Sasse, 1998). Brassinolide was the first brassinosteroid isolated and characterized from plants (Figure 1) (Grove et al., 1979). Since this initial discovery, the structure of over 40 analogues of brassinolide has been elucidated, and brassinosteroids are believed to be ubiquitous in the plant kingdom (Takatsuto, 1994). In view of their structural similarities with animal steroids, it has been proposed that brassinosteroids might interact with a soluble receptor in order to regulate the expression of specific genes (Figure 1). This hypothesis has recently been challenged with the molecular characterization of the *Brassinosteroid-Insensitive-1* (*BRI1*) gene which encodes a putative brassinosteroid receptor (Li and Chory, 1997). The

BRI1 protein belongs to a family of plant receptor-like transmembrane kinases. Members of this family have an extracellular leucine-rich repeat domain, a transmembrane domain, and an intracellular serine/threonine protein kinase domain, suggesting that brassinosteroids mediate their effects at the cell surface. Despite this important discovery, direct or indirect binding of brassinosteroids to the BRI1 receptor has not been demonstrated and their mode of action remains to be characterized.

In mammals, it is well recognized that sulfotransferases play an important role in the modulation of the biological activity of a number of compounds, such as steroid and thyroid hormones, and catecholamine neurotransmitters (Hobkirk, 1993, Strott, 1996, Visser, 1994, Roth, 1986). Sulfonate conjugation not only facilitates transport and excretion of hydrophobic molecules by increasing their water solubility, it abolishes the biological activity of hormones, such as estrogens. Considering the important role of mammalian sulfotransferases in the modulation of the biological activity of hormones, our research interest is to elucidate the function of plant sulfotransferases in growth and development. In this paper, we describe the characterization of one member of a *Brassica napus* sulfotransferase gene family coding for an enzyme that sulfonates brassinosteroids as well as mammalian estrogenic steroids. We also demonstrate that enzymatic sulfonation of 24-epibrassinolide abolishes its biological activity. In addition, our results indicate that the pattern of expression of the *B. napus* steroid sulfotransferase gene is similar to other pathogenesis-related (PR) proteins, *i.e.* having very low constitutive expression in control seedlings and being inducible by salicylic acid (Ward et al., 1991). This represents the first example where the function of a PR protein can be linked to the modulation of the biological activity of a plant hormone by conjugation.

EXPERIMENTAL PROCEDURES

Isolation of the *BNST* genes. A *B. napus* genomic library constructed in pTZ18R was probed with ^{32}P-labeled *ATST1* (*RaR047*) cDNA (Lacomme and Roby, 1996) under conditions of high stringency according to standard procedures (Sambrook et al., 1989). Mapping analysis of two positive clones resulted in the identification of three genes (*BNST1*, *-2* and *-3*), which were subcloned in pBSK$^-$ (Stratagene) and sequenced in an automated system (Applied Biosystems Inc.). The alignment of the deduced amino acid sequences of *BNST1*, *-2*, *-3* and *ATST1* was created with the PILEUP program of the GCG sequence analysis package (Devereux et al., 1984).

Expression of recombinant BNST3 in *Escherichia coli*. Oligonucleotides (5'-CGGGATCCATGTCATCATCATCATCTG-3'; 5'-GGGAAGCTTAAACTATCATCATTAAAGC-3') were designed to introduce *Bam*HI and *Hin*dIII restriction sites at the respective 5' and 3' ends of the coding region of BnST3 by PCR amplification using Vent DNA polymerase (New England Biolabs). This allowed the directional cloning of the *BNST3* coding sequence in the bacterial expression vector pQE30 (Qiagen). The expression of BNST3 in *E. coli* cultures ($O.D._{600} \approx 0.7$) was induced with 1 mM isopropyl ß-D-thio-galactopyranoside for 9 hrs at 22°C. Bacterial cells were collected by centrifugation, resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 14 mM ß-mercaptoethanol and lysed by two passages in a French pressure cell (14 000 psi). The recombinant BNST3 recovered in the soluble fraction was purified by affinity chromatography onto nickel-nitrilotriacetic acid agarose matrix as recommended (Qiagen).

Expression of *BNST* genes and of *ATST1* in plants. *B. napus* cv. Westar and *A. thaliana* ecotype Columbia were grown in vermiculite under continuous white light. The roots of seven-day-old *B. napus* seedlings and of five-week-old *A. thaliana* plants were drenched with an aqueous sodium salicylate solution (10 mM, pH 7.0) and plants were grown for times indicated in the text. Plants were pulverized in liquid nitrogen, then either extracted in phenol/chloroform as described (Cashmore, 1982) to isolate total RNA, or boiled in reducing SDS sample buffer to obtain total proteins. Northern blot analysis of total RNA was achieved under high stringency conditions according to standard procedures (Sambrook et al., 1989) using the ^{32}P-labeled coding region of *BNST1* as a probe. Sulfotransferases in protein extracts were detected by Western blot analysis using anti-BNST1 polyclonal antibodies (dilution 1:3000) and goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase (dilution 1:3000; Bio-Rad). Anti-BNST1 polyclonal antibodies were raised in rabbits using purified recombinant BNST1 expressed in *E. coli* as the antigen. The antibodies were found to cross-react with BNST2 and -3 (data not shown).

Enzymatic assays. Analysis of substrate specificity was performed by testing enzymatic activity with three different concentrations of acceptor substrate: 200, 5 and 0.1 µM. Reaction mixtures (50 µl) also contained 100 pmol ^{35}S-labeled 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Dupont NEN) and approximately 5 µg of affinity purified recombinant BNST3 in 50 mM Tris, pH 7.5. For kinetic analysis, a PAPS concentration of 5 µM was used. Concentrations of 24-epicathasterone were 2, 1, 0.5 and 0.2 µM and of 24-epiteasterone were 50, 20, 10 and 5 µM. Stock solutions (1 mM) of acceptor substrates were prepared in dimethyl sulfoxide or methanol.

Commercial substrates were obtained from Sigma, and desulfoglucosinolates were kindly provided by D. Reed, Plant Biotechnology Institute, Saskatoon. Reactions were allowed to proceed for 10 min at 25°C. Incorporation of the ^{35}S-labeled sulfate was monitored as described (Varin et al., 1987).

Bean second internode test. 24-Epibrassinolide sulfate was produced enzymatically and extracted twice in ethyl acetate as a tetrabutyl ammonium salt (Varin et al., 1987). Test compounds (10 nmol) solubilized in 2 µl methanol were applied at the base of the second internode of seven-day-old bean seedlings as described (Mitchell and Livingston, 1968). Methanol alone (2 µl) was applied on control bean plants. Bean seedlings were grown at 21°C with a 16-hr photoperiod. The length of the second internode was measured 4 days following the application of test compounds.

Synthesis of brassinosteroids. Brassinolide was purchased from Beak Technologies Inc., Brampton, Canada. The following other brassinosteroids were synthesized from ergosterol using published procedures: 24-Epicathasterone and 24-epibrassinolide (McMorris and Patil, 1993), (22*S*, 23*S*)-28-homocastasterone and 28-homobrassinolide (McMorris et al., 1994), 6-deoxo-24-epicastasterone (Spengler et al., 1995), 24-epiteasterone (Voigt et al., 1996), 24-epicathasterone and 22-deoxy-24-epiteasterone (Voigt et al., 1997).

Synthesis of 6-deoxo-24-epicathasterone. A mixture of 24-epicathasterone (47.4 mg, 0.1 mmol), hydrozine-monohydrate (1.5 ml, 80%), KOH (1.4 g) and diethylene glycol (10 ml) was stirred and heated for 2 h at 200°C. After cooling the mixture was diluted with HCl (10 ml, 1.2 N) and the solution extracted with chloroform. Usual workup gave a residue, which was purified by flash chromatography on silica gel. Elution

with n-hexane/ethyl acetate 2:8 v/v furnished 6-deoxo-epicathasterone with m.p. 175-178°C and EI-MS: *m/z* 400 (M+-18). The ^{1}H NMR data are in agreement with the given structure.

RESULTS

Isolation of a family of *Brassica napus* sulfotransferase genes. A clone encoding a putative sulfotransferase of unknown substrate specificity has recently been isolated from an *A. thaliana* cDNA library (Lacomme and Roby, 1996). This clone, *ATST1*, was used as a probe to isolate three intronless genes (*BNST1*, *-2* and *-3*) from a *B. napus* genomic library. The *BNST1*, *-2* and *-3* genes belong to a multigene family comprising at least 12 members, as determined by Southern blot analysis and RT-PCR experiments (data not shown). *BNST1*, *-2* and *-3* encode proteins having a predicted molecular mass of approximately 35 kDa that share 73 to 83% sequence identity (Figure 2). The variations among the three amino acid sequences are mostly observed in the domain that is involved in sulfotransferase acceptor substrate recognition, suggesting that the three enzymes may have distinct substrate specificities (Varin et al., 1995). The BNST amino acid sequences are very similar to *A. thaliana* ATST1, BNST3 being the closest relative with 87% amino acid sequence identity. Thus, it is likely that the *B. napus* sulfotransferase genes are orthologs of *A. thaliana ATST1*. In contrast, the *B. napus* sulfotransferase proteins share 41% sequence identity with the flavonol sulfotransferases of *Flaveria chloraefolia* and 27-30% identity with the mammalian phenol, estrogen and hydroxysteroid sulfotransferases.

The expression of *BNST* genes is induced by salicylic acid. *B. napus* seedlings grown under normal conditions expressed very low to undetectable levels of *BNST*

mRNAs and proteins (Figure 3A and C, lanes 1). However, watering the seedlings with 10 mM salicylic acid resulted in a rapid induction of their expression. Steady-state mRNA levels increased within 6 hours following salicylate treatment (Figure 3A, lane 2) and resulted in the expression of several proteins with apparent molecular mass ranging from 32 to 35 kDa (Figure 3C, lanes 2 and 3). The kinetics of induction of *BNST* genes is comparable to that of PR genes (Ward et al., 1991). Due to their high level of nucleotide- and amino acid sequence identity, analysis of the expression of *BNST* genes using the full-length *BNST1* coding sequence, or polyclonal antibodies raised against the BNST1 protein as probes should identify most, if not all, of the related expressed *BNST* genes. Therefore, the detection of several proteins in Western blots is consistent with *BNST1*, *-2* and *-3* being members of a multigene family. In contrast, the detection of a single polypeptide with an apparent molecular mass of 32.5 kDa in salicylate-treated *A. thaliana* (Figure 3C, lane 4) is in agreement with the report that *ATST1* exists as a single copy gene in this plant (Lacomme and Roby, 1996). The inducible expression of *BNST* genes by salicylate suggests that these genes play a role in the plant defense response. This is further supported by the pattern of expression of *ATST1* in *A. thaliana*, which is preferentially induced by infection with avirulent pathogens (Lacomme and Roby, 1996).

BNST3 is a steroid sulfotransferase. In order to define the role of the BNST proteins, we studied the sulfotransferase activity of purified recombinant BNST3 expressed in *E. coli*. The choice of BNST3 was dictated by the fact that it is expressed in salicylate-treated seedlings as determined by RT-PCR and is the most similar to *A. thaliana* ATST1. Although a variety of acceptor molecules of plant and mammalian origin (e.g. desulfoglucosinolates, gibberellins, phenolic acids, flavonoids, steroids,

phytosterols and terpenoids) were used as substrates, BNST3 catalyzed the transfer of the ^{35}S-labeled sulfonate group from the cosubstrate PAPS to brassinosteroids and estrogenic steroids only (Figure 1) (Table 1). The BNST3 enzyme did not sulfonate the structurally related mammalian steroids estrone, 17α-estradiol and testosterone, the phytosterols campesterol, stigmasterol and ß-sitosterol, nor the ecdysteroids ecdysone or 20-hydroxyecdysone, thus exhibiting specific structural requirements for the sulfonate acceptor substrate. The fact that the BNST3 enzyme accepts 17ß-estradiol 3-methyl ether and not estrone as substrate indicates that sulfonation takes place at position 17 of the estrogenic steroid substrates. Furthermore, the BNST3 enzyme is stereospecific for the 17-hydroxyl group in position ß, since it does not sulfonate the 17α-stereoisomer. It seems that a 17-hydroxyl group attached to a rigid ring system, in estradiol, may limit its sulfonate accepting ability. Substrate-dependent stereoselectivity has also been reported for the rat hydroxysteroid sulfotransferase (STa) and phenol sulfotransferase (AST IV), and was shown to be caused by the degree of steric bulk on the hydroxyl-containing chiral carbon (Rao and Duffel, 1991, Banoglu and Duffel, 1997).

To determine the position of sulfonation and the substrate preference of BNST3, several natural and synthetic brassinosteroids were tested (Figure 1). Of those, BNST3 exhibits the highest affinity for 24-epicathasterone followed by 24-epiteasterone (Tables 1 and 2). The catalytic efficiency (V_{max}/K_m) of the enzyme is 13-fold higher for 24-epicathasterone as compared with 24-epiteasterone (Table 2). Furthermore, 24-epicathasterone is the only substrate for which a significant enzymatic activity is measured at a concentration of 0.1 µM (2.5 pkatal/mg). Although BNST3 also accepts 24-epibrassinolide as a substrate, this activity is only observed at high substrate

concentration (Table 1). The enzyme is also sensitive to the level of oxidation of ring B, as revealed by the low relative activity values obtained with 6-deoxo-24-epicastasterone and 6-deoxo-24-epicathasterone as substrates. The most striking feature of BNST3 substrate specificity is its efficient use of the natural substrate 24-epibrassinolide and the synthetic enantiomer (22*S*, 23*S*)-28-homobrassinolide, whereas it is inactive with the natural substrate brassinolide (Table 1). The fact that the enzyme accepts 24-epicathasterone and 24-epiteasterone but does not accept the synthetic substrate 22-deoxy-24-epiteasterone suggests strongly that sulfonation is taking place at position 22 of the steroid side chain (Table 1). The specificity of the enzyme for position 22 explains why early brassinosteroid intermediates and brassinosteroid precursors lacking a hydroxyl group at this position are not accepted by the enzyme.

Sulfonation of 24-epibrassinolide abolishes its biological activity. It has previously been reported that hydroxylation of brassinosteroid intermediates at position 22 increases considerably their biological activity, suggesting that sulfonation at this position by BNST3 might lead to the loss of their function (Voigt et al., 1996). To test this hypothesis, we compared the ability of 24-epibrassinolide and its sulfonated derivative to promote growth of the bean second internode, a well established biological test for the activity of brassinosteroids (Fujioka et al., 1995). As expected, application of 10 nmol epibrassinolide dramatically induced elongation of the internode (mean = 6.5 cm, s.d. = 1.2 cm, n = 5) (Figure 4A), as compared with control plants (mean = 1.1 cm, s.d. = 0.4 cm, n = 5). However, the addition of 10 nmol epibrassinolide sulfate did not stimulate the internode elongation (mean = 1.5 cm, s.d. = 0.5 cm, n = 5) (Figure 4B) as compared with control bean seedlings.

DISCUSSION

We have isolated three genes (*BNST1*, *-2* and *-3*) from *B. napus* which encode sulfotransferases. They belong to a gene family comprising at least 12 members having a high level of sequence identity with a previously characterized cDNA clone (*ATST1*) from *A. thaliana* (Lacomme and Roby, 1996). According to the guidelines on sulfotransferase nomenclature, *ATST1* and the *BNST* genes fall below the 45% amino acid sequence identity threshold value to be members of the flavonol *SULT3* family (Weinshilboum et al., 1997). Therefore, they represent the first characterized members of the new *SULT4* family. When expressed in *E. coli*, the enzyme encoded by the *BNST3* gene exhibited specificity for steroid substrates and catalyzed the sulfonation of the plant-specific brassinosteroids, and of mammalian estrogens such as 17ß-estradiol (Figure 1) (Table 1). Whereas several studies (Geuns, 1978, Simons and Grinwich, 1989) described the presence in plants of the mammalian steroids estradiol, estrone and testosterone, as well as their physiological effects on growth, development and flowering, the functional significance of estrogen sulfonation by a plant enzyme remains to be elucidated.

Despite the lack of information on the accumulation of 24-epimers of brassinosteroids in *B. napus*, our results suggest that they are in fact synthesized in this plant. These results call for a thorough investigation of brassinosteroids in *B. napus* tissues in order to confirm their natural occurrence in this plant. The differences observed in the domain responsible for sulfotransferase substrate specificity of BNST1, -2, and -3 suggest that they have distinct substrate preferences, and it is possible that other members of the BNST family are involved in the sulfonation of brassinolide and/or its precursors.

BNST3 has the highest affinity for 24-epicathasterone, of the substrates tested. The 13-fold lower V_{max}/K_m value observed for 24-epiteasterone as compared with 24-epicathasterone suggests that the latter might be the only brassinosteroid sulfated *in vivo* by BNST3. The preference of BNST3 for an early precursor, as compared with the final product 24-epibrassinolide is quite surprising considering the fact that the latter is about 1000-fold more active (Fujioka et al., 1995). However, cathasterone is considered to be the first brassinosteroid precursor with significant biological activity, and it has been proposed that its synthesis is the rate-limiting step in the formation of the more potent brassinosteroids (Choe et al., 1998, Fujioka et al., 1995). The substrate preference of BNST3 for 24-epicathasterone may be an efficient mechanism to block its intrinsic activity, and at the same time interfere with the biosynthesis of the more potent final product 24-epibrassinolide.

The fact that the currently used techniques for the analysis of brassinosteroids are not suitable for the preservation of the labile sulfate moiety may explain the lack of reports on the characterization of sulfated brassinosteroids in plant extracts. However, it has been shown that rice seedlings administered labeled cathasterone produce non-glycosidic polar brassinosteroid derivatives which may in fact be sulfate esters (Yokota et al., 1992), based on their chromatographic behavior and their susceptibility to solvolysis.

Our results also demonstrate that sulfonation of 24-epibrassinolide abolishes its biological activity in the bean second internode bioassay suggesting that plants, like mammals, may modulate the biological activity of steroids by sulfonation. The recent finding that *BRI1* codes for a potential transmembrane leucine-rich repeat-receptor kinase, that is required for brassinosteroid perception, suggests that brassinosteroids

mediate their biological activity on the external surface of the cell membrane (Li and Chory, 1997). Furthermore, the fact that the expression of the *CPD* gene is confined to specific cell types of leaves, other than those involved in elongation, suggests that brassinosteroids must be transported from source to target tissues (Mathur et al., 1998). Therefore, the sulfonation of brassinosteroids may interfere with their excretion, their transport or their binding to the BRI1 receptor. The availability of cloned *BNST3* and *ATST1* cDNAs provides the tools for further study of the function of these enzymes in relation to brassinosteroid action. Transgenic *A. thaliana* overexpressing *BNST3* are presently produced in our laboratory.

Another interesting feature of the brassinosteroid sulfotransferases is the induction of their expression following salicylate treatment in both *B. napus* and *A. thaliana* (Lacomme and Roby, 1996), suggesting that the modulation of steroid activity may be part of the defense response of plants to pathogens. The results of several experiments suggest that brassinosteroids stimulate plant growth by increasing the plasticity of the cell wall (Clouse, 1997). In the context of a pathogen infection, this growth mechanism is undesirable since it reduces the protection offered by the cell wall. The sulfonation of brassinosteroids by the salicylate- and pathogen-inducible steroid sulfotransferases provides a mechanism through which brassinosteroid-dependent cell wall hydrolysis might be stopped. Additional mechanisms are likely to parallel steroid sulfotransferase activity to reduce the levels of biologically active brassinosteroids during pathogen infection. A reduced rate of synthesis of brassinosteroids is also expected, since the expression of the Δ^{24}-sterol C-methyltransferase, which catalyzes the first committed step in phytosterol biosynthesis, was reported to decrease following elicitation of soybean

cells (Shi et al., 1996). Furthermore, suppression of phytosterol biosynthesis following elicitor treatment has been reported in parsley, potato, tobacco and *Tabernaemontana divaricata* (Vögeli and Chappell, 1988, Haudenschild and Hartmann, 1995, Brindle et al., 1988, van der Heijden et al., 1989).

The widespread occurence of sulfotransferases and sulfatases in mammalian tissues has led to the hypothesis that the concerted action of both groups of enzymes may regulate the level of active steroids (Hobkirk, 1993). Considering the structural, functional and metabolic conservation of steroids between plants and mammals, it is likely that a common mechanism of modulation of steroid activity has also been conserved through evolution. In support of this hypothesis, we demonstrate the occurrence of steroid sulfotransferases in plants, and that sulfonation of 24-epibrassinolide leads to the loss of its biological activity.

ACKNOWLEDGMENTS

We thank Dr. Dominique Roby for the *A. thaliana ATST1* (*RaR047*) cDNA clone, and Dr. Guy Bellemare for the *B. napus* genomic library. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), and le Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR), Government of Quebec. Dr. Michèle Rouleau was the recipient of an NSERC postdoctoral fellowship and Frédéric Marsolais of an NSERC postgraduate scholarship.

Table 1. Substrate specificity of recombinant BNST3

Substrate	Relative activity in % at 200 µM	Relative activity in % at 5 µM
Brassinolide	< 5	< 5
(22*R*, 23*R*)-28-Homobrassinolide	< 5	< 5
(22*S*, 23*S*)-28-Homobrassinolide	100[a]	15
24-Epibrassinolide	64	15
24-Epicastasterone	27	< 5
(22*S*, 23*S*)-28-Homocastasterone	51	28
24-Epiteasterone	inh.[b]	30
24-Epicathasterone	inh.[c]	100[d]
22-Deoxy-24-epiteasterone	< 5	< 5
6-Deoxo-24-epicathasterone	45	8
6-Deoxo-24-epicastasterone	16	< 5
ß-Estradiol	43	< 5
ß-Estradiol 3-methyl ether	48	< 5

[a] Maximum specific activity equals 11 pkatal/mg.
[b] Substrate inhibition was observed at concentrations above 50 µM.
[c] Substrate inhibition was observed at concentrations above 5 µM.
[d] Maximum specific activity equals 7.0 pkatal/mg.

Table 2. Kinetic parameters of recombinant BNST3 for the preferred brassinosteroid substrates

Substrate	K_m (µM)	V_{max} (pkatal mg^{-1})	V_{max}/K_m (pkatal mg^{-1} µM^{-1})
24-Epicathasterone	1.4	42	31
24-Epiteasterone	4.2	9.6	2.3

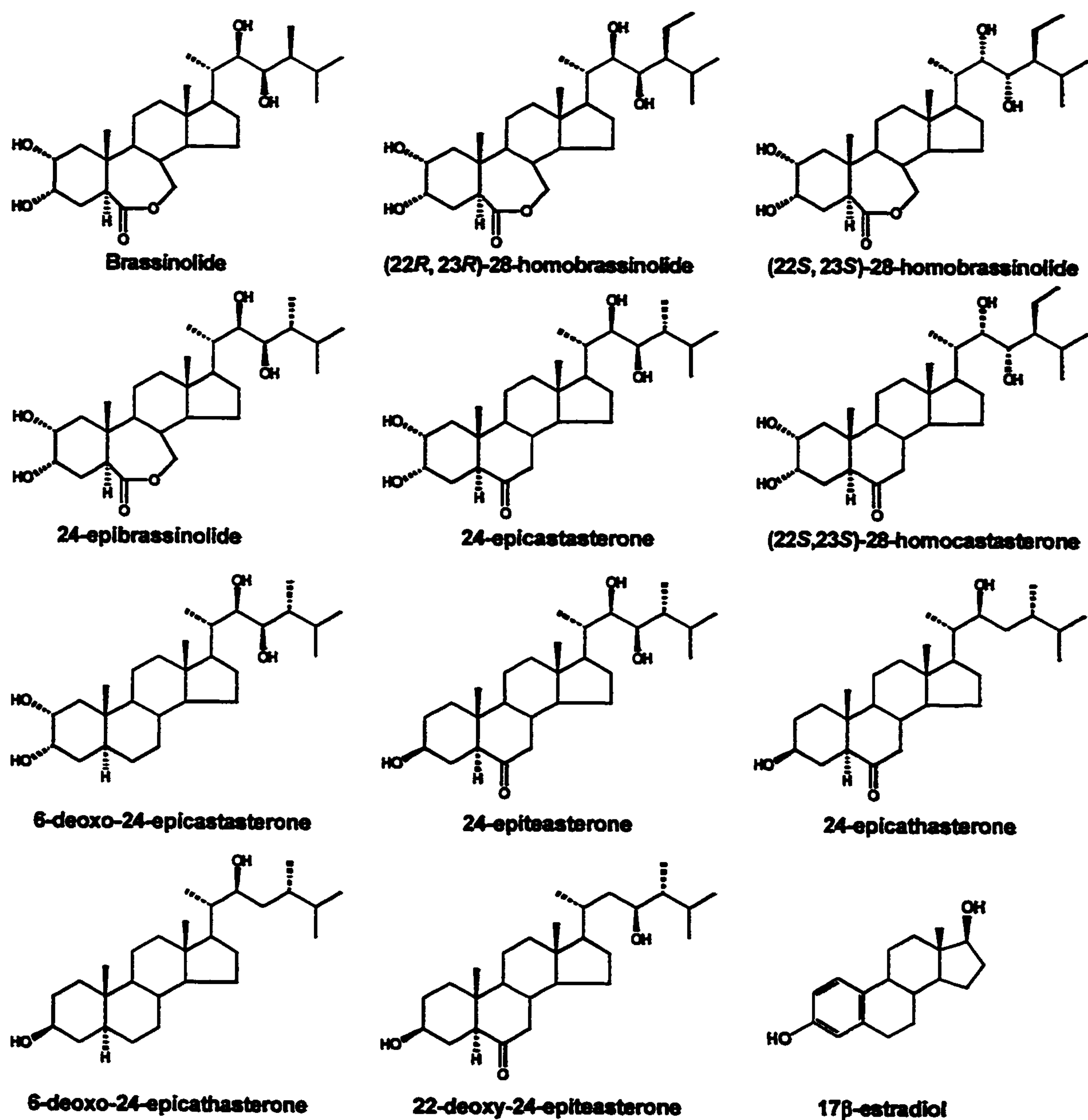


Figure 1. Chemical structure of the steroids used in this study

```
                                                                                          90
BNST1   .MESSSVPVYLKDENLTQETRDLLSSLPSEKGWLVSQMYQFEGIWQTQALVQGIVNCQKHFEANDSDVILATLAKSGTTWLKALLFALIH
BNST2   MSS-----D--R------K-K--I-----------C-----Q-R-H----L---LT-------K---I--V-NP---------V----N
BNST3   MSS-----D--R--K--------I-------------I---Q-R-H-E--L---LT-----K-K---I--V-NP--------S-V----N
ATST1   MSS-----A--G--D------A-I----K-------EI-E-Q-L-H---IL---LI---R---K---I--V-NP---------V---LN

                                                                                         180
BNST1   RHKFPV..SGKHPLLVTNPHSLVPYLEGDYCSSPEVNFAELPSPRLMQTHLTHHSLPVSIKSSSCKIIYCCRNPKDMFVSIWHFGRKLAP
BNST2   ------YSV..II-SCYQSAL---F-GRSLLR--DFD-SQ-S-----N--IS-L---E-V-------V------------L----K----
BNST3   ------.S--D---------L---FM--V-YE--DFD-SL--F----N--IS-L---E-V-----Q-V------------L----K----
ATST1   ------SS--N---------L---F---V-YE--DFD-SS-------N--IS-L---E-V-------V------------L----K----

                                                                                         270
BNST1   EKTAEYPIETAVAAFCKGKFIGGPFWDHVLEYWYESLKNPNKVLFVTYEELKKQTEVEVKRIAEFIGCGFTAEEEVSEIVKLCSFESLSS
BNST2   -E--D----K--E---Q-----------------A--E--------S---P--K-GETI------L---LVG----RA-----------
BNST3   QE--D--L-K--E---Q----A------------A--E-------------------------------------------------R
ATST1   -E--D----K--E---E-----------I-----A-RE-------------------M------LE---IE----R-----------N

                                                          326
BNST1   LEVNRQGKLPNGIESNAFFRKGETGGWRDTLSESLADVIDRTTEQKFGGSGLKFSS
BNST2   -----E----S-M-TR---------V-------T----E-----I-E--Q-------C
BNST3   ----R---------T----------I-------------A------E----------C
ATST1   ----KE---------TKT-------I--------SFE
```

Figure 2. Comparison of the deduced amino acid sequences of *B. napus* sulfotransferase genes and *A. thaliana ATST1* (*RaR047*) (accession number Z46823). Dots indicate gaps introduced for optimal alignment. Regions I and IV, conserved in all cytosolic sulfotransferases are boxed. Residues critical for catalysis and PAPS binding are indicated (Marsolais and Varin, 1995, 1997, Kakuta et al., 1997). Domain II involved in the determination of substrate specificity is underlined (Varin et al., 1995).

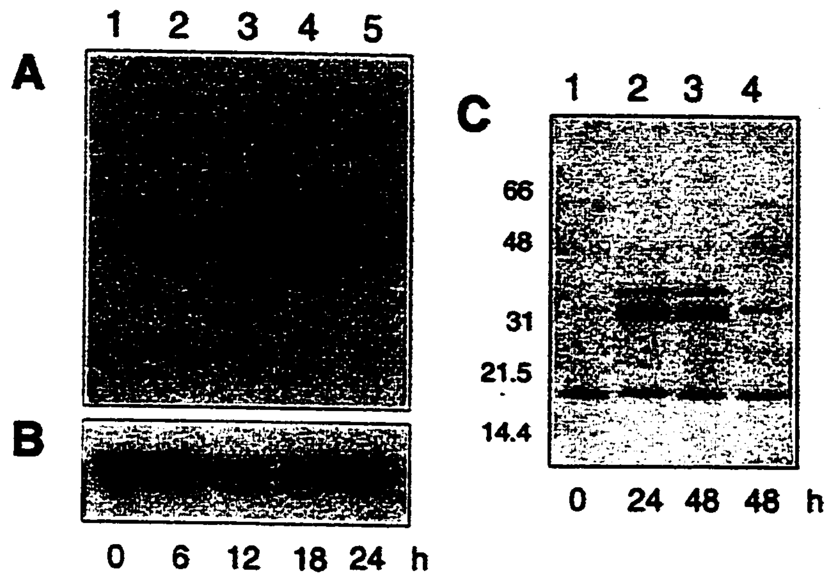


Figure 3. A) Northern analysis of *BNST* gene expression in *B. napus* seedlings treated with 10 mM salicylic acid for the indicated times. The blot was hybridized with the coding region of the *BNST1* gene. B) same blot as in *A* hybridized with an actin probe. C) Western analysis of sulfotransferase gene expression in *B. napus* and *A. thaliana*. Total proteins were extracted from *B. napus* seedlings (lanes 1- 3) and *A. thaliana* (lane 4) that have been incubated with 10 mM salicylic acid for the times indicated. The membrane was incubated with anti-BNST1 antibodies. Size of molecular weight markers is indicated on the left.

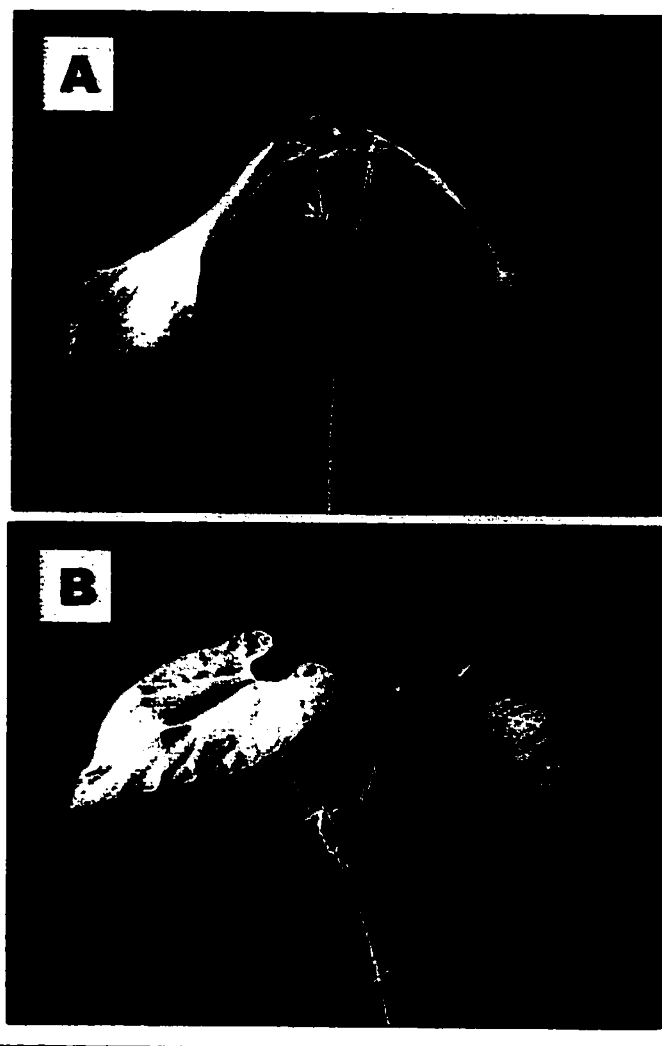


Figure 4. Biological activity of 10^{-10} mol epibrassinolide (A) and 10^{-10} mol epibrassinolide sulfate (B) in the bean second internode bioassay.

CHAPTER 3

Molecular and biochemical characterization of an ethanol-inducible steroid sulfotransferase from *Brassica napus*

In chapter 2, induction of the *BNST* genes by salicylic acid, and their proposed role during the plant defense response against pathogen infection was discussed. In this chapter, induction of the *BNST* genes by ethanol is presented. Ethanol is known to induce several plant cytochrome P450 genes involved in herbicide detoxication (Reichhardt et al., 1979, Potter et al., 1995). In plants, low oxygen stress results in a switch to ethanolic fermentation, and in the induction of several genes encoding anaerobic proteins (Sachs et al., 1996). Most anaerobic proteins are enzymes of glycolysis and ethanolic fermentation, with the exception of a xyloglucan endotransglycosylase (XET). We demonstrate that ethanol treatment of *Brassica napus* seedlings also results in the induction of *ADH* and *XET* genes. Furthermore, endogenous ethanol produced under hypoxia may be responsible for the induction of the steroid sulfotransferases under these conditions. Our results open a new perspective on gene regulation by ethanol in plants.

Molecular and biochemical characterization of an ethanol-inducible steroid sulfotransferase from *Brassica napus*

Frédéric Marsolais, Annie Rousseau and Luc Varin[1]

Department of Biology, Concordia University, 1455 de Maisonneuve Blvd. W., Montreal, Quebec, Canada, H3G 1M8

[1]Author for correspondence: Tel.: (514)-848-3396; Fax: (514)-848-2881;

E-mail: varinl@clone.concordia.ca

Part of this chapter was presented at the Congress of the International Society of Plant Molecular Biology, Quebec, Quebec, Canada, June 18-24, 2000.

This chapter will be submitted for publication to Plant Journal.

SUMMARY

Ethanol treatment of *Brassica napus* seedlings results in an induction of *BNST* genes, encoding steroid sulfotransferases. The *BNST4* cDNA was isolated from a library prepared with ethanol-treated seedlings. Recombinant BNST4 has a similar substrate specificity for 24-epibrassinosteroids as compared to the previously characterized BNST3 (Chapter 2), suggesting that they are isoenzymes. Ethanol treatment also resulted in the induction of alcohol dehydrogenase and xyloglucan endotransglycosylase genes. Furthermore, BNST polypeptides were induced under hypoxic and anoxic conditions. Our results suggest that endogenous ethanol produced under low oxygen stress may act as a signal regulating gene expression. The function of *BNST* induction under these conditions may be the modification of steroid-dependent growth and developmental processes.

INTRODUCTION

Alcohol ingestion results in an increased level of expression in mammalian liver of alcohol dehydrogenase (*ADH*) and of cytochrome P450-2E1 (*CYP2E1*) genes. The toxic effects of ethanol are thought to be mediated through these inducible enzymes. Acetaldehyde produced by ADH and CYP2E1 is rapidly conjugated with glutathione, leading to a depletion of reduced glutathione, while CYP2E1 is thought to be responsible for the production of reactive oxygen species leading to oxidative stress (Peters and Preedy, 1998, Mari and Cederbaum, 2000). Induction of members of the *CYP2B* family by phenobarbital has provided another paradigm for gene regulation by xenobiotics in mammalian liver (Kemper, 1998, Honkasaki and Negishi, 1998). In plants, ethanol treatment results in the induction of various P450s involved in herbicide detoxication (Reichhart et al., 1979, Potter et al., 1995). Some of these enzymes are also inducible by phenobarbital (Zimmerlin and Durst, 1992).

Mammalian class I and plant class P ethanol-active ADH have a different function and evolutionary origin (Shafqat et al., 1996, Martinez et al., 1996, Dolferus et al., 1997). Mammalian ADH is hypothesized to be involved in the detoxication of various alcohols and aldehydes in liver (Yoshida et al., 1991). In contrast, plant ADH participates in the metabolic pathway of ethanolic fermentation. The study of *ADH* genes, and of their role during low oxygen stress caused by flooding constitutes a classical subject of plant molecular biology (Freeling and Bennett, 1985, Drew, 1997). During development of maize and *Arabidopsis thaliana*, *ADH* genes are expressed during seed germination, in the root apex of seedlings, in the roots of mature plants, as well as in pollen grains (Walker et al., 1987, Olive et al., 1990, Dolferus et al., 1994). In maize root tips, hypoxia

and anoxia result in a change from respiratory metabolism to fermentation. During the first 20 minutes, lactic acid production occurs concomitant with a reduction in cytoplasmic pH, followed by the activation of pyruvate decarboxylase (PDC) and a switch to ethanolic fermentation (Roberts et al., 1984a and b). After 90 minutes of anaerobic stress, the selective accumulation of anaerobic proteins (ANPs) can be observed (Sachs et al., 1980). Most ANPs are enzymes of glycolysis or ethanolic fermentation, including PDC and ADH (Ricard et al., 1994, Sachs et al., 1996), with the notable exception of a xyloglucan endotransglycosylase (*XET*) gene, which may be involved in the formation of aerenchyma in root tissue (Saab and Sachs, 1996). In maize, hypoxic induction of *ADH* is tissue-specific, being restricted to cells of the root system (Freeling and Bennett, 1985). A similar pattern of tissue-specific induction is observed in *A. thaliana*, although a low level of induction is found in leaves (Dolferus et al., 1994). In comparison, *ADH* is fully inducible in mature leaves of rice, a flooding-resistant species (Xie and Wu, 1989).

Recent work on root and pollen metabolism points toward an important role of ethanolic fermentation under normal growth conditions (Bucher et al., 1995, Tagede et al., 1997, Tagede et al., 1998a). Although fermentation in the seed and root may be triggered by limited oxygen availability, it occurs during *in vitro* growth of pollen under fully aerobic conditions, and is regulated by the carbon source (Tagede et al., 1997). Despite these findings, *ADH* genes are hypothesized to be not essential, but required for survival under low oxygen stress (Schwartz, 1972). It is generally accepted that in *A. thaliana*, *ADH1* encodes the only ethanol-active ADH (Chang and Meyerowitz, 1986).

Loss of function mutations in *ADH1* have no effect on growth under normal conditions (Jacobs et al., 1988, Dolferus et al., 1990).

We recently reported the characterization of BNST3, a salicylate-inducible steroid sulfotransferase from *Brassica napus* (Chapter 2). BNST3 catalyzes the *O*-sulfonation of brassinosteroids and estrogenic steroids. The enzyme is stereospecific for 24-epibrassinosteroids, with a preference for 24-epicathasterone, an intermediate in the biosynthesis of 24-epibrassinolide. In analogy with the function of mammalian estrogen sulfotransferases (Strott, 1996), BNST3 was proposed to be involved in brassinosteroid inactivation. This is consistent with the inducible pattern of *BNST3* expression, as well as the absence of biological activity of 24-epibrassinolide sulfate in the bean second internode bioassay. In this paper, we describe the characterization of BNST4, an ethanol-inducible steroid sulfotransferase from *B. napus*. *BNST4* belongs to a sulfotransferase gene family comprising the previously characterized *BNST* genes and their *Arabidopsis thaliana* homolog *ATST1* (*RaR047*) (Lacomme and Roby, 1996, Chapter 2). Characterization of the enzymatic activity of recombinant BNST4 using brassinosteroids as substrates suggests that BNST3 and BNST4 are isoenzymes, although their substrate specificity with mammalian sex steroids differs. *BNST* gene expression is inducible by ethanol in a time- and dose-dependent manner. Using RT-PCR, we demonstrate that several *BNST* genes and *ADH* are ethanol-inducible, although their kinetics of transcript accumulation is variable. Our results also indicate that low oxygen stress results in the induction of BNST polypeptides, suggesting that ethanol produced during fermentation may act as a signal to regulate the expression of ethanol-responsive genes.

EXPERIMENTAL PROCEDURES

Plant growth and induction. *B. napus* (cv. Westar) seedlings were grown in Magenta boxes containing 30 ml of germination medium (1XMS salts, 1XB5 vitamins, 1% sucrose, 0.5 g/l 2-[*N*-morpholino]ethane-sulfonic acid, 0.8% phytoagar (Sigma) for six days at 25°C under long day conditions (16 h light, 8 h dark), and treatments were started by adding 2 ml of a 15X solution of alcohol on the surface of the medium. For phenobarbital treatments, roots were drenched in a 4 mM aqueous solution (Reichhart et al., 1979). Hypoxic conditions were generated by complete immersion of the seedlings under distilled water in closed Magenta boxes. For anoxia, the water was saturated with helium at a rate of 12.5 ml/min, in half-closed Magenta boxes.

Isolation of the *BNST4* cDNA. A *B. napus* cDNA library was constructed in lambda ZAPII (Stratagene) with poly-A^+ mRNA isolated from 6-day-old seedlings treated with 2% ethanol for 48 h. The library was probed with anti-BNST1 polyclonal antibodies (Chapter 2). Two identical positive clones were isolated, as estimated by restriction mapping, and one of the clones, *BNST4*, was sequenced manually using a T7 sequencing kit (Promega). Sequence analysis was performed using Blast (Altschul et al., 1990).

Expression of recombinant BNST4 in *Escherichia coli*. An oligonucleotide (5'-ACATGCATGCATGTCATCATCATCATCATC-3') was designed to introduce an *Sph*I restriction site at the 5' end of the *BNST4* coding sequence. This oligonucleotide was used in conjunction with the M13-20 primer to amplify the full-length *BNST4* sequence by the polymerase chain reaction using *Pwo* DNA polymerase (Roche Molecular Biochemicals). The PCR product was phosphorylated using T4 polynucleotide kinase,

digested with *SphI*, and subcloned into the *SphI* and *SmaI* sites in the polylinker of the bacterial expression vector pQE30 (Qiagen). Enzymes used for cloning were from New England Biolabs. The production of BNST4 in *E. coli* cultures ($A_{600} \approx 0.5$) was induced with 1 mM isopropyl ß-*D*-thio-galactopyranoside for 10 h at 22°C. Bacterial cells were collected by centrifugation, resuspended in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 14 mM ß-mercaptoethanol, and lysed by sonication. Cell debris was pelleted by centrifugation at 10000X*g* for 30 min. The recombinant BNST4 present in the supernatant was purified by affinity chromatography on nickel-nitrilotriacetic acid-agarose as recommended (Qiagen). In order to replace the imidazole buffer after purification, the protein was desalted on a PD-10 column (Pharmacia) and eluted with 50 mM Tris, pH 7.5 containing 14 mM ß-mercaptoethanol.

Enzyme assays. Analysis of substrate specificity was performed by testing the enzymatic activity with two different concentrations of acceptor substrates as follows: 200 and 5 µM. Acceptor substrates were dissolved in ethanol or methanol, and the final concentration of the cosolvent in the assay was 5%. Commercial substrates were obtained from Sigma, Aldrich and Steraloids. Reaction mixtures (50 µl) also contained 5 µM ^{35}S-PAPS (NEN Life Science Products) and approximately 1 µg of purified BNST4 in 50 mM Tris, pH 7.5. Reactions were allowed to proceed for 10 min at 25°C. Incorporation of ^{35}S-sulfonate was performed according to a standard assay (Varin et al., 1987). Kinetic analysis was performed with the preferred steroid substrates. The concentrations were 10, 5, 2 and 1 µM for 24-epicathasterone, 10, 8, 4 and 2.5 µM for 24-epiteasterone, 100, 60, 40, 20 and 10 µM for pregnenolone, 100, 60, 40, 20, 10 and 5 µM for dehydroepiandrosterone, and 40, 30, 20, 12 and 8 µM for androstenediol. Kinetic

parameters were determined from double-reciprocal Lineweaver-Burk plots. Tests of inhibition by steroids were performed at a fixed concentration of 10 μM 24-epicathasterone, since BNST4 was subject to substrate inhibition at higher concentrations. 17ß-estradiol and testosterone were assumed to be competitive inhibitors with respect to 24-epicathasterone, and $[I_{0.5}]$ values were deduced from plots of percentage of unhinibited initial velocity versus concentration of inhibitor (Segel, 1975). Concentrations were 100, 80, 60, 40 and 20 μM for testosterone, and 60, 40, 20, 10 and 5 μM for 17ß-estradiol. The K_m value for the cosubstrate was determined at a fixed concentration of 10 μM 24-epicathasterone, and PAPS concentrations of 5, 4, 2, 1 and 0.8 μM.

Expression of *BNST* genes in *Brassica napus* seedlings. *B. napus* seedlings were ground in liquid nitrogen, and either extracted to isolate total RNA (Cashmore, 1982), or boiled in SDS sample buffer to obtain total proteins. Northern blot analysis of total RNA was achieved according to standard procedures (Sambrook et al., 1989) using the ^{32}P-labeled coding regions of *BNST4*, and of *A. thaliana TCH4* (Xu et al., 1996). Protein extracts were separated by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel and transferred to nitrocellulose, and BNSTs were detected using anti-BNST1 polyclonal antibodies (Chapter 2) (dilution 1:1000) and goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase. For RT-PCR experiments, 2.5 μg of total RNA was treated with 20 U 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 the RNA was ethanol precipitated. After resuspension, cDNA was synthesized using Moloney Murine Leukemia Virus reverse

transcriptase (New England Biolabs) as recommended, and 2 μl from the total 25 μl RT reaction was used for the polymerase chain reaction with Ex Taq DNA Polymerase (Takara Biochemicals). Oligonucleotides used for RT-PCR were, for *BNST1*:
5'-GATTACTGCTCATCTCCAGAGGTCAATTTC-3'
and 5'-GAACTCAGCAATTCGCTTAACCTCAAC-3',
for *BNST2*: 5'-GATCTCCAGATTTCGATTTCTCCCAGTTGTC-3'
and 5'-GATCGTCTCTCCGGTTTTTTTCTTGG-3',
for *BNST3*: 5'-GATTTTGATTTCTCCCTCTTGCCTTTTC-3'
and 5'-GAACTCAGCGATTCGCTTAACCTCC-3' (Chapter 2),
and for *BNST4*: 5'-GTCTCCAAATTTCGATTTCACTGAG-3'
and 5'-GAATTCGGCTATTCTCTTAATCGTATC-3'. For *ADH*, primers
(5'-GGATCATTGGTGTTGATCTCAAC-3' and
5'-GAAAGTACCCTTGAGTGTCCTCTC-3') were designed in the conserved regions of the three genes reported from *B. napus* (Brunel et al., 1999). Actin primers,
5'-CTGGTGATGGTGTGTCTCACAC-3' and
5'-GTTGTCTCATGGATTCCAGGAG-3', were also designed in conserved regions of the two sequences reported from this species (Fristensky et al., 1999, and Genbank accession no. AF111812).

RESULTS

A cDNA clone coding for a sulfotransferase, designated *BNST4*, was isolated from a cDNA library prepared from ethanol-treated *B. napus* seedlings by screening with anti-BNST1 polyclonal antibodies (Chapter 2). *BNST4* encodes a protein with a predicted molecular mass of approximately 35 kDa, and is a member of a sulfotransferase gene

family which includes the previously characterized *BNST1*, *-2* and *-3*, and their *A. thaliana* homolog *ATST1* (*RaR047*) (Lacomme and Roby, 1996, Chapter 2). BNST4 displays 87% amino acid sequence identity with BNST3 and ATST1, as compared to 83% and 79% with BNST2 and BNST1 respectively.

In order to define the biochemical function of BNST4, the substrate specificity of the enzyme was characterized. In preliminary experiments, enzymatic activity of the purified recombinant BNST4 expressed in *Escherichia coli* was tested with a variety of steroid molecules, including brassinosteroids, sterols, and mammalian steroids. Sterols were not accepted as substrates, although low but significant enzyme activity was observed with hydroxycholesterols when tested at high concentrations. The apparent K_m value for PAPS was 1.0 μM. Kinetic analysis was performed with the preferred substrates of the enzyme (Table 1). BNST4 displays a substrate specificity with brassinosteroids that is similar to BNST3 (Chapter 2). The enzyme is stereospecific for 24-epimers of brassinosteroids, with a preference for 24-epicathasterone, followed by 24-epiteasterone (Table 1, Figure 1). These molecules are metabolic precursors of the biologically active end product 24-epibrassinolide. Like BNST3, BNST4 does not accept 22-deoxy-24-epiteasterone as substrate, suggesting that sulfonation is taking place at position 22 on the side chain.

The specificity of BNST4 with mammalian estrogens and hydroxysteroids is distinct from that of BNST3 (Chapter 2). Whereas BNST3 sulfonates the 17ß-hydroxyl group of estradiol, the enzymatic activity of BNST4 with dehydroepiandrosterone and pregnenolone suggests that sulfonation is taking place at position 3 of hydroxysteroids (Table 1). A further difference is the inhibition of BNST4 activity by estrogens and

testosterone (Table 2). The major structural determinant of inhibition appears to be the presence of a rigid keto- or phenolic hydroxyl group at position 3 of the steroid nucleus, as compared with the 3ß-hydroxyl group present in the hydroxysteroid substrates (Tables 1 and 2, Figure 1).

The kinetics of induction of *BNST* genes in response to ethanol was studied by Northern and Western blot using the *BNST4* cDNA, or anti-BNST1 polyclonal antibodies as probes. *B. napus* seedlings grown under normal conditions contain very low to undetectable levels of *BNST* mRNAs and proteins (Figure 2). Treatment with 2% ethanol resulted in an increase in the steady-state levels of *BNST* transcripts within one hour after treatment (Figure 2A and C), and in the accumulation of a protein with an apparent molecular mass of approximately 35 kDa (Figure 2D). The kinetics of induction of *BNST* transcripts and proteins is comparable to that of maize ANPs (Sachs et al., 1996). Induction of *XET* genes by ethanol was also tested using *A. thaliana TCH4* as a probe (Xu et al., 1996). The steady-state levels of *XET* transcripts were induced by ethanol, with a faster kinetics than that of *BNST* genes (Figure 2B and C). *BNST* induction was also analyzed in different tissues of the seedling (Figure 3). Treatment with ethanol resulted in the accumulation of two BNST protein bands in roots and hypocotyls, as compared with a single band in cotyledons. This result is consistent with the presence of a multigene family in *B. napus*, and suggests that the pattern of *BNST* induction by ethanol is tissue-specific. Furthermore, the timing of BNST accumulation in cotyledons is delayed as compared with that in roots and hypocotyls. This difference may be related to the rate of ethanol uptake and transport from the root to the cotyledon.

The specificity of BNST induction by ethanol was analyzed by comparing the effects of aliphatic alcohols and phenobarbital. Ethanol was the most potent BNST inducer, while methanol resulted in a weaker induction of a single BNST protein band (Figure 4). Propanol had very little effect, while butanol treatment was toxic and resulted in extensive protein degradation. A similar response to different alcohols was noted for the induction of cytochrome P450 content and activity towards herbicides in Jerusalem artichoke tubers (Reichhart et al., 1979). A slight induction of BNST proteins was also observed after treatment with phenobarbital (Figure 5).

The dose-response relationship of BNST induction by ethanol was characterized by Western blot. Induction of BNST proteins occurred at 0.1% ethanol, and reached a maximum level at 1.6% (Figure 6). These doses may be compared to those used to induce other ethanol-responsive genes in plants. Induction of P450s in Jerusalem artichoke tuber slices was found to be maximum at ca. 1.2% ethanol (Reichhart et al., 1979), while induction of P450s in etiolated maize seedlings was performed by subirrigating with 10% ethanol (Potter et al., 1995). An ethanol-inducible system of expression was recently developed for use in transgenic plants (Caddick et al., 1998). This system makes use of the ethanol-inducible promoter of the *alcA* gene from *Aspergillus nidulans*, which encodes alcohol dehydrogenase I. The dose requirement for induction has been determined in transgenic tobacco plants expressing the *alc* system (Salter et al., 1998). In hydroponically-grown seedlings, expression of a chloramphenicol acetyltransferase reporter gene was induced at 0.01% ethanol, and reached a maximum at 0.1%. Leaf-spraying of mature plants required doses of 2.5 to 20%, while root drenching of soil-grown plant required at least 0.5% ethanol to achieve significant induction.

To evaluate the biological significance of ethanol induction of *BNST* genes, the impact of low oxygen stress was analyzed. Hypoxic conditions were generated by complete submergence of the seedlings, and anoxic conditions were obtained by saturation of the aqueous medium with helium. Both conditions resulted in a limited induction of sulfotransferase proteins as compared to a treatment with 2% ethanol (Figure 7). This phenomenon may be dependent on the lower levels of ethanol accumulated within the tissue under hypoxic and anoxic conditions.

Gene-specific primers were designed in order to study ethanol induction of individual *BNST* transcripts by RT-PCR. The specificity of the primers was confirmed in control experiments where cross-amplification of the various DNA templates was tested. *BNST1* expression was not enhanced by ethanol (Figure 8). However, *BNST2*, *-3* and *-4* were inducible, but the kinetics of accumulation of their mRNAs was different. *BNST4* displayed the fastest response, with a maximal level at 1 h, followed by a steady decline. *BNST2* and *BNST3* showed slower kinetics, with peak levels at 4 to 6 h, reflecting the results obtained in Northern blot (Figure 2A and B). Induction of *ADH* by ethanol was also tested. Primers were designed in the conserved regions of three *ADH* sequences reported from *B. napus* (Brunel et al., 1999). The level of *ADH* mRNAs increases in response to ethanol, with a kinetics that is comparable to those of *BNST2* and *BNST3* (Figure 8). This result is in agreement with reports that plant ADH enzyme activity is inducible by ethanol (App and Meiss, 1958, Kollöfel, 1968).

DISCUSSION

The biochemical characterization of BNST4 indicates that it shares with BNST3 the same substrate specificity for brassinosteroids. However, the two steroid

sulfotransferases differ in their behavior towards estrogens and hydroxysteroids. Our results suggest that BNST4 sulfonates 24-epibrassinosteroids at position 22, whereas hydroxysteroids are sulfonated at position 3. This difference in the position of sulfonation implies a different orientation of the steroid molecules at the active site of the enzyme. This phenomenon is known to occur in mammalian hydroxysteroid sulfotransferases which catalyze the sulfonation of testosterone at position 17, and of androsterone or dehydroepiandrosterone at position 3 (Park et al., 1999, Falany, 1997).

Although a large number of studies have described the occurrence, the metabolic interconversions, and the biological effects on plants of estrogens and androgens (Geuns, 1978), the results of these studies are highly controversial (Leighton Jones and Roddick, 1988). The enzymatic activity of BNST3 and BNST4 with mammalian steroids may simply be related to a conservation of biochemical function between plant and mammalian steroid sulfotransferases. Functional conservation has been well documented for the plant and mammalian steroid 5α-reductases, involved in brassinosteroid and sex steroid hormone biosynthesis, respectively (Li et al., 1997). Another interesting example comes from the recent characterization of a Δ^5-3ß-hydroxysteroid dehydrogenase from *Digitalis lanata* which catalyzes the conversion of pregnenolone to progesterone (Finsterbusch et al., 1999). These steroid molecules are known to occur in *Digitalis* species as metabolic precursors of cardenolides (Nes, 1977). Since its enzymatic activity was detected in plants devoid of cardenolides, the Δ^5-3ß-hydroxysteroid dehydrogenase was hypothesized to be involved in the conversion of teasterone to 3-dehydroteasterone during brassinosteroid biosynthesis (Finsterbusch et al., 1999).

The function of BNST induction during low oxygen stress may be to inhibit brassinosteroid-dependent growth processes. By acting on 24-epibrassinolide precursors, BNST enzymes may block further biosynthesis. A similar function was proposed for BNST3 induction during pathogen infection (Chapter 2). Under the developmental and metabolic conditions encountered during low oxygen stress, the stimulation of cell elongation by brassinosteroids may not be advantageous. Plants respond to low oxygen stress caused by flooding by a number of metabolic and anatomical adaptations (Drew, 1997, Crawford and Braendle, 1996). Among these adaptations, the development of aerenchyma in the root system increases the capacity to avoid oxygen deficits, and the survival in flooded soils. This phenomenon may be incompatible with brassinosteroid-dependent growth processes. Mechanisms other than sulfonation are likely to regulate the biological activity of brassinosteroids. The *BAS1* gene from *A. thaliana* encodes CYP72B1 which catalyzes the C26-hydroxylation of brassinolide (Neff et al., 1999). Overexpression of *BAS1* in transgenic *A. thaliana* and tobacco leads to a phenotype similar to that of brassinosteroid-deficient or insensitive mutants. *BAS1* is expressed in hypocotyl and rosette leaves of *A. thaliana*, and is involved in the light regulation of hypocotyl elongation.

In mammalian liver, treatment with ethanol, or other xenobiotics such as phenobarbital induces the expression of different subgroups of detoxication enzymes, including cytochrome P450s, glutathione *S*-transferases, UDP-glucoronosyltransferases and sulfotransferases (Koop et al., 1985, Nebert, 1991, Runge-Morris et al., 1998). Similar responses are observed in plants, where ethanol induces the expression of several cytochrome P450s involved in herbicide detoxication, and of a glutathione *S*-transferase

subunit (Reichhart et al., 1979, Potter et al., 1995). Ethanol induction of plant cytochrome P450s has been interpreted as a response to a general chemical stress, leading to the activation of detoxication mechanisms (Potter et al., 1995). However, plants are not normally exposed to xenobiotics, therefore the identity of the physiological substrates of the inducible enzymes represents a crucial problem (Zimmerlin and Durst, 1992). Although we cannot eliminate a broad function in detoxication of steroid-like molecules for the BNST enzymes, BNST3 and BNST4 display a high degree of specificity for steroid substrates in vitro, including 24-epibrassinosteroids (Chapter 2). Our results suggest that the significance of the ethanol response in plants is related to specific physiological stress, such as oxygen deficit.

The question of dosage is critical to evaluate the biological significance of *BNST* induction by ethanol. The doses applied in the treatments were similar to those used for cytochrome P450 induction in Jerusalem artichoke tuber slices (Reichhart et al., 1979). In addition, in the *alc* system of inducible expression, comparable doses were used to induce the fungal *alcA* promoter when soil-grown transgenic tobacco plants were treated by root drenching (Salter et al., 1998). The doses that were used may also be compared with the high levels of ethanol produced during pollen germination, that reach concentrations exceeding 100 mM (ca. 0.4 %) in the growth medium (Bucher et al., 1995). The results obtained on hypoxic and anoxic stress suggest that *BNST* induction may be mediated by endogenous ethanol produced during fermentation. This interpretation is in agreement with the observation that the *alc* system of expression is induced in transgenic tobacco under anoxia (Salter et al., 1998).

The results obtained on *ADH* and *XET* induction raise the possibility that ethanol may act as an inducer of genes encoding ANPs. The early accumulation of ethanol during low oxygen stress is consistent with this hypothesis. One important question is whether the levels of endogenous ethanol increase sufficiently for significant induction. During flooding, ethanol levels may be regulated through diffusion from the roots, at least in tolerant species (Drew, 1997, Crawford and Braendle, 1996). Ethanol may also be reconverted to acetaldehyde, and subsequently to acetate and acetyl-CoA. This pathway of ethanol metabolism is known to operate when plants are returned to aerobic conditions (Cossins, 1978, Waters et al., 1989). An important experiment to evaluate the role of ethanol in *ADH* induction was performed with transgenic tobacco leaves expressing a bacterial *PDC* gene (Bucher et al., 1994). In wild-type tobacco leaves, *ADH* is inducible by anoxia. In transgenic leaves, treatment with respiration inhibitors under aerobic conditions led to the accumulation of ethanol, but the steady state levels of *ADH* mRNA did not increase. This result led the authors to propose that plants perceive low oxygen stress directly through an oxygen-sensing mechanism. Further experiments using null *adh* mutant backgrounds, or inhibitors of ADH enzyme activity may be needed to evaluate the role of ethanol in the induction of ANPs.

ACKNOWLEDGEMENTS

We thank Dr. Janet Braam for the gift of the *TCH4* cDNA. Frédéric Marsolais and Annie Rousseau were the recipients of postgraduate scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC). This work was supported by NSERC.

Table 1. Kinetic parameters of recombinant BNST4 for the preferred steroid substrates

	K_m (μM)	V_{max} (pkatal mg^{-1})	V_{max}/K_m (pkatal mg^{-1} μM^{-1})
24-Epicathasterone	4.9	22	4.5
24-Epiteasterone	3.0	7.9	2.6
Pregnenolone	14	39	2.8
Androstenediol	12	44	3.7
Dehydroepiandrosterone	20	28	0.7

Table 2. BNST4 inhibition by mammalian sex steroids.

Inhibitor	Inhibition (%) [I]= 5μM[a]	Inhibition (%) [I]=100μM	$[I]_{0.5}$ (μM)
17ß-Estradiol	49	87	5.5
17α-Estradiol	24	64	n. d.
17ß-Estradiol 3-methyl ether	28	73	n. d.
Estrone	0	30	n. d.
Testosterone	n. d.	50	100

[a] Inhibition was determined at a concentration of 24-epicathasterone equal to 10 μM, and a PAPS concentration of 5 μM. 100% Enzyme activity was equal to 12 picokatal/mg. n. d., not determined.

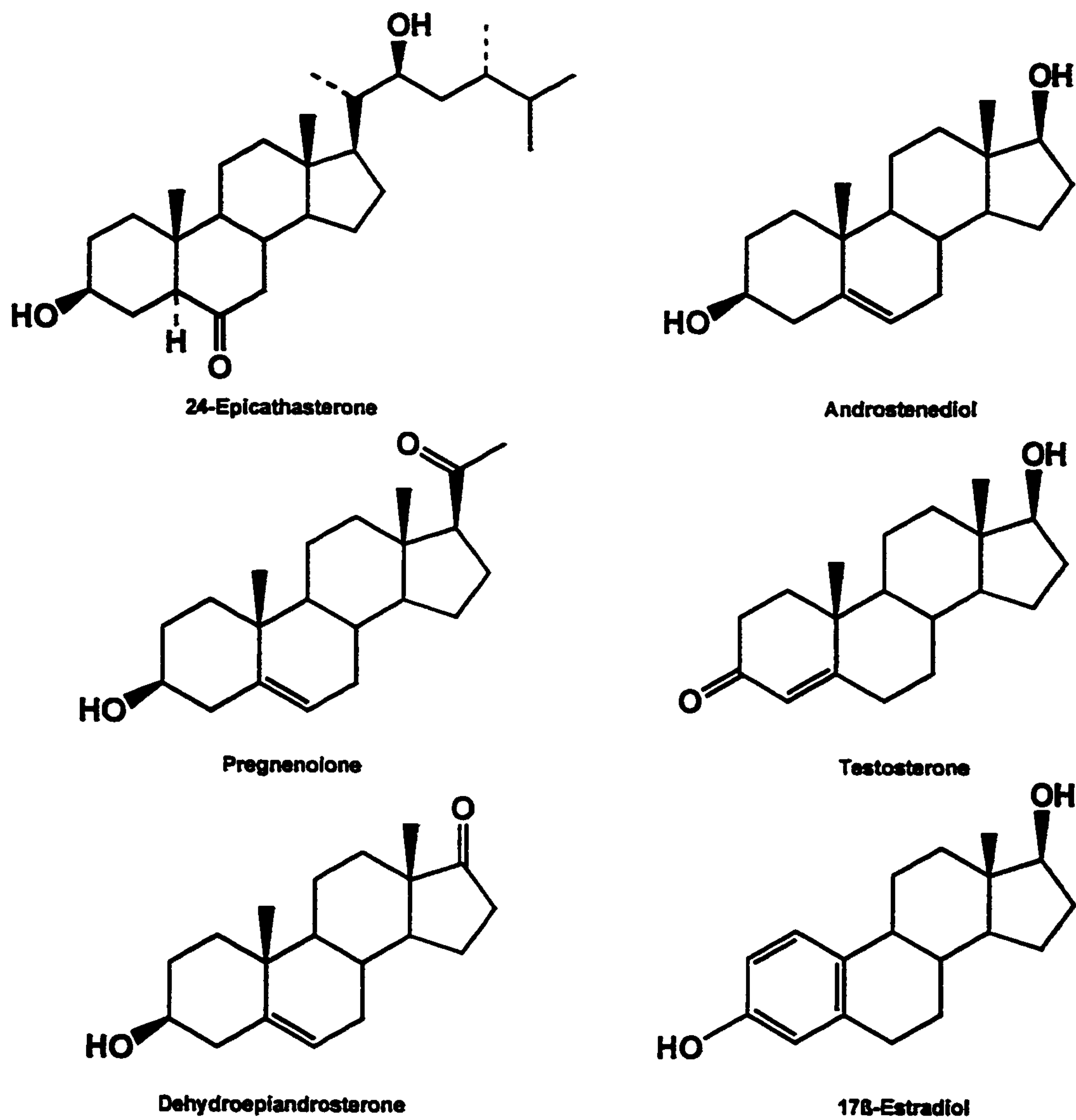


Figure 1. Chemical structure of the steroids used in this study.

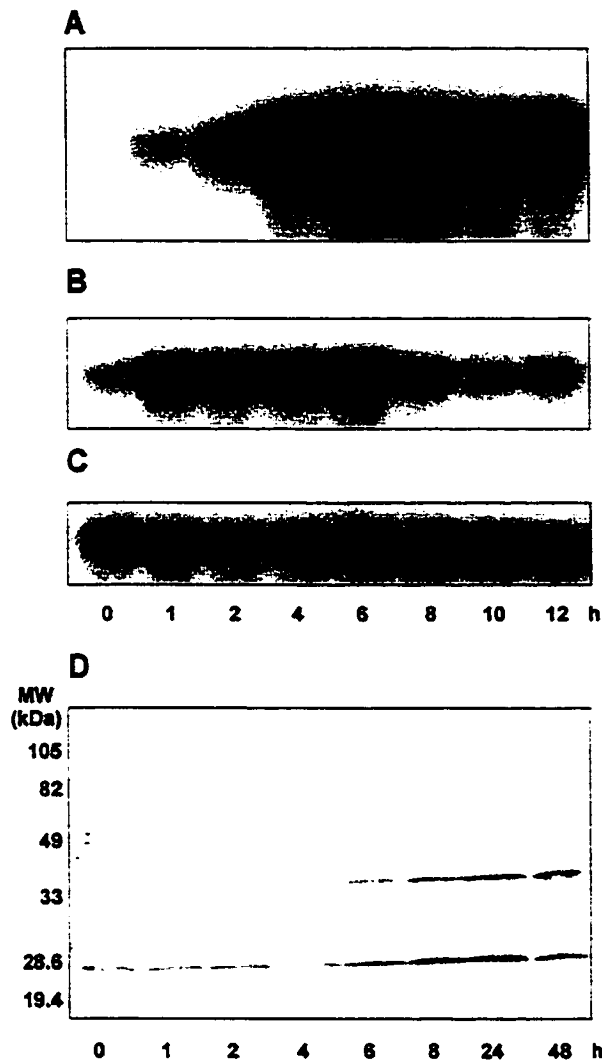


Figure 2. Time-course of *BNST* expression in *B. napus* seedlings treated with 2% ethanol. A) Northern analysis of *BNST* gene expression. The blot was hybridized with the coding region of the *BNST4* cDNA. B) same blot hybridized with *A. thaliana TCH4*. C) same blot hybridized with an actin probe. D) Western analysis of BNST expression. The membrane was incubated with anti-BNST1 antibodies. Size of molecular weight markers is indicated on the left.

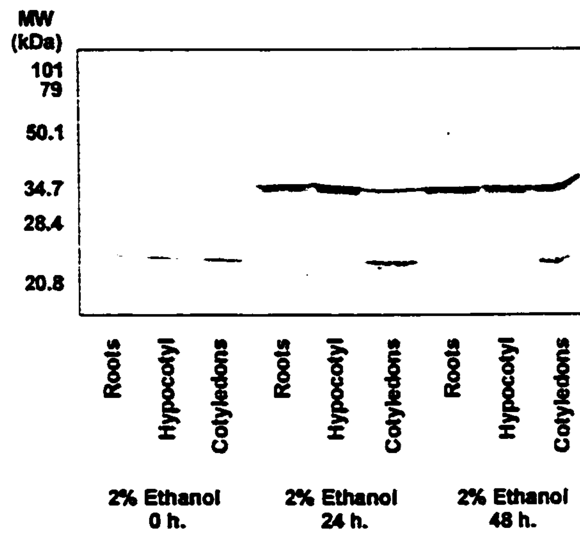


Figure 3. Western analysis of BNST induction by treatment with ethanol in different tissues of the seedling.

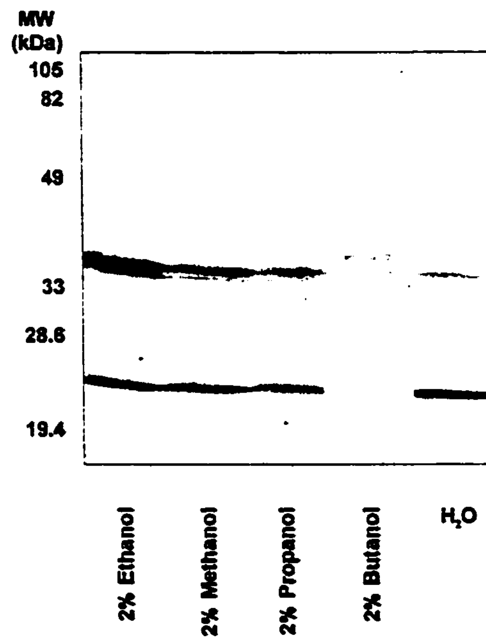


Figure 4. Western analysis of BNST induction in response to treatment with different alcohols.

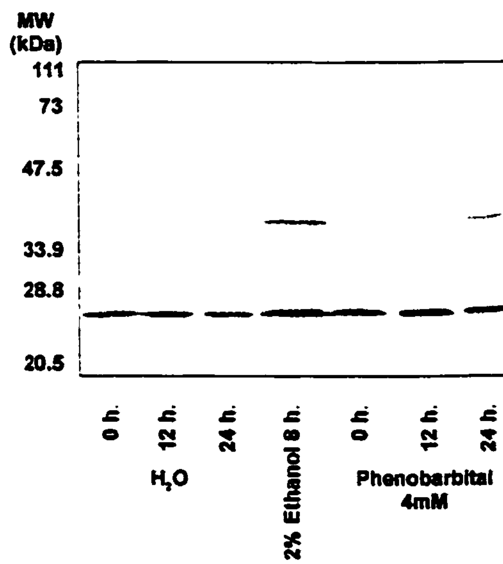


Figure 5. Western analysis of BNST induction by treatment with phenobarbital.

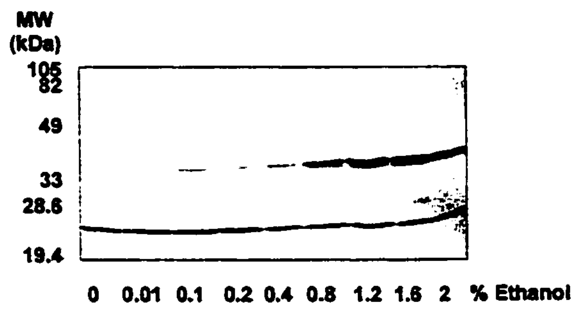


Figure 6. Western analysis of the dose of ethanol required for BNST induction.

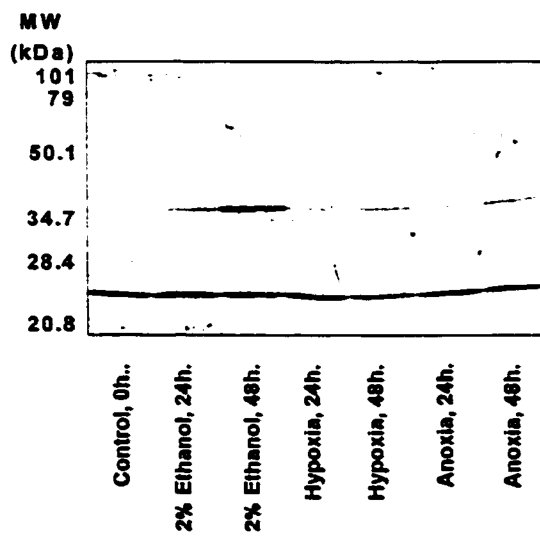


Figure 7. Western analysis of BNST induction during hypoxia and anoxia.

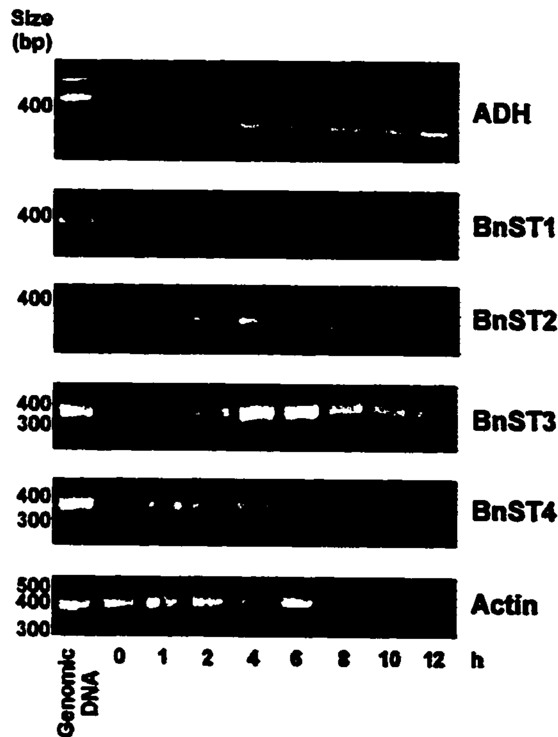


Figure 8. RT-PCR analysis of *ADH* and *BNST* induction by treatment with ethanol. For *ADH*, primers were designed in the conserved regions of the three genes reported from *B. napus*. For *BNST* genes, specific primers were designed to amplify individual transcripts. The specificity of the primers was confirmed in control experiments where cross-amplification of the different templates was tested.

CHAPTER 4

Regulation of steroid sulfotransferase genes from *Brassica napus* in response to salicylic acid and during development

In chapter 3, the induction of *BNST* genes by ethanol was characterized. Using RT-PCR, we demonstrated that *BNST2*, *-3* and *-4* are inducible by ethanol, but that *BNST1* is not. Furthermore, the kinetics of induction of the *BNST* genes by ethanol are variable, and *BNST4* displays the fastest response. In addition, ethanol treatment results in the induction of BNST polypeptides in all tissues of the seedling, and the pattern of BNST isoform accumulation is tissue-specific. In this chapter, we applied the same experimental approach to further study the induction of *BNST* genes by salicylic acid. The same *BNST* genes are inducible by ethanol and salicylic acid. However, the kinetics of induction of *BNST* genes by salicylic acid show an inverse relationship with those observed for ethanol induction, with *BNST4* having the slowest response. Salicylate treatment also results in the accumulation of BNST polypeptides in all tissues of the seedling, and their pattern of accumulation is tissue-specific. The tissue localization of *BNST2* and *-3* expression during development was studied in transgenic *Arabidopsis thaliana* plants expressing promoter-GUS fusions. The results of these experiments are interpreted in relation with the tissue localization of brassinosteroid biosynthetic genes and the proposed functions of the steroid sulfotransferases.

Regulation of steroid sulfotransferase genes from *Brassica napus* in response to salicylic acid and during development

Frédéric Marsolais, Cinta Hernández Sebastià, Samer Hossein and Luc Varin[1]

Department of Biology, Concordia University, 1455 de Maisonneuve Blvd. W., Montreal, Quebec, Canada H3G 1M8

[1]Author for correspondence: Tel.: (514)-848-3396; Fax: (514)-848-2881; E-mail: varinl@clone.concordia.ca

SUMMARY

The *BNST* genes encoding steroid sulfotransferases from *Brassica napus* are inducible by salicylic acid. To further understand their regulation, the induction of individual *BNST* genes by salicylic acid was characterized by RT-PCR. The *BNST1* gene, which is the most distant to other members of the gene family is not inducible. In contrast, the *BNST2*, *-3* and *-4* genes are inducible, but variable kinetics of transcript accumulation were observed. We also demonstrate that BNST polypeptides are inducible by salicylic acid in all tissues of the seedling, but the pattern of accumulation of BNST isoforms is tissue-specific. The tissue localization of expression of the *BNST2* and *-3* genes was studied in transgenic *Arabidopsis thaliana* expressing promoter-GUS fusions. Expression was observed in mature cells of leaf organs and of the transition region between the root and hypocotyl. The expression in fully differentiated cells of these tissues is compatible with a function of the steroid sulfotransferases in brassinosteroid inactivation. However, the expression in a region of leaf tissue which develops an early competence for the export of photosynthates suggests a possible function of sulfonation in the transport of brassinosteroid intermediates.

INTRODUCTION

BNST genes constitute a family of steroid sulfotransferase genes in *Brassica napus* comprising at least four members (*BNST1* to *-4*). A single homolog of the *BNST* genes, *RaR047*, is present in the genome of *Arabidopsis thaliana* (Lacomme and Roby, 1996). *RaR047* was recently renamed *ATST1* to simplify the nomenclature of sulfotransferase-coding genes in *A. thaliana*. BNST3, BNST4 and ATST1 catalyze the sulfonation of 24-epibrassinosteroids, with a preference for 24-epicathasterone, followed by 24-epiteasterone (Chapters 2 and 3, Annex I). The preferred substrates of these enzymes are intermediates in the biosynthesis of 24-epibrassinolide. 24-Epibrassinolide sulfate produced *in vitro* is inactive in the bean second internode bioassay, a standard test for the biological activity of brassinosteroids (Chapter 2). *BNST* genes and *ATST1* are inducible by salicylic acid, suggesting that they play a role in the plant defense response against pathogen infection (Lacomme and Roby, 1996, Chapter 2). Based on the lack of biological activity of 24-epibrassinolide sulfate, and the inducible pattern of expression of *BNST* genes, brassinosteroid sulfonation was proposed to constitute a mechanism of hormone inactivation. A similar function has been demonstrated for the sulfonation of steroid hormones in mammals (Strott, 1996).

In plants, physiological adaptations to environmental conditions often require changes in hormone action. During the plant defense response to pathogens, molecules such as H_2O_2, salicylic acid, jasmonic acid and ethylene act as chemical signals in the signal transduction pathways leading to the establishment of physical and chemical barriers against infection. During this response, energy that is normally used for growth has to be reallocated to defense mechanisms. For instance, processes of cell wall

reinforcement are incompatible with hormone-stimulated cell division and expansion. In support of this hypothesis, it has recently been demonstrated that a H_2O_2-activated protein kinase cascade down-regulates the expression of auxin-responsive genes (Kovtun et al., 2000).

Besides alterations in hormone signal transduction pathways, modification of endogenous hormone levels is another mechanism that may be used to modulate hormone action. Pathogen infection is known to result in a coordinated decrease in expression of genes involved in sterol biosynthesis, such as squalene synthase and sterol C24-methyltransferase (Devarenne et al., 1998, Shi et al., 1996). In several species, the reduction in sterol biosynthesis is coupled with an induction of sesquiterpene phytoalexin biosynthesis. Both biosynthetic pathways use squalene as a common intermediate. In addition to the channeling of intermediates to a defense pathway, the reduction in sterol biosynthesis may provide a mechanism to reduce the endogenous levels of brassinosteroid hormones.

The study of brassinosteroid-deficient mutants of *A. thaliana* has allowed a better understanding of the regulation and spatial organization of brassinosteroid biosynthesis. The first reaction of side-chain hydroxylation catalyzed by DWF4 is the rate-limiting step in the pathway (Sakurai, 1999, Clouse and Feldmann, 1999). DWF4 is a cytochrome P450-dependent monooxygenase which catalyzes the hydroxylation of campestanol at position 22, resulting in the formation of cathasterone (Choe et al., 1998). The following step of hydroxylation at position 23 to form teasterone is catalyzed by CPD, another cytochrome P450-dependent monooxygenase (Szekeres et al., 1996). *CPD* transcription is under negative feedback regulation by end products of the brassinosteroid pathway

(Mathur et al., 1998). This feedback mechanism is dependent on Brassinosteroid-Insensitive 1 (BRI1), a leucine-rich receptor protein kinase which is involved in the brassinosteroid response (Li and Chory, 1997). *A. thaliana bri1* mutants accumulate high levels of end products of the brassinosteroid pathway (Noguchi et al., 1999b). However, nothing is known about the regulation of brassinosteroid biosynthesis under stress conditions, such as pathogen infection.

Brassinosteroid biosynthesis appears to be compartmented between different tissues. *CPD* is expressed in leaves and cotyledons, but not in the elongation zones of the root and hypocotyl, which are major target tissues of brassinosteroid action (Mathur et al., 1998). In comparison, the tomato *DWARF* gene, which is involved in the conversion of 6-deoxocastasterone to castasterone, the immediate precursor of brassinolide, has been reported to be expressed in zones of cell elongation (Lenton, 1998). Therefore, transport of brassinosteroid intermediates to their site of action has been proposed.

In this paper, the regulation of *BNST* expression has been further characterized. Salicylate induction of individual *BNST* genes was studied by RT-PCR. Three members of the gene family are inducible, but their kinetics of mRNA accumulation are variable. Salicylate induction was observed in all tissues of the seedlings, as determined by Western blot, but the pattern of accumulation of BNST isoforms was tissue-specific. In transgenic *A. thaliana* plants expressing *BNST2* or *BNST3* promoter-GUS fusions, expression was observed in leaves and cotyledons, as well as at the root-hypocotyl junction. This pattern of expression is comparable to that of *CPD*, supporting the hypothesis that BNST3 and CPD might share the same substrate.

EXPERIMENTAL PROCEDURES

Plant growth and induction. Seeds from *Arabidopsis thaliana* (ecotype C24) and derived transgenic plants were grown on germination medium (1XMS salts, 1XB5 vitamins, 1% sucrose, 0.5 g/l 2-[*N*-morpholino]ethane-sulfonic acid, 0.8% agar) at 23°C under long day conditions (16 h light, 8 h dark). Seedlings of *Brassica napus* (cv. Westar) were grown for 6 days in vermiculite and treated with 10 mM salicylate by root drenching.

Expression of *BNST* genes in *Brassica napus* seedlings. *B. napus* seedlings were ground in liquid nitrogen, and either extracted to isolate total RNA (Cashmore, 1982), or boiled in SDS sample buffer to extract total protein. Protein extracts were separated by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel and transferred to nitrocellulose. BNSTs were immunodetected using anti-BNST1 polyclonal antibodies (Chapter 2) (dilution 1:1000) and goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase.

For RT-PCR experiments, 2.5 μg of total RNA was treated with 20 U 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 the RNA was ethanol precipitated. After resuspension, cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase (New England Biolabs) as recommended, and 2 μl from the total 25 μl RT reaction was used for the polymerase chain reaction with Ex Taq DNA Polymerase (Takara Biochemicals).

Oligonucleotides used for RT-PCR were, for *BNST1*:

5'-GATTACTGCTCATCTCCAGAGGTCAATTTC-3'

and 5'-GAACTCAGCAATTCGCTTAACCTCAAC-3',

for *BNST2*: 5'-GATCTCCAGATTTCGATTTCTCCCAGTTGTC-3'

and 5'-GATCGTCTCTCCGGTTTTTTTCTTGG-3',

for *BNST3*: 5'-GATTTTGATTTCTCCCTCTTGCCTTTTC-3'

and 5'-GAACTCAGCGATTCGCTTAACCTCC-3',

and for *BNST4*: 5'-GTCTCCAAATTTCGATTTCACTGAG-3'

and 5'-GAATTCGGCTATTCTCTTAATCGTATC-3'. For the chitinase class IV gene (*LSC222*), primers used were: 5'-CCTGATCCTAACCGTTTCCAAACCG-3' and 5'-GCCCGATTTAAAGCTGGAGCGAAC-3' (Hanfrey et al., 1996). Primers for actin, 5'-CTGGTGATGGTGTGTCTCACAC-3' and 5'-GTTGTCTCATGGATTCCAGGAG-3', were designed in conserved regions of the two sequences reported from *B. napus* (Fristensky et al., 1999, and Genbank accession no. AF111812).

Expression of *BNST* promoter-GUS fusions in transgenic *Arabidopsis thaliana*. Constructs were prepared bearing translational fusions of GUS (Jefferson et al., 1987) with a 1081 bp promoter of *BNST2*, and a 1001 bp promoter of *BNST3*. The resulting GUS fusion proteins have 9 additional amino acid residues at the NH_2-terminal. Oligonucleotide primers were designed to introduce *Hind*III and *Bam*HI sites at the respective 5' and 3' ends of the promoter fragments by the polymerase chain reaction using Vent DNA polymerase (New England Biolabs). The original *BNST* clones in vector pTZ18R were used as templates for the polymerase chain reaction. Primers used for the promoter of *BNST2* were 5'-CCCAAGCTTCAACAATTCGCCAAAATAGC-3', and

5'-CGGGATCCCATTGCTAAGAGTAAAGAGC-3' (translation initiation codon underlined), and for the promoter of *BNST3*,

5'-CCCAAGCTTCAGATTCACAGGGAACTGTTGC-3' and

5'-CGGGATCCCATTGCAAGAGTAAAGATC-3'. After restriction endonuclease digestion, PCR fragments were ligated into the *Hin*dIII and *Bam*HI sites in the polylinker of the binary vector pBI101 (Clontech). Positive clones were analyzed by restriction endonuclease digestion. The junctions between the promoters and the GUS coding sequence were sequenced to confirm that the translation initiation codon of the *BNST* genes was in frame with that of the GUS gene.

Constructs bearing the promoter-GUS fusions were transformed into *Agrobacterium tumefaciens* strain LBA4404 using a freeze-thaw method (An et al., 1988). Transgenic *A. thaliana* plants were generated using the root explant method (Valvekens et al., 1988). Transgenic plants were selected on medium containing 50 mg/l kanamycin. For the analysis of independent transgenic lines, genomic DNA from pools of T2 plants was analyzed by Southern blot. For genomic DNA extraction, tissue was ground in liquid nitrogen and homogenized in extraction buffer [3% hexadecyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 52 mM ß-mercaptoethanol, 20 mM EDTA and 100mM Tris-HCl pH 8.0] preheated at 60°C. The extract was incubated for 30 min at 60°C, with occasional shaking. The aqueous phase was extracted twice with chloroform, and precipitated with isopropanol. The precipitate was washed in 95% ethanol, containing 10 mM ammonium acetate, for 20 min. Genomic DNA was further purified by RNase and proteinase K digestions, followed by phenol-chloroform extraction. For Southern blot, 10 µg of genomic DNA was digested with *Eco*RI. The full-length

promoter fragments were used as probes, and the blots were hybridized under stringent conditions according to standard procedures (Sambrook et al., 1989). All enzymes were from New England Biolabs and were used according to the manufacturer's recommendations.

Histochemical GUS assays. Histochemical localization of GUS activity was performed according to Jefferson et al. (1987) with slight modifications. Plants were incubated for 16 h with 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucoronide (X-gluc), in 50 mM sodium phosphate pH 7.2, 0.5% Triton X-100, at 37°C with agitation. X-Gluc was dissolved as a 20X stock in dimethylformamide.

RESULTS

Salicylate induction of *BNST* genes in *Brassica napus* seedlings. The *BNST* genes are inducible by salicylic acid (Chapter 2). Their *A. thaliana* homolog, *ATST1*, is also inducible by salicylic acid, as well as after treatment with virulent and avirulent pathogens (Lacomme and Roby, 1996). Furthermore, *ATST1* is induced preferentially with avirulent pathogens which elicit a resistance response. In order to study salicylate induction of the individual *BNST* genes, gene-specific primers were designed. The specificity of the primers was confirmed in control experiments where cross-amplification of the various DNA templates was tested. *BNST1* is not induced by salicylic acid (Figure 1). However, an increase in the steady-state mRNA levels was observed for *BNST2*, *-3* and *-4*. *BNST2* and *-3* showed a similar response and their kinetics of transcript accumulation was biphasic, with peak levels at 6 and 18 or 24 h. In contrast, *BNST4* induction was slower, with a maximum mRNA level at 12 hours. The *LSC222* gene, coding for a pathogenesis-related protein (class IV chitinase), was used as a

positive control for salicylate induction (Hanfrey et al., 1996). Induction of *LSC222* was slow but sustained, with maximal levels at 12 to 18 h.

The tissue specificity of BNST induction by salicylate in *B. napus* seedlings was tested by Western blot. Treatment with 10 mM salicylic acid resulted in the accumulation in hypocotyl and cotyledons of three protein bands with apparent molecular mass of 32 to 35 kDa, corresponding to the predicted molecular mass of BNST proteins (Figure 2). In contrast, roots were found to accumulate only the two protein bands having a lower molecular mass. The levels of BNST proteins were higher in roots as compared with hypocotyl and cotyledons. This difference may be related to the rate of salicylate uptake and transport from the root to aerial tissues.

Localization of expression in transgenic *Arabidopsis thaliana* expressing *BNST2* or *BNST3* promoter-GUS fusions. Transgenic *A. thaliana* plants expressing fusions of the *BNST2* or *BNST3* promoter with *GUS* were characterized. Promoter sequences extending from the -1081 position to the +3 position of *BNST2*, and from the -1001 to the +3 position of *BNST3* were used for this analysis. Four independent transgenic lines for each construct were initially characterized by Southern blot. Genomic DNA isolated from pools of T2 plants was digested with *Eco*RI. Only one *Eco*RI site is present in the T-DNAs of each construct, downstream from the *GUS* gene. Blots were probed with the promoter sequences present in the original constructs. For transgenic plants expressing *BNST2* promoter fusions, lines 4-1 and 4-5 were found to contain a single insert, whereas lines 4-2 and 4-3 had three copies (Figure 3A). For transgenic plants expressing fusions with the *BNST3* promoter, lines 1-2, 1-4 and 1-5 had a single insert, as compared to three copies for line 1-1 (Figure 3B). The presence of a single

insertion was confirmed by testing the segragation ratios of the selectable marker for kanamycin resistance at the T2 generation.

In preliminary experiments, the histochemical localization of GUS activity was tested in seedlings of six independent lines for each promoter. The localization was found to be similar for all six lines. Two of these lines, 4-2 for the *BNST2* promoter, and 1-1 for the *BNST3* promoter, were selected for further characterization.

Localization of expression was followed throughout development. Two days after seed imbibition, the promoter of *BNST3* directed expression of the GUS reporter gene in the primary root near the junction with the hypocotyl (Figure 4). In seedlings, expression in this zone of the root was observed in root hairs and epidermis. Expression at the root-stem junction was observed throughout later stages of development, including in four week-old mature plants. Transient expression was observed in the meristematic zone of the root, between three and four days post imbibition. An interesting pattern of expression was observed during the maturation of cotyledons, rosette leaves and cauline leaves. In young organs, expression was observed exclusively at the apex. In later stages of maturation, expression was observed at the margin of the organ. In mature cotyledons, expression often extended to the whole blade.

A similar pattern of expression was observed in the leaf organs of transgenic plants expressing a *BNST2* promoter-GUS fusion. However, expression in mature cotyledons extended to the petiole (Figure 5). Expression was not observed at the junction between the root and hypocotyl, or at the root apex of seedlings. Instead, diffuse staining of the root epidermis was sometimes observed during the first week of growth.

DISCUSSION

Salicylate treatment of *B. napus* seedlings leads to the induction of three *BNST* genes. The fourth one, *BNST1*, which is the most distant member of the family, is not salicylate-inducible. The kinetics of induction of *BNST4* was comparable to that of the pathogenesis-related gene coding for a class IV chitinase which was used as a positive control for salicylate induction. In comparison, *BNST2* and *BNST3* had faster kinetics of induction. In Western blot experiments, salicylate treatment resulted in the accumulation in hypocotyl and cotyledons of three protein bands having the expected molecular weight of BNSTs. In root extracts, only two of these bands were present, suggesting that salicylate induction is tissue-specific for at least one *BNST* gene.

Interesting parallels can be made between these results and those obtained following ethanol treatment (Chapter 3). As was observed for salicylic acid, *BNST1* is not induced by ethanol. In addition, ethanol treated seedlings accumulated two protein bands corresponding to BNSTs in hypocotyl and cotyledons, but only one band was present in root extracts. The kinetics of induction of *BNST* genes by salicylic acid showed an inverse relationship with those observed for ethanol induction. *BNST4* is induced slowly by salicylic acid, but has the fastest response to ethanol treatment. In mammals, the ethanol-inducible cytochrome P4502E1 is also inducible by salicylic acid (Damme et al., 1996, Dupont et al., 1999). However, its mechanism of induction by both compounds is unknown.

In order to study the localization of *BNST2* and *BNST3* expression, transgenic *A. thaliana* plants expressing fusions of their promoters with the GUS reporter gene were characterized. Plant promoters of a 1 kb length usually contain all the elements of

regulation needed for proper localization of expression, as well as those necessary for the modulation of the level of expression by hormones or environmental signals (Clouse and Feldmann, 1999). The *BNST* promoters were introduced into a heterologous species, *A. thaliana*, which has a different plant architecture as compared with *B. napus*. However, the two species are genetically related, and they belong to the same family, the Brassicaceae. Therefore, regulatory elements present in the *BNST* promoters that govern the tissue localization of expression in *B. napus* may be expected to be functional in *A. thaliana*.

The two promoters gave a similar pattern of expression in leaf organs (Figures 4 and 5). In young leaves, expression was restricted to the apex. During leaf development, this region of the tissue is the first one to become competent for the export of photosynthates, and is therefore associated with an early development of photosynthetic capacity, and of the anatomical features necessary for this function, such as the minor veins of the vascular system (Esau, 1977). The apex of the leaf is therefore a region of early differentiation and maturation. These observations are also relevant to the cotyledon, which develops photosynthetic capacity in *A. thaliana*.

The junction between the root and hypocotyl is more complex anatomically. During early seedling development, the hypocotyl elongates from its base. However, the region at the root-hypocotyl junction where expression was observed is not a site of cell elongation. The boundary between the root and hypocotyl is the transition region, where the connection between the distinct vascular systems of the root and the shoot is gradual (Esau, 1977). In seedlings, the zone of the root proximal to the hypocotyl is associated with early maturation. This region of the root is localized in the zone of specialization,

and is the first one where root hair cells differentiate. In both roots and leaf organs, *BNST* promoter-driven GUS expression is associated with regions of cell differentiation.

The localization of expression observed for *BNST2* and *BNST3* may be compared with that of the *CPD* gene of *A. thaliana* (Mathur et al., 1998). *CPD* is expressed in the parenchyma of the leaves and cotyledons. When the leaf organs reach the stage of senescence, *CPD* expression becomes restricted to the margin of the blade. The absence of *CPD* expression in the zones of elongation of the shoot and root suggests that brassinosteroid intermediates need to be transported to their sites of action. This spatial organization of brassinosteroid biosynthesis may be required due to the negative feedback mechanism exerted on *CPD* by end products of the pathway. Higher content of biologically active brassinosteroids in the zones of elongation may prevent the expression of *CPD*. Although the impact of the *cpd* mutation on 24-epibrassinosteroid content has not been examined, CPD is probably active with 24-epicathasterone as substrate. The specificity of the biosynthetic enzymes is probably limited, since brassinosteroids with a large diversity in side-chain structure accumulate in plants. Moreover, 24-epibrassinolide is active in the transcriptional repression of *CPD* (Mathur et al., 1998).

The common localization of expression between the *BNST* genes and *CPD* in leaves suggests that the steroid sulfotransferases may catalyze the sulfonation of 24-epicathasterone *in vivo*. Based on the localization of their expression, two different functions may be considered for the steroid sulfotransferases. The sulfonation of 24-epicathasterone in leaves may be required for its transport to the zones of cell elongation of the roots and shoot. In support of this hypothesis, the apical zone of the leaf is the first region to become competent for the export of photosynthates to other parts of the plant.

Alternatively, the sulfonation of 24-epicathasterone may prevent the formation of biologically active 24-epibrassinolide. In leaves, *BNST* expression is associated with mature tissue, such as the apex. Expression in the transition region of seedlings and in the zone of specialization of the root may prevent 24-epibrassinolide-stimulated cell elongation. *BNST* induction during pathogen infection may also prevent cell elongation, which is incompatible with cell wall reinforcement processes that take place during the defense response. Under these conditions, the specificity of the sulfotransferases for 24-epicathasterone may be advantageous if the feedback mechanism regulating *CPD* expression becomes non-functional (Figure 6).

In future work, the hypotheses on the biological function of the steroid sulfotransferases may be tested in transgenic plants expressing sense or antisense steroid sulfotransferase genes. It would also be interesting to determine the impact of salicylate treatment or pathogen infection on the content of endogenous brassinosteroids, and on the expression of brassinosteroid biosynthetic genes.

ACKNOWLEDGMENTS

We thank Dr. Michèle Rouleau for the Western blot experiment, and Catherine Béliveau for the production of *A. thaliana* transgenic plants. We also thank Dr. Pierre-Mathieu Charest for helpful discussions. Frédéric Marsolais was the recipient of a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC). This work was supported by NSERC.

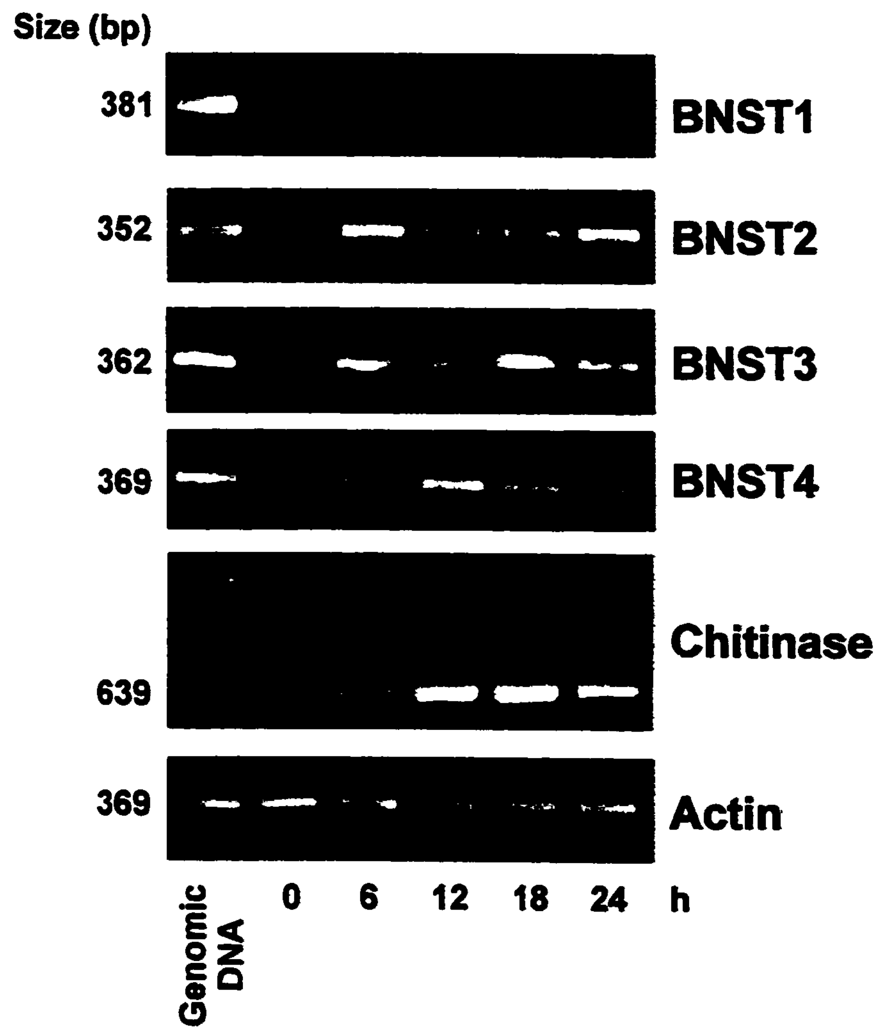


Figure 1. RT-PCR analysis of *BNST* expression in *Brassica napus* seedlings treated with salicylic acid.

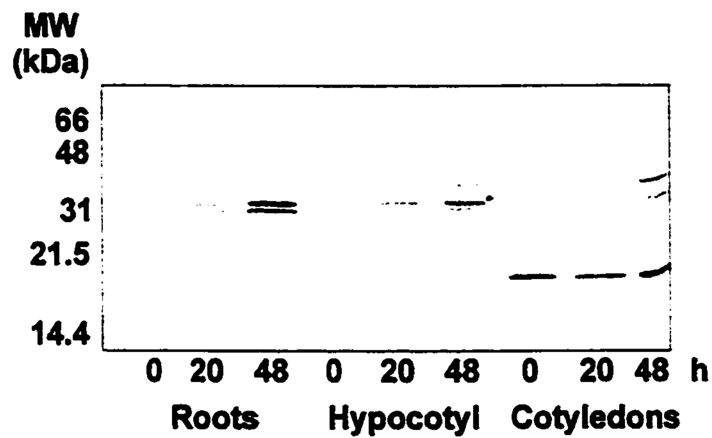


Figure 2. Western analysis of BNST expression in *Brassica napus* seedlings treated with salicylic acid. The membrane was incubated with anti-BNST1 antibodies.

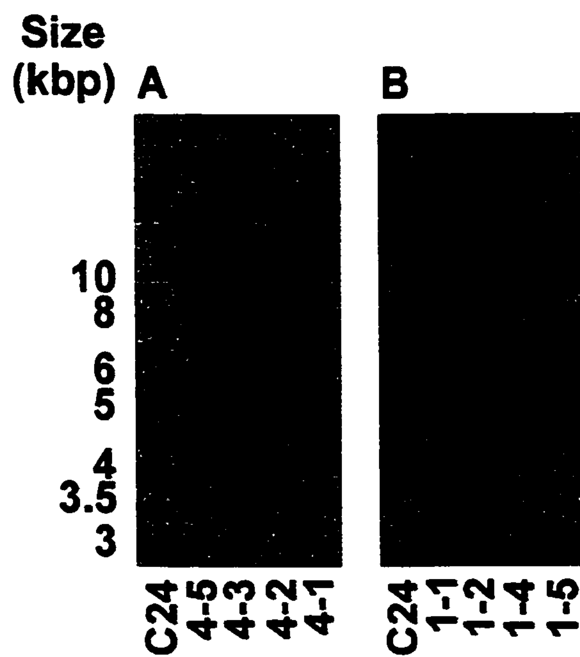


Figure 3. Southern analysis of independent transgenic lines of *Arabidopsis thaliana* expressing *BNST2* (A) or *BNST3* (B) promoter-GUS fusions. The blots were probed with the full-length promoter sequences present in the original constructs.

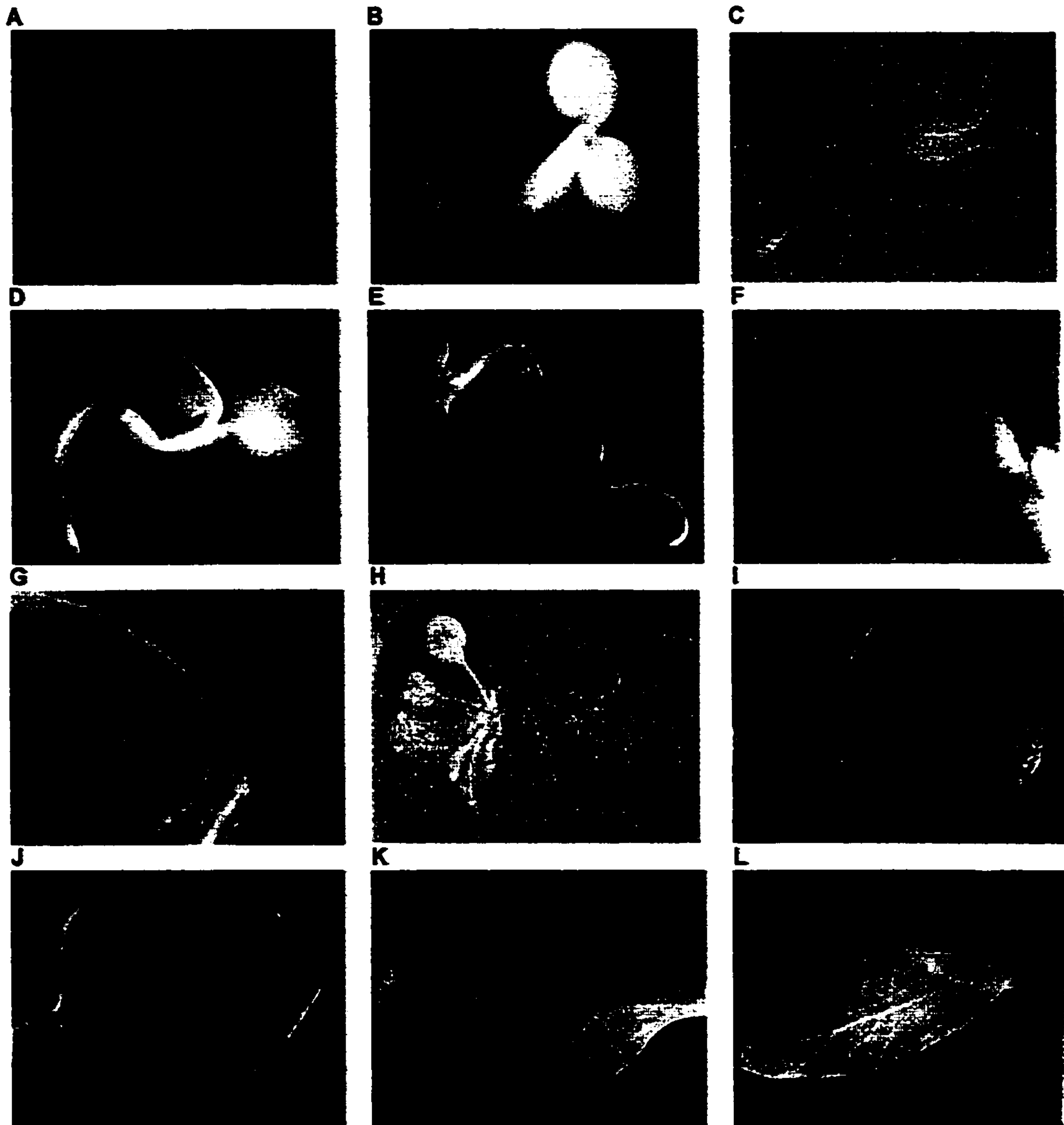


Figure 4. Histochemical localization of *BNST3* promoter-GUS expression in transgenic *Arabidopsis thaliana.* A) Embryos. B) Seedling, 2 days after seed imbibition. C) 3 days. D) 4 days. E) 6 days. F) Detail of cotyledon at 6 days. G) Detail of the transition zone between root and hypocotyl at 6 days. H) 2 weeks. I) Detail of cotyledon at 2 weeks. J) Detail of rosette leaf at 2 weeks. K) Detail of rosette leaf at 5 weeks. L) Detail of cauline leaf at 5 weeks.

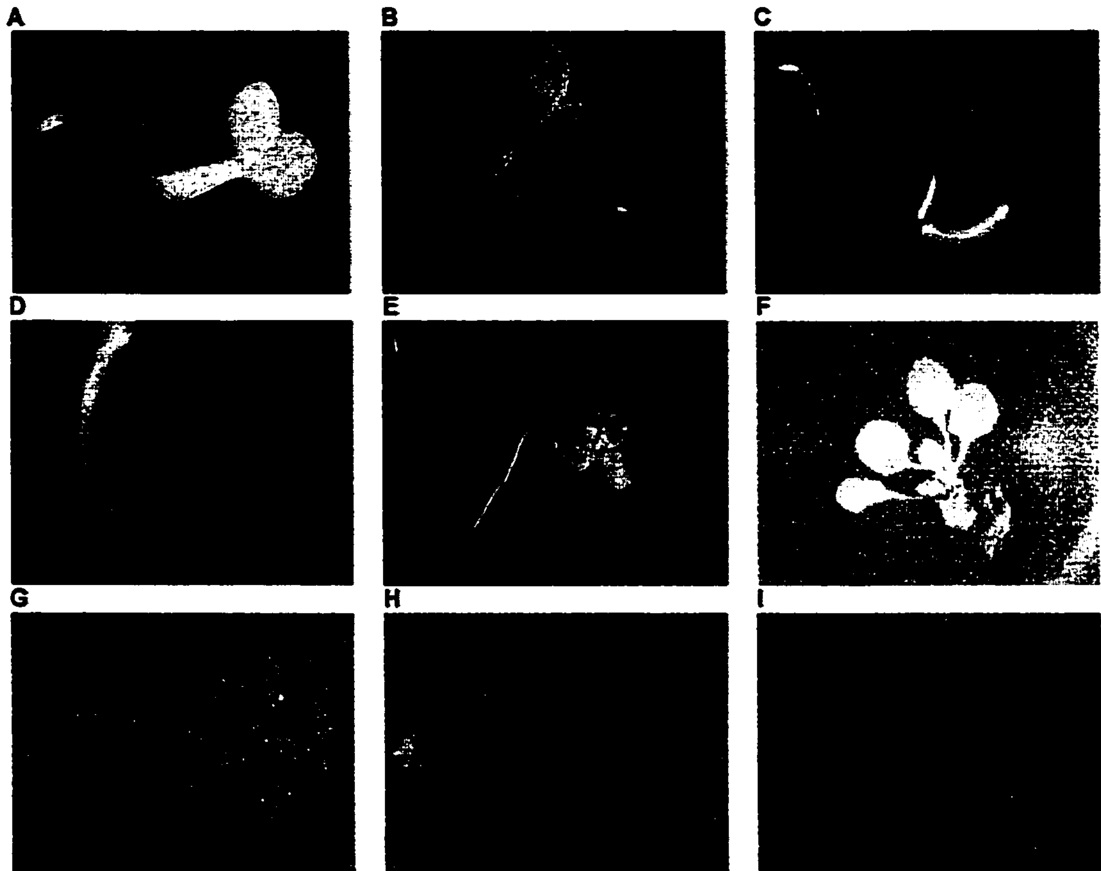


Figure 5. Histochemical localization of *BNST2* promoter-GUS expression in transgenic *Arabidopsis thaliana*. A) 3 days after seed imbibition. B) 4 days. C) 6 days. D) Detail after 6 days. E) 8 days. F) 2 weeks. G) 3 weeks. H) 4 weeks. I) 5 weeks.

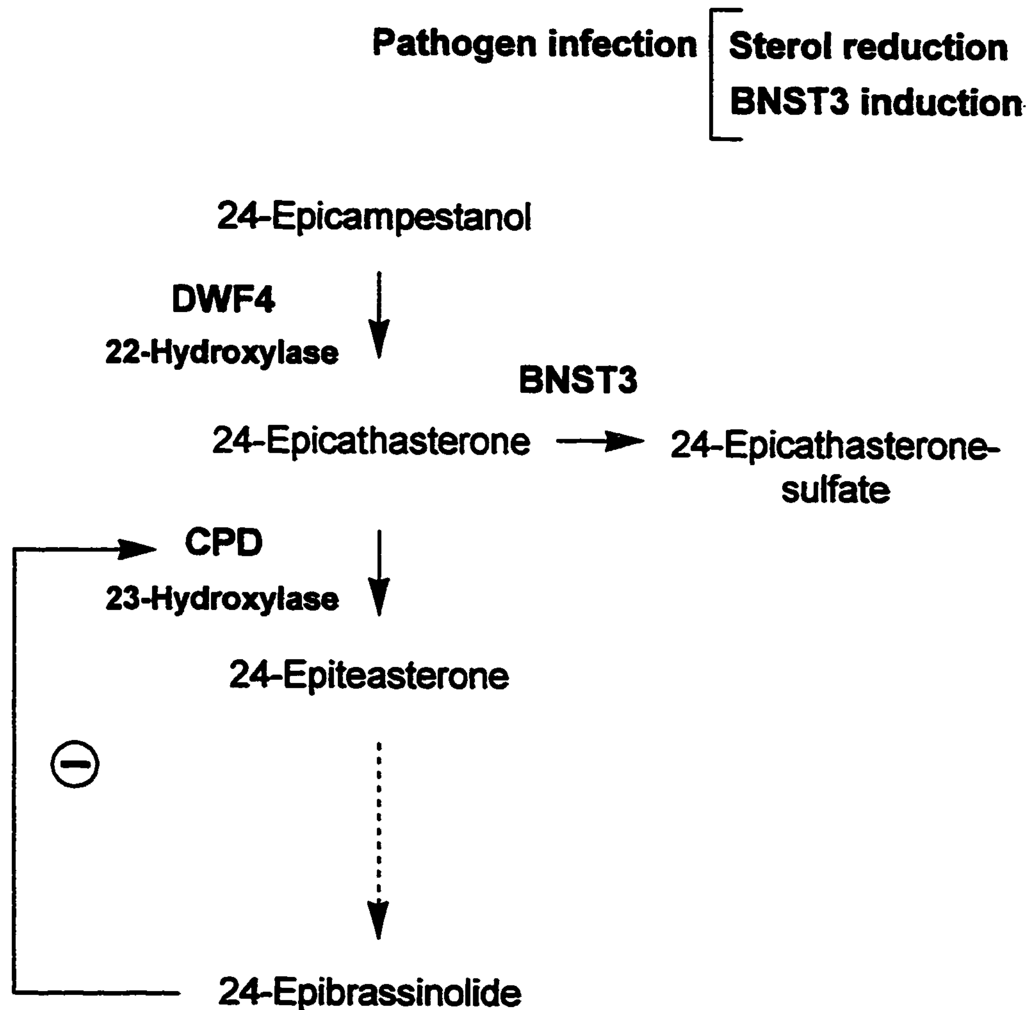


Figure 6. Function of the BNST steroid sulfotransferases in brassinosteroid metabolism. The reaction catalyzed by DWF4 (campestanol 22-hydroxylase) is the rate-limiting step in biosynthesis. The following step, catalyzed by CPD (cathasterone 23-hydroxylase) is regulated by a negative feedback mechanism by end products of the brassinosteroid biosynthetic pathway.

CHAPTER 5

Expression of the *BNST3* steroid sulfotransferase gene in transgenic *Arabidopsis thaliana* and purification of an endogenous substrate from *Brassica napus*

In chapter 4, we have further studied the regulation of the *BNST* genes in response to salicylic acid. We have shown that the *BNST* genes are induced in all tissues of the seedling, and that only the *BNST1* gene, which is the most distant to other members of the gene family, is not salicylate inducible. The tissue localization of *BNST2* and *-3* expression was studied in transgenic *Arabidopsis thaliana* expressing promoter-GUS fusions. The patterns of expression that were observed were compatible with a function of the sulfotransferases in brassinosteroid inactivation or transport. In this chapter, we tested the hypothesis that the sulfotransferases are involved in brassinosteroid inactivation by expressing the *BNST3* gene in transgenic *A. thaliana* under the control of the CaMV35S promoter. The transgenic plants had a similar growth and development as compared with wild-type plants. Furthermore, transgenic lines expressing *BNST3* had a similar response to treatment with exogenous 24-epiteasterone and 24-epibrassinolide. These results suggest that the BNST3 enzyme may accept other untested substrates *in vivo*. We initiated experiments to characterize endogenous substrates of the steroid sulfotransferases that are present in extracts of *Brassica napus* seedlings.

Expression of the *BNST3* steroid sulfotransferase gene in transgenic *Arabidopsis thaliana* and purification of an endogenous substrate from *Brassica napus*

Frédéric Marsolais, Cinta Hernández Sebastià, Samer Hossein and Luc Varin[1]

Department of Biology, Concordia University, 1455 de Maisonneuve Blvd. W., Montreal, Quebec, Canada, H3G 1M8

[1]Author for correspondence: Tel.: (514)-848-3396; Fax: (514)-848-2881; E-mail: varinl@clone.concordia.ca

SUMMARY

The *BNST3* gene from *Brassica napus* encodes a sulfotransferase which catalyzes the transfer of a sulfonate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to brassinosteroids and mammalian estrogens. The BNST3 enzyme is stereospecific for 24-epibrassinosteroids, with a preference for 24-epicathasterone, an intermediate in the biosynthesis of 24-epibrassinolide. Based on the inducible pattern of expression of *BNST3* in response to salicylic acid, and the lack of biological activity of 24-epibrassinolide sulfate in the bean second internode bioassay, brassinosteroid sulfonation was proposed to function as a mechanism of hormone inactivation. This hypothesis was tested in transgenic *Arabidopsis thaliana* expressing *BNST3* under the control of the CaMV35S promoter. No effect of the transgene on plant growth was observed. Furthermore, the response of the transgenic plants to exogenous 24-epiteasterone or 24-epibrassinolide was similar to that of wild-type plants. These results suggest that the BNST3 enzyme is inactive with these brassinosteroids in vivo. Alternative experimental approaches will be needed to understand the biological function of the *BNST3* gene. The results of preliminary experiments aimed at the characterization of an endogenous substrate of the steroid sulfotransferase are discussed.

INTRODUCTION

The *BNST3* gene from *Brassica napus* is part of a multigene family of steroid sulfotransferases comprising at least four members, designated *BNST1* to *-4*. In *Arabidopsis thaliana*, *RaR047* is the unique homolog of the *BNST* genes (Lacomme and Roby, 1996). *RaR047* was renamed *ATST1* in order to simplify the nomenclature of sulfotransferase genes in *A. thaliana* (Marsolais et al., 2000). BNST3, -4 and ATST1 accept brassinosteroids and mammalian steroids as substrates *in vitro* (Chapters 2 and 3). They are stereospecific for 24-epibrassinosteroids, and display a substrate preference for 24-epicathasterone, followed by 24-epiteasterone, which are intermediates in the biosynthesis of 24-epibrassinolide. *BNST3* and *-4* are inducible by salicylic acid, a chemical signal in the defense response against pathogen infection (Chapters 2 and 4). *ATST1* is also inducible by salicylic acid, as well as after infection with virulent and avirulent pathogens (Lacomme and Roby, 1996).

In analogy with the function of mammalian steroid sulfotransferases, the plant steroid sulfotransferases may be involved in brassinosteroid inactivation. This hypothesis is supported by the lack of biological activity of 24-epibrassinolide sulfate in the bean second internode bioassay (Chapter 2), and by the inducible pattern of expression of the sulfotransferases. Induction of the steroid sulfotransferases by pathogen infection may provide a mechanism to stop brassinosteroid-dependent cell elongation, which is incompatible with cell wall reinforcement processes occurring during the defense response. The sulfonation of 24-epibrassinolide precursors may prevent further biosynthesis.

In transgenic *A. thaliana* plants expressing a fusion of a 1kb promoter of *BNST3* with *GUS*, expression was observed in leaves and cotyledons, and in roots near the junction with the hypocotyl (Chapter 4). There was a correlation between the localization of expression of *BNST3* and that of *CPD* (cathasterone 23-hydroxylase) in leaves (Mathur et al., 1998), suggesting that the sulfotransferase may be active with 24-epicathasterone *in vivo*. *BNST3* expression was associated with mature cells of the root, and of the leaf apex, which is compatible with a function of the sulfotransferase in brassinosteroid inactivation.

In this paper, we describe the characterization of *A. thaliana* transgenic plants expressing *BNST3*. The morphology and growth of the transgenic plants were comparable to those of wild-type plants. In addition, the transgenic plants were not altered in their responses to the exogenous application of 24-epiteasterone or 24-epibrassinolide, as compared to the wild-type, suggesting that BNST3 may not accept these molecules as substrates *in vivo*. Alternative experimental approaches will be required to study the biological function of *BNST3*. Finally, we describe experiments performed in order to purify and characterize a substrate of BNST3 present in *B. napus* extracts.

EXPERIMENTAL PROCEDURES

Preparation of transgenic *Arabidopsis thaliana* plants expressing *BNST3*. *Nco*I and *Bam*HI sites were introduced by PCR at the respective 5' and 3' ends of the *BNST3* coding sequence using oligonucleotides 5'-CATGCCATGGCAATGTCATCATCGTCATC-3' (translation initiation codon of *BNST3* underlined) and 5'-CGGGATCCCAGCAAAGTCGCAAGATTG-3'. The PCR product was digested with *Nco*I and *Bam*HI, and ligated into the corresponding sites of

the polylinker of the pBI525 vector (Datla et al., 1993). In the resulting construct, the translation initiation codon of *BNST3* is in frame with the ATG present at the *Nco*I site of the polylinker, resulting in the production of a protein having two additional amino acids at its NH_2-terminal. The cassette of the pBI525-BNST3 construct was removed by digestion with *Hin*dIII and *Eco*RI, and religated in the corresponding sites of the binary vector pBI101 (Clontech). The junction between the CaMV35S promoter and the *BNST3* coding sequence was sequenced to confirm that the translation initiation codon of the gene was in frame with that present in the polylinker.

The pBI525/101-BNST3 construct was transformed in *Agrobacterium tumefaciens* strain LBA4404 using a freeze-thaw method (An et al., 1988). Transgenic *Arabidopsis thaliana* (ecotype C24) plants were generated using the root explant method (Valvekens et al., 1988). Transgenic plants were selected on medium containing kanamycin at a concentration of 50 mg/l.

Characterization of *Arabidopsis thaliana* transgenic lines expressing *BNST3*. To determine the number of inserts present in independent transgenic lines, genomic DNA from pools of T2 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 ß-mercaptoethanol, 20 mM EDTA and 100 mM Tris-HCl of 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 in 95% ethanol, containing 10 mM ammonium acetate, for 20 min. Genomic DNA was further purified by RNase and proteinase K digestions, followed by phenol-chloroform extraction. For Southern blot

analysis, 10 μg of genomic DNA was digested with *Eco*RI. The *Hin*dIII-*Bgl*II fragment of the pBI524 vector, containing a double copy of the minimal CaMV35S promoter, was used as a probe. The blot was hybridized under stringent conditions according to standard procedures (Sambrook et al., 1989). All enzymes were from New England Biolabs, and were used according to the manufacturer's recommendations.

For the analysis of BNST3 expression in independent transgenic lines, T2 plants were pooled, ground in liquid nitrogen, and the powder was boiled in SDS sample buffer. Protein extracts were separated by SDS-PAGE on a 12% polyacrylamide gel and transferred to nitrocellulose. BNST3 was immunodetected using anti-BNST1 polyclonal antibodies (Chapter 2) (dilution 1:1000) and goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase.

Bioassays with 24-epibrassinosteroids. Seeds were germinated *in vitro* on germination medium (MS salts, B5 vitamins, 1% sucrose, 0.5g/l MES) containing various concentrations of 24-epicathasterone, 24-epiteasterone, or 24-epibrassinolide dissolved in 95% ethanol. Control plates contained ethanol only. The final concentration of ethanol in the growth medium was 0.1%. The plants were grown under long day conditions (16 h light, 8 h dark) at 23 °C. Seedlings used for root measurements were grown vertically. Root elongation was measured after 5 days using a caliper. Hypocotyl measurements were performed after 7 days, using a dissecting microscope equipped with an ocular micrometer. The precision of the measurements was ± 0.5 mm for root length, and ± 0.2 mm for hypocotyl length.

Experimental design and statistical analyses. Hypocotyl or root length analyses were carried as 2 factorial analyses of variance (ANOVA), 3X4, with line and 24-

epibrassinosteroid concentration as factors. Lines were wild-type, 2-2 and 2-12, and 24-epibrassinosteroid concentrations were 0, 0.01, 0.1 and 1 μM. The experimental unit was a petri dish with 5 seedlings. There were 2 replications of the experimental unit distributed in a randomized complete block. ANOVA were conducted using the SuperANOVA statistical program (Abacus Concepts Inc.). To satisfy the assumptions of the ANOVA test, the homogeneity of the variances was checked using residual graphic analysis. When variances were not homogeneous, the variables were transformed as log (x+1) according to the distribution of residuals. In cases where the homogeneity of the variances was not acceptable after mathematical transformation of the values, we applied the equivalent of non-parametrical statistics, in the form of the rank transformation of means in the parametric test ANOVA [Rank (x, Allrows) for the SuperANOVA program] (Conover, 1980). In this case the assumption of the normal distribution of the errors is no longer required. Means in the graphs correspond to untransformed values.

Expression of recombinant sulfotransferases in *Escherichia coli*. Recombinant BNST3, -4 and ATST1 were produced in *Escherichia coli* cells expressing the corresponding genes cloned in the expression vector pQE30 (Qiagen). The expression of the sulfotransferases in *E. coli* cultures ($A_{600} \approx 0.5$) was induced with 1 mM isopropyl ß-D-thio-galactopyranoside for 9 h at 22°C. Bacterial cells were collected by centrifugation, resuspended in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl, 14 mM ß-mercaptoethanol, and lysed by sonication. The recombinant sulfotransferases recovered in the soluble fractions were purified by affinity chromatography onto nickel-nitrilotriacetic acid-agarose matrix as recommended (Qiagen).

Preparation of *Brassica napus* extracts and enzyme assays. 6-day-old *Brassica napus* seedlings (cv. Westar) were ground in liquid nitrogen, and the powder was extracted in 75% methanol (approximately 10 ml/g of tissue). Methanol was evaporated, and the aqueous phase was extracted successively with chloroform, ethyl acetate and butanol. The extracts were lyophilized and resuspended in DMSO. The final concentration of DMSO in the assay was 5%. Reaction mixtures (50 µl) also contained 5 µM ^{35}S-PAPS (NEN Life Science Products) and 1-5 µg of purified recombinant sulfotransferase in 50 mM Tris-HCl, pH 7.5. Reactions were allowed to proceed for 10 min at 25°C. Incorporation of the ^{35}S-sulfonate and extraction of the ^{35}S-labeled sulfated product was performed according to a standard assay (Varin et al., 1987). After extraction with ethyl acetate, sulfated products were lyophilized and resuspended in methanol.

Purification of sulfated products by HPLC. Sulfated products were purified by reverse phase HPLC on a Novapack C18 column (Waters), equilibrated with solvent A (20% methanol, 0.5% acetic acid, 10 mM TBADP). The column was washed for 5 min in solvent A. Sulfated products were eluted with a linear gradient of 50 min of solvent A to solvent B (100% methanol, 0.5% acetic acid, 10 mM TBADP), followed by 10 min of solvent B, at a flow rate of 0.8 ml/min. Elution of estradiol 3-sulfate (Sigma) was monitored by measuring the absorbance at 280 nm using a Waters 486 tunable absorbance detector. Elution of labeled sulfated products was monitored by measuring the ^{35}S present in each fraction. Subsequently, 100 µl of the total 800 µl were submitted to scintillation counting. Approximately 100 000 dpm of ^{35}S-labeled sulfated product was injected for each chromatography. The chromatography experiments were performed with a Waters 625 LC HPLC system.

RESULTS

In order to test the hypothesis that *BNST3* is involved in 24-epibrassinosteroid inactivation, this gene was expressed in transgenic *A. thaliana* under the control of the CaMV35S promoter. The number of inserts present in five independent transgenic lines was analyzed by Southern blot (Figure 1). Lines 2-2 and 2-12 were found to contain a single copy of the insert. These results were confirmed by the analysis of segregation ratios of the selectable marker for kanamycin resistance at the T2 generation. The levels of BNST3 expression in eight independent transgenic lines were analyzed by Western blot. Plants from lines 2-2 and 2-12 were found to accumulate a protein having the expected molecular mass of BNST3, of approximately 35 kDa (Figure 2). Lines 2-2 and 2-12 were therefore selected for further phenotypic characterization. T3 seeds of each line that were homozygous for the transgenic insertion were used in further experiments.

The growth of the transgenic plants expressing *BNST3* was compared with that of the wild-type under various growth conditions. Plants were grown under both long day (16 h light, 8 h dark) and short day conditions (8 h light, 16 h dark). There was no difference between the growth of the transgenic lines and that of the wild-type under these conditions (Figure 3). Several growth parameters were measured after 5 weeks, including the height of the main inflorescence, the number of rosette leaves, the number of inflorescences, and the number of flowers and siliques (data not shown). In addition, there was no effect of the transgene on hypocotyl length under dark growth conditions. Root growth and root morphology of seedlings was also compared. In all instances, there was no significant difference between the transgenic lines expressing *BNST3* and the wild-type.

To further characterize the transgenic plants expressing *BNST3*, experiments were designed to test their resistance to exogenous 24-epibrassinosteroids when grown *in vitro*. Brassinolide is known to consistently inhibit root growth of *A. thaliana* seedlings at concentrations of 1 and 0.1 μM (Clouse et al., 1993). In addition, treatment of *A. thaliana* seedlings with exogenous brassinolide results in a significant promotion of hypocotyl elongation (Neff et al., 1999). The effect of 24-epicathasterone, 24-epiteasterone and 24-epibrassinolide on root elongation of transgenic lines was tested. For hypocotyl growth, only 24-epibrassinolide was tested, since its precursors were found to be biologically inactive in preliminary experiments.

There was no significant difference in the root and hypocotyl response to exogenous 24-epibrassinolide between the two transgenic lines expressing *BNST3* and the wild-type at any hormone concentration (Figure 4A and B). Treatment with increasing concentrations of 24-epibrassinolide resulted in a stimulation of hypocotyl elongation for the transgenic lines and wild-type ($P = 0.0001$). Furthermore, 24-epibrassinolide at concentrations of 0.1 and 1 μM promoted hypocotyl growth as compared with the control for both transgenic lines and wild-type ($P \leq 0.026$) (Figure 4A). Similar results were obtained for the effect of 24-epibrassinolide on root growth (Figure 4B). The increase of 24-epibrassinolide concentration resulted in a similar inhibition of root elongation for the transgenic lines and wild-type, after rank transformation of the values ($P = 0.0047$). Treatment with 1 μM 24-epibrassinolide resulted in root inhibition for line 2-2 and the wild-type ($P < 0.049$). For line 2-12, there was a tendency towards a significant inhibitory effect of 1 μM 24-epibrassinolide ($P = 0.075$). The weaker effect observed on line 2-12 may be due to a slight delay in germination, which was not observed in previous

experiments. The differences observed between the mean root lengths of the trangenic lines and the wild-type in the absence of 24-epibrassinolide were not statistically significant.

The effect of 24-epicathasterone and of 24-epiteasterone on root growth of the transgenic lines was also tested, since these intermediates are the preferred substrates of BNST3 (Chapter 2). 24-Epicathasterone had no effect on root growth of the transgenic and wild-type lines at the concentrations that were tested (Figure 5A). The root growth of the transgenic lines did not differ significantly from that of the wild-type in response to exogenous 24-epiteasterone. The increase in 24-epiteasterone concentration resulted in inhibition of root elongation of the transgenic lines and wild-type, after log (x+1) transformation of the values (Figure 5B). As with 24-epibrassinolide, treatment with 1 μM 24-epiteasterone had a significant inhibitory effect on root growth as compared with the control for line 2-2 and wild-type ($P \leq 0.014$). However, inhibition of line 2-12 at this concentration was not significant, which may also be due to the slight delay in germination.

The absence of conclusive results from the experiments with the transgenic plants prompted us to search for other endogenous substrates of BNST3 present in *B. napus* extracts. As a preliminary experiment, a chromatographic method using reverse phase HPLC was designed to efficiently separate various sulfated steroids produced *in vitro* (Table 1). The retention time of 17ß-estradiol sulfate produced with BNST3 (35 min) was slightly different from that of authentic 17ß-estradiol 3-sulfate (37 min). This result is consistent with the hypothesis that BNST3 catalyzes the transfer of the sulfonate group to position 17 of 17ß-estradiol (Chapter 1). The differences in the retention times of the

various sulfated steroids is related to the polarity of the molecules. The presence of an apolar methyl group in 17ß-estradiol 3-methyl ether sulfate results in an increase in retention time (46 min), as compared with that of 17ß-estradiol sulfate (35 min). In contrast, the presence of a polar hydroxyl group at position 2 and of a lactone function in ring B of 24-epibrassinolide sulfate results in a slight decrease in retention time (43 min) as compared with that of 24-epiteasterone sulfate (45 min). The products of *in vitro* sulfonation of 24-epicathasterone by BNST3, BNST4 or ATST1 had identical retention times (45 min), suggesting that the three recombinant enzymes may form the same product from this substrate.

Enzymatic activity of BNST3 with the chloroform, ethyl acetate, butanol and aqueous extracts of *B. napus* seedlings was tested. Enzyme activity was detected only with the butanol extract. The product formed from the butanol extract was purified by HPLC. Its retention time (38 min), resembled those of sulfated steroids purified in this study, such as 17ß-estradiol 3-sulfate and (22*S*, 23*S*)-28-homobrassinolide sulfate (Table 1). In comparison, the product formed from the butanol extract by BNST4 eluted at 56 min, suggesting a more hydrophobic molecule.

DISCUSSION

In this study, we tested the hypothesis that *BNST3* is involved in brassinosteroid inactivation by expressing this gene in transgenic *A. thaliana*. This experimental approach provides a functional test for enzymes involved in plant hormone inactivation, and has been used successfully for the *BAS-1* gene of *A. thaliana*, encoding a cytochrome P450-dependent monooxygenase which catalyzes the 26-hydroxylation of brassinolide

(Neff et al., 1999). Overexpression of *BAS-1* in *A. thaliana* and tobacco resulted in a dwarf phenotype, similar to that of brassinosteroid-deficient and -insensitive mutants.

Expression of *BNST3* in transgenic *A. thaliana* did not lead to an observable phenotype, even though the transgenic lines that were selected for phenotypic characterization accumulated detectable levels of a protein having the expected molecular mass of BNST3, as determined by Western blot. Attempts to measure the steroid sulfotransferase activity in protein extracts from transgenic plants were unsuccessful, since this activity is very labile, and could not be detected from *B. napus* seedlings induced with salicylic acid or ethanol either. However, transgenic tobacco BY-2 cells transformed with the same construct used for *A. thaliana* transformation had low but detectable steroid sulfotransferase activity using 17ß-estradiol as substrate.

The *BNST3* transgene may have an impact on the pool of 24-epibrassinolide, but not on those of other brassinosteroid hormones, such as brassinolide. 24-Epibrassinolide has been detected in seeds of *A. thaliana* (Schmidt et al., 1997). However, the relative contribution of 24-epibrassinolide to the total content of brassinosteroid hormones in this species is unknown. If 24-epibrassinolide is a minor brassinosteroid hormone in *A. thaliana*, a reduction of its levels may have a limited impact on plant growth.

The lack of effect of *BNST3* expression in transgenic plants may be due to a mechanism of compensation involving increased brassinosteroid biosynthesis. Overexpression in transgenic *A. thaliana* of a pumpkin GA 20-oxidase involved in the production of inactive tricarboxylic acid gibberellins had minor effects on plant growth (Xu et al., 1999). In this experiment, the effect of the transgene was compensated for by an increased expression of GA 20-oxidase and 3ß-hydroxylase genes involved in

biosynthesis. A lack of effect of overexpression in transgenic plants has been observed for several genes involved in brassinosteroid or gibberellin biosynthesis (Szekeres et al., 1996, Bishop et al., 1999, Sun and Kamiya, 1994). This outcome is expected if the gene that is overexpressed is involved in a non-rate-limiting step of biosynthesis.

To further test the function of *BNST3* in 24-epibrassinosteroid inactivation, the resistance of the transgenic plants to treatment with exogenous 24-epibrassinosteroids was tested. This experimental approach circumvents the ambiguities associated with metabolic compensation or limiting substrate pools, and it has been used to confirm the function of genes involved in indole 3-acetic acid metabolism in *A. thaliana*. Overexpression of *NIT2*, encoding a nitrilase involved in the conversion of indole 3-acetonitrile to indole 3-acetic acid did not result in a phenotype, but the transgenic plants were more sensitive to exogenous indole 3-acetonitrile (Schmidt et al., 1996, Grsic et al., 1998). Similarly, overexpression of the *IAR3* gene, encoding an indole 3-acetic acid-alanine hydrolase, had no effect on plant growth, but the transgenic plants were more sensitive to root inhibition by the corresponding amino acid conjugate (Davies et al., 1999).

The hypocotyl and root responses of two transgenic lines expressing *BNST3* to exogenous 24-epibrassinolide were similar to those of the wild-type. These results are consistent with the low affinity of BNST3 for this substrate (Chapter 2). 24-Epicathasterone, which is the preferred substrate of BNST3 (Chapter 2), was biologically inactive in the range of concentrations that were tested. This result may be explained by the low biological activity of 24-epicathasterone. Its 24-epimer, cathasterone, exhibits 1000-fold less activity as compared with brassinolide in the rice lamina inclination

bioassay (Fujioka et al., 1995). The response of the transgenic lines to 24-epiteasterone did not differ significantly from that of the wild-type. This result is consistent with the 13-fold lower V_{max}/K_m value determined for 24-epiteasterone as compared with 24-epicathasterone (Chapter 2).

A possible explanation for the lack of effect of *BNST3* in transgenic *A. thaliana* is that the level of expression of the transgene may not be sufficiently high in the main target tissues of brassinosteroid action, which are the elongation zones of the root and shoot. Another possible reason is related to the mode of action of brassinosteroids. The results of the characterization of the putative brassinosteroid receptor, BRI1, suggest that the plant steroid hormones are perceived at the cell surface (Li and Chory, 1997). In the experiments performed with exogenous 24-epibrassinolide, the intracellular localization of the sulfotransferase may not allow the modulation of hormone action.

An alternative hypothesis to explain the results obtained from the *A. thaliana* transgenic plants is that BNST3 may sulfonate a biologically inactive product of brassinosteroid metabolism. Overexpression of an enzyme that is not directly involved in hormone inactivation is not expected to have an impact on plant growth. In the formation of 25- or 26-glucosyl conjugates of 24-epicastasterone and 24-epibrassinolide, hydroxylation at position 25 or 26 is rate-limiting and results in hormone inactivation (Hai et al., 1996, Adam and Schneider, 1999, Neff et al., 1999). Another important reaction in the metabolism of 24-epicastasterone and 24-epibrassinolide is the epimerization of the 3-hydroxyl group to the 3ß position. This reaction occurs in tomato and *Ornithopus sativus* (Hai et al., 1996, Kolbe et al., 1998), and is a common step in brassinosteroid metabolism resulting in inactivation (Suzuki et al., 1995b, Kim, 1991).

Epimerization at position 3 is an absolute prerequisite for glucosyl or acyl conjugation at this position (Kolbe et al., 1998). BNST3 has a substrate preference for 24-epibrassinosteroids having a 3ß-hydroxyl group, such as 24-epicathasterone (Chapter 2). Therefore, 3, 24-diepicastasterone and 3, 24-diepibrassinolide represent attractive candidate substrates for this enzyme. In *O. sativus*, epimerization of the 3-hydroxyl group of 24-epicastasterone and 24-epibrassinolide may be followed by side-chain cleavage, resulting in the production of pregnane metabolites (Kolbe et al., 1994, 1995). These metabolites are similar in structure to progesterone, one of the preferred substrates of BNST4 (Chapter 3).

The results obtained with the *A. thaliana* transgenic plants did not allow us to reach a conclusion on the biological function of *BNST3*. Alternative experimental approaches will be needed, and the biochemical characterization of an endogenous substrate of the enzyme may be critical to address this question. Preliminary experiments were performed in order to purify a substrate of BNST3 present in the butanol extract of *B. napus* seedlings. In reverse phase HPLC, the retention time of the sulfated product of BNST3 was in the range of those determined for several sulfated steroids in this study. However, the retention time of this product did not correspond to those determined for 24-epicathasterone sulfate and 24-epiteasterone sulfate. These results indicate that the substrate of BNST3 that is present in the butanol extract is distinct from 24-epicathasterone and 24-epiteasterone. In addition, the product formed from the butanol extract by BNST4 had a different retention time as compared to that of the product of BNST3, suggesting that the two enzymes may have different substrates *in vivo*.

The experimental approach aiming at the purification of a substrate of BNST3 from *B. napus* extracts has a few limitations. In the crude metabolite fractions that were used, the presence of inhibitors may not allow the detection of enzymatic activity with the best substrate. In addition, the enzymatic activity observed with a substrate purified from the plant does not prove that this substrate is sulfated *in vivo*. Therefore, the approach of substrate purification should be combined with the identification of steroid sulfate esters present in *B. napus* seedlings.

ACKNOWLEDGMENTS

We thank Dr. Günter Adam for the brassinosteroids, Dr. Narinder Kapoor for the ocular micrometer, Dr. Paul Widden for the dissecting microscope, and Dr. Bill Crosby for the pBI524 and -5 vectors. We also thank Catherine Béliveau for preparing the *A. thaliana* transgenic plants, and Dr. Daphne Fairbairn for her help with statistical analyses. Frédéric Marsolais was the recipient of a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC). This work was supported by NSERC.

Table 1. Retention times of sulfated products in HPLC

Sulfate product	Enzyme	R_t (min)
17ß-Estradiol sulfate	BNST3	35
Authentic 17ß-estradiol 3-sulfate	-	37
17ß-Estradiol 3-methyl ether sulfate	BNST3	46
(22*S*, 23*S*)-28-Homobrassinolide sulfate	BNST3	37
24-Epibrassinolide sulfate	BNST3	43
24-Epiteasterone sulfate	BNST3	45
24-Epicathasterone sulfate	BNST3	45
Sulfated product of butanol extract	BNST3	38
24-Epicathasterone sulfate	BNST4	45
Sulfated product of butanol extract	BNST4	56
24-Epicathasterone sulfate	ATST1	45

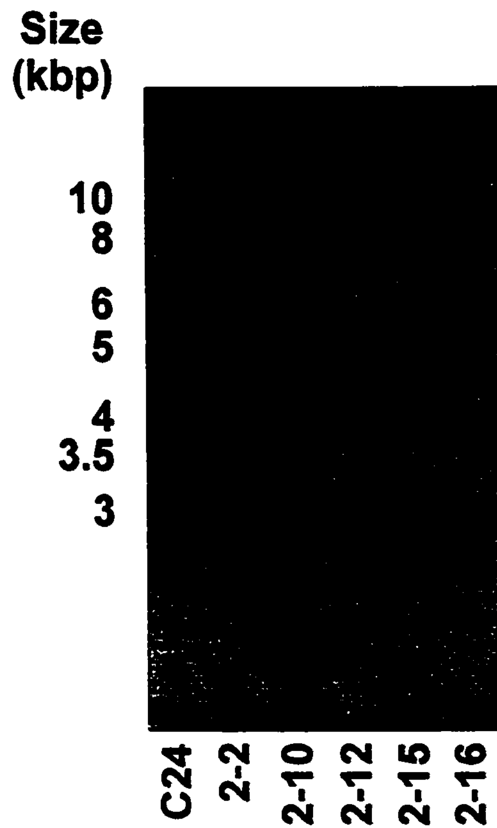


Figure 1. Southern blot of independent transgenic lines of *Arabidopsis thaliana* expressing *BNST3*. Genomic DNA was digested with *Eco*RI, and the blot was probed with a ^{32}P-labeled fragment derived from the pBI524 vector, containing a double copy of the minimal CaMV35S promoter (Datla et al., 1993). The size of markers is indicated on the left.

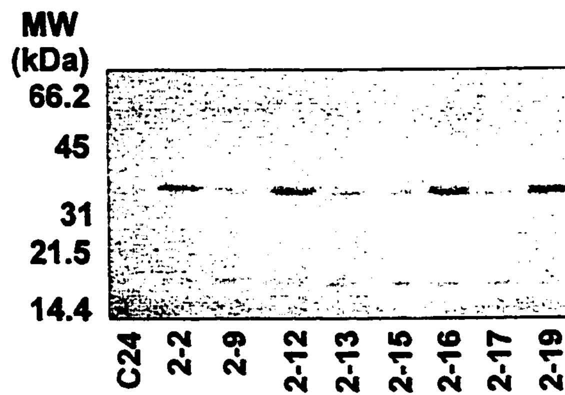


Figure 2. Western blot of independent transgenic lines of *Arabidopsis thaliana* expressing *BNST3*. Immunodetection was performed with anti-BNST1 polyclonal antibodies (Chapter 2). The size of molecular weight markers is indicated on the left.

Figure 3. Wild-type *Arabidopsis thaliana* and transgenic lines expressing *BNST3*. From left to right, two wild-type *A. thaliana* plants (ecotype C24), two plants from line 2-2, and two plants from line 2-12. Plants were 3 week-old, and were grown under long day conditions.

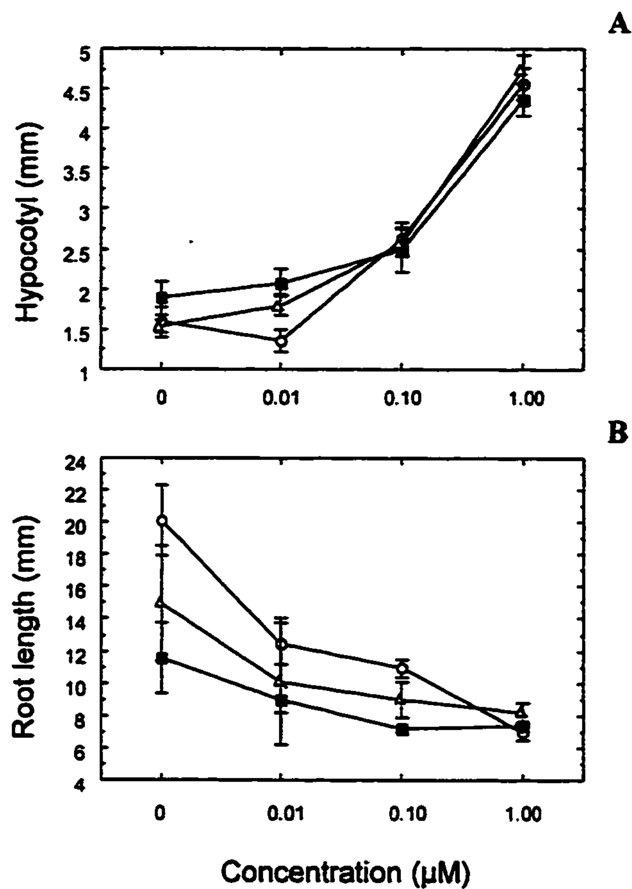


Figure 4. Response of wild-type *Arabidopsis thaliana* and transgenic lines expressing *BNST3* to treatment with exogenous 24-epibrassinolide (■ 2-12, Δ 2-2 and o C24 wild type). A) Hypocotyl elongation. B) Root elongation. Bars correspond to Standard Errors.

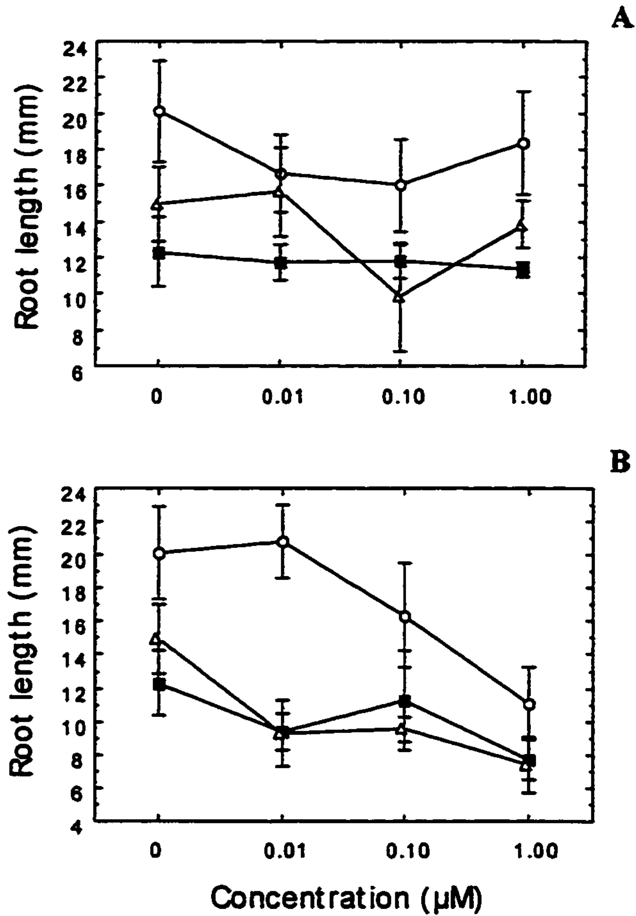


Figure 5. Root elongation of wild-type *Arabidopsis thaliana* and transgenic lines expressing *BNST3* in response to treatment with exogenous A) 24-epicathasterone; B) 24-epiteasterone (■ 2-12, Δ 2-2 and o C24 wild type). Bars correspond to Standard Errors.

CONCLUSION

In this thesis, we present the first evidence that, like in mammals, steroid sulfotransferases exist in plants. We have characterized four genes encoding steroid sulfotransferases from *Brassica napus*, designated *BNST1* to *-4*. A single homolog of *BNST* genes, *RaR047* (Lacomme and Roby, 1996), is present in *Arabidopsis thaliana* (Marsolais et al., 2000). *RaR047* was later renamed *ATST1*, in order to simplify the nomenclature of sulfotransferase-coding genes in *A. thaliana* (Marsolais et al., 2000). The recombinant BNST3, BNST4 and ATST1 catalyze the sulfonation of brassinosteroids and of mammalian steroids (Chapters 2 and 3, Annex I, Tables 1 and 2). The enzymes are stereospecific for 24-epibrassinosteroids, with a substrate preference for 24-epicathasterone, a precursor of 24-epibrassinolide. The steroid sulfotransferases also exhibit enzymatic activity with synthetic (22*S*, 23*S*)-28-homobrassinosteroids.

The recombinant steroid sulfotransferases are highly specific for steroid substrates, and interesting relationships between the steroid structure and enzymatic activity were observed. BNST3 and BNST4 do not accept the synthetic analog 22-deoxy-24-epiteasterone as substrate, suggesting that sulfonation takes place at position 22 of 24-epibrassinosteroids. Furthermore, the substrate specificity of BNST3 for estrogens suggests that the sulfonate group is transferred to position 17 of 17ß-estradiol.

Estrogens and testosterone act as inhibitors of BNST4. The major structural determinant of inhibition is the presence of a rigid keto- or phenolic hydroxyl group at position 3 of the steroid nucleus. In contrast, BNST4 is active with hydroxysteroids having a 3ß-hydroxyl group, such as pregnenolone, dehydroepiandrosterone and androstenediol. The enzymatic activity observed with these substrates suggests that

BNST4 catalyzes the transfer of the sulfonate group to position 3 of hydroxysteroids. The difference between the positions of sulfonation expected for brassinosteroids and hydroxysteroids implies that the two types of steroid substrates bind in a different orientation at the active site of the enzyme. Such a phenomenon has been described for mammalian hydroxysteroid sulfotransferases, which catalyze the sulfonation of testosterone at position 17, and of androsterone or dehydroepiandrosterone at position 3 (Falany, 1997, Park et al., 1999).

The substrate specificity of BNST2 could not be determined since the recombinant protein produced in *Escherichia coli* was insoluble. In addition, the biochemical function and the regulation of expression of *BNST1* remain to be characterized. *BNST1* is the most distant member of the *BNST* gene family, and in contrast with other *BNST* genes, it is not inducible by salicylic acid or ethanol. The recombinant BNST1 enzyme displayed enzymatic activity with several brassinosteroids and 17ß-estradiol, but only at high concentration.

The substrate specificity of ATST1 is broader as compared with those of BNST3 and -4. Recombinant ATST1 has a low but significant enzymatic activity with 22-deoxy-24-epiteasterone at high concentration (Annex I, Table 1). In addition, ATST1 catalyzes the sulfonation of all estrogens and hydroxysteroids that were tested. Unlike BNST3, ATST1 does not display stereospecificity for the 17-hydroxyl group of estradiol, and accepts estrone as substrate.

The enzymatic activities of BNST3, BNST4 and ATST1 with estrogens and hydroxysteroids suggest a conservation of biochemical function between the plant and mammalian steroid sulfotransferases. Functional conservation between plant and

mammalian steroid biosynthetic enzymes has been demonstrated for the steroid 5α-reductase (Li et al., 1997) and the Δ^5-3ß-hydroxysteroid dehydrogenase (Finsterbusch et al., 1999). The enzymatic activity of the human steroid sulfotransferases was tested with brassinosteroids. The recombinant estrogen sulfotransferase is inactive, since this enzyme is specific for steroids having a phenolic ring A. However, the recombinant hydroxysteroid sulfotransferase catalyzes the sulfonation of several brassinosteroids (Annex I, Table 3). The enzyme exhibits a substrate preference for brassinosteroids having a 3ß-hydroxyl group, as is found in its natural substrate dehydroepiandrosterone.

According to a proposed molecular classification of soluble sulfotransferases from eukaryotes, enzymes belonging to the same family share at least 45% sequence identity (Weinshilboum et al., 1997). The plant *SULT3* family comprises the previously characterized flavonol 3- and 4′-sulfotransferases from *Flaveria* species, and a sulfotransferase of unknown biochemical function from *F. bidentis* (FBSTX) (Varin et al., 1997) (Annex I, Figure 1). With the near completion of the *A. thaliana* genome project, all sulfotransferase-coding genes are known from a single plant species. BNST proteins and ATST1 form a new family of plant sulfotransferases (Marsolais et al., 2000) (Annex I, Figure 1). This new family includes another sulfotransferase from *A. thaliana*, ATST6, which is the most distant member. The presence of an *ATST1*-related gene in *A. thaliana* is very interesting from a biochemical point of view. Considering that *A. thaliana* accumulates both 24-epibrassinosteroids and their 24-epimers (Schmidt et al., 1997), the strict stereospecificity of ATST1 is intriguing. A sulfotransferase having a stereospecificity complementary to that of ATST1 may be expected to exist in *A. thaliana*, and this enzyme may be ATST6.

In the molecular classification of cytochrome P450s, plant enzymes are divided in two classes (Durst and Nelson, 1995). Class A P450s perform plant-specific reactions of secondary metabolism. The non-class A P450s are more similar to enzymes from other kingdoms, and belong to common metabolic pathways, such as lipid or steroid biosynthesis. Although mammalian and plant soluble sulfotransferases are related phylogenetically, we do not observe the same relationships as those seen for cytochrome P450s (Annex I, Figure1). Plant steroid sulfotransferases cluster with other plant enzymes, and not with mammalian steroid sulfotransferases, despite their related biochemical functions.

In mammals, sulfonation of estrogens and androgens results in a loss of biological activity (Roy, 1992, Strott, 1996). 24-Epibrassinolide sulfate produced by enzymatic sulfonation with BNST3 is inactive in the bean second internode bioassay, a standard test for the biological activity of brassinosteroids (Chapter 2). Based on this result, as well as the inducible pattern of expression in response to salicylic acid, our main hypothesis was that BNST3 is involved in brassinosteroid inactivation. However, the low affinity of BNST3 for 24-epibrassinolide suggests that the enzyme is not catalytically active with this substrate *in vivo*, but the sulfonation of a 24-epibrassinolide precursor, 24-epicathasterone, probably prevents further biosynthesis.

Two other hypotheses may be considered concerning the function of BNST3. Sulfonation of 24-epibrassinolide precursors may be required for their transport. In humans, dehydroepiandrosterone produced in the adrenal gland is sulfated by the hydroxysteroid sulfotransferase and transported through the circulation (Hobkirk, 1993). Dehydroepiandrosterone sulfate may be used to synthesize estrogens and androgens, after

removal of the sulfate group by a sulfatase. Brassinosteroids probably need to be transported from leaves to the zones of elongation of root and stem, as deduced from the tissue localization of *CPD* expression in *A. thaliana* (Mathur et al., 1998). The sulfonation of 24-epibrassinolide precursors may also be involved in their storage. In plants, auxin-amino acid conjugates stored in the seed are gradually released in their free form during seedling germination, before the capacity for auxin biosynthesis is established (Sembdner et al., 1995). This type of function might be considered for brassinosteroid sulfonation if the genes encoding the sulfotransferases were expressed in reproductive tissues, or during seed maturation.

One important aspect of the formation of steroid sulfate conjugates in mammals is the reversibility of the reaction. Sulfatases have not been characterized from plants, and there is no sequence in the *A. thaliana* database sharing significant homology with arylsulfatase genes from mammals or *Chlamydomonas reinhardtii.* Plant steroid sulfonation may, therefore, constitute a terminal metabolic reaction. The fate of sulfated metabolites in the plant cell is also unknown. Several glucoside and glutathione conjugates of herbicides and endogenous metabolites are transported to the vacuole (Kreuz et al., 1996, Coleman et al., 1997), and a vacuolar transporter of sulfate conjugates has been recently characterized at the biochemical level (Klein et al., 1997). The sulfate conjugate transporter, like that for glutathione conjugates (Lu et al., 1997), is probably non-specific, although its specificity remains to be fully characterized.

The induction of *BNST3* and *ATST1* (Lacomme and Roby, 1996) by salicylic acid allowed us to establish a link between the biochemical function of the sulfotransferases and the physiological response resulting from pathogen infection. During the defense

response, the formation of a physical barrier against pathogen infection through the reinforcement of the cell wall is incompatible with brassinosteroid-stimulated cell expansion. Based on the induction by salicylic acid, a function of BNST3 in brassinosteroid transport would, therefore, be very unlikely. BNST3 induction during the defense response may lead to a decrease in 24-epibrassinolide biosynthesis. To our knowledge, this is the first time that an enzyme involved in hormone metabolism has been linked to physiological changes associated with the plant defense response. It is clear that other mechanisms may be used to modulate hormone responses during pathogen infection. In support of this hypothesis, it has recently been demonstrated that activation of a protein kinase cascade by H_2O_2 leads to a repression of auxin-inducible genes in *A. thaliana* (Kovtun et al., 2000).

In chapter 3, the induction of steroid sulfotransferases by ethanol has been characterized. Ethanol is known to induce several plant cytochrome P450 genes involved in herbicide detoxication (Reichhardt et al., 1979, Potter et al., 1995). In plants, low oxygen stress results in a switch from respiratory metabolism to ethanolic fermentation, and in the induction of several genes encoding anaerobic proteins (Sachs et al., 1996). Most anaerobic proteins are enzymes of glycolysis and ethanolic fermentation, with the exception of a xyloglucan endotransglycosylase (XET). Interestingly, ethanol treatment of *B. napus* seedlings also results in the induction of *ADH* and *XET* genes. Furthermore, endogenous ethanol produced under hypoxia may be responsible for the induction of the steroid sulfotransferases under these conditions. These results open a new perspective on gene regulation by ethanol in plants. In future work, the status of ethanol as a chemical signal during low oxygen stress should be evaluated.

In chapter 4, the tissue localization of *BNST2* and *-3* expression was determined in transgenic *A. thaliana* expressing promoter-GUS fusions. The main features of the tissue-specific expression are a localization in the root near the junction with the hypocotyl, and at the apex of leaf organs. There is a correlation between the pattern of *BNST* expression in leaves and that of *CPD*, encoding cathasterone 23-hydroxylase (Mathur et al., 1998). These results suggest that BNST3 may be active with 24-epicathasterone *in vivo*. The pattern of expression that was observed, especially in leaves, is compatible with a function of the steroid sulfotransferases in either brassinosteroid inactivation or transport.

In chapter 5, the main hypothesis that *BNST3* is involved in brassinosteroid inactivation was tested by expressing this gene in transgenic *A. thaliana* under the control of the CaMV35S promoter. No effect of the transgene was observed on the growth and development of the transgenic plants. In addition, transgenic lines expressing *BNST3* had a similar response to exogenous 24-epiteasterone or 24-epibrassinolide as compared with the wild-type. These results suggest that BNST3 may not be active with these brassinosteroids in vivo. Several reasons may explain the results obtained with the transgenic plants expressing *BNST3*. Increased biosynthesis of 24-epibrassinolide may compensate for the activity of the steroid sulfotransferase. Metabolic compensation has been well documented in experiments aiming at the manipulation of the levels of biologically active gibberellins in transgenic plants (Xu et al., 1999). In addition, 24-epibrassinolide may represent a minor fraction of the total active brassinosteroids, so that a reduction in its levels may have a weak effect on plant growth. Finally, the steroid sulfotransferase may not be expressed in sufficient levels in the tissues where brassinosteroids accumulate, such as the zones of elongation of the root and hypocotyl.

Based on the characterization of the putative brassinosteroid receptor BRI1 (Li and Chory, 1997), the plant steroid hormones may be perceived at the cell surface. In the experiments performed with exogenous 24-epibrassinolide, the intracellular localization of the sulfotransferase may not allow the modulation of hormone action.

The lack of effect of *BNST3* expression in transgenic *A. thaliana* did not allow us to reach a conclusion on the function of the steroid sulfotransferase. However, in future work, the possible function of the steroid sulfotransferases in brassinosteroid transport should be tested in transgenic *A. thaliana* plants expressing the *ATST1* gene in antisense orientation, or with T-DNA tagged knock-out mutants of *ATST1*.

Alternative experimental approaches may be required to understand the function of the steroid sulfotransferases. In chapter 5, we have presented the results of preliminary experiments performed in order to characterize endogenous substrates of the sulfotransferases. The possibility that the enzymes accept a different physiological substrate from those characterized *in vitro* should be seriously considered. Several biologically inactive brassinosteroid metabolites, such as 3, 24-diepibrassinolide and 3, 24-diepicastasterone, as well as pregnanes produced after side chain cleavage, represent attractive candidate substrates, as discussed in chapter 5. In future work, the purification and structural characterization of endogenous substrates should be combined with the identification of steroid sulfate esters present in aqueous extracts of *B. napus* seedlings.

REFERENCES

Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D. and Shinozaki, K. (1997) Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell* **9**, 1859-1868.

Adam, G. and Schneider, B. (1999) Uptake, transport and metabolism. In *Brassinosteroids: Steroidal Plant Hormones* (Sakurai, A., Yokota, T. and Clouse, S. D., eds.). Tokyo: Springer-Verlag, pp. 113-136.

Alfenito, M. R., Souer, E., Goodman, C. D., Buell, R., Mol, J., Koes, R. and Walbot, V. (1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione S-transferases. *Plant Cell* **10**, 1135-1149.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

An, G., Ebert, P. R., Mitra, A. and Ha, S. B. (1988) Binary vectors. In *Plant Molecular Biology Manual* (Gelvin, S. B. and Schilperoort, R. A., eds.). Dordrecht: Kluwer Academic, pp. 1-19.

Ananvoranich, S., Varin, L., Gulick, P. and Ibrahim, R. K. (1994) Cloning and regulation of flavonol 3-sulfotransferase in cell suspension cultures of *Flaveria bidentis*. *Plant Physiol.* **106**, 485-491.

App, A. A. and Meiss, A. N. (1958) Effect of aeration on rice alcohol dehydrogenase. *Arch. Biochem. Biophys.* **77**, 181-190.

Atwell, B. J., Drew, M. C. and Jackson, M. B. (1988) The influence of oxygen deficiency on ethylene synthesis, 1-aminocyclopropane-1-carboxylic acid levels and aerenchyma formation in roots of *Zea mays* L. *Physiol. Plant.* **72**, 15-22.

Bailey-Serres, J. and Dawe, R. K. (1996) Both 5' and 3' sequences of maize *adh1* mRNA are required for enhanced translation under low-oxygen conditions. *Plant Physiol.* **112**, 685-695.

Bailey-Serres, J. and Freeling, M. (1990) Hypoxic stress-induced changes in ribosomes of maize seedlings. *Plant Physiol.* **94**, 1237-1243.

Banoglu, E. and Duffel, M.W. (1997) Studies on the interactions of chiral secondary alcohols with rat hydroxysteroid sulfotransferase STa. *Drug Metab. Dispos.* **25**, 1304-1310.

Beato, M., Herrlich, P. and Schütz, G. (1995) Steroid hormone receptors: many actors in search of a plot. *Cell* **83**, 851-857.

Benveniste, P. (1986) Sterol biosynthesis. *Annu. Rev. Plant Physiol.* **37**, 275-308.

Bishop, G. J., Nomura, T., Yokota, T., Harrison, K., Noguchi, T., Fujioka, S., Takatsuto, S., Jones, J. D. G. and Kamiya, Y. (1999) The tomato DWARF enzyme catalyses C-6 oxidation in brassinosteroid biosynthesis. *Proc. Natl. Acad. Sci. USA* **96**, 1761-1766.

Bowman, K. G. and Bertozzi, C. R. (1999) Carbohydrate sulfotransferases: mediators of extracellular communication. *Chem. Biol.* **6**, R9-R22.

Brindle, P. A., Kuhn, P. J. and Threlfall, D. R. (1988) Biosynthesis and metabolism of sesquiterpenoid phytoalexins and triterpenoids in potato cell suspension culture. *Phytochemistry* **27**, 133-150.

Brunel, D., Froger, N. and Pelletier, G. (1999) Development of amplified consensus genetic markers (ACGM) in *Brassica napus* from *Arabidopsis thaliana* sequences of known biological function. *Genome* **42**, 387-402.

Bucher, M., Brändle, R. and Kuhlemeier, C. (1994) Ethanolic fermentation in transgenic tobacco expressing *Zymomonas mobilis* pyruvate decarboxylase. *EMBO J.* **13**, 2755-2763.

Bucher, M., Brander, K. A., Sbicego, S., Mandel, T. and Kuhlemeier, C. (1995) Aerobic fermentation in tobacco pollen. *Plant Mol. Biol.* **28**, 739-750.

Caddick, M. X., Greenland, A. J., Jepson, I., Krause, K.-P., Qu, N., Riddell, K. V., Salter, M. G., Schuch, W., Sonnewald, U. and Tomsett, A. B. (1998) An ethanol inducible gene switch for plants used to manipulate carbon metabolism. *Nature Biotechnol.* **16**, 177-180.

Cao, H. and Dong, X. (1998) Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proc. Natl. Acad. Sci. USA* **95**, 6531-6536.

Cao, H., Bowling, S. A., Gordon, A. S. and Dong, X. (1994) Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6**, 1583-1592.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S. and Dong, X. (1997) The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88**, 57-63.

Cashmore, A. R. (1982) The isolation of poly A^+ messenger RNA from higher plants. In *Methods in Chloroplast Molecular Biology*. (Edelman, M., Hallick, R. B. and Chua, N.-H., eds.) New York: Elsevier Biomedical Press, pp. 387-392.

Catala, C., Rose, J. and Bennett, A. (1997) Auxin-regulation and spatial localization of an endo-1, 4-ß-D-glucanase and a xyloglucan endotransglycosylase in expanding tomato hypocotyls. *Plant J.* **12**, 417-426.

Cerana, R., Bonetti, A., Marre, M. T., Romani, G., Lado, P. and Marre E. (1983) Effects of a brassinosteroid on growth and electrogenic proton extrusion in azuki bean epicotyls. *Physiol. Plant.* **59**, 23-27.

Cerana, R., Lado, P., Anastasia, M., Ciuffreda, P. and Allevi, P. (1984) Regulating effects of brassinosteroids and of sterols on growth and H^+ secretion in maize roots. *J. Plant Physiol.* **114**, 221-225.

Chamnongpol, S., Willekens, H., Moeder, W., Langerbartels, C., Sandermann, H. Jr, Van Montagu, M., Inze, D. and Van Camp, W. (1998) Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic tobacco. *Proc. Natl. Acad. Sci. USA* **95**, 5818-5823.

Chan, J., Song, C. S., Matusik, R. J., Chatterjee, B. and Roy, A. K. (1998) Inhibition of androgen action by dehydroepiandrosterone sulfotransferase transfected in PC-3 prostate cancer cells. *Chem. Biol. Interact.* **109**, 267-278.

Chang, C. and Meyerowitz, E. M. (1986) Molecular cloning and DNA sequence of the Arabidopsis thaliana alcohol dehydrogenase gene. *Proc. Natl. Acad. Sci. USA* **83**, 1408-1412.

Chatterjee, B., Song, C.-S., Kim, J. M. and Roy, A. K. (1994) Androgen and estrogen sulfotransferases in the rat liver: physiological function, molecular cloning, and in vitro expression. *Chem. Biol. Interact.* **92**, 273-279.

Chen, W. and Singh, K. B. (1999) The auxin, hydrogen peroxide and salicylic acid induced expression of the Arabidopsis GST6 promoter is mediated in part by an ocs element. *Plant J.* **19**, 667-677.

Chen, W., Chao, G. and Singh, K.B. (1996) The promoter of a H2O2-inducible, Arabidopsis glutathione S-transferase gene contains closely linked OBF- and OBP1-binding sites. *Plant J.* **10**, 955-966.

Cho, H.-T. and Kende, H. (1997) Expression of expansin genes is correlated with growth in deepwater rice. *Plant Cell* **9**, 1661-1671.

Choe, S., Dilkes, B. P., Fujioka, S., Takatsuto, S., Sakurai, A. and Feldmann, K. A. (1998) The *DWF4* gene of Arabidopsis encodes a cytochrome P450 that mediates multiple 22α-hydroxylation steps in brassinosteroid biosynthesis. *Plant Cell* **10**, 231-244.

Choe, S., Dilkes, B. P., Gregory, B. D., Ross, A. S., Yuan, H., Noguchi, T., Fujioka, S., Takatsuto, S., Tanaka, A., Yoshida, S., Tax, F. E. and Feldmann, K. A. (1999a) The Arabidopsis *dwarf1* mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. *Plant Physiol.* **119**, 897-907.

Choe, S., Noguchi, T., Fujioka, S., Takatsuto, S., Tissier, C. P., Gregory, B. D., Ross, A. S., Tanaka, A., Yoshida, S., Tax, F. E. and Feldmann, K. A. (1999b) The Arabidopsis *dwf7/ste1* mutant is defective in the delta7 sterol C-5 desaturation step leading to brassinosteroid biosynthesis. *Plant Cell* **11**, 207-221.

Choe, S., Tanaka, A., Noguchi, T., Fujioka, S., Takatsuto, S., Ross, A. S., Tax, F. E., Yoshida, S. and Feldmann, K. A. (2000) Lesions in the sterol delta reductase gene of Arabidopsis cause dwarfism due to a block in brassinosteroid biosynthesis. *Plant J.* **21**, 431-443.

Choi, D., Ward, B. L. and Bostock, R. M. (1992) Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. *Plant Cell* **4**, 1333-1344.

Chory, J. and Li, J. (1997) Gibberellins, brassinosteroids and light-regulated development. *Plant Cell Environ.* **20**, 801-806.

Chory, J., Nagpal, P. and Peto, C. A. (1991) Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in Arabidopsis. *Plant Cell* **3**, 445-459.

Chory, J., Chatterjee, M., Cook, R. K., Elich, T., Fankhauser, C., Li, J., Nagpal, P., Neff, M., Pepper, A., Poole, D., Reed, J. and Vitart, V. (1996) From seed development to flowering, light controls plant development via the pigment phytochrome. *Proc. Natl. Acad. Sci. USA* **93**, 12066-12071.

Clouse, S. D. (1997) Molecular genetic analysis of brassinosteroid action. *Physiol. Plant.* **100**, 702-709.

Clouse, S. D. and Feldmann, K. A. (1999) Molecular genetics of brassinosteroid action. In *Brassinosteroids: Steroidal Plant Hormones* (Sakurai, A., Yokota, T. and Clouse, S. D., eds.). Tokyo: Springer-Verlag, pp. 163-190.

Clouse, S. and Sasse, J. (1998) Brassinosteroids: essential regulators of plant growth and development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 427-451.

Clouse, S. D., Hall, A. F., Langford, M., McMorris, T. C. and Baker, M. E. (1993) Physiological and molecular effects of brassinosteroids on *Arabidopsis thaliana*. *J. Plant Growth Regul.* **12**, 61-66.

Clouse, S. D., Langford, M. and McMorris, T. C. (1996) A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. *Plant Physiol.* **111**, 671-678.

Coleman, J. O. D., Blake-Kalff, M. M. A. and Emyr Davies, T. G. (1997) Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends Plant Sci.* **2**, 144-151.

Coles, J. P., Phillips, A. L., Croker, S. J., Garcia-Lepe, R., Lewis, M. J. and Hedden, P. (1999) Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. *Plant J.* **17**, 547-556.

Cossins, E. A. (1978) Ethanol metabolism in plants. In *Plant Life in Anaerobic Environments* (Hook, D. D. and Crawford, R. M. M., eds.). Ann Arbor: Ann Arbor Science Publishers, pp. 169-202.

Crawford, R. M. M. and Braendle, R. (1996) Oxygen deprivation stress in a changing environment. *J. Exp. Bot.* **47**, 145-159.

Cutler, H. G., Yokota, T. and Adam G. (eds.) (1991) *Brassinosteroids: Chemistry, Bioactivity and Applications*. Washington, DC: American Chemical Society, ACS Symposium Series **474**.

Damme, B., Darmer, D. and Pankow, D. (1996) Induction of hepatic cytochrome P4502E1 in rats by acetylsalicylic acid or sodium salicylate. *Toxicology* **106**, 99-103.

Darras, V. M., Hume, R. and Visser, T. J. (1999) Regulation of thyroid hormone metabolism during fetal development. *Mol. Cell. Endocrinol.* **151**, 37-47.

Datla, R. S. S., Bekkaoui, F., Hammerlindl, J. K., Pilate, G., Dunstan, D. I. and Crosby, W. L. (1993) Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. *Plant Sci.* **94**, 139-149.

Davies, D. D. (1980) Anaerobic metabolism and the production of organic acids. In *The Biochemistry of Plants* **2** (Stumpf, P. K. and Conn, E. E., eds.). New York: Academic Press, pp. 581-611.

Davies, D. D., Grego, S. and Kenworthy, P. (1974) The control of the production of lactate and ethanol by higher plants. *Planta* **118**, 297-310.

Davies, R. T., Goetz, D. H., Lasswell, J., Anderson, M. N. and Bartel, B. (1999) *IAR3* encodes an auxin conjugate hydrolase from Arabidopsis. *Plant Cell* **11**, 365-376.

De Bruxelles, G. L., Peacock, W. J., Dennis, E. S. and Dolferus, R. (1996) Abscisic acid induces the alcohol dehydrogenase gene in Arabidopsis. *Plant Physiol.* **111**, 381-391.

Deepak, D., Khare, A. and Khare, M. P. (1989) Plant pregnanes. *Phytochemistry* **28**, 3255-3263.

Delong, A., Calderon-Urrea, A. and Dellaporta, S. (1993) Sex determination gene *TASSELSEED2* of maize encodes short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. *Cell* **74**, 757-768.

Després. C., Delong, C., Glaze, S., Liu, E. and Fobert, P. R. (2000) The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* **12**, 279-290.

Devarenne, T. P., Shin, D. H., Back, K., Yin, S. and Chappell, J. (1998) Molecular characterization of tobacco squalene synthase and response to fungal elicitor. *Arch. Biochem. Biophys.* **349**, 205-215.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**, 387-395.

Diener, A. C., Li, H., Zhou, W.-x., Whoriskey, W. J., Nes, W. D. and Fink, G. R. (2000) *STEROL METHYLTRANSFERASE 1* controls the level of cholesterol in plants. *Plant Cell* **12**, 853-870.

Dolferus, R., Van den Bossche, D. and Jacobs, M. (1990) Sequence analysis of two null-mutant alleles of the single *Arabidopsis* Adh locus. *Mol. Gen. Genet.* **224**, 297-302.

Dolferus, R., Jacobs, M., Peacock, W. J. and Dennis, E. S. (1994) Differential interactions of promoter elements in stress responses of the *Arabidopsis Adh* gene. *Plant Physiol.* **105**, 1075-1087.

Dolferus, R., Osterman, J. C., Peacock, W. J. and Dennis, E. S. (1997) Cloning of the *Arabidopsis* and rice formaldehyde dehydrogenase genes: implication for the origin of plant ADH enzymes. *Genetics* **146**, 1131-1141.

Drew, M. C. (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 223-250.

Du, L. and Halkier, B. (1996) Isolation of a microsomal enzyme system involved in glucosinolate biosynthesis from seedlings of *Tropaeolum majus* L. *Plant Physiol.* **111**, 831-837.

Dupont, I., Berthou, F., Bodenez, P., Bardou, L., Guirriec, C., Stephan, N., Dreano, Y. and Lucas, D. (1999) Involvement of cytochrome P-450 2E1 and 3A4 in the 5-hydroxylation of salicylate in humans. *Drug Metab. Dispos.* **27**, 322-326.

Eisenhofer, G., Coughtrie, M. W. and Goldstein, D. S. (1999) Dopamine sulfate: an enigma resolved. *Clin. Exp. Pharmacol. Physiol. Suppl.* **26**, S41-S53.

Esau, K. (1977) *Anatomy of Seed Plants*. New York: John Wiley and Sons.

Falany, C. N. (1997) Enzymology of human cytosolic sulfotransferases. *FASEB J.* **11**, 206-216.

Falany, J. L. and Falany, C. N. (1996) Regulation of estrogen sulfotransferase in human endometrial adenocarcinoma cells by progesterone. *Endocrinology* **137**, 1395-401.

Falany, J. L. and Falany, C. N. (1997) Regulation of estrogen activity by sulfation in human MCF-7 breast cancer cells. *Oncol. Res.* **9**, 589-596.

Faulkner, I. J. and Rubery, P. H. (1992) Flavonoids and flavonol sulfates as probes of auxin-transport regulation in *Cucurbita pepo* hypocotyl segments and vesicles. *Planta* **186**, 618-625.

Feldmann, K. A., Marks, M. D., Christianson, M. L. and Quatrano, R. S. (1989) A dwarf mutant of *Arabidopsis* generated by T-DNA insertion mutagenesis. *Science* **243**, 1351-1354.

Fennoy, S. L. and Bailey-Serres, J. (1995) Post-transcriptional regulation of gene expression in oxygen-deprived roots of maize. *Plant J.* **7**, 287-295.

Finsterbusch, A., Lindemann, P., Grimm, R., Eckerskorn, C. and Luckner, M. (1999) Δ^5-3ß-Hydroxysteroid dehydrogenase from *Digitalis lanata* Ehrh. - a multifunctional enzyme in steroid metabolism? *Planta* **209**, 478-486.

Frear, D. S., Swanson, H. R. and Tanaka, F. S. (1993) Metabolism of flumetsulam (DE-498) in wheat, corn and barley. *Pestic. Biochem. Physiol.* **45**, 178-192.

Freeling, M. and Bennett, D. C. (1985) Maize *Adh1*. *Annu. Rev. Genet.* **19**, 297-323.

Fristensky, B., Balcerzak, M., He, D.-F. and Zhang, P. (1999) Expressed sequence tags from the defense response of *Brassica napus* to *Leptosphaeria maculans*. *Molecular Plant Pathology On-Line*, http://www.bspp.org.uk/mppol/1999/0301Fristensky

Fujioka, S. (1999) Natural occurrence of brassinosteroids in the plant kingdom. In *Brassinosteroids: Steroidal Plant Hormones* (Sakurai, A., Yokota, T. and Clouse, S. D., eds.). Tokyo: Springer-Verlag, pp. 21-45.

Fujioka, S. and Sakurai, A. (1997) Brassinosteroids. *Nat. Prod. Rep.* **14**, 1-10.

Fujioka, S., Inoue, T., Takatsuto, S., Yanagisawa, T., Yokota, T. and Sakurai, A. (1995) Biological activities of biosynthetically-related congeners of brassinolide. *Biosci. Biotechnol. Biochem.* **59**, 1973-1975.

Fujioka, S., Li, J., Choi, Y. H., Seto, H., Takatsuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J. and Sakurai, A. (1997) The Arabidopsis *deetiolated2* mutant is blocked early in brassinosteroid biosynthesis. *Plant Cell* 9, 1951-1962.

Geuns, J. M. C. (1978) Steroid hormones and plant growth and development. *Phytochemistry* 17, 1-14.

Griffiths, P. G., Sasse, J. M., Yokota, T. and Cameron, D. W. (1995) 6-Deoxotyphasterol and 3-dehydro-6-deoxoteasterone, possible precursors to brassinosteroids in the pollen of *Cupressus arizonica*. *Biosci. Biotechnol. Biochem.* 59, 956-959.

Grove, M. D., Spencer, G. F., Rohwedder, W. K., Mandava, N. B., Worley, F. J., Warthen, J. D., Steffens, G. L., Flippen-Anderson, J. L. and Cook, J. C. (1979) Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. *Nature* 281, 216-217.

Grsic, S., Sauerteig, S., Neuhaus, K., Albrecht, M., Rossiter, J. and Ludwig Mſller, J. (1998) Physiological analysis of transgenic *Arabidopsis thaliana* plants expressing one nitrilase isoform in sense or antisense direction. *J. Plant Physiol.* 153, 446-456.

Gu, Y. Q., Yang, C., Thara, V. K., Zhou, J. and Martin, G. B. (2000) *Pti4* is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell* 12, 771-786.

Guo, D.-a., Jia, Z. and Nes, W. D. (1996) Phytosterol biosynthesis: isotope effects associated with biomethylation formation to 24-alkene sterol isomers. *Tetrahedron Lett.* 37, 6823-6826.

Hai, T., Schneider, B. and Adam, G. (1995) Metabolic conversion of 24-*epi*-brassinolide into pentahydroxylated brassinosteroid glucosides in tomato cell cultures. *Phytochemistry* 40, 443-448.

Hai, T., Schneider, B., Porzel, A. and Adam, G. (1996) Metabolism of 24-*epi*-castasterone in cell suspension cultures of *Lycopersicon esculentum*. *Phytochemistry* 41, 197-201.

Hammond-Kosack, K. E. and Jones, J. D. G. (1996) Resistance gene-dependent plant defense responses. *Plant Cell* 8, 1773-1791.

Hanai, H., Nakayama, D., Yang, H., Matsubayashi, Y., Hirota, Y. and Sakagami, Y. (2000) Existence of a plant tyrosylprotein sulfotransferase: novel plant enzyme catalyzing tyrosine O-sulfation of preprophytosulfokine variants in vitro. *FEBS Lett.* 470, 97-101.

Hanfrey, C., Fife, M. and Buchanan-Wollaston, V. (1996) Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins. *Plant Mol. Biol.* 30, 597-609.

Hannoufa, A., Varin, L. and Ibrahim, R. K. (1991) Spatial distribution of flavonoid conjugates in relation to glucosyltransferase and sulfotransferase activities in *Flaveria bidentis*. *Plant Physiol.* **97**, 259-263.

Haudenschild, C. and Hartmann, M.-A. (1995) Inhibition of sterol biosynthesis during elicitor-induced accumulation of furanocoumarins in parsley cell suspension cultures. *Phytochemistry* **40**, 1117-1124.

He, C. J., Drew, M. C. and Morgan, P. W. (1994) Induction of enzyme associated with lysigenous aerenchyma formation in roots of *Zea mays* during hypoxia or nitrogen starvation. *Plant Physiol.* **105**, 861-865.

He, C. J., Finlayson, S. A., Drew, M. C., Jordan, W. R. and Morgan, P. W. (1996) Ethylene biosynthesis during aerenchyma formation in roots of *Zea mays* subjected to mechanical impedence and hypoxia. *Plant Physiol.* **112**, 1679-1685.

He, Z., Wang, Z.-Y., Li, J., Zhu, Q., Lamb, C., Ronald, P. and Chory, J. (2000) Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science* **288**, 2360-2363.

Hedden, P. and Kamiya, Y. (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 431-460.

Hedden, P. and Phillips, A. L. (2000) Manipulation of hormone biosynthetic genes in transgenic plants. *Curr. Opin. Biotechnol.* **11**, 130-137.

Hedden, P., Phillips, A. L., Coles, J., Thomas, S., Appleford, N., Ward, D., Beale, M. and Lenton, J. (1999) Gibberellin biosynthesis: genes, regulation and genetic manipulation. *Riken Rev.* **21**, 29-30.

Hobkirk, R. (1985) Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. *Can. J. Biochem. Cell. Biol.* **63**, 1127-1144.

Hobkirk, R. H. (1993) Steroid sulfation. *Trends Endocrinol. Metab.* **4**, 69-74.

Hoeren, F. U., Dolferus, R., Wu, Y., Peacock, W. J. and Dennis, E. S. (1998) Role for AtMYB2 in the induction of the Arabidopsis alcohol dehydrogenase gene (*ADH1*) by low oxygen. *Genetics* **149**, 479-490.

Hoffman-Benning, S. and Kende, H. (1992) On the role of abscisic acid and gibberellin in the regulation of growth in rice. *Plant Physiol.* **99**, 1156-1161.

Holt, B. F. 3rd, Mackey, D. and Dangl, J. L. (2000) Recognition of pathogens by plants. *Curr. Biol.* **10**, R5-R7.

Honkakosi, P. and Negishi, M. (1998) Regulatory DNA elements of phenobarbital-responsive cytochrome P450 *CYP2B* genes. *J. Biochem. Mol. Toxicol.* **12**, 3-9.

Horvath, D. M., Huang, D. J. and Chua, N.-H. (1998) Four classes of salicylate-induced tobacco genes. *Mol. Plant-Microbe Interact.* **11**, 895-905.

Huang, S., Raman, A. S., Ream, J. E., Fujiwara, H., Cerny, R. E. and Brown, S. M. (1998) Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in Arabidopsis. *Plant Physiol.* **118**, 773-781.

Huxtable, J. R. (1986) *Biochemistry of Sulfur*. New York: Plenum Press.

Iwasaki, T., and Shibaoka, H. (1991) Brassinosteroids act as regulators of tracheary-element differentiation in isolated *Zinnia* mesophyll cells. *Plant Cell Physiol.* **32**, 1007-1014.

Jackson, M. B. (1985) Ethylene and responses of plants to soil waterlogging and submergence. *Annu. Rev. Plant Physiol.* **36**, 145-174.

Jackson, M. B., Fenning, T. M., Drew, M. C. and Saker, L. R. (1985) Stimulation of ethylene production and gas-space (aerenchyma) formation in adventitious roots of *Zea mays* L. by small partial pressures of oxygen. *Planta* **165**, 486-492.

Jacobs, M. and Rubery, P. H. (1988) Naturally occurring auxin transport regulators. *Science* **241**, 346-349.

Jacobs, M., Dolferus, R. and Van Den Bossche, D. (1988) Isolation and biochemical analysis of ethyl methanesulfonate-induced alcohol dehydrogenase null mutants of *Arabidopsis thaliana* (L.) Heynh. *Biochem. Genet.* **26**, 105-122.

Jang, J.-C., Fujioka, S., Tasaka, M., Seto, H., Takatsuto, S., Ishii, A., Aida, M., Yoshida, S. and Sheen, J. (2000) A critical role of sterols in embryonic patterning and meristem programming revealed by the *fackel* mutants of *Arabidopsis thaliana*. *Genes Dev.* **14**, 1485-1497.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. (1987) GUS fusions: *β*-glucoronidase as a sensitive and versatile fusion marker in higher plants. *EMBO J.* **6**, 3901-3907.

Jupin, I. and Chua, N.-H. (1996) Activation of the CaMV *as-1 cis*-element: Differential DNA-binding of a factor related to TGA1a. *EMBO J.* **15**, 5679-5689.

Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M. and Pedersen, L. C. (1997) Crystal structure of estrogen sulphotransferase. *Nat. Struct. Biol.* **4**, 904-908.

Katsumi, M. (1991) Physiological modes of brassinolide action in cucumber hypocotyl growth. In *Brassinosteroids: Chemistry, Bioactivity and Applications* (Cutler, H. G., Yokota, T. and Adam G., eds.). Washington, DC: American Chemical Society, ACS Symposium Series **474**, pp. 246-254.

Kauschmann, A. Jessop, A. Koncz, C., Szekeres, M., Willmitzer, L. and Altmann, T. (1996) Genetic evidence for an essential role of brassinosteroids in plant development. *Plant J.* **9**, 701-713.

Kehoe, J. W. and Bertozzi, C. R. (2000) Tyrosine sulfation: a modulator of extracellular protein-protein interactions. *Chem. Biol.* **7**, R57-R61.

Kemper, B. (1998) Regulation of cytochrome P450 gene transcription by phenobarbital. *Prog. Nucleic Acids Res. Mol. Biol.* **61**, 23-64.

Kester, M. H., Bulduk, S., Tibboel, D., Meinl, W., Glatt, H., Falany, C. N., Coughtrie, M. W., Bergman, A., Safe, S. H., Kuiper, G. G., Schuur, A. G., Brouwer, A. and Visser, T. J. (2000) Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. *Endocrinology* **141**, 1897-1900.

Kim, G.-T., Tsukaya H. and Uchimiya, H. (1998) The *ROTUNDIFOLIA3* gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells. *Genes Dev.* **12**, 2381-2391.

Kim, G.-T., Tsukaya, H., Saito, Y. and Uchimiya, H. (1999) Changes in the shapes of leaves and flowers upon overexpression of cytochrome P450 in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **96**, 9433-9437.

Kim, S.-K. (1991) Natural occurrences of brassinosteroids. In *Brassinosteroids: Chemistry, Bioactivity and Applications* (Cutler, H. G., Yokota, T. and Adam G., eds.). Washington DC: American Chemical Society, ACS Symposium Series **474**, pp. 246-254.

Kimmerer, T. W. and Kozlowski, T. T. (1982) Ethylene, ethane, acetaldehyde and ethanol production by plants under stress. *Plant Physiol.* **69**, 840-847.

Klahre, U., Noguchi, T., Fujioka, S., Takatsuto, S., Yokota, T., Nomura, T., Yoshida, S. and Chua, N.-H. (1998) The Arabidopsis *DIMINUTO/DWARF* gene encodes a protein involved in steroid synthesis. *Plant Cell* **10**, 1677-1690.

Klein, M., Martinoia, E. and Weissenbock, G. (1997) Transport of lucifer yellow CH into plant vacuoles -- evidence for direct energization of a sulphonated substance and implications for the design of new molecular probes. *FEBS Lett.* **420**, 86-92.

Koka, C. V., Cerny, R. E., Gardner, R. G., Noguchi, T., Fujioka, S., Takatsuto, S., Yoshida, S. and Clouse, S. D. (2000) A putative role for the tomato genes *DUMPY* and *CURL-3* in brassinosteroid biosynthesis and response. *Plant Physiol.* **122**, 85-98.

Kolbe, A., Schneider, B., Porzel, A., Voigt, B., Krauss, G. and Adam, G. (1994) Pregnane-type metabolites of brassinosteroids in cell suspension cultures of *Ornithopus sativus*. *Phytochemistry* **36**, 671-673.

Kolbe, A., Schneider, B., Porzel, A. and Adam, G. (1995) Acyl-conjugated metabolites of brassinosteroids in cell suspension cultures of *Ornithopus sativus*. *Phytochemistry* **38**, 633-636.

Kolbe, A., Schneider, B., Porzel, A. and Adam, G. (1996) Metabolism of 24-*epi*-castasterone and 24-*epi*-brassinolide in cell suspension cultures of *Ornithopus sativus*. *Phytochemistry* **41**, 163-167.

Kolbe, A., Porzel, A., Schneider, B. and Adam, G. (1997) Diglycosidic metabolites of 24-epi-teasterone in cell suspension cultures of *Lycopersicon esculentum* L. *Phytochemistry* **46**, 1019-1022.

Kolbe, A., Schneider, B., Porzel, A. and Adam, G. (1998) Metabolic inversion of the 3-hydroxy function of brassinosteroids. *Phytochemistry* **48**, 467-470.

Kollöfel, C. (1968) Activity of alcohol dehydrogenase in the cotyledons of peas germinated under different environmental conditions. *Acta Bot. Neerl.* **17**, 70-77.

Kovtun, Y., Chiu, W.-L., Tena, G. and Sheen, J. (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. USA* **97**, 2940-2945.

Kreuz, K., Tommasini, R. and Martinoia, E. (1996) Old enzymes for a new job: herbicide detoxification in plants. *Plant Physiol.* **111**, 349-353.

Labrie, Y., Couet, J., Simard, J. and Labrie, F. (1994) Multihormonal regulation of dehydroepiandrosterone sulfotransferase messenger ribonucleic acid levels in adult rat liver. *Endocrinology* **134**, 1693-1699.

Lachaise, F. and Lafont, R. (1984) Ecdysteroid metabolism in a crab: *Carcinus maenas*. *Steroids* **43**, 243-259.

Lacomme, C. and Roby, D. (1996) Molecular cloning of a sulfotransferase in *Arabidopsis thaliana* and regulation during development and in response to infection with pathogenic bacteria. *Plant Mol. Biol.* **30**, 995-1008.

Lamb, C. and Dixon, R. A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 251-275.

Lange, T., Hedden, P. and Graebe, J. E. (1994) Expression cloning of a gibberellin 20-oxidase, a multifunctional enzyme involved in gibberellin biosynthesis. *Proc. Natl. Acad. Sci. USA* **91**, 8552-8556.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J. and Ward, E. (1998) Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. *Plant J.* **16**, 223-233.

Lebel-Hardenack, S. and Grant, S. R. (1997) Genetics of sex determination in flowering plants. *Trends Plant Sci.* **2**, 130-136.

Leighton Jones, J. and Roddick, J. G. (1988) Steroidal estrogens and androgens in relation to reproductive development in higher plants. *J. Plant Physiol.* **133**, 156-164.

Lenton, J. (1998) Plant hormones on the move! *Trends Plant Sci.* **3**, 457-458.

Lester, D. R., Ross, J. J., Smith, J. J., Elliott, R. C. and Reid, J. B. (1999) Gibberellin 2-oxidation and the *SLN* gene of *Pisum sativum*. *Plant J.* **19**, 65-73.

Li, J. and Chory, J. (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90**, 929-938.

Li, J. and Chory, J. (1999) Brassinosteroid actions in plants. *J. Exp. Bot.* **50**, 275-282.

Li, J., Nagpal, P., Vitart, V., McMorris, T. C. and Chory, J. (1996) A role for brassinosteroids in light-dependent development of *Arabidopsis*. *Science* **272**, 398-401.

Li, J., Biswas, M. G., Chao, A., Russel, D. W. and Chory, J. (1997) Conservation of function between mammalian and plant steroid 5α-reductases. *Proc. Natl. Acad. Sci. USA* **94**, 3554-3559.

Löve, A. and Löve, D. (1945) Experiments on the effects of animal sex hormones on dioecious plants. *Ark. Botan.* **32**, 1-60.

Lu, Y. P., Li, Z. S. and Rea, P. A. (1997) *AtMRP1* gene of Arabidopsis encodes a glutathione S-conjugate pump: isolation and functional definition of a plant ATP-binding cassette transporter gene. *Proc. Natl. Acad. Sci. USA* **22**, 8243-8248.

Mancini, M. A., Song, C.-S., Rao, T. R., Chatterjee, B. and Roy, A. K. (1992) Spatio-temporal expression of estrogen sulfotransferase within the hepatic lobule of male rats: implication of in situ estrogen inactivation in androgen action. *Endocrinology* **131**, 1541-1546.

Mandava, N. B. (1988) Plant growth-promoting brassinosteroids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 23-52.

Mari, M. and Cederbaum, A. I. (2000) *CYP2E1* overexpression in HepG2 cells induces glutathione synthesis by transcriptional activation of gamma-glutamylcysteine synthetase. *J. Biol. Chem.* **275**, 15563-71.

Marrs, K. A., Alfenito, M. R., Lloyd, A. M. and Walbot, V., (1995) A glutathione *S*-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. *Nature*. **375**, 397-400.

Marsolais, F. and Varin, L. (1995) Identification of amino acid residues critical for catalysis and cosubstrate binding in the flavonol 3-sulfotransferase. *J. Biol. Chem.* **270**, 30458-30463.

Marsolais, F. and Varin, L. (1997) Mutational analysis of domain II of flavonol 3-sulfotransferase. *Eur. J. Biochem.* **247**, 1056-1062.

Marsolais, F., Gidda, S. K., Boyd, J. and Varin, L. (2000) Plant soluble sulfotransferases: structural and functional similarity with mammalian enzymes. *Rec. Adv. Phytochem.* **34**, 433-456.

Martin, D. N., Proebsting, W. M. and Hedden, P. (1999) The *SLENDER* gene of pea encodes gibberellin 2-oxidase. *Plant Physiol.* **121**, 775-781.

Martínez, M. C., Achkor, H., Persson, B., Fernández, M R., Shafqat, J., Farrés, J., Jörnvall, H. and Parés, X. (1996) *Arabidopsis* formaldehyde dehydrogenase. *Eur. J. Biochem.* **241**, 849-857.

Mathur, J., Molnar, G., Fujioka, S., Takatsuto, S., Sakurai, A., Yokota, T., Adam, G., Voigt, B., Nagy, F., Maas, C., Schell, J., Koncz, C. and Szekeres, M. (1998) Transcription of the *Arabidopsis CPD* gene, encoding a steroidogenic cytochrome P450, is negatively controlled by brassinosteroids. *Plant J.* **14**, 593-602.

Mayumi, K. and Shibaoka, H. (1995) A possible double role for brassinolide in the re-orientation of cortical microtubules in the epidermal cells of azuki bean epicotyls. *Plant Cell Physiol.* **36**, 173-181.

Mayumi, K. and Shibaoka, H. (1996) The cyclic reorientation of cortical microtubules on walls with a crossed polylamellate structure: effects of plant hormones and an inhibitor of protein kinases on the progression of the cycle. *Protoplasma* **195**, 112-122.

McDowell, J. M. and Dangl, J. L. (2000) Signal transduction in the plant immune response. *Trends Biochem. Sci.* **25**, 79-82.

McMorris, T. C. and Patil, P. A. (1993) Improved synthesis of 24-epibrassinolide from ergosterol. *J. Org. Chem.* **58**, 2338-2339.

McMorris, T. C., Patil, P. A., Chavez, R. G., Baker, M. E. and Clouse, S. D. (1994) Synthesis and biological activity of 28-homobrassinolide and analogs. *Phytochemistry*, **36**, 585-589.

Mitchell, J. W. and Livingston, G. A. (1968) Methods of studying plant hormones and plant growth-regulating substances. In *Agriculture Handbook* No. **336**. Agricultural Research Service, United States Department of Agriculture. Washington, DC: United States Government Printing Office, pp. 26-28.

Monk, L. S., Fagerstedt, K. V. and Crawford, R. M. M. (1989) Oxygen toxicity and superoxide dismutase as an anti-oxidant in physiological stress. *Physiol. Plant.* **76**, 456-459.

Moreland, D. E., Corbin, F. T., Fleischmann, T. J. and MacFarland, J. E. (1995) Partial characterization of microsomes isolated from mung bean cotyledons. *Pestic. Biochem. Physiol.* **52**, 98-108.

Mulder, G. J. and Jakoby, W. B. (1990) Sulfation. In *Conjugation Reactions in Drug Metabolism* (Mulder, G. J., ed.). London: Taylor and Francis, pp. 107-161.

Muñoz, F. J., Labrador, E. and Dopico, B. (1998) Brassinolides promote the expression of a new *Cicer arietinium* β-tubulin gene involved in epicotyl elongation. *Plant Mol. Biol.* **37**, 807-817.

Mushegian, A. and Koonin, E. V. (1995) A putative FAD-binding domain in a distinct group of oxidases including a protein involved in plant development. *Protein Sci.* **4**, 1243-1244.

Nebert, D W. (1991) Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions. *Mol. Endocrinol.* **5**, 1203-1214.

Neff, M., Nguyen, E., Malancharuvil, S., Fujioka, T., Noguchi, H., Seto, M., Tsubuki, T., Honda, S., Takatsuto, S., Yoshida, S. and Chory, J. (1999) *BAS1*: A gene regulating brassinosteroid levels and light responsiveness in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **96**, 15316-15323.

Nes, W. D. (1990) Control of sterol biosynthesis and its importance to developmental regulation and evolution. *Rec. Adv. Phytochem.* **24**, 283-327.

Nes, W. D., Janssen, G. G. and Bergenstrahle, A. (1991) Structural requirements for the transformation of substrates by the (S)-adenosyl-L-methionine:delta 24(25)-sterol methyl transferase. *J. Biol. Chem.* **266**, 15202-15212.

Nes, W. R. and McKean, M. L. (1977) *Biochemistry of Steroids and Other Isopentenoids.* Baltimore: University Park Press.

Newman, J. D. and Chappell, J. (1999) Isoprenoid biosynthesis in plants: carbon partitioning within the cytoplasmic pathway. *Crit. Rev. Biochem. Mol. Biol.* **34**, 95-106.

Niehrs, C., Beisswanger, R. and Huttner, W. B. (1994) Protein tyrosine sulfation, 1993 - an update. *Chem. Biol. Interact.* **92**, 257-271.

Niggeweg, R., Thurow, C., Kegler, C. and Gatz, C. (2000) Tobacco transcription factor 2.2 is the main component of ASF-1/SARP and is involved in salicylic acid- and auxin-inducible expression of as-1-containing target promoters. *J. Biol. Chem.* **275**, 19897-19905.

Noguchi, T., Fujioka, S., Takatsuto, S., Sakurai, A., Yoshida, S., Li, J. and Chory, J. (1999a) Arabidopsis *det2* is defective in the conversion of (24*R*)-24-methylcholest-4-en-3-one to (24*R*)-24-methyl-5α-cholestan-3-one in brassinosteroid biosynthesis. *Plant Physiol.* **120**, 833-839.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K. A. and Tax, F. E. (1999b) Brassinosteroid-insensitive dwarf mutants of *Arabidopsis* accumulate brassinosteroids. *Plant Physiol.* **121**, 743-752.

Nomura T., Nakayama, M., Reid, J. B., Takeuchi, Y. and Yokota, T. (1997) Blockage of brassinosteroid synthesis and sensitivity causes dwarfism in garden pea. *Plant Physiol.* **113**, 31-37.

Nomura, T., Kitasaka, Y., Takatsuto, S., Reid, J. B., Fukami, M. and Yokota, T. (1999) Brassinosteroid/sterol synthesis and plant growth as affected by *lka* and *lkb* mutations of pea. *Plant Physiol.* **119**, 1517-1526.

Oh, M.-H., Romanow, W. G, Smith, R. C., Zamski, E., Sasse, J. and Clouse, S. D. (1998) *BRU1* encodes a xyloglucan endo-transglycosylase that is expressed in inner and outer tissues of elongating soybean epicotyls. *Plant Cell Physiol.* **39**, 124-130.

Ohme-Takagi, M. and Shinshi, H. (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* **7**, 173-182.

Olive, M. R., Peacock, W. J. and Dennis, E. S. (1991) The anaerobic responsive element contains two GC-rich sequences essential for binding a nuclear protein and hypoxic activation of the maize *Adh1* promoter. *Nucl. Acids Res.* **19**, 7053-7060.

Park, B. C., Lee, Y. C. and Strott C. A. (1999) Testosterone sulfotransferase: evidence in the guinea pig that this reaction is carried out by 3α-hydroxysteroid sulfotransferase. *Steroids* **64**, 510-517.

Perata, P., Geshi, N., Yamaguchi, J. and Akazawa, T. (1993) Effect of anoxia on the induction of α-amylase in cereal seeds. *Planta* **191**, 402-408.

Peters, T. J. and Preedy, V. R. (1998) Metabolic consequences of alcohol ingestion. *Novartis Found. Symp.* **216**, 19-24.

Phillips, A. L., Ward, D. A., Uknes, S., Appleford, N. E. J., Lange, T., Huttly, A. K., Gaskin, P., Graebe, J. E. and Hedden, P. (1995) Isolation and expression of three gibberellin 20-oxidases cDNA clones from Arabidopsis. *Plant Physiol.* **108**, 1049-1057.

Pieterse, C. M. and van Loon, L. C. (1999) Salicylic acid-independent plant defence pathways. *Trends Plant Sci.* **4**, 52-58.

Potter, S., Moreland, D. E., Kreuz, K. and Ward, E. (1995) Induction of cytochrome P450 genes by ethanol in maize. *Drug Metab. Drug Interact.* **12**, 317-327.

Qian, Y., Deng, C. and Song, W. C. (1998) Expression of estrogen sulfotransferase in MCF-7 cells by cDNA transfection suppresses the estrogen response: potential role of the enzyme in regulating estrogen-dependent growth of breast epithelial cells. *J. Pharmacol. Exp. Ther.* **286**, 555-560.

Rao, S. I. and Duffel, M.W. (1991) Benzylic alcohols as stereospecific substrates and inhibitors for aryl sulfotransferase. *Chirality* **3**, 104-111.

Raskin, I. and Kende, H. (1984a) Regulation of growth in stem sections of deep-water rice. *Planta* **160**, 66-72.

Raskin, I. and Kende, H. (1984b) Role of gibberellin in the growth response of submerged deep water rice. *Plant Physiol.* **76**, 947-950.

Reichhart, D., Salaun, J.-P., Benveniste, I. and Durst, F. (1979) Induction by manganese, ethanol, phenobarbital, and herbicides of microsomal cytochrome *P*-450 in higher plant tissues. *Arch. Biochem. Biophys.* **196**, 301-303.

Ricard, B., Couée, I., Raymond, P., Saglio, P. H., Saint-Ges, V. and Pradet, A. (1994) Plant metabolism during hypoxia and anoxia. *Plant Physiol. Biochem.* **32**, 1-10.

Richberg, M. H., Aviv, D. H. and Dangl, J. L. (1998) Dead cells do tell tales. *Curr. Opin. Plant Biol.* **1**, 480-485.

Richman, A. S., Gijzen, M., Starratt, A. N., Yang, Z. and Brandle, J. E. (1999) Diterpene synthesis in *Stevia rebaudiana*: recruitment and up-regulation of key enzymes from the gibberellin biosynthetic pathway. *Plant J.* **19**, 411-421.

Roberts, J. K. M., Callis, J., Jardetzky, O., Walbot, V. and Freeling, M. (1984a) Cytoplasmic acidosis as a determinant of flooding intolerance in plants. *Proc. Natl. Acad. Sci. USA* **81**, 6029-6033.

Roberts, J. K. M., Callis, J., Wemmer, D., Walbot, V. and Jardetzky, O. (1984b) Mechanism of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. *Proc. Natl. Acad. Sci. USA* **81**, 3379-3383.

Roddick, J. G. and Guan, M. (1991) Brassinosteroids and root development. In *Brassinosteroids: Chemistry, Bioactivity and Applications* (Cutler, H. G., Yokota, T. and Adam, G., eds.). Washington, DC: American Chemical Society, ACS Symposium Series **474**, pp. 231-245.

Roth, J. A. (1986) Sulfoconjugation: role in neurotransmitter and secretory protein activity. *Trends Pharmacol. Sci.* **7**, 404-407.

Roy, A. K. (1992) Regulation of steroid hormone action in target cells by specific hormone-inactivating enzymes. *Proc. Soc. Exp. Biol. Med.* **199**, 265-272.

Runge-Morris, M. A. (1997) Regulation of expression of the rodent cytosolic sulfotransferases. *FASEB J.* **11**, 109-117.

Runge-Morris, M. and Vento, C. (1995) Effects of streptozotocin-induced diabetes on rat liver sulfotransferase expression. *Drug Metab. Dispos.* **23**, 455-459.

Runge-Morris, M., Rose, K., Falany, C. N. and Kocarek, T. A. (1998) Differential regulation of individual sulfotransferase isoforms by phenobarbital in male rat liver. *Drug Metab. Drug Interact.* **26**, 795-801.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.-Y., Johnson, J., Delaney, T. P., Jesse, T., Vos, P. and Uknes, S. (1997) The *Arabidopsis thaliana* NIM1 protein shows homology to the mammalian transcription factor inhibitor IkB. *Plant Cell* **9**, 425-439.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H.-Y. and Hunt, M. D. (1996) Systemic acquired resistance. *Plant Cell* **8**, 1809-1819.

Saab, I. N. and Sachs, M. M. (1996) A flooding-induced xyloglucan endo-transglycosylase homolog in maize is responsive to ethylene and associated with aerenchyma. *Plant. Physiol.* **112**, 385-391.

Sachs, M. M., Freeling, M. and Okimoto, R. (1980) The anaerobic proteins of maize. *Cell* **20**, 761-767.

Sachs, M. M., Subbaiah, C. C. and Saab, I. N. (1996) Anaerobic gene expression and flooding tolerance in maize. *J. Exp. Bot.* **47**, 1-15.

Saden-Krehula, M., Tajic M. and Kolbah, D. (1979) Sex hormones and corticosteroids in pollen of *Pinus nigra* Ar. *Phytochemistry* **18**, 345-346.

Saden-Krehula, M., Kolbah, D., Tajic, M. and Blazevic, N. (1983) 17-Ketosteroids in *Pinus nigra* Ar.: steroid hormones in the pollen of pine species. *Naturwissenschaften* **70**, 250-252.

Sakurai, A. (1999) Biosynthesis. In *Brassinosteroids: Steroidal Plant Hormones* (Sakurai, A., Yokota, T. and Clouse, S. D., eds.). Tokyo: Springer-Verlag, pp. 91-111.

Salter, M. G., Paine, J. A., Riddell, K. V., Jepson, I., Greenland, A. J., Caddick, M. X. and Tomsett, A. B. (1998) Characterisation of the ethanol-inducible *alc* gene expression system for transgenic plants. *Plant J.* **16**, 127-132.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sasse, J. (1999) Physiological actions of brassinosteroids. In *Brassinosteroids: Steroidal Plant Hormones* (Sakurai, A., Yokota, T. and Clouse, S. D., eds.). Tokyo: Springer-Verlag, pp. 137-161.

Sauter, M. and Kende, H. (1992) Gibberellin-induced growth and regulation of the cell division cycle in deepwater rice. *Plant Sci.* **38**, 129-134.

Sauter, M., Seagull, R. W. and Kende, H. (1993) Internodal elongation and orientation of cellulose microfibrils and microtubules in deepwater rice. *Planta* **190**, 354-362.

Scheel, D. (1998) Resistance response physiology and signal transduction. *Curr. Opin. Plant Biol.* **1**, 305-310.

Schmidt, J., Spengler, B., Yokota, T. and Adam, G. (1993) The co-occurence of 24-*epi*-castasterone and castasterone in seeds of *Ornithopus sativus*. *Phytochemistry* **32**, 1614-1615.

Schmidt, J., Altmann, T. and Adam, G. (1997) Brassinosteroids from seeds of *Arabidopsis thaliana*. *Phytochemistry* **45**, 1325-1327.

Schmidt, R. C., MĴller, A., Hain, R., Bartling, D. and Weiler, E. W. (1996) Transgenic tobacco plants expressing the *Arabidopsis thaliana* nitrilase II enzyme. *Plant J.* **9**, 683-691.

Schneider, B., Kolbe, A., Porzel, A. and Adam, G. (1994) A metabolite of 24-*epi*-brassinolide in cell suspension cultures of *Lycopersicon esculentum*. *Phytochemistry* **36**, 319-321.

Schrick, K., Mayer, U., Horrichs, A., Kuhnt, C., Bellini, C., Dangl, J., Schmidt, J. and Jürgens, G. (2000) FACKEL is a sterol C-14 reductase required for organized cell division and expansion in *Arabidopsis* embryogenesis. *Genes Dev.* **14**, 1471-1484.

Schumacher, K. and Chory, J. (2000) Brassinosteroid signal transduction: still casting the actors. *Curr. Opin. Plant Biol.* **3**, 79-84.

Schumacher, K., Vafeados, D., McCarthy, M., Sze, H., Wilkins, T. and Chory, J. (2000) The *Arabidopsis det3* mutant reveals a central role for the vacuolar H^+-ATPase in plant growth and development. *Genes Dev.* **13**, 3259-3270.

Schwartz, D. (1972) An example of gene fixation resulting from selective advantage in suboptimal conditions. *Am. Nat.* **103**, 479-481.

Segel, I. H. (1975) *Enzyme Kinetics*. New York: John Wiley and Sons.

Sembdner, G., Atzorn, R. and Schneider, G. (1994) Plant hormone conjugation. *Plant Mol. Biol.* **26**, 1459-1481.

Shafqat, J., El-Ahmad, M., Danielsson, O., Martínez, M. C., Persson, B., Parés, X. and Jörnvall, H. (1996) Pea formaldehyde-active class III alcohol dehydrogenase: common derivation of the plant and animal forms but not of the corresponding ethanol-active forms (classes I and P). *Proc. Natl. Acad. Sci. USA* **93**, 5595-5599.

Shah, J. and Klessig, D. F. (1999) Salicylic acid: signal perception and transduction. In *Biochemistry and Molecular Biology of Plant Hormones* (Hooykaas, P. J. J., Hall, M. A. and Libbenga, K. R., eds.). Amsterdam: Elsevier, New Comprehensive Biochemistry **33**, pp. 513-541.

Shi, J., Gonzales, R. A. and Bhattacharyya, M. K. (1996) Identification and characterization of an S-adenosyl-L-methionine: Δ24-sterol-*C*-methyltransferase cDNA from soybean. *J. Biol. Chem.* **271**, 9384-9389.

Simons, R.G. and Grinwich, D.L. (1989) Immunoreactive detection of four mammalian steroids in plants. *Can. J. Bot.* **67**, 288-296.

Soeno, K., Kyokawa, Y., Natsume, M. and Abe, H. (2000) Teasterone-3-O-beta-D-glucopyranoside, a new conjugated brassinosteroid metabolite from lily suspension cultures and its identification in lily anthers. *Biosci. Biotechnol. Biochem.* **64**, 702-709.

Song, C.-S., Kim, J. M., Roy, A. K. and Chatterjee, B. (1990) Structure and regulation of the senescence marker protein 2 gene promoter. *Biochemistry* **29**, 542-541.

Song, W. C., Moore, R., McLachlan, J. A. and Negishi, M. (1995) Molecular characterization of a testis-specific estrogen sulfotransferase and aberrant liver expression in diabetogenic C57BL/KsJ-db/db mice. *Endocrinology* **136**, 2477-2484.

Spengler, B., Schmidt, J., Voigt, B. and Adam, G. (1995) 6-Deoxo-28-norcastasterone and 6-deoxo-24-epicastasterone: two new brassinosteroids from *Ornithopus sativus*. *Phytochemistry*, **40**, 907-910.

Strange, C., Ramirez, I., Gomez, I., Jordana, X. and Holuigue, L. (1997) Phosphorylation of nuclear proteins directs binding to salicylic acid-responsive elements. *Plant J.* **11**, 1315-1324.

Strott, C. A. (1996) Steroid sulfotransferases. *Endocr. Rev.* **17**, 670-697.

Sugano, S., Miura, R. and Morishima, N. (1996) Identification of intermediates in the conversion of cholesterol to pregnenolone with a reconstituted cytochrome p-450ssc system: accumulation of the intermediate modulated by the adrenodoxin level. *J. Biochem.* **120**, 780-787.

Sun, T.-p. and Kamiya, Y. (1994) The Arabidopsis *GA1* locus encodes the cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. *Plant Cell* **6**,1509-1518.

Suzuki, H., Kim, S.-K., Takahashi, N. and Yokota, T. (1993) Metabolism of castasterone and brassinolide in mung bean explant. *Phytochemistry* **33**, 1361-1367.

Suzuki, H., Inoue, T., Fujioka, S., Takatsuto, T., Yanagisawa, T., Yokota, T., Murofushi, N. and Sakurai, A. (1994) Possible involvement of 3-dehydroteasterone in the conversion of teasterone to typhasterol in cultured cells of *Catharanthus roseus*. *Biosci. Biotechnol. Biochem.* **58**, 1186-1188.

Suzuki, H., Inoue, T., Fujioka, S., Saito, T., Takatsuto, S., Yokota, T., Murofushi, N., Yanagisawa, T. and Sakurai, A. (1995a) Conversion of 24-methylcholesterol to 6-oxo-24-methylcholestanol, a putative intermediate of the biosynthesis of brassinosteroids, in cultured cells of Catharanthus roseus. *Phytochemistry* **40**, 1391-1397.

Suzuki, H., Fujioka, S., Takatsuto, S., Yokota, T., Murofushi, N. and Sakurai, A. (1995b) Biosynthesis of brassinosteroids in seedlings of *Catharanthus roseus*, *Nicotiana tabacum*, and *Oryza sativa*. *Biosci. Biotechnol. Biochem.* **59**, 168-172.

Szekeres, M., Németh, K., Koncz-Kálmán, Z., Mathur, J., Kauschmann, A., Altmann, T., Redei, G. P., Nagy, F., Schell, J. and Koncz, C. (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. *Cell* **85**, 171-182.

Tagede, M. and Kuhlemeier, C. (1997) Aerobic fermentation during tobacco pollen development. *Plant Mol. Biol.* **35**, 343-354.

Tagede, M., Brändle, R. and Kuhlemeier, C. (1998) Anoxia tolerance in tobacco roots: effects of overexpression of pyruvate decarboxylase. *Plant J.* **14**, 327-335.

Tagede, M., Bucher, M., Stähli, W., Suter, M., Dupuis, I. and Kuhlemeier, C. (1998) Activation of plant defense responses and sugar efflux by expression of pyruvate decarboxylase in potato leaves. *Plant J.* **16**, 661-671.

Tagede, M., Dupuis, I. and Kuhlemeier, C. (1999) Ethanolic fermentation: new functions for an old pathway. *Trends Plant Sci.* 4, 320-325.

Takatsuto, S. (1994) Brassinosteroids: distribution in plants, bioassays and microanalysis by gas chromatography-mass spectrometry. *J. Chromatogr.* 658, 3-15.

Takatsuto, S. and Yokota, T. (1999) Biochemical analysis of natural brassinosteroids. In *Brassinosteroids: Steroidal Plant Hormones* (Sakurai, A., Yokota, T. and Clouse, S. D., eds.). Tokyo: Springer-Verlag, pp. 47-68.

Thomas, S. G., Phillips, A. L. and Hedden, P. (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. *Proc. Natl. Acad. Sci. USA* 96, 4698-4703.

Thomma, B. P. H. J., Eggermont , K., Penninckx, I. A. M. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A. and Broekaert, W. F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in arabidopsis are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. U S A.* 95, 15107-15111.

Thummel, C. S. (1995) From embryogenesis to metamorphosis: the regulation and function of Drosophila nuclear receptor superfamily members. *Cell* 83, 871-877.

Tominaga, R., Sakurai, N. and Kuraishi, S. (1994) Brassinolide-induced elongation of inner tissues of segments of squash (*Cucurbita maxima* Duch.) hypocotyls. *Plant Cell Physiol.* 35, 1103-1106.

Tomizuka, T., Oeda, T., Tamura, Y., Yoshida, S. and Strott, C. A. (1994) Characterization of guinea pig estrogen sulfotransferase expressed by Chinese hamster ovary cell-K1 stable transfectants. *Endocrinology* 135, 938-943.

Ulmasov, T., Ohmiya, A., Hagen, G. and Guilfoyle, T. (1995) The soybean GH2/4 gene that encodes a glutathione S-transferase has a promoter that is activated by a wide range of chemical agents. *Plant Physiol.* 108, 919-927.

Urao, T., Yamaguchi-Shinozaki, K., Urao, S. and Shinozaki, K. (1993) An Arabidopsis MYB homolog is induced by dehydration stress and its gene product binds to the conserved Myb recognition sequence. *Plant Cell* 5, 1529-1539.

Uozu, S., Tanaka-Ueguchi, M., Kitano, H., Hattori, K. and Matsuoka., M. (2000) Characterization of XET-related genes of rice. *Plant Physiol.* 122, 853-859.

Valvekens, D., van Montagu, M. and van Lijsebettens, M. (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. USA* 85, 5536-5540.

van der Heijden, R., Threlfall, D. R., Verpoorte, R. and Whitehead, I. M. (1989) Regulation and enzymology of pentacyclic triterpenoid phytoalexin biosynthesis in cell suspension cultures of *Tabernaemontana divaricata*. *Phytochemistry* **28**, 2981-2988.

Varin, L. (1992) Flavonoid sulfation: phytochemistry, enzymology and molecular biology. *Rec. Adv. Phytochem.* **26**, 233-253.

Varin, L., Barron, D. and Ibrahim, R. K. (1987) Enzymatic assay for flavonoid sulfotransferase. *Anal. Biochem.* **161**, 176-180.

Varin, L., DeLuca, V., Ibrahim, R. K. and Brisson, N. (1992) Molecular characterization of two plant flavonol sulfotransferases. *Proc. Natl. Acad. Sci. USA* **89**, 1286-1290.

Varin, L., Marsolais, F. and Brisson, N. (1995) Chimeric flavonol sulfotransferases define a domain responsible for substrate and position specificities. *J. Biol. Chem.* **270**, 12498-12502.

Varin, L., Marsolais, F., Richard, M. and Rouleau, M. (1997) Biochemistry and molecular biology of plant sulfotransferases. *FASEB J.* **11**, 517-525.

Visser, T. J. (1994) Role of sulfation in thyroid hormone metabolism. *Chem. Biol. Interact.* **92**, 293-303.

Vogeli, U. and Chappell, J. (1988) Induction of sesquiterpene cyclase and suppression of squalene synthase activities in plant cell cultures treated with fungal elicitor. *Plant Physiol.* **88**, 1291-1296.

Voigt, B., Schmidt, J. and Adam, G. (1996) Synthesis of 24-epiteasterone, 24-epityphasterol and B-homo-6a-oxalactones from ergosterol. *Tetrahedron* **52**, 1997-2004.

Voigt, B., Porzel, A., Buhn, C., Wagner, C., Merzweiler, K. and Adam, G. (1997) Synthesis of 24-epicathasterone and related brassinosteroids with modified side chain. *Tetrahedron* **53**, 17039-17054.

Walker, J. C., Howard, E. A., Dennis, E. S. and Peacock, W. J. (1987) DNA sequences required for anaerobic expression of maize *Adh1* gene. *Proc. Natl. Acad. Sci. USA* **84**, 6624-6629.

Wang, Z.-Y. and Chory, J. (2000) Recent advances in molecular genetic studies of the functions of brassinolide, a steroid hormone in plants. *Rec. Adv. Phytochem.* **34**, 409-431.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Métraux, J.-P. and Ryals, J. A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* **3**, 1085-1094.

Waters, I., Armstrong, W., Thompson, C. J., Setter, T. L., Adkins, S., Gibbs, J. and Greenway, H. (1989) Diurnal changes in radial oxygen loss and ethanol metabolism in roots of submerged and non-submerged rice seedlings. *New Phytol.* **113**, 439-451.

Webster, C., Gaut, R. L., Browning, K. S., Ravel, J. M. and Roberts, J. K. M. (1991) Hypoxia enhances phosphorylation of eukaryotic initiation factor 4A in maize root tips. *J. Biol. Chem.* **266**, 23341-23346.

Weinshilboum, R. M., Otterness, D. M., Aksoy, I. A., Wood, T. C., Her, C. and Raftogianis, R. B. (1997) Sulfotransferase molecular biology: cDNAs and genes. *FASEB J.* **11**, 3-14.

Whitnall, M. H., Driscoll, W. J., Lee, Y. C. and Strott, C. A. (1993) Estrogen and hydroxysteroid sulfotransferases in guinea pig adrenal cortex: cellular and subcellular distributions. *Endocrinology* **133**, 2284-2291.

Windsor, M. L. and Zeevart, J. A. D. (1997) Induction of ABA 8'-hydroxylase by (+)-*S*-, (−)-*R*- and 8', 8', 8'-trifluoro-*S*-abscisic acid in suspension cultures of potato and *Arabidopsis*. *Phytochemistry* **45**, 931-934.

Winter, J., Schneider, B., Strack, D. and Adam G. (1997) Role of a cytochrome P450-dependent monooxygenase in the hydroxylation of 24-*epi*-brassinolide. *Phytochemistry* **45**, 233-237.

Xiang, C., Miao, Z. H. and Lam, E. (1996) Coordinated activation of as-1-type elements and a tobacco glutathione S-transferase gene by auxins, salicylic acid, methyl-jasmonate and hydrogen peroxide. *Plant Mol. Biol.* **32**, 415-426.

Xie, Y. and Wu, R. (1989) Rice alcohol dehydrogenase genes: anaerobic induction, organ specific expression and characterization of cDNA clones. *Plant Mol. Biol.* **13**, 53-68.

Xu, W., Campbell, P., Vargheese, A. K. and Braam, J. (1996) The *Arabidopsis XET*-related gene family: environmental and hormonal regulation of expression. *Plant J.* **9**, 879-889.

Xu, Y. L., Li, L., Gage, D. A. and Zeevart, J. A. D. (1999) Feedback regulation of *GA5* expression and metabolic engineering of gibberellin levels in Arabidopsis. *Plant Cell* **11**, 927-935.

Yamamoto, R., Demura, T. and Fukuda, H. (1997) Brassinosteroids induce entry into the final stage of tracheary element differentiation in cultured *Zinnia* cells. *Plant Cell Physiol.* **38**, 980-983.

Yokota, T. (1997) The structure, biosynthesis and function of brassinosteroids. *Trends Plant Sci.* **2**, 137-143.

Yokota, T. (1999) Brassinosteroids. In *Biochemistry and Molecular Biology of Plant Hormones* (Hooykaas, P. J. J., Hall, M. A. and Libbenga, K. R., eds.). Amsterdam: Elsevier, New Comprehensive Biochemistry **33**, pp. 277-293.

Yokota, T., Ogino, Y., Suzuki, H., Takahashi, N., Saimoto, H., Fujioka, S. and Sakurai, A. (1991) Metabolism and biosynthesis of brassinosteroids. In *Brassinosteroids: Chemistry, Bioactivity and Applications* (Cutler, H. C., Yokota, T. and Adam, G., eds.). Washington, DC: American Chemical Society, ACS Symposium Series **474**, pp. 86-96.

Yokota, T., Higuchi, K., Kosaka, Y. and Takahashi, N. (1992) Transport and metabolism of brassinosteroids in rice. In *Progress in Plant Growth Regulation* (Karssen, C. M., van Loon, L. C. and Vreugdenhil, D., eds.). Dordrecht: Kluwer, pp. 298-305.

Yoshida, A., Hsu, L. C. and Yasunami, M. (1991) Genetics of human alcohol-metabolising enzymes. *Prog. Nucleic Acids Res. Mol. Biol.* **40**, 255-287.

Yoshioka, H., Yamada, N. and Doke, N. (1999) cDNA cloning of sesquiterpene cyclase and squalene synthase, and expression of the genes in potato tuber infected with *Phytophthora infestans*. *Plant Cell Physiol.* **40**, 993-998.

Young, I. J., Knights, B. A. and Hillman, J. R. (1977) Estradiol and its biosynthesis in *Phaseolus vulgaris* L. *Nature* **267**, 429.

Young, I. J., Hillman, J. R. and Knights, B. A. (1978) Endogenous estradiol-17β in *Phaseolus vulgaris*. *Z. Pflanzenphysiol.* **95**, 307-316.

Zhang, Y., Fan, W., Kinkema, M., Li, X. and Dong, X. (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. USA* **96**, 6523-6528.

Zhou, J., Tang, X. and Martin, G. B. (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *EMBO J.* **16**, 3207-3218.

Zhou, J. M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J. and Klessig, D. F. (2000) NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Mol. Plant Microbe Interact.* **13**, 191-202.

Zimmerlin, A. and Durst, F. (1992) Aryl hydroxylation of the herbicide diclofop by a wheat cytochrome P-450 monooxygenase. *Plant Physiol.* **100**, 874-881.

Zurek, D. M. and Clouse, S. D. (1994) Molecular cloning and characterization of a brassinosteroid-regulated gene from soybean (*Glycine max* L.) epicotyls. *Plant Physiol.* **104**, 161-170.

ANNEX I

Table 1. Relative activity of recombinant ATST1 with steroid substrates.

Substrate[a]	Relative activity at 5 μM (%)	Relative activity at 200 μM (%)
24-Epicathasterone	100[b]	inh.[c]
6-Deoxo-24-Epicathasterone	41	49
24-Epiteasterone	47	34
22-Deoxy-24-Epiteasterone	12	21
24-Epicastasterone	12	<10
(22*S*, 23*S*)-28-Homobrassinolide	16	100[d]
(22*S*, 23*S*)-28-Homocastasterone	32	84
Pregnenolone	34	48
Dehydroepiandrosterone	16	51
Androstenediol	37	52
Testosterone	18	<10
17α-Estradiol	15	13
17ß-Estradiol	16	11
17ß-Estradiol 3-methyl ether	13	22
Estrone	13	<10

[a] The concentration of PAPS in the assay was equal to 5 μM. [b] Relative activity of 100% was equal to 13 picokatal/mg. [c] inh.: substrate inhibition was observed at this concentration. [d] Relative activity of 100% was equal to 20 picokatal/mg.

Table 2. Kinetic parameters of recombinant ATST1 for steroid substrates.

Substrate	K_m (μM)	V_{max} (picokatal mg^{-1})	V_{max}/K_m (picokatal mg^{-1} μM^{-1})
24-Epicathasterone	6.9	57	8.3
6-Deoxo-24-Epicathasterone	1.9	4.4	2.2
17ß-estradiol	3.0	1.6	0.5
Dehydroepiandrosterone	1.1	1.3	1.2
Pregnenolone	13	5.9	0.5

Table 3. Enzymatic activity of the recombinant human hydroxysteroid sulfotransferase with brassinosteroids.

Substrate[a]	Relative activity (%)
Dehydroepiandrosterone[b]	100[c]
6-Deoxo-24-epicathasterone	54
22-Deoxy-24-Epiteasterone	78
(22*S*, 23*S*)-22, 23-Epoxy-24-Epiteasterone	52
24-Epiteasterone	32

[a] The kinetic parameters determined for the recombinant enzyme were a K_m value for dehydroepiandrosterone of 5.0 µM, a K_m value for PAPS of 2.0 µM, and a V_{max} value of 40 picokatal/mg. [b] Acceptor substrates were assayed at a concentration of 10 µM. PAPS concentration in the assay was 5 µM [c] Relative activity of 100% was equal to 30 picokatal/mg.

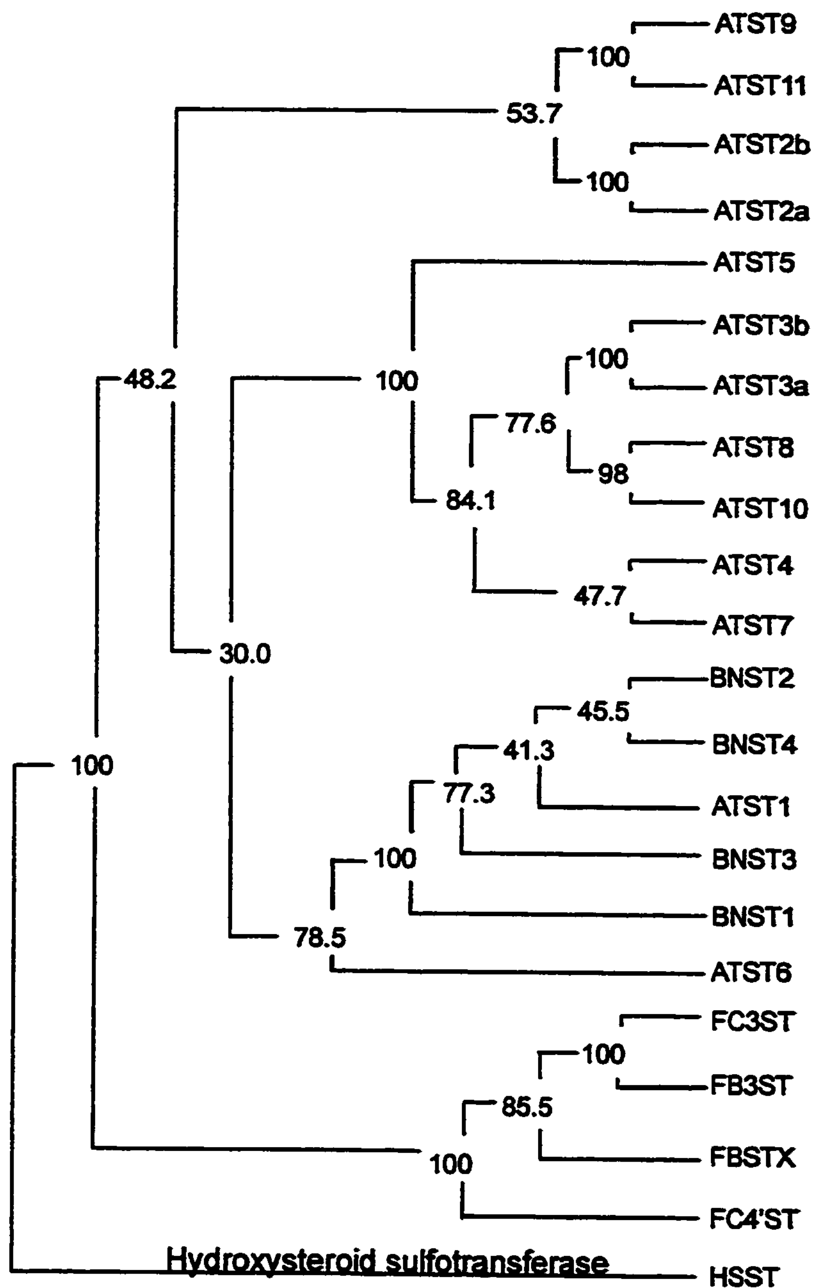


Figure 1. Phylogenetic reconstruction of plant soluble sulfotransferases prepared using the PHYLIP package (Felsenstein, 1993). Numbers indicate bootstrap values of branches. Accession numbers of sulfotransferase genes are: HSST (X84816), FC4'ST (M84136), FBSTX (U10277), FB3ST (U10275), FC3ST (M84135), ATST6 and ATST5 (ACO005836), BNST1 (AF000305), BNST3 (AF000307), ATST1 (Z23001), BNST2 (AF000306), ATST7 (ABO26651), ATST4 (ACO05396), ATST10 (ACO49171), ATST8 (ACO06232), ATST3a and -b (AI138649), ATST2a and -b (ABO10697), ATST9 and -11 (ACO16662).