

## Abstract

KOKA, CHALAPATHI RAO VENKATA. Physiological and Molecular Characterization of a Brassinosteroid-Deficient Tomato Mutant (Under the direction of Dr. Steven D. Clouse.)

Brassinosteroids (BR) are a newly recognized class of phytohormones that are structurally very similar to the steroid hormones found in animals and insects. While a large number of physiological studies pointed to the importance of BRs, the analysis of BR-related mutants in *Arabidopsis* and pea provided convincing genetic evidence for an essential role of these steroids in plant growth and development. BR-mutants are of two types: *BR-deficient* mutants which are rescued by exogenous application of BR and are mainly due to lesions in genes involved in the BR biosynthetic pathway: and *BR-insensitive* mutants which fail to perceive exogenous BRs and are likely due to aberrations in genes involved in a signal-transduction pathway.

We found that a dwarf tomato (*Lycopersicon esculentum* Mill.) mutant, *dumpy* (*dpy*), was BR-deficient and was also rescued by application of all C-23-hydroxylated, 6-deoxo intermediates of brassinolide biosynthesis, as well as castasterone and brassinolide. However, 6-deoxocathasterone and all other upstream pathway precursors failed to rescue the mutant, suggesting that the *dpy* mutation is affected in the conversion of 6-deoxocathasterone to 6-deoxoteasterone, similar to the *Arabidopsis* *constitutive photomorphogenesis and dwarfism* (*cpd*) mutant. Measurements of endogenous levels of BR intermediates were consistent with this hypothesis. However, transformation of *dpy* with *Arabidopsis* *CPD* did not complement the mutation. Western analysis

also showed elevated levels of *CPD* protein in *dpy* leaves compared to wild-type leaves. These observations suggest that there might be another gene product involved in the conversion of 6-deoxocathasterone to 6-deoxoteasterone.

Another approach to understanding the mode of action of BR is to isolate BR-regulated genes and determine the mechanism of gene regulation by BR. We exploited the *dpy* mutant to isolate BR-regulated genes by subtractive hybridization since the expression levels of these genes in the untreated mutant is likely to be low due to BR-deficiency. In our studies we focused on isolation of early responsive genes (within 4 h after BR-treatment) and used poly A<sup>+</sup> RNA from BR-treated or untreated meristem and young expanding leaf tissues for subtraction. We report here the isolation and characterization of two BR-regulated genes *LeBR1*, a novel xyloglucan endotransglycosylase (XET) and *LeBR2*, a novel serine/threonine kinase. Further expression analysis of *LeBR1* demonstrated that the mRNA levels of this gene are high in BR-treated samples at all the time-points tested (2-24 h). The endogenous levels of this gene were also found to be lower in *dpy* when compared to the wildtype. Analysis of *LeBR2* also showed that mRNA levels of this gene are reduced in the *dpy* background when compared to the wildtype plants. Further analysis of endogenous levels of *LeBR2* mRNA in different wild-type tissues showed that both young and mature leaves had high expression levels, but that some *LeBR2* expression was present in all the tissue types examined. Computer (PSORT) analysis of the *LeBR2* sequence suggests that the protein is likely localized to the inner mitochondrial

membrane or transported into this organelle, as it contains a possible intra-mitochondrial signal and a putative transmembrane domain.

**Physiological and Molecular Characterization of a Brassinosteroid-  
Deficient Tomato Mutant**

by

**Chalapathi Rao Venkata Koka**

A dissertation submitted to the Graduate Faculty of  
North Carolina State University in partial fulfillment  
of the requirement for the Degree of  
**Doctorate of Philosophy**

**Horticultural Sciences**

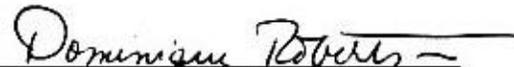
**Raleigh**

**1999**

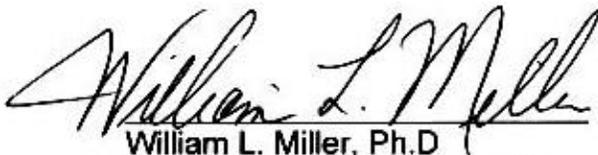
**Approved by:**



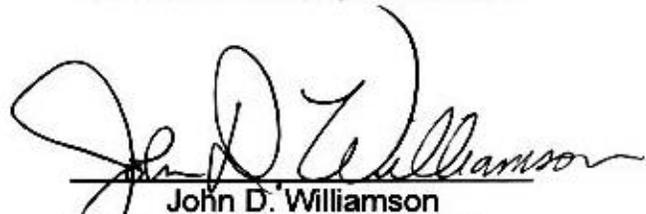
Steven D. Clouse, Ph.D.  
Chair of Advisory Committee



Dominique Robertson, Ph.D.  
Member of Advisory Committee



William L. Miller, Ph.D.  
Member of Advisory Committee



John D. Williamson  
Member of Advisory Committee

## DEDICATION

To my beloved wife, Lucia and  
to my parents, Gangadhara Rao and Uma Rao  
for their love, blessings and support

## BIOGRAPHY

Chalapathi Rao V. Koka was born on March 26, 1969 in Vijayawada, Andhra Pradesh, India. He grew up in Bangalore, Karnataka, India. He received his primary education from St. Anthony's Primary School and secondary education from St. Joseph's India High School. He majored in Sciences (Physics, Chemistry, Mathematics and Biology) and graduated from high school in 1987.

He later pursued an undergraduate degree in Agricultural Sciences at the University of Agricultural Sciences, Bangalore, India. During his undergraduate degree program he majored in Plant Breeding and Genetics and minored in Agricultural Economics. He graduated with a Bachelor of Science degree in 1991. After graduating with the Bachelors degree, he worked as a Research Assistant with the Plant Breeding R&D group within the Seeds Division of Parry's India Limited company.

After a year, in December, 1992, he quit the job to pursue higher education and enrolled in the Master's program in the Department of Biology at Utah State University, Logan, UT. He graduated with a Master of Science degree in Plant Molecular Biology during the summer of 1995. His MS thesis was titled 'Gene Regulation in Maize Silk during Pollen Tube Growth', under the direction of Dr. Elizabeth Hood.

In the fall of 1995 he was admitted to North Carolina State University and pursued a Doctorate of Philosophy degree in Plant Physiology/Molecular Biology in the department of Horticultural Sciences.

## ACKNOWLEDGMENTS

I would like to thank my advisory committee, Drs. Steve Clouse, Niki Robertson, William Miller, and John Williamson for their guidance, assistance, encouragement, criticism and support. I am especially indebted to my advisor Dr. Steve Clouse for his role in developing the research project, in providing the financial support, and for his patience in handling my shortcomings, which have helped me immensely towards developing my research skills.

I greatly appreciate the day to day assistance and support from Mr. Gregory Scott, the technician in the laboratory, without whom the experiments could not have been completed. I offer my sincere gratitude to my laboratory peers, Dr. Rebecca Torisky, Dr. Man-Ho Oh, Ms. Jingrui Jiang, and Mr. Eric Cerny for sharing their opinions, ideas, and experiences. I am especially grateful to Mr. Eric Cerny, for identifying the BR-deficient mutant in tomato, which has been very useful in achieving my research objectives.

I am very thankful to Dr. Shozo Fujioka of The Institute of Physical and Chemical Research (RIKEN) in Saitama, Japan for providing the brassinolide biosynthetic intermediates. I am also thankful to Dr. Csaba Koncz of the MaxPlanck Research Institute in Germany for providing the antibodies for the Arabidopsis CPD protein and the Agrobacterium strain containing the CPD cDNA. I would also like to thank Drs. Jonathan Jones and Gerard Bishop of the John Innes Center in Norwich, UK for supplying the tomato transposable element lines.

I was very grateful for the encouragement and support of my wife, Lucia Villavicencio and my parents, Koka Gangadhara Rao and Koka Uma Rao who have been very patiently waiting for me to be gainfully employed. Finally, I would like to thank all my fellow graduate students for the friendship and making my stay at North Carolina State University very enjoyable.

## TABLE OF CONTENTS

List of Tables.....	ix
List of Figures.....	x
<b>Character One: Literature Review: Brassinosteroids, a New Class of Phytohormone.....</b>	<b>1</b>
Background and History.....	2
Chemical Structure.....	2
Brassinosteroid prevalence in the Plant Kingdom.....	3
Bioassays for Brassinosteroids.....	4
Physiological Effects of Brassinosteroids.....	5
Cell Elongation.....	6
Cell Division.....	7
Development.....	7
Vascular Differentiation.....	8
Flowering.....	9
Stress Responses.....	9
Biosynthetic Pathways.....	10
Brassinosteroid Metabolism.....	12
Brassinosteroid Mutants.....	13
Brassinosteroid-deficient Mutants.....	13
Brassinosteroid-insensitive Mutants.....	19
Brassinosteroid-regulated Genes.....	22
Research Objectives.....	23
References.....	25

<b>Chapter Two: Characterization of ‘<i>dumpy</i>’, a Brassinosteroid-deficient Tomato Mutant.....</b>	<b>36</b>
Introduction.....	37
Materials and Methods.....	40
Feeding Experiments using Brassinosteroid Biosynthesis Intermediates.....	40
Quantitative Analysis of Endogenous Brassinosteroids and Sterols.....	41
Dark-grown Seedling Studies.....	41
Protein Extraction.....	42
Western Blot Analysis.....	43
Transformation of <i>dpy</i> with the Arabidopsis CPD Gene.....	43
Transposon Tagging.....	45
Results.....	46
Phenotype of <i>dpy</i> and its Response to BR.....	46
The <i>dpy</i> Mutant is BR Deficient.....	47
<i>dpy</i> May Not be a Homologue of the Arabidopsis <i>CPD</i> .....	48
Cloning of <i>dpy</i> Mutation Using Transposon Tagging.....	49
Discussion.....	49
Acknowledgments.....	55
Literature Cited.....	55
Tables.....	60
Figures.....	62
<b>Chapter Three: Identification and Characterization of Brassinosteroid-regulated genes.....</b>	<b>77</b>
Introduction.....	78
Materials and Methods.....	80
Subtractive Hybridization.....	80

Differential Display.....	81
RNA Isolation and Analysis.....	81
Probe Preparation.....	82
Northern Analysis.....	82
Hybridization Conditions.....	83
DNA Extraction.....	83
Southern Blot Analysis.....	84
Synthesis of Anti-sense RNA probes.....	85
RNA Protection Assay.....	86
RACE-PCR based cloning of Full-length cDNA.....	87
Results.....	88
Cloning of a BR-regulated XET-like gene from <i>dpy</i> .....	88
Cloning and molecular characterization of a BR-regulated putative kinase from <i>dpy</i> .....	89
<i>dpy</i> has reduced expression levels of <i>LeBR2</i> .....	91
Discussion.....	92
References.....	96
Figures.....	100

## List of Tables

### Chapter II

1. Endogenous levels of sterol precursors and BR biosynthetic intermediates in *dpy* and wild-type tomatoes

## List of Figures

### Chapter II

1. Brassinolide biosynthetic pathway displaying both early and late C6 oxidation.
2. Characterization of light-grown *dumpy* (*dpy*) mutant.
3. Characterization of dark grown *dpy* mutant.
4. Feeding experiments with light-grown *dpy* plants.
5. Feeding experiments with dark-grown *dpy* plants.
6. Western Blot analysis of *dpy* and wild-type tomato leaves.
7. Experimental strategy for the generation of transposon tagged *DPY* gene.

### Chapter III

1. Representative differential display gel.
2. Northern analysis of *LeBR1*.
3. Nucleotide sequence of the partial cDNA of *LeBR1* with derived amino acid sequence.
4. Multiple sequence alignment of *LeBR1* with other XETs.
5. RNA-Protection Assay of *dpy* total RNAs with antisense <sup>32</sup>P-labeled *LeBR2*.RNA probe.
6. RNA-Protection Assay of wild type total RNAs with antisense <sup>32</sup>P-labeled *LeBR2*.RNA probe.
7. Nucleotide sequence of the full-length cDNA of *LeBR2* with derived amino acid sequence.
8. Multiple sequence alignment of *LeBR2* with other serine/threonine kinases.
9. Southern analysis of *LeBR2*..
10. Tissue-specific analysis of endogenous levels of *LeBR2* mRNA using RNA-Protection Assay.

11. A) Hydropathy plot of the translated *LeBR2* polypeptide based on the algorithm of Kyte and Doolittle (1982).

B) Schematic representation of different domains of *LeBR2*

## **Chapter 1**

# **Review of Brassinosteroids, a New Class of Phytohormone**

## 1.1. Background and History

Brassinosteroids (BRs) are a group of naturally occurring plant growth regulators that are wide spread in the plant kingdom. BRs were so named because the first compound discovered in this group was from the pollen of *Brassica napus* L. Prior to the determination of BR structure, they were referred to as *Brassins* (Mitchell *et al.*, 1970). Mitchell and Gregory (1972) demonstrated that brassins could have tremendous influence on yield, photosynthetic efficiency and seed vigor in various crop plants. These observations lead the USDA to undertake the task of purifying and characterizing the active compound. In 1979, the compound was successfully purified and determined to be a steroidal lactone and was termed brassinolide (Mandava, 1988).

The successful extraction and purification of BRs depends on partitioning by solvents and use of chromatographic techniques for further separations. Selection of appropriate fractions is mainly guided by bioassays. Presently, Gas Chromatography-Mass Spectroscopy (GC-MS) is the primary method employed to analyze the purified fractions (Adam *et al.*, 1996; Takasuto, 1994).

## 1.2. Chemical Structure

Brassinolide (BL) the first BR isolated, was crystallized and its structure was determined by X-ray diffraction to be (22R, 23R, 24S)-2 $\alpha$ -3 $\alpha$ , 22, 23-tetrahydroxy-24-methyl-6, 7-s-5 $\alpha$ -cholestano-6, 7-lactone (Grove *et al.*, 1979). All BRs have a steroid nucleus comprised of four rings (A, B, C, and D), with a side chain at C17. Structure/function studies of BR have shown that there are certain

requirements for these compounds to have high biological activity. They are i) Oxygen function at C-6 in the form of a ketone or a lactone. ii) Cis-glycol function at C-2 and C-3 in ring A. iii) Cis-hydroxyls at C-22 and C-23. iv) trans-A/B ring junction. v) Substitution at C-24 (reviewed in Mandava, 1988).

Subsequently, a second BL-related compound called castasterone (CS) was isolated and its structure was determined by spectroscopic methods to be (22R, 23R, 24S)-2 $\alpha$ -3 $\alpha$ , 22,23-tetrahydroxy-24-methyl-5 $\alpha$ -cholestano-6-one (Yokota et al., 1982). To date >40 BRs have been isolated and their structures determined (Yokota, 1997).

### 1.3. Brassinosteroid prevalence in the Plant Kingdom

BL is present at a concentration of approximately 0.1  $\mu\text{g}/\text{kg}$  in *Brassica napus* pollen, the first species evaluated (Mandava, 1988). Since then a number of other species have been assayed for the presence of BRs. These studies have revealed that BRs are widely distributed in the plant kingdom. They are present in all plant species examined, in both higher and lower plants (Takatsuto, 1991). Based on these observations BRs are considered to be ubiquitous throughout the plant kingdom.

Among the tissue types in the plant, pollen is the richest source of BRs. It is present in lower amounts in shoots and leaves. Another rich source of BRs is the crown gall cell of *Catharanthus roseus* (Park et al., 1989). This system has been exploited to determine the biosynthetic pathway of BRs in the cell. Also,

immature tissues are likely to have higher concentration of BRs compared to mature tissues.

#### **1.4. Bioassays for Brassinosteroids**

During the isolation and purification of the active principle in the 'Brassin activity', a highly sensitive and specific bioassay was absolutely essential to keep track of the fraction containing the active component. Studies of structure–activity relationships also required such bioassays. There are three main bioassays for BRs:

(1) Bean Second-Internode Bioassay: The internodes of plants grown at 25-27°C in 12h day-length are treated with lanolin paste with or without the test compound. The increase in internode length is measured after four days. If BL is present, there is not only elongation but also swelling, curvature, and splitting of the internodes, depending on the concentration of BL. Gibberellic Acids also influence internode elongation, but do not result in swelling, curvature and splitting of the second internode (Thompson *et al.*, 1981).

(2) Rice Lamina Inclination Test: This was originally developed as an auxin bioassay (Wada *et al.*, 1981), but was found to be highly sensitive and specific for BR bioassays. For this assay, seven-day old etiolated rice seedlings of cultivars Arborio J-1 and Nihonbare are used. The leaf segments consisting of the second leaf lamina, the lamina joint and the leaf sheath are excised and floated on distilled water for 24h. Uniformly bent segments are selected and incubated in 2.5-mM aqueous dipotassium maleate solution containing BR, for

48h at 29°C, in the dark. The magnitude of the angle between the leaf and sheath is measured (Takatsuto, 1991). This test is linear between  $5 \times 10^{-3}$  and  $5 \times 10^{-5}$  µg/ml BL and CS. In the case of Indole-acetic acid (IAA), results are weaker in magnitude by five times.

(3) Wheat Leaf Unrolling Test: This test was developed by Wada *et al.* (1985). Leaf segments 1.5-3.0 cm from the tip of six-day-old etiolated wheat seedlings (cv. Norin No. 61) grown at 26°C are excised under a green safe light and incubated in 2.5 mM dipotassium maleate solution containing the test compound for 24h at 30°C in the dark. The unrolling of the leaf segment is determined by measuring their width with calipers. This test has one-tenth the sensitivity of the rice lamina-inclination test.

## 1.5. Physiological Effects of Brassinosteroids

Based on the pleiotropic phenotypic and physiological effects observed in BR mutants (discussed later), it is quite clear the BRs play a role in promoting cell expansion, leaf senescence, flowering and, in repressing light- and stress-regulated gene expression.

Initially, BRs were thought to act synergistically with auxins. Subsequent evaluation has shown that BRs can have an independent mode of action, although there could be cross talk between different plant growth regulators (reviewed in Mandava, 1988).

### 1.5.1. Cell Elongation

The primary effect of BRs is promoting cell growth (elongation). This response is predominantly in young tissue, particularly in the meristem. In fact, BRs have little or no effect in mature tissue. Use of an ATPase inhibitor, dicyclohexyl carbodiimide, has demonstrated that elongation induced by BR involves ATPases (Katsumi, 1985). Since other plant growth regulators, such as auxins and GAs also promote cell elongation, the regulation of some auxin-regulated genes (GH1, SAUR and JCW) was studied for response to BR (Clouse *et al.*, 1992). This experiment showed that, although GH1 and SAUR genes are up regulated by BR, the kinetics of regulation is very different when compared to their regulation by auxin. This correlates with the different kinetics of elongation mediated by auxins versus BRs. In general, BR-related responses are more delayed as opposed to auxin-related responses that seem to be fairly rapid (Clouse and Sasse, 1998). This was the first molecular evidence suggesting that BRs are likely to have an independent mode of action. Oh and Clouse (1998), using *Petunia hybrida* mesophyll protoplasts, showed that BR promoted the frequency of cell divisions and also accelerated the time of first cell division when the protoplasts were grown under sub-optimal levels of auxin and cytokinin. Under optimal conditions BR only accelerated the time of first cell division, but had no effect on the final cell division frequencies.

However, synergistic interactions between BR and other hormones have been observed (reviewed in Mandava, 1988), and so it is very likely that there are physiological effects that are due to these interactions. One such interaction

is enhancement of elongation of azuki bean epicotyl segments that were induced by indole acetic acid (IAA) (Mayumi and Shibaoka, 1995). This BR-requiring step is affected by inhibitors of microtubule filament formation and by inhibitors of cellulose biosynthesis. Hence, the authors speculate that BRs enhance only the longitudinal elongation and suppress the lateral elongation by organizing the cortical microtubules along the cell axis, thereby depositing cellulose microfibrils in the same orientation (Mayumi and Shibaoka, 1995). This synergism between BR and IAA was also observed in dwarf pea and in bean hypocotyl hooks (Yopp *et al.*, 1981), bean epicotyl (Cohen and Meudt, 1983), and in cucumber hypocotyl (Katsumi, 1985).

### **1.5.2. Cell Division**

From histological studies of the BR mutant, *cbb*, it was concluded that the phenotype was mainly due to lack of cell elongation or expansion and that there were adequate numbers of cells, suggesting that cell division was not inhibited (Kauschmann *et al.*, 1996). The fact that there are reports of BR having inhibitory effects on cell division (reviewed in Sasse, 1997) also supported this idea. However, there are reports where BR has promotive effects on cell division and differentiation. In protoplasts of Chinese cabbage, 24-epibrassinolide increased cell division rates. Also, dedifferentiation of protoplast and regeneration of the cell wall were enhanced (Nakajima *et al.*, 1996)

### **1.5.3. Development**

Pleiotropic effects of BR mutants suggest that BRs are involved in growth and development. Asakawa *et al.* (1996) have reported that there are increases

in the levels of free BRs during pollen maturation. This, along with male sterility of BR mutants, suggests that a sufficient level of BR in pollen is very important for fertilization in plants. Spraying of BL on the stigma has also been shown to induce formation of haploid seeds (Kitani, 1994).

BRs may also be involved in regulation of lateral root formation since the use of uniconazole, a BR biosynthesis inhibitor, resulted in many stunted lateral roots in *Lotus japonicus*, and the numbers were restored to normal levels by exogenous BL, but not GA (Kawaguchi *et al.*, 1996). Consistent with the observation that BR mutants have delayed senescence, He *et al.*, (1996) demonstrated that 24-epiBL accelerated senescence in vitro. They also observed that there was increased activity of peroxidase and higher levels of malondialdehyde, and decreased activity of superoxide dismutase. This suggests BR-effects are mediated through 'activated oxygen'.

#### **1.5.4. Vascular Differentiation**

As early as 1991, it was demonstrated that BR increased tracheary element differentiation in *Zinnia elegans* mesophyll cells (Iwasaki and Shibaoka, 1991) and in *Helianthus tuberosus* explants (Clouse and Zurek, 1991). Using the *Zinnia* system, in which single mesophyll cells can differentiate directly into tracheary elements, Iwasaki and Shibaoka (1991) also showed that external application of the BR biosynthesis inhibitor, uniconazole, prevented transdifferentiation from taking place. However, this did not inhibit cell division. This inhibition could be released by external application of BR. This system is

being further used to elucidate the role of BR in the formation of tracheary elements (Yamamoto *et al.*, 1997).

### **1.5.5. Flowering**

Very little is known about the role of BR in flowering. The growth of pollen tubes *in vitro* has been found to be enhanced by 24-epiBL (Hewitt *et al.*, 1985). Application of BR to the staminate inflorescence of *Luffa cylindrica* induced changes in the treated flower from: staminate to bisexual, bisexual to pistillate, and pistillate to vegetative shoot (Suge, 1986). The author also found that BR promoted flowering only in radish plants from non-vernalized seeds and not from vernalized seeds. GA<sub>3</sub>, on the other hand, induced flowering in plants from both vernalized and non-vernalized seeds. Exogenous BR application also induced parthenogenic seed formation in *Arabidopsis* and *Brassica juncea* (Kitani, 1994). In the case of *cpd*, it was suggested that sterility is due to lack of elongation of the pollen tube and fertility can be restored by exogenous BR (Szekeres *et al.*, 1996).

These observations, along with the fact that BR mutants exhibit male sterility, indicate that BRs have a role to play during fertilization. The actual role that BRs play needs to be further evaluated.

### **1.5.6. Stress Responses**

There are a number of papers that show that BRs enable plants in the field to tolerate stresses better, resulting in higher yields (Meudt *et al.*, 1983; Hamada, 1986). Protection from cold stress in the form of chlorophyll

biosynthesis and membrane stability has been reported in rice, maize, and cucumber (He *et al.*, 1991; Katsumi, 1991).

Wilén *et al.* (1995) have looked into possible regulation of the heat-shock protein (hsp90) gene in bromegrass cell cultures by BR and abscisic acid, and have reported that freezing tolerance was enhanced by BR only, but heat tolerance was enhanced by both BR and Abscisic acid. However, the regulatory effects of these two hormones on hsp90 transcript levels was very different.

Most of the observations based on field tests done, mainly in developing countries, are not repeatable in developed countries. The current understanding is that BR's beneficial effects to mediate stress by plants is more likely to occur when plants are grown under sub-optimal conditions rather than under optimal conditions (reviewed in Sasse, 1999).

## **1.6. Biosynthetic Pathways**

Mandava (1988) suggested that BRs might be derived from plant sterols. The research groups led by T. Yokota, S. Fujioka and A. Sakurai were the first to provide experimental evidence for the biosynthetic pathway. They have been successful in delineating the major steps of the BR biosynthetic pathways that are prevalent in plants (Abe *et al.*, 1994; Choi, Y -H. *et al.*, 1993, 1996, 1997, Fujioka and Sakurai, 1995, 1997a, b; Sakurai and Fujioka, 1993, 1997a, b; Suzuki *et al.*, 1993a, 1994a, b, 1995a, b; Yokota, 1997; Yokota *et al.*, 1990a). These advances in understanding the biosynthesis and metabolism of BRs have been achieved mainly by employing cell culture systems of *Catharanthus roseus*

and other plant species. Cell culture systems have a two-fold advantage. First, C<sub>28</sub>-BR levels, like brassinolide, castasterone, etc., were found to be comparable to levels in natural brassinosteroid-rich tissues such as pollen (Choi *et al.*, 1993, Fujioka *et al.*, 1995a, Park *et al.*, 1989, Suzuki *et al.*, 1994a). Second, the cell cultures have a very effective means of assimilating the substrates as the need for translocation is eliminated.

In animals and insects, cholesterol is the precursor for biosynthesis of steroids. Campesterol (CS) and its analogues are believed to be the precursors in plants. Feeding experiments with labeled CS have confirmed this fact (reviewed by Fujioka and Sakurai, 1997). These experiments resulted in identification of campestanol (CN), 6 $\alpha$ -hydroxycampestanol, and 6-oxo-campestanol (6-oxoCN) as intermediates downstream of CS (Suzuki *et al.*, 1995b).

6-oxoCN can enter either of two alternative pathways: the Early C6-oxidation pathway, in which oxidation at C6 occurs before the introduction of vicinal hydroxyls at C22 and C23, or the Late C6-oxidation pathway, in which C6 is oxidized after the introduction of the hydroxyls at the side chain and C2 of the A ring (Choi *et al.*, 1996, 1997). Using feeding experiments with labeled substrates, the sequence of biosynthesis was demonstrated. The sequence is from 6-oxo-campestanol to cathasterone, to teasterone, to 3-dehydroteasterone, to typhasterol, to castasterone, and eventually to brassinolide (reviewed in Fujioka and Sakurai, 1997). Initially, it was thought that there was only one BR biosynthetic pathway. However, evidence of naturally occurring 6-deoxo-BRs

(Abe *et al.*, 1995a, b, Giffiths *et al.*, 1995, Schmidt *et al.*, 1995, Spengler *et al.*, 1995, Yokota *et al.*, 1994) in many plant species, created a need to reevaluate the pathway. This resulted in the discovery of 6-deoxocastasterone (6-deoxoCT), 6-deoxotyphasterol (6-deoxoTY) and 6-deoxoteasterone (6-deoxoTE) (Choi *et al.*, 1996, 1997). Feeding experiments using labeled substrates have shown that 6-deoxoteasterone is converted to 6-deoxoTY, as was 3-dehydro-6-deoxoTE (Choi *et al.*, 1996). 6-deoxocastasterone (6-deoxoCS) is converted to castasterone (CS) and brassinolide (BL) (Choi *et al.*, 1996).

## 1.7. Brassinosteroid Metabolism

Like many other hormones, BRs also have been isolated in conjugated forms. In mung bean, BL is converted to a glycoside, 23-O- $\beta$ -D-glycopyranosyloxy-brassinolide. As there was high activity in the rice lamina inclination test, this compound is considered to be a storage form of BR (Suzuki *et al.*, 1993b). Isolation of similar glycosides from seeds of *Phaseolus vulgaris* also supports this idea (Kim, S. -K., 1991). It was demonstrated that epibrassinolide is metabolized to tetrahydroxy lactone and other compounds that are supposed to be conjugated with fatty acids and/or sugars (Nishikawa *et al.*, 1995).

Hydroxylation at C-25 and C-26 positions have been shown to be deactivation steps (Kauschmann *et al.*, 1997). These reactions require two different enzymes (Winter *et al.*, 1996, 1997). Hydroxylation at C-25 is thought to involve cytochrome P450, as the P450 inhibitors, clotrimazole and ketoconazole,

block this step. The cytochrome P450 involved is very specific, accepting only BL and epibrassinolide as substrates. Metabolites containing disaccharides were also found (Kolbe *et al.*, 1997). Two principal pathways of brassinosteroid metabolism have been proposed (Adam *et al.*, 1996), both of which are likely to be inactivation processes: (i) oxidation of the side chain followed by glucosylation (ii) degradation of the side chain by hydroxylation followed by cleavage. This is likely to be followed by epimerization at C-3 and sometimes conjugation with fatty acids or glucose.

## 1.8. Brassinosteroid Mutants

Much of the biosynthetic pathway, at the molecular level, is being confirmed by the study of brassinosteroid mutants. There are two primary types of mutants. Mutations in the genes resulting in the loss of biosynthesis of BRs are termed *deficient* mutants, and they can be rescued by external application of BR. In contrast, mutations in genes involved in signal transduction that result in the loss of perception of BR are called *insensitive* mutants, and these cannot be rescued by external application of BRs.

### 1.8.1 Brassinosteroid-deficient Mutants

To date several mutants, such as *deetiolated2* (*det2*), *constitutive photomorphogenesis and dwarfism* (*cpd*) and *dwarf4* (*dwf4*) have been shown to be defective in the brassinosteroid biosynthetic pathway (Li *et al.*, 1996, 1997; Szekeres *et al.*, 1996; Choe *et al.*, 1998). These mutants have characteristic features: In the light, they are dwarfs with very dark-green and curled leaves,

short stems, delayed senescence and flowering, and reduced male fertility. In the dark, they have a de-etiolated phenotype: short hypocotyls and expanded cotyledons.

*det2*, an arabidopsis mutant, was initially identified as a photomorphogenic mutant that grows in the darkness as if in the light (Chory *et al.*, 1991). *DET2* has been cloned and the encoded protein has homology to mammalian steroid 5 $\alpha$ -reductases. The DET2 protein has 38-40% identity to human steroid 5 $\alpha$ -reductase at the amino acid level with 80% identity when comparing the conserved amino acid residues (Li *et al.*, 1996). The human homologue of DET2 encodes an enzyme that catalyses reduction of 3-oxo,  $\Delta$  4,5 steroids. Since a similar reaction is required to convert campesterol to campestanol, the authors investigated the possibility of the *det2* mutation disrupting the biosynthetic pathway to BRs, the only known steroids in plants. It was demonstrated that recombinant DET2 protein expressed in human cells could perform the 5 $\alpha$ -reduction of testosterone to dihydrotestosterone. Furthermore, *det2* is specifically rescued by external application of BR (Li *et al.*, 1996); *det2* can also be rescued by complementing with the human steroid 5 $\alpha$ -reductase gene (Li *et al.*, 1997). Based on the fact that DET2 acts only on 3-oxo,  $\Delta$  4,5 steroids, it is proposed that there must be another enzyme that converts campesterol, which is a 3 $\beta$ -hydroxy,  $\Delta$  4,5 steroid to its 3-oxo,  $\Delta$  4,5 isomer.

GC-MS analysis of *det2* showed that there was no accumulation of campesterol when compared to the wildtype. Also campesterol has 3 $\beta$ -hydroxyl,

5,6 structure and needs to be converted to a 3-oxo,  $\Delta$  4,5 structure, the substrate for 5 $\alpha$ -reductase. This suggested that there are one or more intermediate compounds between campesterol and campestanol. Using GC-MS, the intermediates have been identified as (24R)-24-methylcholest-4-en-3-one and (24R)-24-methyl-5 $\alpha$ -cholestan-3-one (Noguchi *et al.*, 1999). The *dwf6* Arabidopsis mutant, isolated by T-DNA tagging, is allelic to *det2* (Azpiroz *et al.*, 1998). A dwarf mutant of pea (*lk*) is also thought to perform the same 5 $\alpha$  reduction as DET2, since it has reduced levels of castasterone, 6-deoxocastasterone, and campestanol (Yokota *et al.*, 1997). The *dwarf1* (*dwf1*) Arabidopsis mutant was first described as a T-DNA tagged mutation (Feldmann *et al.*, 1989) and the *DWF1* gene was cloned. Since then Takahashi *et al.*, (1995) isolated a second T-DNA tagged allele, *dimunito* (*dim1*), and sequence analysis indicated that it contained a putative nuclear localization signal, and so might not be involved in any biosynthetic pathway of plant hormones. However, more recently, further characterization of DWF1 was reported. Analysis of the amino acid sequence using PSORT software (Nakai and Kanehisa, 1992) suggested that it is likely to be localized to the endomembrane system with one transmembrane domain (Choe *et al.*, 1999a). This result correlates with the fact that most of the steroid biosynthetic enzymes need to be localized to membranes for proper functioning. The severity of different *dwf1* alleles is directly correlated to the location of the mutation in relation to the FAD-binding domain. Mutations before that domain were more severe than mutations after the domain. Also the

residues in the FAD-binding domain were highly conserved, suggesting that it is very important for function (Choe *et al.*, 1999b).

Kauschmann *et al.* (1996) later isolated another allele, *cbb1* (*dwf1-6*) and showed that the mutant phenotype was rescued by feeding BRs. Feeding experiments with BR intermediates indicated that *dwf1* is blocked before campesterol. Using deuterium labeled 24-methylenecholesterol and 24-methylidestosterone, Klahre *et al.* (1997) showed that both these compounds accumulated and concluded that reduction to campesterol is the step that is blocked in the *dwf1* mutant, and that in fact *DWF1* encodes a biosynthetic enzyme. *lkb*, a garden pea BR-deficient mutant also accumulates high levels of 24-methylenecholesterol. Nomura *et al.* (1999) have demonstrated that the dwarfism of *lkb* is due to BR deficiency caused by blocked synthesis of campesterol from 24-methylenecholesterol. This suggests that the *LKB* gene is a homolog of *DWF1* (Nomura *et al.*, 1997, Nomura *et al.*, 1999).

The *dwf4* mutant of *Arabidopsis* is a typical BR mutant with short hypocotyls, open cotyledons and no apical hook in the dark. The phenotype seems to be mainly due to decreased or blocked cell elongation. The *Dwarf4* gene has been cloned and the sequence encodes a cytochrome P-450 (Choe *et al.*, 1998). It has 43% identity to CPD (Szekeres *et al.*, 1996), and belongs to the CYP90 family of P-450s and can probably hydroxylate various substrates through its monooxygenase activity (reviewed in West, 1980). Only compounds containing 22 $\alpha$ -hydroxyls rescue the *dwf4* mutant during feeding experiments. This confirms that *DWF4* is a 22 $\alpha$ -hydroxylase. The 22 $\alpha$ -hydroxylation was

suggested to be the rate-limiting step for biosynthesis of BR (Fujioka *et al.*, 1995b). Based on the endogenous level of intermediates and their corresponding biological activity, Choe *et al.* (1998) also proposed that the step catalyzed by DWF4 is the rate-limiting step in synthesis of BR.

Another *Arabidopsis* mutant, *constitutive photomorphogenesis and dwarfism (cpd)*, displaying skotomorphogenesis similar to *det2* (Chory *et al.*, 1991), was not rescued by cathasterone but was by teasterone (Szekeres *et al.*, 1996). Like other BR mutants in the dark, *cpd* also develops short hypocotyls, no apical hook, open cotyledons, and has extended leaf primordia. Szekeres *et al.* (1996) have also demonstrated that some of the light regulated gene expression patterns were altered, when compared to the wildtype, both in light and dark growth conditions. Also, stress-related gene expression patterns were altered. *CPD* has been cloned and the sequence encodes a protein belonging to a new class (CYP90) of the cytochrome P-450 superfamily (Szekeres *et al.*, 1996). Employing BR feeding studies, they showed that *CPD* was likely to encode a 23 $\alpha$ -hydroxylase since the *cpd* mutation was rescued to wildtype only by BR intermediates hydroxylated at the C23 position. This has been demonstrated to be the case with both early and late-C6 oxidation pathway intermediates. Other alleles of *cpd* in *Arabidopsis* are *cabbage3 (cbb3)*, and *dwarf3 (dwf3)* (Kauschmann *et al.*, 1996; Choe *et al.*, 1998).

Another *Arabidopsis* mutant, *dwarf7 (dwf7)*, originally identified in a T-DNA tagged screen, has also recently been characterized (Choe *et al.*, 1999). This is a loss-of-function allele of *sterol1 (ste1)* identified as a mutant that has 30% of

the levels of C-5-desaturated sterols compared to the wildtype (Gachotte *et al.*, 1995). *ste1*, however, did not have any phenotype and so it was proposed that 30% levels of the sterols were sufficient for normal growth. *dwf7* and its alleles have a less severe phenotype when compared to the other BR mutants. Rescue of the phenotype by external application of BR suggested that this mutation is involved in BR biosynthesis. GC-MS analysis of the mutant showed that it was very low in sterols such as 24-methylenecholesterol (24-MC), CR and CN. Using <sup>13</sup>C-labeled mevalonic acid (MVA) and compactin, a MVA inhibitor, along with GC-MS, it was determined that there was accumulation of <sup>13</sup>C<sub>5</sub>-dehydroepisterol in *dwf7* (Choe *et al.*, 1999).

The *dwf7* alleles (*dwf7-1* and *dwf7-2*) have been cloned, confirming that they are null alleles of *STE1*, which encodes a 7 sterol C-5 desaturase (Gachotte *et al.*, 1996). Both contain a base change from guanine to an adenine, converting tryptophan to a stop codon (Choe *et al.*, 1999).

The tomato *dwarf* (*d*) mutation has been used to create dwarf tomato varieties (Graham, 1959). Genetics studies have uncovered several alleles of *d* (Rick, 1954) that are classified as strong, intermediate or weak, with the strong alleles contributing towards the dwarf phenotype. Homozygous *extreme dwarf* (*d<sup>x</sup>*) plants have reduced cell size and number (Nadzhimov *et al.*, 1988). *d<sup>x</sup>* mutants responded to GA application by increase in plant height alone. Except for that, there is no other phenotypic change in *d<sup>x</sup>* and hence it was argued that *d<sup>x</sup>* is not a GA-deficient mutant.

The *Dwarf* gene has been clone by transposon tagging (Bishop, *et al.*, 1996). The sequence shows that it encodes a cytochrome P-450 that acts as a terminal oxidases in the electron transport chain, belonging to the CYP90 superfamily of P-450s. Feeding experiments using  $d^x$  in different experiments showed that the DWARF protein is responsible for the conversion of 6-deoxoCS to CS (Bishop *et al.*, 1999).

Characterization of these different mutants from Arabidopsis, tomato and pea, has provided very strong evidence for the important role of BRs during plant growth and development.

### **1.8.2. Brassinosteroid-insensitive Mutants**

The importance of steroid hormones for growth and development of animals has been well documented (Evans, 1988). In animals, the model for steroid hormone action involves an intracellular steroid receptor, which behaves as a ligand-dependent transcription factor. These receptors usually have a ligand-binding domain, a DNA-binding domain, a nuclear-localization signal, and transcriptional-activation functions. Upon binding to the ligand, they regulate gene expression (Beato *et al.*, 1995). There are also several steroid-receptors that are membrane localized in animals, but not much is known about their mode of action (reviewed in McEwen, 1991).

In plants, there are a number of insensitive mutants that fail to respond to different growth hormones. These mutations are likely due to aberrations in a signal transduction pathway. Characterization of these mutations is very

important for delineating the signal transduction pathway, as has been demonstrated in case of ethylene (Ecker, 1995) and abscisic acid (Finkelstein and Zeevart, 1994). *ETR1*, an ethylene-insensitive locus, encodes an ethylene receptor, belonging to a class of regulatory proteins known as sensor histidine protein kinases (Chang *et al.*, 1993; Schaller and Bleecker, 1995). This approach has also been useful to further the understanding of BR signal transduction. Since BRs have an inhibitory effect for root elongation *brassinosteroid-insensitive 1 (bri1)* mutant, of *Arabidopsis*, was first identified by screening for plants whose roots elongate normally in presence of BR (Clouse *et al.*, 1993). *bri1* is an extreme dwarf, exhibits de-etiolation, is male sterile and has very delayed senescence suggesting that the *BRI1* protein plays a very important role in BR signal transduction (Clouse *et al.*, 1996). Other alleles of *bri1* have been isolated: *cbb2 (bri1-2)* (Kauschmann *et al.*, 1996), *dwf2-1/5 (bri1-3/7)* (Feldmann and Azpiroz, 1994), and *bin1-1/18 (bri1-101/118)* (Li and Chory, 1997).

Based on the understanding of steroid signal transduction in animals and the structural similarities of plant and animal steroid compounds, it was proposed that plants are likely to possess members of the steroid-receptor superfamily (Clouse, 1996; Clouse *et al.*, 1996). However, when the *BRI1* gene was cloned, the sequence showed strong homology to leucine-rich repeat (LRR) receptor kinases, which are localized to cell membrane to transduce extracellular signals into the cells (Li and Chory, 1997). This gene has an extracellular domain, a trans-membrane domain, an intracellular kinase domain and a signal peptide that targets the protein to the cell membrane. Of the five alleles sequenced, 4 of them

had mutations in the kinase domain, and one had a mutation in the putative extracellular ligand-binding domain (Li and Chory, 1997).

LRRs are seen in many different proteins, and are thought to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994). *BRI1* has a unique 70-amino acid island within the LRR. This is required for function as mutation in this region contributes towards the *bri1* phenotype (Li and Chory, 1997). The kinase domain contains all of the 11 conserved subdomains that are characteristic of almost all eukaryotic protein kinases. It also has conserved domains that are indicative of it being a serine/threonine kinase, as opposed to a tyrosine kinase (Hanks and Quinn, 1991).

*BRI1* has high identity scores with other LRR receptor kinase in plants, such as *ERECTA* (Torii *et al.*, 1996) and *CLAVATA1 (CLV1)* (Clark *et al.*, 1997). Besides, *ERECTA* and *CLV1* genes, *BRI1* is closely related to *SERK*, expressed during somatic-embryogenesis in carrot cells (Schmidt *et al.*, 1997), *Xa21*, a disease-resistance gene from rice (Song *et al.*, 1995), and *RLK5* (Walker, 1993) and *TMK1* (Chang *et al.*, 1992), LRR-receptor kinases with unknown function.

The pleiotropic effects of all the BR mutants that were discussed here suggest that BRs are involved in several processes throughout plant growth and development, including the promotion of cell elongation, light- and stress-regulated gene expression, leaf and chloroplast senescence, and fertility. In other words, BR is absolutely required for normal plant growth and development.

## 1.9. Brassinosteroid Regulated Genes

The other aspect of BRs that researchers are concentrating on is the elucidation of the mode of action for these steroids in plants. Much is known about the mode of action of steroids in animals.

The first BR-regulated gene, *BRU1* (BR Upregulated-1), was isolated from soybean epicotyl (Zurek and Clouse, 1994). It encodes a xyloglycan endotransglycosylase (XET), having highest homology to *meri-5* from *Arabidopsis thaliana* (Medford *et al.*, 1991) and an XET from nasturtium seeds (de Silva *et al.*, 1993). Sequence analysis suggests that there is a signal peptide targeting the protein for secretion from the cell, similar to all known XETs (de Silva *et al.*, 1993). It is suggested that expansins are responsible for loosening the cell walls to make room for cell expansion and that XETs are involved in synthesis of new xyloglucans for reattaching them to other xyloglucans, to reinforce cell wall rigidity (Cosgrove, 1997). Zurek and Clouse (1994) have also demonstrated that BR regulation of *BRU1* gene is post-transcriptional.

*TCH4*, one of the genes that is up-regulated in response to touch in *Arabidopsis*, was shown to also be a BR-regulated XET (Xu *et al.*, 1995). Besides touch and BR, this gene also responds to dark, heat shock, cold stress, and auxin. Even in this case, the kinetics of response to auxin and BR are different. Response to auxin is very rapid as opposed to response to BR. In contrast to *BRU1*, the *TCH4* gene is transcriptionally regulated by BR.

Mathur *et al.* (1998), have demonstrated that BRs negatively affect *CPD* gene expression. This is suggestive of the presence of feedback regulation that controls the biosynthesis at this step in the pathway. In this case, it was shown that *de novo* protein synthesis was required for repression to occur, indicating that it is a secondary effect of BR. Other intermediates like TE and CS also inhibited the expression of the *CPD* gene, but it is not clear if they need to be converted to BL before inhibition can occur.

### **1.10. Research Objectives**

Our laboratory is mainly interested in elucidating the role and mode of action of BR in plants. The importance of BR has been recently confirmed and, since this is the only group of compounds that are similar to animal steroids, there is lot of interest in them. When we started this research project the importance of BR in plant growth and development was just beginning to be elucidated.

The objectives of my doctoral research project were two fold. Firstly, to further characterize the tomato mutant, *dumpy* (*dpy*) (Cerny, 1997) that was previously characterized as a putative BR-deficient mutant. I used experimentation with BR biosynthetic intermediates to determine what step in the pathway was interrupted by the mutation. Following these results we tried to clone the gene by using the Ac/Ds transposon tagging system and also tried to

complement the mutation by transformation with a possible homologue of *dpy* in *Arabidopsis* (*CPD*).

Secondly, we tried to clone early BR-responsive genes to contribute to elucidating the signal transduction pathway involving BRs. Initially we used differential display techniques using wild-type *Arabidopsis* (ecotype Columbia), with no success. However, when *dumpy* was identified in the lab (Cerny, 1997), we used it as the source tissue with the assumption that *dpy* is likely to have lower levels of endogenous BR activity, since it is a BR deficient mutant. Not having much success with this either, we changed from differential display to a PCR-based cDNA subtraction technique.

Although the characterization of the *dpy* mutant of tomato was not to completion (the mutated gene has not been cloned yet) we have made good progress in describing the mutation in detail that should be very useful for future work. Also, there is a good potential of learning more about the mode of action of BR by using the two BR-regulated genes that were characterized in tomato during this research project.

## References

- Abe, H., Honjo, C., Kyokawa, Y., Asakawa, S., Natsume, M., and Narushima, M.** (1994). 3-Oxoteasterone and the epimerization of teasterone: identification in lily anthers and *Distylium racemosum* leaves and its biotransformation into typhasterol. *Biosci. Biotech. Biochem.* 58: 986-989.

- Abe, H., Takasuto, S., Nakayama, M., and Yokota, T.** (1995a). 28-homotyphasterol, a new natural brassinosteroid from rice (*Oryza sativa* L.) bran. *Biosci. Biotech. Biochem.* 59: 176-178.
- Abe, H., Takasuto, S., Okuda, R., and Yokota, T.** (1995b). Identification of castasterone, 6-deoxocastasterone, and typhasterol in the pollen of *Robinia pseudo-acacia* L. *Biosci. Biotech. Biochem.* 59: 309-310.
- Adam, G., Porzel, A., Schmidt, J., Schneider, B., and Voigt, B.** (1996). New developments in brassinosteroid research. In "Studies in Natural product Chemistry" (Atta-ur-Rahman, /ed.) Vol. 18. Pp. 495-549. Amsterdam: Elsevier.
- Asakawa, S., Abe, H., Nishikawa, N., Natsume, M., and Koshioka, M.** (1996). Purification and identification of new acyl-conjugated teasterone in lily pollen. *Biosci. Biotech. Biochem.* 60: 1416-1420.
- Azpiroz, R., Wu, Y., LoCascio, J.C., and Feldmann, K.** (1998). An *Arabidopsis* brassinosteroid-dependent mutant is blocked in cell elongation. *Plant Cell* 10: 219-230.
- Beato, M., Herrlich, P., and Schutz, G.** (1995). Steroid hormone receptors: Many actors in search of a plot. *Cell* 83: 851-857.
- Bishop, G.J., Harrison, K., and Jones, J.D.G.** (1996). The tomato *Dwarf* gene isolated by heterologous transposon tagging encodes the first member of a new cytochrome P450 family. *Plant Cell* 8: 959-969.
- Bishop, G.J., Nomura, T., Yokota, T., Harrison, K., Noguchi, T., Fujioka, S., Takasuto, 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.
- Cerny, R.E.** (1997). Physiological and molecular analysis of Brassinosteroid mutants and developmental genes in *Lycopersicon esculentum*. MS thesis. North Carolina State Univ.
- Chang, C., Schaller, G.E., Patterson, S.E., Kwok, S.F., Meyerowitz, E.M., and Bleecker, A.B.** (1992). The *TMK1* gene from *Arabidopsis* codes for a protein with structural and biochemical characterization of a receptor protein kinase. *Plant Cell* 4: 1263-1271.
- Chang, C., Kwok, S.F., Bleeker, A.B., and Meyerowitz, E.M.** (1993). *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. *Science* 262: 539-544.
- Choe, S., Dilkes, B.P., Fujioka, S., Takasuto, S., Sakurai, A., and Feldmann, K.A.** (1998). The *DWF4* gene of *Arabidopsis* encodes a phytochrome P450

- that mediates multiple 22 $\alpha$ -hydroxylation steps in brassinosteroid biosynthesis. *Plant Cell* 10: 231-243.
- Choe, S., Dilkes, B.P., Gregory, B.D., Ross, A.S., Takasuto, S., Tanaka, A., Yoshida, S., Tax, F.E., and Feldmann, K.,** (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., Takasuto, S., Tissier, C., Gregory, B.D., Ross, A.S., Tanaka, A., Yoshida, S., Tax, F.E., and Feldmann, K.,** (1999b). The Arabidopsis *dwf7/ste1* mutant is defective in the  $\Delta 7$  sterol C-5 desaturation step leading to brassinosteroid biosynthesis. *Plant Cell.* 11: 207-221.
- Choi, Y.H., Inoue, T., Fujioka, S. Saimoto, H. and Sakurai, A.** (1993). Identification of brassinosteroid-like active substances in plant cell cultures. *Biosci. Biotech. Biochem.* 57: 860-861.
- Choi, Y.-H., Fujioka, S., Harada, A., Yokota, T., Takasuto, S., and Sakurai, A.** (1996). A brassinolide biosynthetic pathway via 6-deoxocastasterone. *Phytochemistry* 43: 593-596.
- Choi, Y.-H., Fujioka, S., Nomura, T., Harada, A., Yokota, T., Takasuto, S., and Sakurai, A.** (1997). An alternative brassinolide biosynthetic pathway via late C6-oxidation. *Phytochemistry* 44: 609-613.
- 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.
- Clark, S.E., Williams, R.W., and Meyerowitz, E.M.** (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell.* 89: 575-585.
- Clouse, S.D.** (1996). Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. *Plant J.* 10: 1-8.
- Clouse, S.D.** (1997). Molecular genetic analysis of brassinosteroid action. *Physiol. Plant* 100: 702-709.
- Clouse, S.D., and Zurek, D.** (1991). Molecular analysis of brassinolide action in plant growth and development. In *Brassinosteroids*. Cutler, H.G., Yokota, T., and Adam, G. (Eds) pp. 112-140. American Chemical Society, Washington DC.
- Clouse, S.D., and Sasse, J.M.** (1998). Brassinosteroids: Essential regulators of plant growth and development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49: 427-451.

- 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). Springer-Verlag, Tokyo, pp 163-190.
- Clouse, S.D., Zurek, D.M., McMorris, T.C., and Baker, M.E.** (1992). Effect of brassinolide on gene expression in elongating soybean epicotyls. *Plant Physiol.* 100: 1377-1383.
- 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.
- Cohen, J.D. and Meudt, W.** (1983). Investigations on the mechanism of the brassinosteroid response. I. Indole-3-acetic acid metabolism and transport. *Plant Physiol.* 72: 691-694
- Cosgrove, D.** (1997). Relaxation in a high-stress environment: the molecular basis of extensible cell walls and enlargement. *Plant Cell* 9: 1031-41.
- De Silva, J., Jarman, C.D., Arrowsmith, DA, Stronach, M.S., Chengappa, S., Sidebottom, C., and Reid, J.S.** (1993). Molecular characterization of a xyloglucan-specific *endo*-(1,4)- $\beta$ -D-glucanase (xyloglucan *endo*-transglycosylase) from nasturtium seeds. *Plant J.* 3: 701-711.
- Ecker, J.** (1995). The ethylene signal transduction pathway in plants. *Science* 240: 667-675.
- Evans, R.M.,** (1988). The steroid and thyroid hormone receptor super-family. *Science* 240: 889-895.
- Feldmann, K.A., and Azpiroz, R.** (1994). Primary dwarfs. In: J. Bowman (Ed) *Arabidopsis: an atlas of morphology and development*, Springer-Verlag, New York, pp. 82-85.
- 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.
- Finkelstein, R., and Zeevart, J.** (1994). Gibberellin and abscisic acid biosynthesis and response. In: E. Meyerowitz, C. Sommerville (Eds) *Arabidopsis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp 523-554.
- Fujioka, S and Sakurai, A.** (1995). Biosynthesis of brassinosteroids and its regulation. *Chem. Regul. Plants* 30: 137-141.

- Fujioka, S and Sakurai, A.** (1997a). Biosynthesis and metabolism of brassinosteroids. *Physiologia Plantarum* 100: 710-715.
- Fujioka, S and Sakurai, A.** (1997b). Brassinosteroids. *Nat. Prod. Rep.* 14: 1-10.
- Fujioka, S., Inoue, T., Takasuto, S., Yanagisawa, T., Yokota, T. and Sakurai, A.** (1995a). Identification of a new brassinosteroid, cathasterone, in culture cells of *Catharanthus roseus* as a biosynthetic precursor of teasterone. *Biosci. Biotech. Biochem.* 59: 1543-1547.
- Fujioka, S., Inoue, T., Takasuto, S., Yanagisawa, T., Yokota, T. and Sakurai, A.** (1995b). Biological activities of biosynthetically-related congeners of brassinolide. *Biosci. Biotech. Biochem.* 59: 1973-1975.
- Fujioka, S., Li, J., Choi, Y.-H., Seto, H., Takasuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J., and Sakurai, A.** (1997). The *Arabidopsis deetiolated2* mutant is blocked early in the brassinosteroid biosynthesis. *The Plant Cell* 9: 1959-1962.
- Gachotte, D., Meens, R., and Benveniste, P.** (1995). An *Arabidopsis* mutant deficient in sterol biosynthesis: Heterologous complementation by ERG3 encoding a  $\Delta 7$ -sterol-C-5-desaturase from yeast. *Plant J.* 8: 407-416.
- Gachotte, D., Hüsselstein, T., Bard, M., Lacroute, F., and Benveniste, P.** (1996). Isolation and characterization of an *Arabidopsis thaliana* cDNA encoding a  $\Delta 7$ -sterol-C-5-desaturase by functional complementation of a defective yeast mutant. *Plant J.* 9: 391-398.
- Giffiths, P.G., Sasse, J.M., Yokota, T., and Cameron, D. W.** (1995). 6-Deoxyphasterol and 3-dehydro-6-deoxoteasterone, possible precursors to brassinosteroids in the pollen of *Cupress arizonica*. *Biosci. Biotech. Biochem.* 59: 956-959.
- Graham, T.O.** (1959). Impact of recorded Mendelian factors on the tomato, 1929-59. *Rep. Tomato Genet. Coop.* 9: 37.
- Grove, M.D., Spencer, G.F., Rohwedder, W.K. Mamdava, N. Worley, J.F., and Warhen, J.D.**(1979). Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. *Nature* 281: 216-17.
- Hamada, K.** (1986). Brassinolide in crop cultivation. In: McGregor, P. (Ed) *Plant Growth Regulators in Agriculture*. FFTC, Taiwan, pp. 190-196.
- Hanks, S.K., and Quinn, A.M.** (1991). Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Meth. Enzymol.* 200: 38-62.
- He, R., Wang, G., and Wang, X.** (1991). Effects of brassinolide on growth and chilling resistance of maize seedlings. In: Cutler, H.G., Yokota, T., and Adam,

- G. (Eds) *Brassinosteroids: Chemistry, Bioactivity, and Applications*. ACS Symp Series 474. Amer. Chem. Soc., Washington, DC, pp. 220-230.
- He, Y.-J., Xu, R.-J., and Zhao, Y.-J.** (1996). Enhancement of senescence by epibrassinolide in leaves of mung bean seedling. *Acta Phytophysiol. Sin.* 22: 58-62.
- Hewitt, F.R., Hough, T.O., O'Neill, P., Sasse, J.M., Williams, E.G., and Rowan, K.S.** (1985). Effect of brassinolide and other growth regulators on the germination and growth of pollen tubes of *Prunus avium* using a multiple hanging drop assay. *Aust. J. Plant Physiology* 12: 201-211.
- 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.
- Katsumi, M.** (1985). Interaction of a brassinosteroid with IAA and GA<sub>3</sub> in the elongation of cucumber hypocotyl sections. *Plant Cell Physiol.* 26: 615-625.
- Katsumi, M.** (1991). Physiological modes of brassinolide action in cucumber hypocotyl growth. In: Cutler, H.G., Yokota, T., and Adam, G. (Eds) *Brassinosteroids: Chemistry, Bioactivity, and Applications*. ACS Symp Series 474. Amer. Chem. Soc., Washington, DC, 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
- Kauschmann, A., Adam, G., Lichtblau, D., Mussig, C., Schimdt, J., Vioigt, B., Willmitzer, L., and Altmann, T.** (1997). Molecular/genetic analysis of brassinosteroid synthesis and action. *Proc. Plant Growth Regul. Soc. Am.* 24: 95-96.
- Kawaguchi, M., Imaizumi-Anraku, H., Fukai, S. and Syono, K.** (1996). Unusual branching in the seedlings of *Lotus japonicus* – gibberellins reveal the nitrogen-sensitive cell divisions within the pericycle on roots. *Plant Cell Physiol.* 37: 461-470.
- Kim, S.-K.** (1991). Natural occurrences of brassinosteroids. In "Brassinosteroids. Chemistry, Bioactivity, and Applications. ACS Symposium Series" (H.G. Cutler, T. Yokota, and G. Adams, Eds.) Vol. 474, pp. 26-35. American Chemical Society, Washington.
- Kitani, T.** (1994). Induction of parthenogenic haploid plants with brassinolide. *Jpn. J. Genet.* 69: 35-39.
- Klahre, U., Noguchi, T., Fujioka, S., Takatsuto, s., Yokota, T., Nomura, T., Yoshid, S., Chua, N.-H.** (1998). The *Arabidopsis Diminuto/DWARF1* gene encodes a protein involved in steroid synthesis. *Plant Cell* 10: 1677-1690.

- Kobe, B., and Deisenhofer, J.** (1994). The leucine-rich repeat: a versatile binding motif. *Trends Biochem. Sci.* 19: 415-421.
- Kolbe, A., Porzel, A., Schneider, B., and Adams, G.,** (1997). Diglycosidic metabolites of 24-epi-teasterone in cell suspension cultures of *Lycopersicon esculentum* L. *Phytochemistry* 46: 1019-1022.
- Li, J.M., and Chory, J.** (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 90: 929-938.
- Li, J.M., 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.M., Biswas, M.G., Chao, A., Russell, D.W., and Chory, J.** (1997). Conservation of function between mammalian and plant steroid 5 $\alpha$ -reductases. *Proc. Natl. acad. Sci. USA* 94: 3534-3539.
- Mandava, N.B.** (1988). Plant growth-promoting brassinosteroids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39:23-52.
- Mathur, J., Molnar, G., Fujioka, S., Takasuto, 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 of brassinolide in reorientation of cortical microtubules in the epidermal cells of Azuki bean epicotyls. *Plant Cell Physiol.* 36: 173-181.
- McEwens, B.S.** (1991). Nongenomic and genomic effects of steroids on neural activity. *Trends Pharma. Sci.* 12: 141-147.
- Medford, J.I., Elmer, J.S., and Klee, H.J.** (1991). Molecular cloning and characterization of genes expressed in shoot apical meristems. *Plant Cell* 3: 359-370.
- Meudt, W.J., Thompson, M.J., Bennett, H.W.** (1983). Investigations on the mechanism of the brassinosteroid response. III. Techniques for potential enhancement of crop production. *Proc. Plant Growth Regul. Soc. Amer.* 10: 312-318.
- Mitchell, J.W. and Gregory, L.E.** (1972). Enhancement of overall growth, a new response to brassins. *Nature* 239: 254.
- Mitchell, J.W., Mandava, N.B. Worley, J.F., Plimmer, J.R., Smith, M.V.** (1970). Brassins: a new family of plant hormones from rape pollen. *Nature.* 225: 1065-66.

- Nadzhimov, U.K., Jupe, S.C., Jones, M.G., and Scott, I.M.,** (1988). Growth and gibberellin relations of the extreme *dwarf d<sup>x</sup>* mutant. *Physiol. Plant.* 73: 252-256.
- Nakai, K., and Kanehisa, M.** (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* 14: 897-911.
- Nakajima, N., Shida, A., and Toyama, S.** (1996). Effects of brassinosteroid on cell division and colony formation of chinese cabbage mesophyll protoplasts. *Jpn. J. Crop. Sci.* 65: 114-118.
- Nishikawa, N., Abe, H., Hatsume, M., Shida, A., and Toyama, S.** (1995). Epimerization and conjugation of <sup>14</sup>C labeled epibrassinolide in cucumber seedlings. *J. Plant Physiology.* 147: 294-300.
- Noguchi, T., Fujioka, S., Takasuto, S., Sakurai, A., Yoshida, S., Li, J.M., and Chory, J.** (1999). Arabidopsis *det2* is defective in the conversion of (24*R*)-24-methylcholest-4-en-3-one to (24*R*)-24-methyl-5 $\alpha$ -cholestan-3-one in brassinosteroid biosynthesis. *Plant Physiol.* 120: 833-839.
- Nomura, T., Nakayama, M., Reid, J.B., Takeuchi, Y., and Yokota, T.** (1997). Blockage of brassinosteroid biosynthesis 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., and Clouse, S.D.** (1998). Brassinolide affects the rate of cell division in isolated leaf protoplasts of *Petunia hybrida*. *Plant Cell Reports.* 17: 921-924.
- Park, K.-H., Saimoto, H., Nakagawa, S., Sakurai, A., Yokota, T., Takahashi, N., and Shono, K.** (1989). Occurrence of brassinolide and castasterone in crown gall cells of *Catharanthus rosues*. *Agric. Biol. Chem.* 53: 805.
- Rick, C.M.,** (1954). *Extreme dwarf*, a new allele at the *d* locus. *Rep Tomato. Genet. Coop.* 4: 16-17.
- Sakurai, A and Fujioka, S.** (1993). The current status of physiology and biochemistry of brassinosteroids. *Plant Growth Regul.* 13: 147-159.
- Sakurai, A and Fujioka, S.** (1997a). Biosynthesis of steroidal plant hormones, brassinosteroids. *Baiosaiensu to Indasutori* 55: 38-40.
- Sakurai, A and Fujioka, S.** (1997b). Studies on biosynthesis of brassinosteroids. *Biosci. Biotech. Biochem.* 61: 757-762.
- Sakurai, A, Fujioka, S. and Saimoto, H.** (1991). Production of brassinosteroids in plant-cell cultures. In "Brassinosteroids. Chemistry, Bioactivity, and Applications. ACS Symposium Series" (H.G. Cutler, T. Yokota, and G. Adams, Eds.) Vol. 474, pp. 97-106.

- Sasse, J.** (1997). Recent progress in brassinosteroid research. *Physiologia Plantarum* 100: 696-701.
- Sasse, J.** (1999). Physiological actions of brassinosteroids. In: *Brassinosteroids, steroidal plant hormones*. Sakurai, A., Yokota, T., and Clouse, S.D. (Eds) Springer-Verlag, Tokyo. pp 137-161.
- Sasse, J. M.** (1985). The place of brassinolide in the sequential response to plant growth regulators in elongating tissue. *Physiol. Plant.* 63: 303-308.
- Schaller, G.E., and Bleecker, A.B.** (1995). Ethylene-binding sites generated in yeast expressing the Arabidopsis *ETR1* gene. *Science* 270: 1809-1811.
- Schmidt, J.S., Voigt, B. and Adam, G.** (1995). 2-Deoxybrassinolide—a naturally occurring brassinosteroid from *Apium graveolens*. *Phytochemistry* 40: 1041-1043.
- Schmidt, E., Guzzo, F., and Toonen, M.** (1997). A leucine-rich repeat containing receptor-like kinase marks somatic plant cells component to form embryos. *Development* 124: 2049-2062.
- Song, W., Wang, G., Chen, L.-L., Kim, H.-S., Pi, L.-Y., Holsten, T., Gardner, J., Wang, B., Zhao, W.-X., Zhu, L.-H., Fauquet, C., and Ronald, P.** (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science* 270: 1804-1806.
- Spengler, B., Schmidt, J., Voigt, B., and Adams, G.** (1995). 6-Deoxo-28-norcastasterone and 6-oxo-24-epicastasterone – two new brassinosteroids from *Ornithopus sativus* – *Phytochemistry* 40: 907-910.
- Suge, H.** (1986). Reproductive development of higher plants as influenced by brassinolide. *Plant Cell Physiol.* 27: 199-205.
- Suzuki, H., Fujioka, S., Takasuto, S., Yokota, T., Murofushi, N and Sakurai, A.** (1993a). Biosynthesis of brassinolide from castasterone in cultured cells of *Catharanthus rosues*. *Plant Growth Reg.* 12: 101-106.
- Suzuki, H., Kim, S.-K., Takahashi, N., and Yokota, T.** (1993b). Metabolism of castasterone and brassinolide in mung bean explant. *Phytochemistry* 33: 1361-1368.
- Suzuki, H., Fujioka, S., Takasuto, S., Yokota, T., Murofushi, N and Sakurai, A.** (1994a). Biosynthesis of brassinolide from teasterone via typhasterol and castasterone in cultured cells of *Catharanthus rosues*. *J. Plant Growth Regul.* 13: 21-26.
- Suzuki, H., Fujioka, S., Yokota, T., Murofushi, N and Sakurai, A.** (1994b). Identification of brassinolide, castasterone, typhasterol, and teasterone from the pollen of *Lilium elegans*. *Biosci. Biotech. Biochem.* 58: 1186-1188.
- Suzuki, H., Fujioka, S., Yokota, T., Murofushi, N and Sakurai, A.** (1995a). Biosynthesis of brassinosteroids in seedlings of *Catharanthus rosues*, *Nicotiana tabacum*, and *Oryza sativa*. *Biosci. Biotech. Biochem.* 59, 168-172.

- Suzuki, H., Inoue, T., Fujioka, S., Saito, T., Takasuto, S., Yokota, T., Murofushi, N., Yangisawa, T., and Sakurai, A.,** (1995b). Conversion of 24-methylcholesterol to 6-oxo-24-methyl-cholestanol, a putative intermediate of the biosynthesis of brassinosteroids, in cultured cells of *Catharanthus roseus*. *Phytochemistry* 40: 1391-1397.
- Szekeress, M., Nemeth, K., Koncz-Kalman, 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.
- Takahashi, T., Gasch, A., Nishizawa, N., and Chua, N.-H.** (1995). The DIMINUTO gene of *Arabidopsis* is involved in regulating cell elongation. *Genes & Devel.* 9: 97-107.
- Takatsuto, S.** (1991). Microanalysis of Naturally Occurring Brassinosteroids. *Brassinosteroids: Chemistry, Bioactivity, and Application*. American Chemical Society pp.107-19.
- Takatsuto, S.** (1994). Brassinosteroids: distribution in plants, bioassays and microanalysis by gas chromatography-mass spectrometry. *J. Chromatogr.* 658: 3-15.
- Thompson, M.J., Mandava, N.B., Meudt, W.J., Lusby, W.R., and Spaulding, D.W.** (1981). *Steroids*. 38: 567.
- Torii, K.U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R.F., and Komeda, Y.** (1996). The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell*. 8: 735-746.
- Wada, K., Marumo, S., Ikekawa, N., Morisaki, M., and Mori, K.**(1981) Brassinolide and Homobrassinolide promotion of lamina inclination of rice seedlings. *Plant Cell Physiology*. 22: 323-325
- Wada, K., Kondo, H., and Marumo, S.** (1985). A simple bioassay for brassinosteroids: a wheat leaf-unrolling test. *Agric. Biol. Chem.* 49: 2249-2251.
- Walker, J.** (1993). Receptor-like protein kinase genes of *Arabidopsis thaliana*. *Plant J.* 3: 451-456.
- West, C.A.** (1980). Hydroxylases, monooxygenases, and cytochrome P-450. In *Biochemistry of Plants: A Comprehensive Treatise*, Vol 2. Metabolism and respiration, D.D. Davies, Ed (New York: Academic Press), pp. 317-365.
- Wilens, R.W., Sacco, M., Gusta, L.V., and Krishna, P.** (1995). Effects of 24-epibrassinolide on freezing and thermotolerance of bromegrass (*Bromus inermis*) cell cultures. *Physiol. Plantarum*. 95: 195-202.
- Winter, J., Schneider, B., Strack, D., and Adam, G.,** (1996). Involvement of cytochrome P450 in the metabolism of 24-epi-brassinolide in tomato cell cultures. *Proc. Plant Growth Regul. Soc. Am.* 23: 290.

- Winter, J., Schneider, B., Strack, D., and Adam, G.,** (1997). Role of cytochrome P450-dependent monooxygenase in the hydroxylation of 24-epibrassinolide. *Phytochemistry* 45: 233-237.
- Xu,-W., Purugganan, M.M., Polisensky, D.H., Antosiewicz, D.M., Fry, S.C., and Braam, J.** (1995). *Arabidopsis TCH4*, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. *Plant Cell*. 7: 1555-1567.
- 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.** (1987). *Chemical Regulation of Plants*. 22: 10.
- Yokota, T.** (1997). The structure, biosynthesis and function of brassinosteroids. *Trends Plant Sci*. 2: 137-143.
- Yokota, T., Arima, M. and Takashashi, N.** (1982). *Tetrahedron Letters*. 23: 1275.
- Yokota, T., Nakayama, M., and Wakisaka, T.** (1994). 3-Dehydroteasterone, a 3,6-diketobrassinosteroid as a possible biosynthetic intermediate of brassinolide from wheat grain. *Biosci, Biotech, Biochem*. 58: 1183-1185.
- Yokota, T., Nomura, T., and Kitasaka, Y.** (1997). Biosynthetic lesions in brassinosteroid-deficient pea mutants. *Proc. Plant Growth Reg. Soc. Amer*. 24: 99.
- Yokota, T., Ogino, Y., Takahashi, N., Saimoto, H., Fujioka, S., and Sakurai, A.** (1990a). Brassinolide is biosynthesized from castasterone in *Catharanthus roseus* gall cells *Agric. Biol. Chem*. 54: 1107-1108.
- Yopp, J.H., Mandava, N.B., and Sasse, J.M.** (1981). Brassinolide, a growth-promoting steroidal lactone. I. Activity in selected auxin bioassays. *Physiol. Plant*. 53: 445-452.
- Zurek, D.M., and Clouse, S.D.** (1994). Molecular cloning and characterization of a brassinosteroid-regulated gene from elongating soybean epicotyls. *Plant Physiol*. 104: 161-170.

## Chapter 2

# Characterization of '*dumpy*', a Brassinosteroid-deficient Tomato Mutant

## Introduction

Brassinosteroids (BRs) are a group of naturally occurring phytohormones that have structural similarity to animal steroids. In the last few years there has been tremendous progress in elucidating the role and mode of action of these compounds and they are now considered to be the sixth class of plant hormones (Clouse, 1996; Yokota, 1997). In plants, BRs have been shown to have an influence on cell division, cell elongation, senescence, and male fertility. Genetic analysis of different mutants has provided convincing evidence that BRs are essential phytohormones (reviewed in Clouse and Sasse, 1998). As described in Chapter One, elucidation of the biosynthetic pathway (Fujioka and Sakurai, 1997a), along with the characterization of different BR-biosynthetic and BR-insensitive mutants in *Arabidopsis thaliana* (Clouse and Feldmann, 1999), has contributed greatly towards understanding the mode of action of BRs.

Current understanding of the control of biosynthesis and signal transduction is largely based on the characterization of Arabidopsis mutants such as, *det2*, *cpd*, *bri1*, *dwf1* and *dwf4*, (Clouse and Feldmann, 1999; Yokota, 1997; Choe *et al.*, 1998, 1999a). Szekeres *et al.*, (1996) have demonstrated that the *CPD* sequence encodes a protein belonging to a new class (CYP90) of the cytochrome P450 superfamily. In animals, there are at least six different cytochrome P450 genes whose expression is highly regulated by signaling mechanisms (Waterman and Bischof, 1997). Mathur *et al.* (1998) have shown that there is feedback regulation of the *CPD* gene by BRs that requires the synthesis and/or processing of a negative regulatory factor. Other alleles of *cpd*

in *Arabidopsis* include *cabbage3* (*cbb3*), and *dwarf3* (*dwf3*) (Kauschmann, *et al.*, 1996; Choe *et al.*, 1998). *CPD* is thought to encode a  $23\alpha$ -hydroxylase that catalyzes the conversion of cathasterone (CT) or 6-deoxocathasterone (6-deoxoCT) to teasterone (TE) or 6-deoxoteasterone (6-deoxoTE), respectively.

In *Arabidopsis*, all of the BR deficient and insensitive mutants have characteristic features, as these mutations have pleiotropic effects. In the light, they are dwarfs with very dark-green and curled leaves, short stems, delayed senescence and flowering, and reduced male fertility. In the dark, they have a de-etiolated phenotype: short hypocotyls and expanded cotyledons (Clouse and Feldmann, 1999).

Tomato (*Lycopersicon esculentum* Mill.) is another plant system that has been widely used as a model system for genetic and biochemical research. There are many natural mutants in tomato that are due to lesions in genes involved in hormone biosynthesis and signal transduction. This has resulted in extensive characterization of mutants with altered fruit physiology. The *Never ripe* (*Nr*), *non-ripening* (*nor*), and *ripening inhibitor* (*rin*) mutants which exhibit abnormal fruit ripening process have been well characterized (DellaPenna *et al.*, 1989). The *Nr* gene has been cloned (Wilkinson *et al.*, 1995) and was demonstrated to be a homolog of the *Arabidopsis ETR1* gene (Chang *et al.*, 1993), which encodes an ethylene receptor. Other tomato mutants involving growth hormones include the auxin insensitive mutant, *diageotropica* (*dgt*) (Kelly and Bradford, 1986); ABA deficient mutants, *flacca* (*flc*), *notabilis* (*not*), and *sitiens* (*sit*) (Taylor *et al.*, 1988; Parry *et al.*, 1992); and GA deficient mutants,

*gib1*, *gib2* and *gib3* (Knorrneef *et al.*, 1990). All of these are being further studied to better understand hormone action in plants.

To date, only the *dwarf* mutant of tomato has been classified as a BR deficient mutant (Bishop *et al.*, 1999). The *DWARF* gene shares sequence similarity to the *CPD* gene of *Arabidopsis* (Bishop *et al.*, 1996; Szekeres *et al.*, 1996), but feeding experiments with BR biosynthetic intermediates shows that the *DWARF* gene product is likely to be involved in conversion of 6-deoxoCS to CS (Figure 1; Bishop *et al.*, 1999).

In our laboratory, natural tomato dwarf mutant lines were obtained from the Tomato Genetics Resource Center (TGRC), Davis CA. In all, 27 non-allelic single gene mutants were screened for responses to exogenous application of BR (Cerny, 1997). A *Lycopersicon esculentum* tomato mutant, *dumpy* (*dpy*), was shown to be BR deficient since the phenotype was rescued by exogenous application of BR (Cerny, 1997). Besides *dpy*, the above screening also identified *extreme dwarf* (*d<sup>x</sup>*) (Bishop *et al.*, 1999) as a BR-deficient mutant. The objective of the present study was to further characterize the *dpy* mutant by feeding BR biosynthetic intermediates to identify the step in the BR biosynthetic pathway that is blocked in the mutant. We also tried to clone the *DPY* gene by utilizing transposon tagging. This approach, using the maize transposons *Activator* and *Dissociation* (*Ac/Ds*), has been successfully used in cloning the tomato *DWARF* gene (Bishop *et al.*, 1996), *N* from tobacco (Whitham *et al.*, 1994), *L6* from flax (Lawrence *et al.*, 1995), and *Cf-9* from tomato (Jones *et al.*, 1994). We report here physiological and biochemical evidence that the *Lycopersicon esculentum*

mutant *dpy* is likely to result in BR-deficiency due to lesions in a gene involved in the conversion of 6-deoxoCT to 6-deoxoTE, but is unlikely to be the homologue of the Arabidopsis *CPD* gene.

## Materials and Methods

### Feeding Experiments using Brassinosteroid Biosynthesis Intermediates

'*dumpy*' seeds were sown in 'Fafard 4P' potting media. After two weeks they were transplanted into 12.5 cm Azalea pots and grown in a greenhouse under natural light.

Twenty one days after seeding, the plants were divided into 17 treatments of 3 pots each. Each set of 3 pots was treated with foliar spray application. The treatments consisted of campesterol (CR), campestanol (CN), 6-hydroxy-campestanol (6-OH CN), 6-oxocampestanol (6-OH CN), cathasterone (CT), 6-deoxocathasterone (dCT), teasterone (TE), 6-deoxoteasterone (dTE), 3-dehydroteasterone (3DT), 3-dehydro-6-deoxoteasterone (d3DT), typhasterol (TY), 6-teoxotyphasterol (dTY), castasterone (CS), 6-deoxocastasterone (dCS), and brassinolide (BL), along with one untreated control and one treated with solvent control (0.001% ethanol; 0.1% Tween 20). The above intermediates were sprayed at 1.0  $\mu\text{M}$  concentration, except for dCS, CS, and BL, which were sprayed at 0.1  $\mu\text{M}$  concentration. Plants were sprayed with respective intermediates twice a day for 21 days, using sufficient volume to wet the leaf surface thoroughly. Upon completion, they were photographed under natural light in the greenhouse.

## **Quantitative Analysis of Endogenous Brassinosteroids and Sterols**

Thirty plants each of 'Alisa Craig' (wildtype) and *dpy* tomatoes were grown for 6 weeks in the greenhouse. The upper 4 nodes of each plant were excised and the material from each genotype was pooled, weighed, frozen in liquid nitrogen and lyophilized. Determination of endogenous BR levels in *dpy* and wildtype was performed on extracts of pooled individuals of each genotype spiked with internal deuterated standards of the respective compound being analyzed, which is widely accepted as the most accurate method of BR determination (Fujioka *et al.*, 1997; Choe *et al.*, 1999a, 1999b).

## **Dark-grown Seedling Studies**

BR feeding experiments were performed in the dark by using sterilized seeds in 100% ethanol for 15 min, followed by 30% commercial bleach solution (1.05% w/v hypochlorite) for 40 min.. Seeds were then rinsed in sterile water, incubated overnight at 4°C, and then were placed in a straight line in 150 X 15 mm Perti plates containing 1% agar, 2 or 3% sucrose, and half-strength MS media (Murashige and Skoog, 1962) at pH 5.7. Different BR biosynthetic intermediates were added to the sterile, cooled media just before pouring plates. All intermediates were used at 1.0 µM except for BL, which was used at 0.2 or 0.5 µM. Plates were incubated vertically under continuous darkness at 25°C in a growth chamber. Seedlings were removed from the growth chamber after 9 or 11 d and photographed. Hypocotyl length of 10 seedlings for each treatment was measured to the nearest mm.

For the dark studies in liquid culture, seeds were sterilized as above and placed in 250 ml flasks containing 25 ml of half-strength MS media, pH 5.7, with 3% sucrose. BL and intermediates were added to the cooled medium at the same final concentrations used for the agar plate experiment. Flasks were incubated in a dark chamber at 24°C with constant shaking at 75 rpm. Hypocotyl length of 10 seedlings for each treatment was measured to the nearest mm after 9 d of culture.

### **Protein Extraction**

One gram of leaf tissue was mixed with 4 ml of fresh, ice-cold extraction buffer (50 mM MOPS, pH 7.5; 5 mM MgCl<sub>2</sub>; 5 mM DTT; 1 mM EDTA; 0.1 mM PMSF) and pinch of sand was added (to facilitate grinding). The tissue was then ground with a pestle in a mortar on ice. The sample was transferred to a microfuge tube, centrifuged for 2 min @ 16,000x g and the supernatant was poured into a fresh tube. The concentration of protein was estimated using Bradford method (Bradford, 1976). Proteins (15 µg per sample) were separated using standard SDS-PAGE (Gallagher and Smith, 1994). The proteins were transferred onto a Nitrocellulose membrane by using a Semi-dry Blotting method. Transfer Buffer was prepared by dissolving 5.82g Tris, 2.93g glycine, and 3.75 ml of 10% SDS in dH<sub>2</sub>O; adding 200 ml of methanol; and adjusting the volume to 1 liter with dH<sub>2</sub>O, for a final pH of 9.2. The gel was soaked in transfer buffer for 30 min. Transfer was done at 12 V for 15 min.

## Western Blot Analysis

After proteins were transferred onto the nitrocellulose membrane, the membrane was incubated in Blocking Solution (PBST + 5% Milk Powder) at 4°C, with shaking, over-night. PBST is 80 mM Na<sub>2</sub>HPO<sub>4</sub>; 20 mM NaH<sub>2</sub>PO<sub>4</sub>; 10 mM NaCl; 0.1% Tween 20; pH 7.5. Membranes were washed twice with PBST, for 5 min each, followed by incubation with primary antibody obtained from Dr. Koncz's lab (1:750 dilution) in a total volume of 15 ml PBST at room temperature for 90 min. Membranes were washed again twice with PBST, for 5 min each.

The ECL Western blotting analysis system (Amersham Pharmacia Biotech, NJ, USA) was used to detect the primary antibodies. This system is a light emitting non-radioactive method for detection of immobilized specific antigens, conjugated with horseradish peroxidase-labelled antibodies. The protocol recommended by the manufacturer was followed.

## Transformation of *dpy* with the Arabidopsis CPD gene

The *CPD* cDNA cloned into the BamH-I site of pPCV701, and driven by the *mas 2'* promoter (Szekeres *et al.*, 1996), was used to complement the *dpy* mutation, by Agrobacterium-mediated transformation of tomato. A GV3101 Agrobacterium strain containing pBI121 plasmid (Clontech, Palo Alto, CA) alone was used as a transformation control.

Seeds of *dpy* and wildtype were sterilized, placed in sterile magenta jars containing MS media, and grown under the same conditions as mentioned earlier. Agrobacterium-mediated transformation was performed as described by

Koncz *et al.* (1994). The *dpy* seedlings were fed sterile BL ( $10^{-6}$  M) so that the cotyledons would be less curled for ease in preparing the explants for transformation, since the *dpy* cotyledons are heavily curled. *Agrobacterium* strains were cultured in 50 mL of LB liquid media containing Kanamycin (100  $\mu$ g/ml) grown at 28°C, overnight. The feeder layer consisted of 1 mL of fine tobacco suspension culture on plates containing MS medium with 1.0 mg/L 2,4-D, 0.6% agarose spread to an even layer. Plates were incubated unsealed and stacked in low light overnight.

Cotyledons were used as explants and were cut into two pieces of approximately 0.5 cm with two cut ends. The explants were blotted on sterile filter paper and placed on the feeder plates, which had a Whatman no.1 filter paper spread on top of the feeder layer. Unsealed petri dishes were stacked and incubated overnight at 25°C, under low light.

*Agrobacterium* cultures were collected and resuspended in MS medium containing 3% sucrose to an OD<sub>590</sub> of 0.4-0.5. The explants were immersed in this suspension for a few seconds and returned to the feeder plate after blotting on a sterile filter paper to remove the excess suspension. The feeder plates were returned to the incubator under the same conditions as before. Co-cultivation was done for 40 hrs. We tried transforming wildtype with GV3101+pBI121 and *dpy*, with both GV3101+pBI121 and GV3101+CDP, each.

After 40 hrs, the explants were placed on plates containing regeneration media (1x MS salts, 100 mg/L myo-inositol, 20 g/L sucrose, 2 g/L Gelrite (Kelco Co. San Diego, CA), 1x Nitsch vitamins, pH 6.0; and 2 mg/L of filter sterilized

Zeatin riboside-trans-isomer) containing antibiotics Kanamycin (100 µg/mL), Carbenicillin (200 µg/mL) and Mefoxin (300 µg/mL), all added after autoclaving and cooling of the media. Nitsch vitamins contained 0.5 mg/L thiamine, 2.0 mg/L glycine, 5.0 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.5 mg/L folic acid, and 0.05 mg/L biotin. Explants were transferred to fresh medium every 2-3 weeks. When the regenerating callus was too large for a petri plate, it was moved into a magenta jar containing the regeneration media.

Shoots were cut from the explants and placed into rooting medium (0.5x MS medium, 5 g/L Sucrose, 2 g/L Gelrite, pH 6.0) with reduced antibiotic concentrations of Carbenicillin (100 µg/mL) and Kanamycin (50 µg/mL). Shoots that did not root were recut at the base of the shoot and placed in fresh medium.

After washing away all of medium, rooted shoots were transferred to soil. Plants were maintained under high humidity, initially, which was gradually reduced. Eventually, the plants were moved into the greenhouse.

### **Transposon Tagging**

We used line SLJ10512, which contains a stabilized *Ac* source (*sAc*, Scofield *et al.*, 1992), that was obtained from Jonathan Jones, John Innes Institute, England (Thomas *et al.*, 1994). The *Ac* element was made mobile by crossing this line with line SLJ1561, which contains a *Ds* element (Thomas *et al.*, 1994). The F<sub>1</sub> progeny of this cross was used as the female parent in crossing with homozygous *dpy* lines. The progeny of this cross were screened for plants with *dpy*-like phenotype, which is likely to result when the *Ac* element

introgressed in the *DPY* gene of the female parent used in crossing with *dpy* pollen.

## Results

### Phenotype of *dpy* and its response to BR

A natural recessive tomato mutant of spontaneous origin, *dumpy* (*dpy*) has greatly condensed, downward curling dark green rugose leaves that are well spaced along the stem (Hernandez-Bravo, 1967). *dpy* plants are also shorter than wildtype, lack axillary branching and show reduced fertility and delayed senescence. Figure 2a shows the phenotype of a *dpy* plant. The phenotype of *dpy*, can be rescued almost completely (but only in the new growth) by exogenous application of BL (Fig. 2b). When exogenous BR supply is withheld, the new growth reverts back to the *dpy* phenotype (data not shown). Nearly complete rescue of other wild-type characteristics including normal plant stature, axillary branching, and increased fertility, was also observed when *dpy* plants were treated with exogenous BL (Fig 2a). There was a response to exogenous application of GAs in terms of increased internode lengths (data not shown), however there was no effect on leaf morphology.

In the dark, *dpy* has short, thick hypocotyls similar in size to *dpy* grown in the light (Fig 3). However, *dpy* seedlings retain a pronounced apical hook and closed cotyledons and hence do not exhibit complete deetiolated phenotype in the dark, unlike *Arabidopsis* BR mutants.

### The *dpy* mutant is BR deficient

The rescue of *dpy* by exogenous application of BL suggests that the mutated gene encodes a BR biosynthetic enzyme. To determine the putative enzyme whose activity is disrupted in *dpy*, intermediates of the BR biosynthetic pathway (Fig. 1), obtained from S. Fujioka (RIKEN Institute, Japan) were applied, twice daily, for 21 d (starting 21 days after planting) to a set of 51 plants.

Application of BL and CS shows that there was nearly complete rescue of *dpy* to wild-type phenotype (Fig. 4). Intermediates in the Late C6-oxidation pathway from 6-deoxoTE and downstream had effects on the phenotype of *dpy* plants, with the effect increasing as we proceeded towards BL in the pathway (Fig 1). Intermediates upstream of 6-deoxoTE did not have any effect on the phenotype. Effects of intermediates from the Early C6-oxidation pathway were similar, although not as dramatic as the Late C6-oxidation pathway counterparts (data not shown).

The dark-grown *dpy* seedlings treated with exogenous BL and its intermediates showed similar rescue patterns, 6-deoxoTE and downstream intermediates rescue the *dpy* phenotype (Fig 5). Similar results were obtained when plants were grown on agar medium or in a liquid culture. However, the mean hypocotyl length was greater in liquid culture. This observation suggests that an enzyme responsible for conversion of 6-deoxoCT to 6-deoxoTE might be one that is mutated in *dpy*.

This observation was further confirmed by measuring and comparing the endogenous levels of BR intermediates by GC-MS with internal deuterated

standards in 42-d-old *dpy* and wild-type plants grown in the greenhouse under identical conditions. Both *dpy* and wild-type plants had similar levels of BR precursors 24-methylenecholesterol, CR and CN (Table 1). However, *dpy* plants contained twice the level of 6-deoxoCT as wildtype and less than half the amount of 6-deoxoTE. Moreover, *dpy* had 25-fold less of 6-deoxoxCS and CS was below the limit of detection in the mutant when compared to the wildtype. The biochemical data also suggests interference in the conversion of 6-deoxoCT to 6-deoxoTE in *dpy*.

### ***dpy* may not be a homologue of Arabidopsis CPD**

Based on the results of the feeding experiments and endogenous levels of BRs in *dpy* and wildtype, we assumed that *dpy* might be a homologue of Arabidopsis CPD. Contrary to expectation, western blot analysis of total protein in *dpy* and wild-type tomato with polyclonal antibodies raised against Arabidopsis CPD protein, showed that the mutant has more protein that is cross-reactive to CPD antibodies than the wildtype (Fig. 6). In both *dpy* and wildtype, the older leaves had more CPD protein than the younger leaves. However, a definitive conclusion can be drawn only by asserting that the protein detected by the CPD antibodies is functionally active. However, CPD encodes a P450 enzyme, which belongs to a large gene family and so to assay for the activity of a specific member, especially without knowing the substrate, is very difficult.

Instead, we attempted to transform *dpy* with the CPD cDNA to determine if it complements the mutation. Of the three combinations of transformation (see

Materials and Methods section) *dpy* transformed with GV3101+pBI121 did not produce any viable callus at all, even when exogenous BR was supplied.

Wildtype and *dpy* transformed with GV3101+CPD produced a good number of calli. Eventually, three independent lines of plants from each transformation were regenerated. However, there were no observable phenotypic differences between the transformed and untransformed lines (data not shown).

### **Cloning of *dpy* mutation using transposon tagging**

We were not successful in recovering a transposon tagged *DPY* gene. As mentioned in the Materials and Methods, we used the heterologous (maize) *Ac/Ds* system that has been shown to be very successful in cloning genes in tomato (Bishop *et al.*, 1996). We screened more than 12,000 plants from the cross involving the *Ac/Ds* progeny with homozygous *dpy* plants without recovering any with a *dpy* phenotype (Fig. 7).

## **Discussion**

By searching the Tomato Genetic Resource Center (TGRC), a set of natural dwarf tomato mutants was identified and screened to determine if any of them were due to BR deficiency. The *dpy* tomato mutant showed dramatic response to exogenous application of BR (Cerny, 1997), resulting in nearly complete rescue of the mutant to wild-type phenotype (Fig. 2), suggesting that the *DPY* gene is involved in BR biosynthesis. Lack of complete rescue of *dpy* to wildtype in response to exogenous application of BR was also observed in

another BR-deficient tomato mutant, *dwarf* (Bishop *et al.*, 1996, Bishop *et al.*, 1999). The authors attributed this observation to inefficient BR transport in tomato. This could also be due to the exacting requirements in terms of both temporal and spatial distribution of BR during growth and development, which would be difficult to meet by exogenous application.

In *dpy*, we have observed that only immature and probably undifferentiated cells can respond to BR. Mature or differentiated cells don't have any response to exogenous BR. BR-deficiency of *dpy* was also confirmed by comparing endogenous levels of BR and its intermediates in the mutant with the levels in wildtype plant of same age and grown under identical conditions, using GC-MS (Table 1). 6-deoxoCS was 25-fold less and CS was below detectable levels in *dpy* when compared with wildtype plants.

Two alternate pathways of BL biosynthesis, termed early and late C-6 oxidation, were identified by feeding experiments in cell cultures of *Catharanthus roseus* (Fig 1.; Choi *et al.*, 1996, 1997). BR biosynthetic intermediates have also been used in feeding experiments to locate the putative site of mutation in the pathway of different BR deficient mutants. Application of both early and late C-6 oxidation pathway intermediates suggested that an enzyme activity involving the conversion 6-deoxoCT to 6-deoxoTE might be the site of mutation in *dpy*. This is because the deoxoTE and other downstream intermediates showed effects of rescue of the *dpy* mutation, with the intensity of rescue increasing as we proceed to the end of the pathway. This is consistent with earlier observations using bioassays, that the biological activity of the intermediates increases with position

along the pathway (Fujioka *et al.*, 1995), and also that the efficiency of rescue of deficient mutants increases as the intermediates approach more closely the structure of BL (Choe *et al.*, 1998, 1999a, 1999b; Fujioka *et al.*, 1997; Klahre *et al.*, 1998; Nomura *et al.*, 1999).

In the case of *Arabidopsis*, both 6-oxo and 6-deoxo intermediates have been detected, indicating that both the early and late C-6 oxidation pathways are active in this species (Choi *et al.*, 1997). Based on observations during feeding experiments it was proposed that late C-6 oxidation might be more active in the light, while early C-6 oxidation is more active in the dark (Choe *et al.*, 1998; Fujioka *et al.*, 1997). However, reports on tomato have shown that the early C-6 oxidation pathway might not be very active in this species (Bishop *et al.*, 1999; Yokota *et al.*, 1997), as intermediates such as TE and TY were not detected. Our observations in feeding experiments involving *dpy* also showed that responses to late C-6 oxidation pathway intermediates were more pronounced than early C-6 oxidation pathway intermediates. The rescue of *dpy* by early C-6 oxidation pathway intermediates was not as dramatic as late C-6 intermediates, although there was slight change in the leaf morphology. GC-MS assays for endogenous levels of intermediates also detected only late C-6 intermediates (Table 1), and early C-6 intermediates such as TE and TY were not detected. In contrast to observations in *Arabidopsis* (Choe *et al.*, 1998; Fujioka *et al.*, 1997), we found that 6-deoxo intermediates were more active than the 6-oxo intermediates in the dark also. This situation is unlikely due to differential stability or uptake, instead it

is likely due to the fact that late C-6 oxidation might be the only active pathway in tomato.

Based on sequence analysis and feeding experiments, the Arabidopsis *cpd* mutant has been proposed to also be due to mutation in the gene responsible for the conversion of CT to TE (Szekeres *et al.*, 1996). It was further proposed that this enzyme is required to hydroxylate 6-deoxoCT to 6-deoxoTE since the mutation of this gene results in a dwarf phenotype, even when both pathways are functional in Arabidopsis. Based on these observations we proposed that *DPY* might be the tomato homologue of *CPD*.

To verify this assumption we transformed *dpy* with *CPD* cDNA to determine if the mutation could be complemented. We used the same construct and Agrobacterium strain that was successfully used to complement the Arabidopsis *cpd* mutation (Szekeres *et al.*, 1996). This experiment resulted in the recovery of transgenic plants that had no phenotypic differences when compared to the untransformed *dpy*. Although it might be premature to draw absolute conclusions since we had only three transgenic lines that were recovered from each transformation, and expression levels of the transformed gene were not experimentally determined. Hence, we cannot rule out the possibility of the tomato protein that cross-reacts with the Arabidopsis CPD antibodies actually being the CPD homologue in tomato and that it might be functionally inactive due to a mutation in the protein. Due to difficulty in evaluating the activity of the CPD protein the best approach to resolve these questions would be to clone the *DPY* gene.

Using antibodies to *CPD* protein, we observed that *dpy* actually had more protein cross-reacting to the *CPD* antibody than the wildtype (Fig 6). The *Arabidopsis CPD* gene has been demonstrated to be negatively regulated by BR (Mathur *et al.*, 1998). This suggests that due to BR deficiency, BR feed back regulation of the *CPD* gene might not be functional and, may result in over accumulation of *CPD* protein in *dpy* when compared with protein levels in the wildtype. Based on these two observations, we propose that *dpy* and *CPD* are unlikely to be homologues; of course the only way to be certain about this is to do protein activity assays. *CPD* is a member of the cytochrome P450 gene family, and activity assays may not be feasible, since this family is large and specificity of the assay is difficult to control.

Instead, *DPY* enzyme might be acting on intermediates one or two steps before the *CPD* enzyme. It is also possible that there are intermediate steps in the conversion of 6-deoxoCT to 6-deoxoTE that have not been demonstrated yet. This idea is encouraged by a recent report showing that the conversion of CR to CN, in the early steps of the pathway, actually has three more intermediate stages than originally thought (Noguchi *et al.*, 1999), and that DET2, which was thought to be solely responsible for converting CR to CN, is only involved in the conversion of 4-en-3-one to 3-one (Noguchi *et al.*, 1999). It is also possible that *DPY* might encode a regulatory protein that is required for the transcription or activity of the true *CPD* homologue.

Since there is no direct evidence for the existence of other intermediates between CT and TE intermediates, cloning and sequence of the *DPY* gene will

go a long way in answering these questions. We have tried to transposon-tag the gene (Fig. 7), but have not been successful in isolating a tagged-allele of *DPY* yet. We have only screened 12,000 seeds to date, and have not yet reached the numbers that increase the odds of find the tagged allele. Other mutants similar to *dpy*, including *dwarf*, have been cloned using this approach (Bishop *et al.*, 1996). The *DWARF* gene encodes a cytochrome P-450 and feeding experiment have shown that this enzyme is responsible for the conversion of 6-deoxoCS to CS (Bishop *et al.*, 1999). Although both *dwarf* and *dpy* have been mapped to similar regions of the long arm of chromosome 2 (Miller and Tanksley, 1990), allelism tests (Yu, 1998) have shown that they are not alleles. Fine mapping of *dpy* might give us better information to use the correct *Ac* tagged line that is most proximal to the *DPY* gene.

However, before continuing the transposon-tagged approach, I would redo the mapping of the *dpy* mutation to confirm the report that it is localized to chromosome 2 specifically. Since working with the *dpy* population, I have observed that there is some segregation with respect to height in the population. This was also observed by Cerny (1997), who identified *dpy* to be BR-deficient. However, he observed that ratio of segregation in the population to be 1: 49 (short: tall). On the contrary, my observation is that the segregation ratio is more like 1:3. To better characterize the genetics of the segregation I did progeny testing to identify homozygous lines within the population that are not segregating. However, progeny testing did not yield a clear picture of the

genetics and the only interpretation I could draw was that the segregation pattern does not fit Mendelian ratios.

In summary, we have documented physiological and biochemical evidence to prove that *dpy* is a BR-deficient mutant. This contributes to the growing evidence that BRs are critical for the growth and development of normal tomato similar to the roles played by BRs in Arabidopsis growth and development. Cloning of the *DPY* gene will be very helpful in further characterizing this mutation, contributing towards better understanding of BR biosynthesis.

### **Acknowledgments**

I would like to thank Dr. Csaba Koncz for providing the Arabidopsis CPD antibodies and the Agrobacterium strain containing the Arabidopsis *CPD* cDNA that was used in the transformation experiments.

I would also like to thank Drs. Jonathan Jones and Gerard Bishop for supplying the transposon lines that were used in the transposon-tagging experiment.

### **Literature cited**

**Bishop, G.J., Harrison, K., and Jones J.D.G.** (1996). The tomato *Dwarf* gene isolated by heterologous transposon tagging encodes the first member of a new cytochrome P450 family. *Plant Cell* 8: 959-969.

**Bishop, G.J., Nomura, T., Yokota, T., Harrison, K., Noguchi, T., Fujioka, S., Takasuto, 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:

- Bradford, M.M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72: 248-254.
- Cerny, R.E.** (1997). Physiological and molecular analysis of Brassinosteroid mutants and developmental genes in *Lycopersicon esculentum*. MS thesis. North Carolina State Univ.
- Chang, C., Kwok, S.F., Bleeker, A.B., and Meyerowitz, E.M.** (1993). *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. *Science* 262: 539-544.
- Choe, S., Dilkes, B.P., Fujioka, S., Takasuto, S., Sakurai, A., and Feldmann, K.A.** (1998). The *DWF4* gene of *Arabidopsis* encodes a phytochrome P450 that mediates multiple 22-hydroxylation steps in brassinosteroid biosynthesis. *Plant Cell* 10: 231-243.
- Choe, S., Dilkes, B.P., Gregory, B.D., Ross, A.S., Takasuto, S., Tanaka, A., Yoshida, S., Tax, F.E., and Feldmann, K.** (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., Takasuto, S., Tissier, C., Gregory, B.D., Ross, A.S., Tanaka, A., Yoshida, S., Tax, F.E., and Feldmann, K.** (1999b). The *Arabidopsis dwarf7/ste1* mutant is defective in the  $\Delta 7$  sterol C-5 desaturation step leading to brassinosteroid biosynthesis. *Plant Cell.* 11: 207-221.
- Choi, Y.-H., Fujioka, S., Harada, A., Yokota, T., Takasuto, S., and Sakurai, A.** (1996). A brassinolide biosynthetic pathway via 6-deoxocastasterone. *Phytochemistry* 43: 593-596.
- Choi, Y.-H., Fujioka, S., Nomura, T., Harada, A., Yokota, T., Takasuto, S., and Sakurai, A.** (1997). An alternative brassinolide biosynthetic pathway via late C6-oxidation. *Phytochemistry* 44: 609-613.
- Clouse, S.D.** (1996). Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. *Plant J.* 10: 1-8.
- Clouse, S.D., and Sasse, J.M.** (1998). Brassinosteroids: Essential regulators of plant growth and development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49: 427-451.
- 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). Springer-Verlag, Tokyo, pp 163-190.

- DellPenna, D., Lincoln, J.E., Fisher, R.L., and Bennett, A.B.** (1989). Transcriptional analysis of polygalacturonase and other ripening associated genes in Rutgers, *rin*, *nor*, and *Nr* tomato fruit. *Plant Physiol.* 90: 1372-1377.
- Fujioka, S and Sakurai, A.** (1997a). Biosynthesis and metabolism of brassinosteroids. *Physiologia Plantarum* 100: 710-715.
- Fujioka, S and Sakurai, A.** (1997b). Brassinosteroids. *Nat. Prod. Rep.* 14: 1-10.
- Fujioka, S., Inoue, T., Takasuto, S., Yanagisawa, T., Yokota, T., and Sakurai, A.** (1995). Biological activities of biosynthetically-related congeners of brassinolide. *Biosci. Biotech. Biochem.* 59: 1973-1975.
- Fujioka, S., Li, J., Choi, Y.-H., Seto, H., Takasuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J., and Sakurai, A.** (1997). The *Arabidopsis deetiolated2* mutant is blocked early in the brassinosteroid biosynthesis. *The Plant Cell* 9: 1959-1962.
- Gallagher, S.R., and Smith, J.A.** (1994). Analysis of proteins. In: *Current protocols in molecular biology*. K. Janssen, F.M. Ausubel, *et al.* (eds). 2: 10.2.1-10.2.21
- Hernandez-Bravo, G.** (1967). Two new dwarf mutants and their linkage relations. *Rep. Tomato Genet. Coop.* 17: 30-31.
- Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J., and Jones, J.D.G.** (1994). Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* 266: 789-793
- 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
- Kelly, M.O., and Bradford, K.J.** (1986). Insensitivity of *diageotropica* tomato mutant to auxin. *Plant Physiol.* 82: 713-717.
- Klahre, U., Noguchi, T., Fujioka, S., Takasuto, S., Yokota, T., Nomura, T., Yoshida, S., Chua, N.-H.** (1998). The *Arabidopsis Diminuto/DWARF1* gene encodes a protein involved in steroid synthesis. *Plant Cell* 10: 1677-1690.
- Knocz, C., Martini, N., Szabados, L., Hroudá, M., Bachmair, A., and Schell, J.** (1994). Specialized vectors for gene tagging and expression studies. In *Plant Molecular Biology Manual*, S.B. Gelvin and R.A. Schilperoort, (eds.) Dordrecht, The Netherlands: Kluwer Academic. B2: pp 1-22.
- Koornneef, M., Bosma, T.D.G., Hanhart, C.J., van der Veen, J.H., and Zeevart, J.A.D.** (1990). The isolation and characterization of gibberellin-deficient mutants in tomato. *Theor. Appl. Genet.* 80: 852-857.

- Lawrence, G.J., Finnegan, E.J., Ayliffe, M.A., and Ellis, J.G.** (1995). The *L6* gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. *Plant Cell* 7: 1195-1206.
- Mathur, J., Molnar, G., Fujioka, S., Takasuto, 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.
- Miller, J.C., and Tanksley, S.D.** (1990). RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon*. *Theor. Appl. Genet.* 80: 437-448.
- Murashige, T., and Skoog, F** (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15: 473-497.
- Noguchi, T., Fujioka, S., Takasuto, S., Sakurai, A., Yoshida, S., Li, J.M., and Chory, J.** (1999). *Arabidopsis det2* is defective in the conversion of (24*R*)-24-methylcholest-4-en-3-one to (24*R*)-24-methyl-5 $\alpha$ -cholestan-3-one in brassinosteroid biosynthesis. *Plant Physiol.* 120: 833-839.
- Nomura, T., Kitasaka, Y., Takasuto, 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.
- Parry, A.D., Griffiths, A., and Horgan, R.** (1992). Abscisic acid biosynthesis in roots. II. The effects of water-stress in wild-type and abscisic-acid-deficient mutant (*notabilis*) plants of *Lycopersicon esculentum* Mill. *Planta* 187: 192-197.
- Scofield, S., Harrison, K., Nurrish, S.J., and Jones, J.D.G.** (1989). Molecular cloning: a laboratory manual, 2<sup>nd</sup> edn. Cold spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Szekeres, M., Nemeth, K., Koncz-Kalman, 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.
- Taylor, I.B., Linforth, R.S.T., Al-Naieb, R.J., Bowman, W.R., and Marple, B.A.** (1988). The wilty tomato mutants *flacca* and *sitiens* are impaired in the oxidation of ABA-aldehyde to ABA. *Plant Cell Environ* 11: 739-745.
- Thomas, C.M., Jones, D.A., English, J.J., Carroll, B.J., Bennetzen, J.L., Harrison, K.A., Burbidge, A., Bishop, G.J., and Jones, J.D.G.** (1994). Analysis of the chromosomal distribution of transposon-carrying T-DNAs in tomato using the inverse polymerase chain reaction. *Mol. Gen. Genet.* 242: 573-585.

- Waterman, M.R., and Bischof, L.J.** (1997). Diveristy of ACTH (cAMP)-dependent transcription of bovine steroid hydroxylase genes. *FASEB J.* 11: 419-427.
- Whithman, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C., and Baker, B.** (1994). The product of the tobacco mosaic virus resistance gene *N*: similarity to Toll and the interleukin-1 receptor. *Cell* 78: 1011-1115.
- Wilkinson, J.Q., Lanahan, M.b., Yen, H., Giovannoni, J.J., and Klee, H.J.** (1995). An ethylene-inducible component of signal transduction encoded by *Never-ripe*. *Science* 270: 1807-1809
- Yokota, T.** (1997). The structure, biosynthesis and function of brassinosteroids. *Trends Plant Sci.* 2: 137-143.
- Yokota, T., Nomura, T., and Nakayama, M.** (1997) Identification of brassinosteroids that appear to be derived from campesterol and cholesterol in tomato shoots. *Plant Cell Physiol.* 38: 1291-1294.
- Yu, M.H.** (1988). Genetic analysis of the dwarf curly leaf tomato mutant via hybridization with *Curl* and *dwarf* mutants

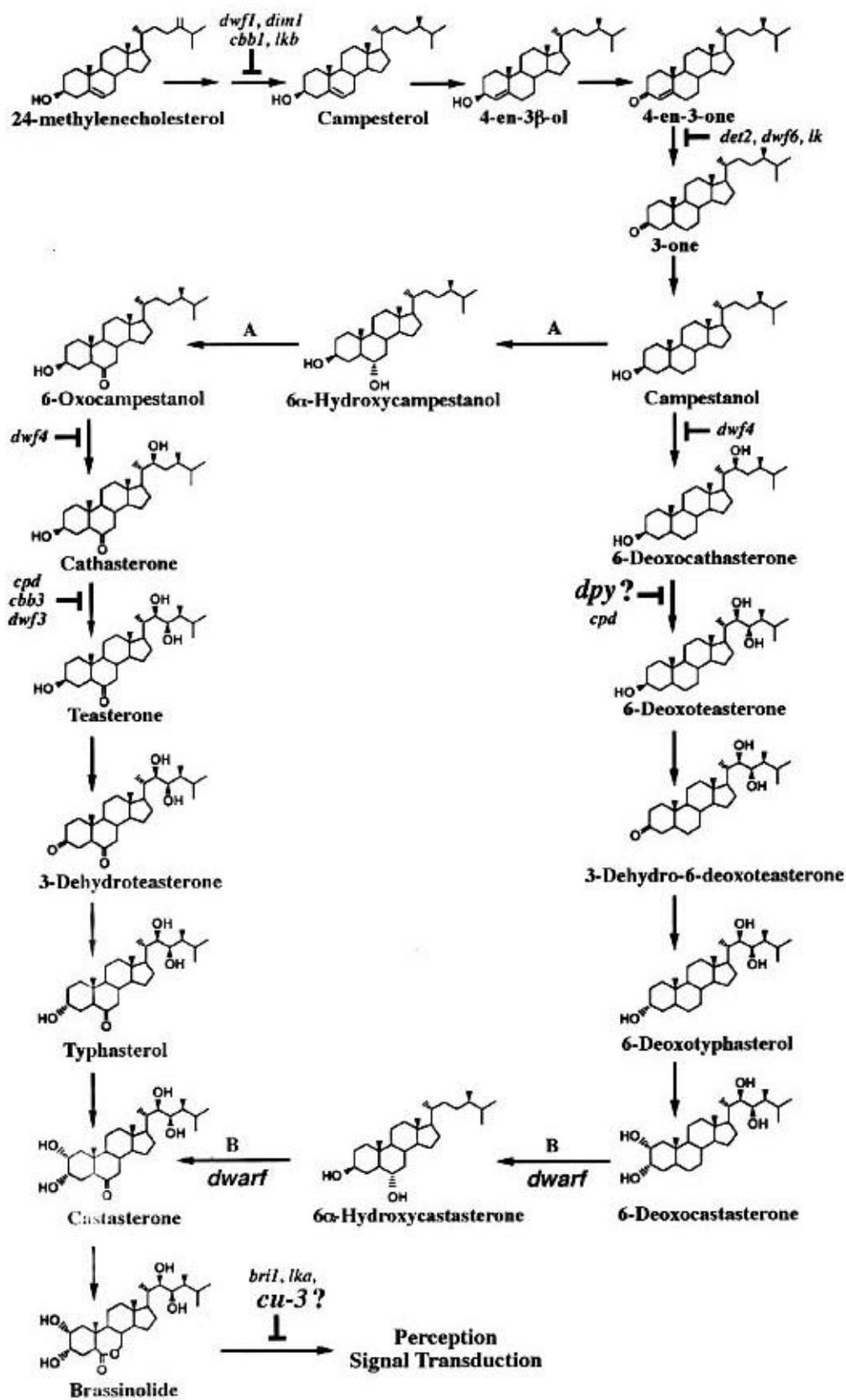
## Tables

**Table I.** *Endogenous levels of sterols and brassinosteroids in dpy and wild-type tomatoes*

Compound	Wildtype	<i>dpy</i>	Wildtype/ <i>dpy</i>
<i>Sterols (ng/g fresh weight)</i>			
24-methylenecholesterol	351	393	0.89
campesterol	2600	2500	1.04
campestanol	33	29	1.14
<i>Brassinosteroids (pg/g fresh weight)</i>			
6-deoxocathasterone	1200	2600	0.46
6-deoxoteasterone	65	27	2.41
6-deoxocastasterone	907	36	25.19
castasterone	66	n.d. <sup>a</sup>	

<sup>a</sup>none detected

## Figures



**Figure 1.** Brassinolide biosynthetic pathway displaying both early (**A**) and late (**B**) C6 oxidation. Putative location of the deficient and insensitive Arabidopsis, pea (*lk*, *lka*, and *lkb*) and tomato (*dpy*, *dwarf* and *cu-3*) mutants are indicated.

A

*dpy**dpy + BL*

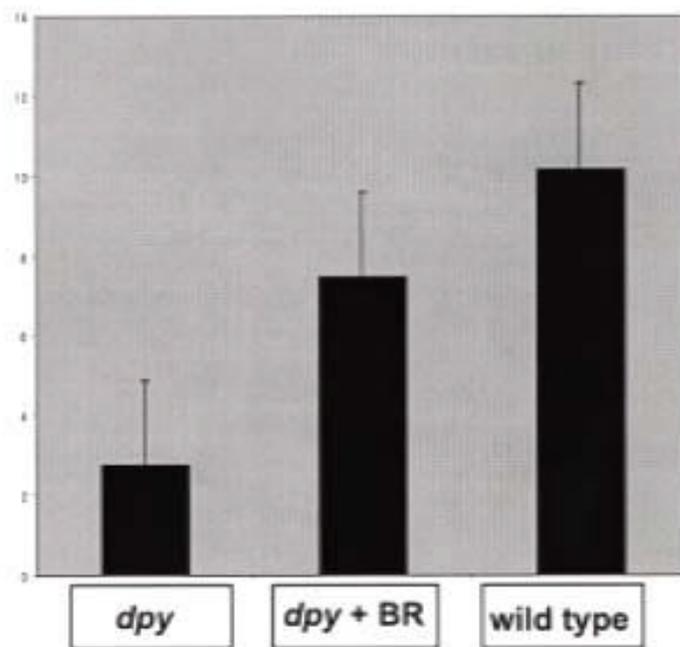
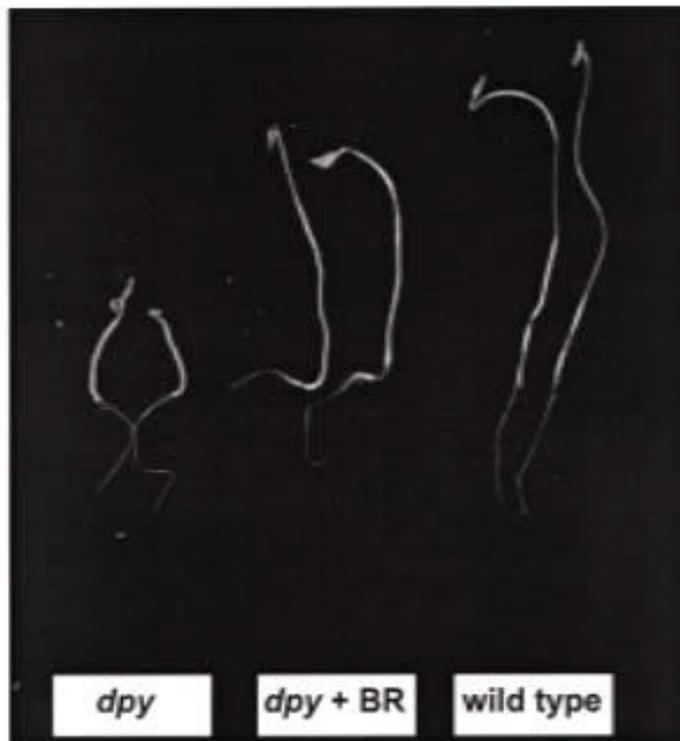
Wildtype

B

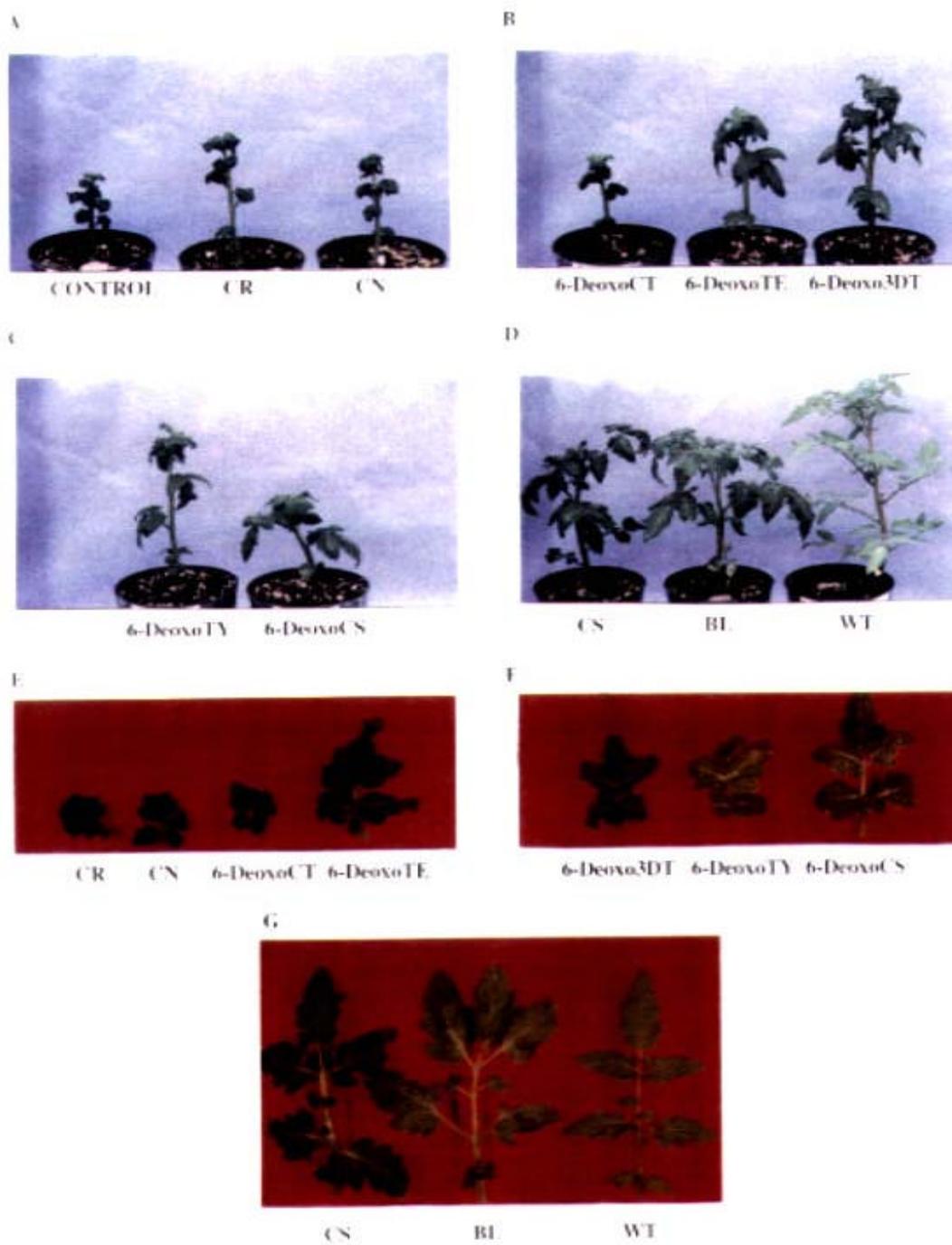
*dpy**dpy + BL*

Wildtype

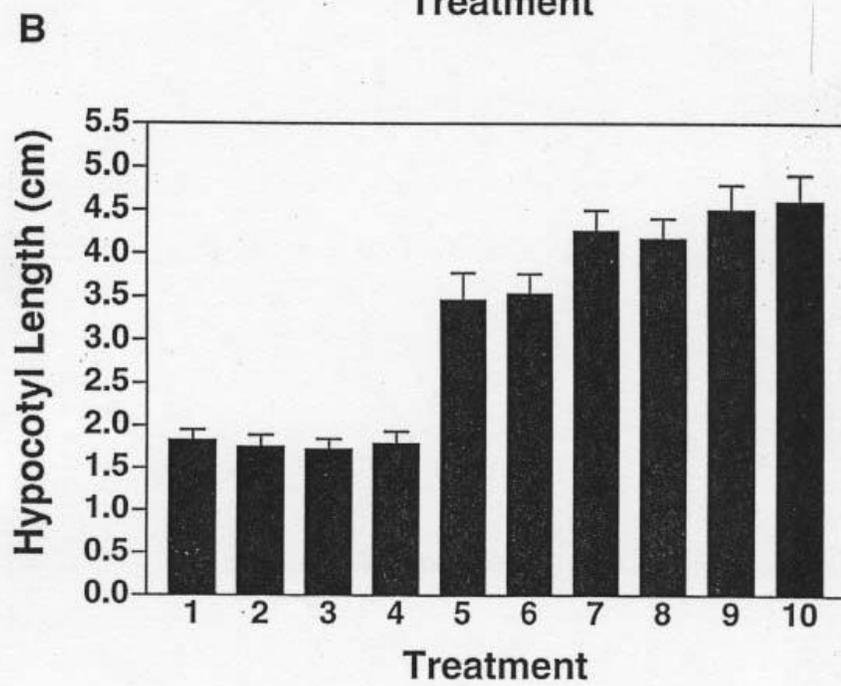
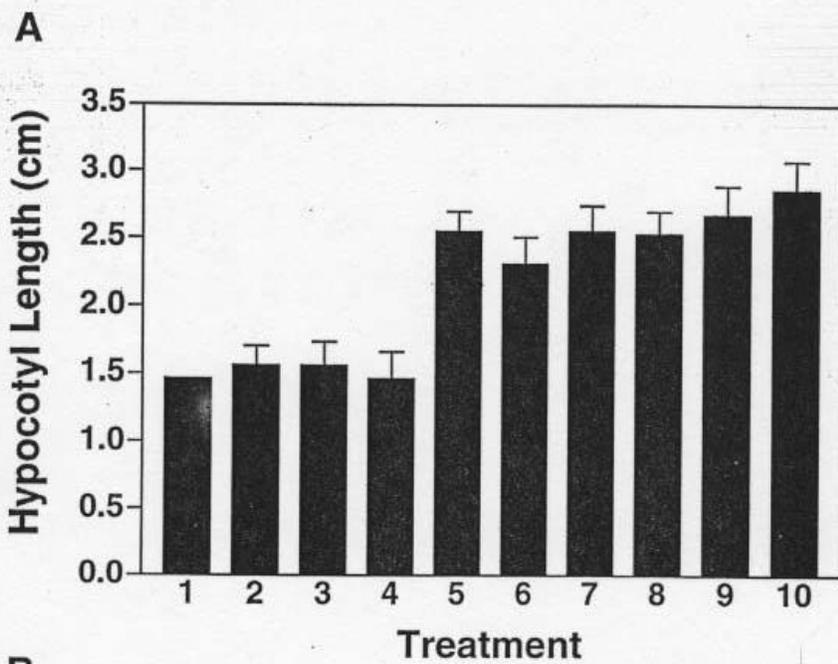
**Figure 2.** Characterization of light-grown *dumpy* (*dpy*) mutant. **(A)** Three-week-old plants were treated with solvent or 0.1  $\mu$ M BL, twice daily for additional 21d, resulting in nearly complete rescue of the *dpy* phenotype to wildtype. **(B)**. Close-up of morphology of the third true leaves from plants treated in (A).



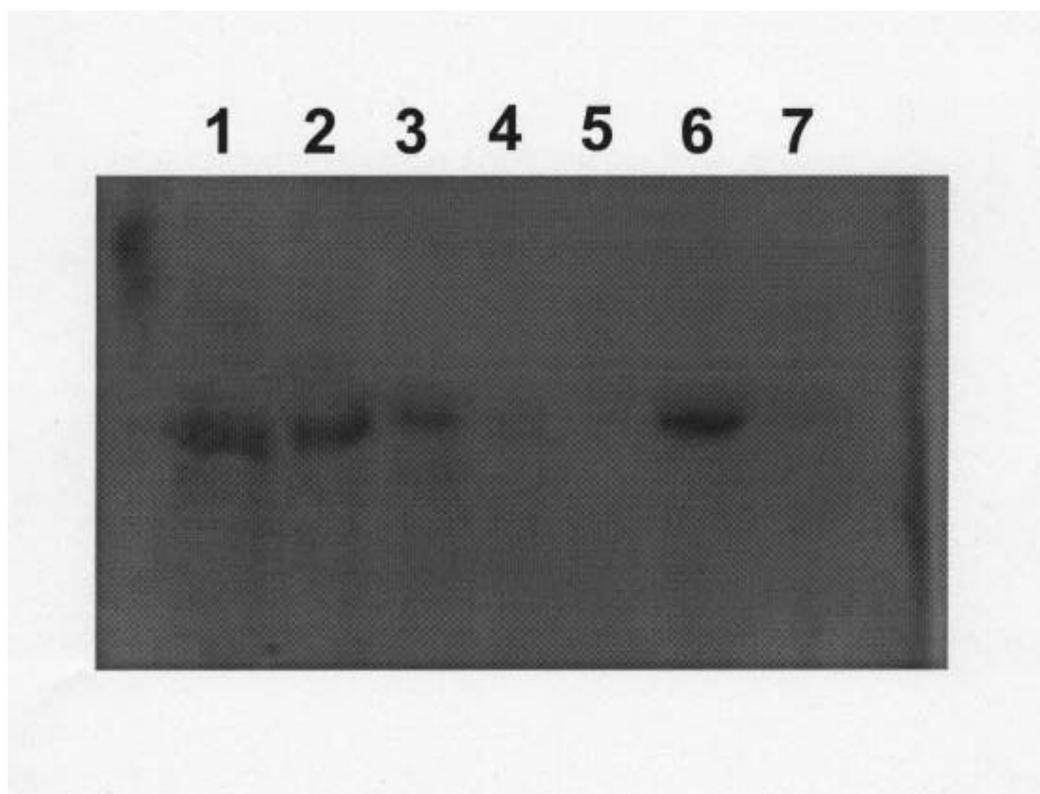
**Figure 3.** Characterization of dark grown *dpy* mutant. **(A)** Seedlings grown in the dark for 11 d on the surface of agar media containing 2% sucrose, half-strength MS and 1% agar in vertically oriented Petri plates, show nearly complete rescue to wild-type length when 0.2  $\mu$ M BL is included in the media. **(B)** Hypocotyl length of 10 seedlings for each treatment described in (A) was measured to the nearest mm +/- SE.



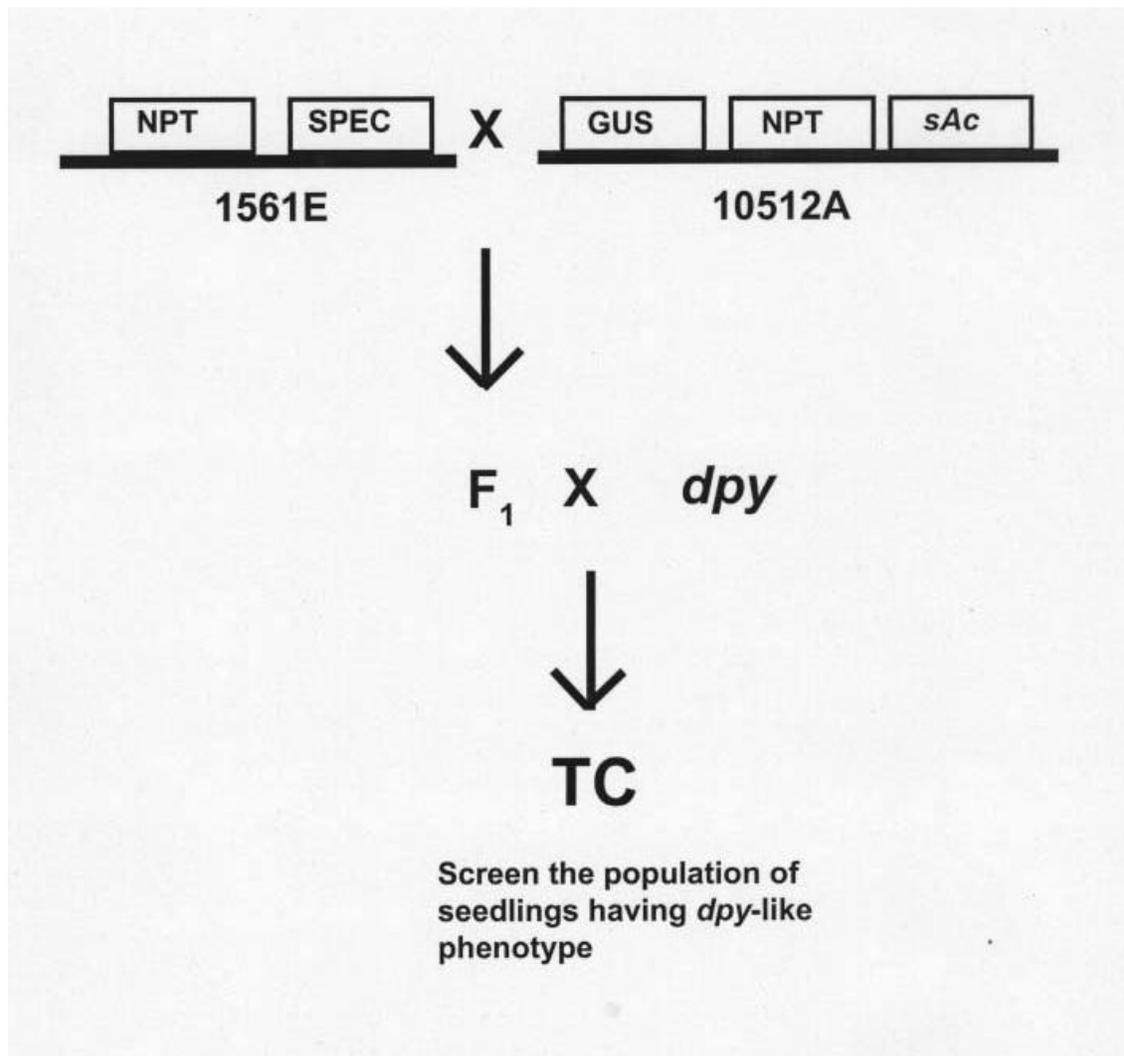
**Figure 4.** Feeding experiments with light-grown *dpy* plants. **(A-D)** Twenty-one days after planting in the greenhouse, intermediates of the BL biosynthetic pathway were applied to *dpy* plants twice daily for an additional period of 21 d. CR through 6-deoxoTY were applied at 1.0  $\mu\text{M}$  and 6-deoxoCS, CS and BL were applied at 0.1  $\mu\text{M}$ . Control and wild-type plants were sprayed with solvent only. **(E-G)** Close-up of the third true leaves from plants in A-D.



**Figure 5.** Feeding experiments with dark-grown *dpy* plants. **(A)** *dpy* seeds were sterilized and plated on vertical Petri plates containing various BL biosynthetic intermediates in 1% agar, 3% sucrose, half-strength MS media, pH 5.7. Seedlings were grown in complete darkness for 9 d. Hypocotyl length of 10 seedlings for each treatment was measured to the nearest mm, +/-SE. **1**, solvent control; **2**, CR; **3**, CN; **4**, 6-deoxoCT; **5**, 6-deoxoTE; **6**, 6-deoxo3DT; **7**, 6-deoxoTY; **8**, 6-deoxoCS; **9**, CS; **10**, BL. Intermediates **2-9** were applied at 1.0  $\mu$ M and BL was applied at 0.5  $\mu$ M. **(B)** Hypocotyl length of 10 seedlings +/- SE grown in shaking liquid culture in the dark for 9 d. Biosynthetic intermediate concentrations are as described in (A).



**Figure 6.** Western Blot analysis of *dpy* and wild-type tomato leaves. Fifteen  $\mu\text{g}$  of tomato protein sample and 20-30  $\mu\text{g}$  of Arabidopsis protein per lane was loaded. After transfer onto the nitrocellulose membrane, the blot was hybridized with polyclonal antibodies raised against Arabidopsis *CPD* protein. Lane **1-3** are loaded with Arabidopsis protein 30, 25 and 20  $\mu\text{g}$ , respectively; Lanes **4** and **5** are wildtype tomato mature leaf and young leaf, respectively; Lanes **6** and **7** are *dpy* tomato mature and young leaf, respectively.



**Figure 7.** Experimental strategy for the generation of transposon tagged *DPY* gene. The *sAc*-containing transgenic line SLJ10512A was crossed with SLJ1561E line containing the *Ds* element (F1). The F1 plants were crossed with homozygous *dpy* plants to yield the Test Cross (TC) progeny, which was screened for plants with *dpy* phenotype.

## **Chapter 3**

# **Cloning and Characterization of Brassinosteroid- regulated Genes from Tomato**

## Introduction

Brassinosteroids (BRs) are a group of naturally occurring plant growth regulators that are structurally similar to steroid hormones in animals and insects (Yokota, 1997). BRs are ubiquitously distributed in the plant kingdom, and when applied exogenously at nanomolar to micromolar levels, they exhibit a wide spectrum of physiological effects, including promotion of cell elongation, cell division, tracheary element differentiation, ethylene biosynthesis and retardation of abscission (Clouse and Sasse, 1998; Yokota, 1997). As described in Chapter One, elucidation of the biosynthetic pathway (Fujioka and Sakurai, 1997), along with the characterization of different BR-biosynthetic and BR-insensitive mutants in *Arabidopsis thaliana* (Clouse and Feldmann, 1999), has contributed towards understanding the mechanism of BR action.

One aspect of BR research that is particularly interesting to us is to elucidate the mode of action and signal transduction for these steroids in plants. Much is known about the mode of action and signal transduction of steroids in animals but little is known as yet about the corresponding role of steroids in plants. To date very few BR-regulated genes have been identified. The first BR-regulated gene to be cloned, *BRU1* (*BR Upregulated-1*), was isolated from soybean epicotyls (Zurek and Clouse, 1994). It encodes a xyloglucan endotransglycosylase (XET), having highest sequence identity to *XTR-7*, *XTR-6* (Xu et al., 1996), and *meri-5* (Medford et al., 1991) from *Arabidopsis thaliana* and an XET from nasturtium seeds (de Silva et al., 1993). It is interesting to note that

the first BR-regulated gene to be isolated is likely involved in cell elongation, the most obvious effect of exogenous application of BR. Zurek and Clouse (1994) have also demonstrated that BR post-transcriptionally regulates expression of *BRU1*.

The Arabidopsis *TCH4* gene, another member of the XET gene family which is up-regulated in response to touch, has also been shown to be up-regulated by BR (Xu *et al.*, 1995). Besides touch and BR, this gene also responds to dark, heat shock, cold stress, and auxin. The kinetics of response of *TCH4* to auxin and BR were different. The response to auxin was very rapid compared to BR. In contrast to *BRU1*, BR transcriptionally regulates expression of *TCH4*.

*CPD*, an Arabidopsis gene that encodes a protein involved in the BR biosynthetic pathway (Szekeres *et al.*, 1996), has been shown to be negatively regulated by BRs (Mathur *et al.* 1998). This suggests that the biosynthetic pathway might be feedback regulated. In this case, it was shown that *de novo* protein synthesis was required for repression to occur, indicating that the BR regulation is a secondary effect.

Genes from differentiating *Zinnia elegans* cells, phenylalanine ammonia-lyase (*ZePAL3*) (Yamamoto *et al.*, 1997), cinnamic acid 4-hydroxylase (*ZC4H*) (Ye *et al.*, 1994), and *ZCP4* encoding a cysteine protease, have been demonstrated to also be regulated by BR (Yamamoto *et al.*, 1997).

The objective of this study was to clone BR-regulated genes from tomato to contribute towards understanding the signal transduction pathway involving BRs. Initially we used differential display with wild-type Arabidopsis (ecotype Columbia) as the model system. Although there were many putative differentially expressed bands, northern analysis using these PCR products as probes revealed that none of the bands were truly regulated by BR. The previously described tomato BR-deficient mutant (*dumpy*) (Cerny, 1997), was used as source tissue since it was likely to have lower levels of endogenous BR activity and so will have very low endogenous expression levels of BR-regulated genes. However, not having much success with differential display, we changed to a PCR-based cDNA subtraction procedure. We report here the cloning and characterization of two BR-regulated genes in tomato (*Lycopersicon esculentum* Mill.).

## **Materials and Methods**

### **Subtractive Hybridization**

Total RNA from the 1 and 4 h control or BR-treated *dpy* tissue, as described in Chapter 2, was used for polyA<sup>+</sup> RNA isolation (Promega PolyA<sup>+</sup> Isolation Kit, Cat. # Z5200, Madison, WI). The 1 and 4h polyA<sup>+</sup> RNA samples corresponding to either control or BR-treated tissue, were pooled and used as driver (control) and tester (BR-treated) following the PCR-Select™ cDNA Subtraction Kit protocol as described by the manufacturer (Catalog # K1804-1, Clontech, Palo Alto, CA). A portion of the subtracted cDNA thus generated was

cloned into the pCR-Script vector using the pCR-Script cloning kit (Stratagene, La Jolla, CA). Differential screening of 96 independent colonies was performed as described in the PCR-Select™ instruction manual.

### **Differential Display**

We used the mRNA Differential Display kit manufactured by GenHunter Inc. (Nashville, TN) following the recommendations of the manufacturer. This method involves reverse transcription of mRNAs with oligo-dT primers anchored to the beginning of the poly (A) tail, followed by a PCR reaction in the presence of a second arbitrary 10mer. The amplified cDNA subpopulations of 3' termini of mRNAs as defined by these pairs of primers are displayed on a DNA sequencing gel. Different primer combinations are used in the PCR reaction to cover the entire poly (A) population. mRNA samples analyzed side by side allow differentially expressed genes to be identified.

### **RNA Isolation and Analysis**

Wildtype or *dpy* seeds were sterilized as described in Chapter 2 and placed in 50 ml of half-strength MS media in Magenta jars. The jars were incubated in a growth chamber at 24°C under 50  $\mu\text{Em}^{-2}\text{s}^{-1}$  light intensity (16h light/8h dark). After 21d, plants were sprayed until run-off with a solvent control (0.001% ethanol, 0.1% Tween 20) or with  $10^{-7}$  M brassinolide (BL). The apices of the plant (the apical meristem and the immature first true leaf) were then harvested at 0, 1, 2, 4, 8, 12, and 24 h after treatment. Total RNA was isolated by

grinding the tissue in liquid nitrogen, followed by homogenization in 5 ml of fresh extraction buffer (1% tri-isopropyl-naphthalene sulphonic acid; 6% p-aminosalicylic acid; 100mM Tris-HCl, pH 7.8; 50 mM EDTA; 10mM NaCl; 1% SDS; 0.1 M  $\beta$ -mercaptoethanol) and 5 ml of phenol: chloroform: isoamyl alcohol (25:24:1). After centrifugation, the aqueous phase was re-extracted with phenol: chloroform: isoamyl alcohol and then subjected to three successive precipitations with ethanol/sodium acetate, 4.0 M LiCl, and ethanol/sodium acetate. The final pellet was resuspended in DEPC-treated water.

### **Probe Preparation**

Stratagene's Random-primer labeling kit was used following the recommended protocol. We used 20-100  $\mu$ g of template DNA along with 50  $\mu$ Ci of  $\alpha$ -<sup>32</sup>P-dCTP (3000 Ci/mmol) per reaction and each reaction was incubated at room temperature for 30-60 min. Probes were purified using Stratagene's Push Column purification system following the manufacturer's recommendations. Specific activity of the probe was determined by Liquid Scintillation Counting.

### **Northern Analysis**

Total RNA was separated on 1.2% Agarose gels containing 3% formaldehyde (37%) in 1x MOPS buffer. Total RNA (15-30  $\mu$ g/lane, depending on the experiment) was mixed with 10-20  $\mu$ l RNA Loading mix (50% formamide, 20% formaldehyde (37%), and 10% 400  $\mu$ g/ml ethidium bromide in 1x MOPS).

This was heated at 60-65°C for 15 min and loaded on to the gel after cooling.

The gel was run at 100-125 V for approximately 3 h.

Total RNA was transferred onto Zeta-probe membranes (Biorad, Richmond, CA) by employing capillary transfer in alkali (50mM NaOH in DEPC H<sub>2</sub>O) for 4-6 h.

### **Hybridization Conditions**

The membrane containing total RNA was incubated in a Hyb-Aid incubator (Midwest Scientific, St. Louis, MO), in 10-20 ml of pre-hybridization solution (50% formamide, 1% SDS, 5x Denhardt's solution, 5x SSC in DEPC H<sub>2</sub>O, 100 µg/ml denatured salmon sperm DNA) for 3 h at 42°C. After pre-hybridization, 1-3 x 10<sup>6</sup> cpm/ml of denatured α-<sup>32</sup>P labeled probe was added to the pre-hybridization solution and incubation was continued at 42°C overnight. The membrane was rinsed once in 2x SSC, 0.1% SDS at room temperature, followed by washes of 2x SSC, 0.1% SDS at room temperature for 20 min, 0.2x SSC, 0.1% SDS at 42°C for 15 min, and a final wash in 0.2x SSC, 0.1% SDS at 55°C for 15 min. The membrane was exposed to X-ray film at -80°C or to phosphor-imager screens at room temperature for detecting the signal.

### **DNA Extraction**

Total genomic DNA was extracted from young expanding tomato leaves following the methods of Thomas et al., (1994) and, Bernatzky and Tanksley (1986). The harvested leaves were ground in liquid nitrogen, using a mortar and

pestle, and transferred to 50-ml centrifuge tubes. The frozen powder was mixed with 10 ml of extraction buffer (100 mM Tris-HCl pH 7.5, 100 mM EDTA pH 7.5 to 8.0, 250 mM NaCl, 1.0%  $\beta$ -mercaptoethanol) and centrifuged at 1,800 x g for 10 min. The aqueous phase was transferred to a fresh tube and extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). DNA was precipitated using 100  $\mu$ l of 3M sodium acetate per ml of aqueous extract, plus 2 volumes of 100% ethanol and centrifuged at 9,200x g for 10 min at 4°C. The pellet was resuspended in 300  $\mu$ l of sterile water with RNase A and incubated at 37°C for 15 min. Later, 300  $\mu$ l of CTAB (200 mM Tris-HCl, pH 7.5; 50 mM EDTA; 2.0 M NaCl; 2% w/v hexadecyltrimethylammonium bromide) was added and samples were incubated at 65°C for 15 min. Samples were then extracted with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) and DNA was precipitated using an equal volume of isopropanol. The pellets were washed in 75% ethanol, dried, and finally resuspended in 200  $\mu$ l of Nano-Pure™ water.

### **Southern Blot Analysis**

Plant DNA (10 $\mu$ g) was digested with restriction endonucleases according to manufacturer's directions (Promega, Madison, WI and New England Biolabs, Beverly, MA) for >2 h. Restriction fragments were separated on a 0.8% Agarose gel by electrophoresis at 35 V. The DNA was then transferred to Zeta-Probe membrane Blotting Membranes (Biorad, Richmond, CA) according to manufacturer's instructions for alkaline blotting. The hybridization conditions were identical to the northern hybridization described above. For low-stringency

hybridization, hybridization temperature was 37°C and the final wash was 42°C. For high-stringency hybridization, hybridization temperature was 42°C and the final wash was 55°C. The blot was exposed to x-ray film at -80 °C.

### **Synthesis of Anti-sense RNA probes**

RNA probes were prepared using the MAXIscript™ Invitro transcription kit (Ambion Inc. Austin, TX), following manufacturer's directions. The template DNA (if a plasmid) was linearized using appropriate restriction endonucleases that would result in a cut distal to either the T3 or T7 promoter, as appropriate. When the template was a PCR product, the T7 promoter was included in the primer in the direction that would yield an anti-sense strand when transcribed by T7 DNA polymerase. [ $\alpha$ -<sup>32</sup>P] UTP at approximately 800 Ci/mmol was used as the labeled nucleotide. The reaction was incubated at room temperature for >3 h, followed by incubation with DNase I at 37°C for 15 min. The probes used for RNA-protection were gel-purified before use.

Gel-purification was done as follows: The reaction was mixed with an equal volume of gel-loading dye, heat denatured at 95°C for 3-4 min, loaded on to a 0.75 mm thick 5% polyacrylamide 8 M urea gel and run for 20-30 min at 200 V. The gel was exposed to x-ray film for 1-2 min and developed. The film was super-imposed on the gel and the gel slice corresponding to the highest band on the film was excised from the gel and transferred to a fresh microcentrifuge tube. Then, 350  $\mu$ l of probe elution buffer was added to the slice followed by incubation at room temperature overnight to elute the probe. The following day, a 1  $\mu$ l

aliquot was mixed with 10 ml of liquid scintillation fluid and specific activity was estimated by liquid scintillation spectrometry.

### **RNA Protection Assay**

The RPA III Ribonuclease Protection Assay kit (Ambion Inc., Austin, TX) was used for RNA protection assays following the manufacturer's instructions. Total RNA samples (5-15  $\mu\text{g}$ ) were used with  $5 \times 10^4$  to  $10 \times 10^5$  cpm of probe per 10  $\mu\text{g}$  of sample RNA. For each experiment, two control tubes containing the same amount of labeled probe mixed with equivalent amounts of yeast RNA were included. The RNA samples and the probe were co-precipitated using 0.5 M  $\text{NH}_4\text{OAc}$ , 2 volumes of 100% ethanol and centrifuged for 15 min at 16,000x g, at 4°C, after incubating the mix at -20°C for at least 20 min. The supernatant was discarded and the pellets were resuspended in 10  $\mu\text{l}$  of hybridization buffer. Samples were then incubated overnight at 42°C following a 3-4 min incubation at 95°C.

RNase Digestion III Buffer was used to prepare a 1:100 dilution of RNase A/RNase T1 mix, and 150  $\mu\text{l}$  of the diluted RNase solution was added to each sample RNA tube and one of the yeast RNA tubes. The other yeast RNA tube was mixed with 150  $\mu\text{l}$  of RNase Buffer without RNase. The tubes were incubated at 37°C for 30 min. The samples were then mixed with 225  $\mu\text{l}$  RNase Inactivation/ Precipitation III solution and incubated at -20°C for 15 min, followed by centrifugation at 16,000 x g for 15 min at 4°C. The supernatant was carefully removed and the pellet was resuspended in 10  $\mu\text{l}$  of gel loading buffer and

denatured by heating to 95°C for 3-4 min. The protected fragments were separated on a 0.75 mm 5% polyacrylamide, 8 M urea gel at 200 V for 20 min-30 min. The gel was exposed to x-ray film or a phospho-imager screen for detection.

### **RACE-PCR based cloning of Full-length cDNA**

Clontech's (Palo Alto, CA) Marathon cDNA Amplification Kit was used for RACE-PCR based cloning. Poly A+ RNA isolated above was used as template for first-strand cDNA synthesis. The double-stranded (ds) cDNA was ligated to an adaptor. Gene-specific primers (5' and 3') were designed based on the partial cDNA sequence of the gene that is known at this point. Gene-specific primers (5' or 3') along with the primer for the adaptor are used in a PCR reaction with the library of adaptor-ligated cDNA (ds) as template for either 5'- or 3'-RACE PCR reaction, respectively. The RACE-PCR fragments were cloned and sequenced to confirm that the fragment is part of the gene of interest. Sequencing was performed at the Iowa State University Sequencing Center, using ABI automated sequencers. Once the sequence of the 5'-RACE, the 3'-RACE products were known, they were aligned with the partial cDNA clone sequence to yield the sequence of the full-length cDNA. Primers were then designed towards the ends of the aligned sequence to yield a PCR product that corresponds to the full-length cDNA. This was confirmed by sequencing the full-length fragment.

## Results

### Cloning of a BR-regulated XET-like gene from the *dpy* background

To identify genes that might possibly be involved in BR signal transduction cascade, we embarked upon cloning genes that respond to exogenous BR within 4 h after application. Initially we used differential display in an attempt to isolate genes that are regulated by BR. We started the study by using wild-type plants treated with or without BR. There were numerous bands that represented putative BR-regulated genes using the differential display technique (Fig 1). However, none of those bands turned out to be truly positive when tested by northern analysis.

Hence, when *dpy* was identified as a BR-deficient mutant that retains BR sensitivity, we exploited it for cloning BR-regulated genes since the reduced levels of endogenous BR's in the *dpy* mutant should accentuate the difference between control and BR-treated tissues. Based on the observation that BR sensitivity is retained only by immature tissue and that only new growth shows the response to BR in *dpy*, we used only the apices (meristem and one expanding leaf close to the meristem) as the tissue source for subtractive hybridization. We successfully cloned two genes whose transcript levels increased when *dpy* was treated with BR.

The first gene isolated, *LeBR1* showed higher levels in BR treated tissue than in the control tissue at all time points examined, from 2 to 24 h after BR application (Fig 2). Furthermore, in untreated tissue, expression of the *LeBR1* gene was reduced in the *dpy* mutant when compared to the wildtype (Fig 2A).

BLAST analysis using the translated sequence of this partial cDNA (Altschul et al., 1997) showed that *LeBR1* has 79% identity and 91% similarity to the amino acid sequence of *BRU1* gene (Fig 3). Interestingly, this gene was previously demonstrated to encode a BR-regulated xyloglucan endotransglycosylase (XET) that was cloned from soybean epicotyl tissue (Zurek and Clouse, 1994). BLAST analysis also showed that *LeBR1* shared significant sequence identity with other XET's in the database, including 75% identity/86% similarity to Arabidopsis *XTR-7* (Xu et al., 1996), 68% identity/80% similarity with *tXET-B1*, 71% identity/83% similarity with *tXET-B2* from tomato (Arrowsmith and de Silva, 1995), 68% identity/78% similarity with Arabidopsis *TCH4* (Xu et a., 1995); and 53% identity/66% similarity to *LeEXT* from tomato (Okazawa et al., 1993). Figure 4 shows the extent of sequence conservation between *LeBR1* and other XET's including the invariant DEIDFLG, which is thought to contain the active site of the enzyme (Borris et al., 1990).

### **Cloning and molecular characterization of a BR-regulated putative kinase from *dpy***

The second gene isolated, *LeBR2* also showed higher transcript levels in BR-treated tissue than in the control tissue at all time points examined, from 2 to 24h after BR-treatment (Fig 5). An RNA-Protection Assay (RPA) had to be used in the analysis of *LeBR2* since repeated attempts to characterize its expression by northern analysis yielded very weak signals. Use of RPA resulted in at least 10-fold increase in sensitivity compared to northern blots and resulted in a good

signal for documentation (Fig 5). The BR-inducibility of *LeBR2* was also evaluated in wild-type tomato. Fig 6 shows that *LeBR2* mRNA levels were higher in the wild-type BR-treated samples 4 to 12 h after BR treatment. It seems that *LeBR2* levels are more tightly regulated in the wild-type background since the degree and the duration of induction of *LeBR2* mRNA is lower and for shorter duration than in the *dpy* background. This tight regulation might be possibly due to the involvement of negative regulators of this gene.

BLAST analysis of the *LeBR2* partial cDNA showed that it likely encodes a serine/threonine protein kinase. Since this gene was of more interest than *LeBR1* (XET), we decided to clone the full-length cDNA of *LeBR2*. To achieve this objective we tried varied approaches, such as screening a tomato cDNA library, which did not yield any positives. A commercially available tomato Bacterial Artificial Chromosome (BAC) library (Research Genetics, Huntsville, AL) was screened which yielded two positive clones that hybridized to the partial cDNA probe. Multiple attempts to sub-clone the BAC fragments were not successful. Later, a tomato genomic library was screened which resulted in one positive clone. Simultaneously we tried the RACE-PCR based approach using Clontech's Marathon™ cDNA Amplification Kit, which finally resulted in the full-length cDNA clone of *LeBR2* (Fig 7). The BLAST analysis of the translated amino acid sequence of *LeBR2* shows highest sequence similarity to two Arabidopsis genomic sequences in the GenBank database. One was a locus located at the top of chromosome-I called ATY12776, (accession number Y12776) (74% identity/83% similarities) and the other was locus IG002N01 located on

chromosome-IV, (accession number AF007269) (73% identity/81% similarities). The genes with highest homology to *LeBR2* among biologically characterized genes are *APK1* from Arabidopsis with 43% identity and 65% similarity (Hirayama and Oka, 1992) and the Arabidopsis *APK2a* that interacts *in vivo* with *AGAMOUS* with 43% identity and 81% similarity (Ito et al., 1997). Fig. 8 shows the extent of sequence conservation among different serine/threonine kinases. We have identified the 11 sub-domains of the kinase domain, with asterisks indicating the most conserved residues (Hanks and Quinn, 1991).

Southern blot analysis of restriction enzyme-digested tomato genomic DNA yielded a very surprising result. Both low- and high-stringency hybridization did not show any cross-hybridization to other known members of the kinase gene family. There was only one band of very low intensity that hybridized with the partial cDNA probe of *LeBR2*, suggesting that there is a single copy of this gene in the genome (Fig 9).

### ***dpy* has reduced expression levels of *LeBR2***

The tissue specific expression patterns of the *LeBR2* gene were also evaluated. Fig 10 shows that this gene is expressed in all of the tissues examined, but that the levels of *LeBR2* in *dpy* are greatly reduced when compared to similar tissues in the wildtype. The expression of this gene in the wildtype was highest in young and mature leaf tissue suggesting that *LeBR2* is most active in the leaves.

In Fig 10A the comparative studies between wild-type and *dpy* plants were done with tissues from mature tomato plants and Fig 10B shows the results of comparative studies between young (3 weeks after planting) wildtype and *dpy* plants. Both results indicate that *dpy* plants have reduced expression of the *LeBR2* gene.

## Discussion

To isolate BR-regulated genes from tomato (*Lycopersicon esculentum*) we took advantage of the BR-deficient *dpy* tomato mutant (Cerny, 1997). Since only the immature tissue shows response to BR, we used only the apices (meristem and the young expanding leaves near it) as the tissue source for subtractive hybridization of cDNAs derived from RNAs from *dpy* tissues treated with or without BR.

We report here the isolation and characterization of two BR-regulated genes from tomato, designated as *LeBR1* and *LeBR2*. The *LeBR1* partial cDNA clone encoded a novel XET whose expression was enhanced by BR at all times tested, from 2 to 24 h. XETs belong to differentially regulated multigene families, whose expression is correlated with cell expansion (Clouse, 1996). Cosgrove (1977) proposed that expansins primarily affect wall relaxation while glucanases and XETs favor cell wall expansion mainly by altering the viscosity of the hemicellulose matrix. But XETs may also be involved in synthesis and incorporation of new xyloglucan into the expanding cell wall (Zurek et al., 1994). BRs also have been reported to influence the abundance of mRNA transcripts of

other XETs in soybean, Arabidopsis and tomato (Catala et al., 1997; Xu et al., 1995; Zurek and Clouse, 1994).

Interestingly, *LeBR1* is more closely related to *BRU1* than to any other XET members from the tomato XET gene family. *BRU1* mRNA transcripts accumulate in soybean epicotyl in response to exogenous BR application and it was shown that the induction of this gene is specific to BR during the early stages of elongation, and that the gene is regulated by BR post-transcriptionally (Zurek and Clouse, 1994). Enzyme activity assays of recombinant BRU1 protein, have shown that the gene encodes a protein with XET activity (Oh et al., 1998). Other BR-regulated XETs such as *TCH4* and *LeEXT* are also regulated by auxins and *TCH4* was regulated transcriptionally by BR. Hence, experiments designed to examine the specificity and mechanism of regulation of *LeBR1* would be very informative.

The *LeBR2* partial cDNA fragment that was cloned by subtractive hybridization encoded a putative serine/threonine kinase. This novel gene had no close homology to any clones that have been biologically characterized. In the past decade there has been tremendous increase in the number of plant protein kinases cloned. Although there are animal and yeast homologues that are well characterized the functions of plant protein kinases are, presently, not well understood. Protein kinases and phosphatases together form a tight regulatory unit. Besides playing a role as regulators of metabolic reactions, there are numerous examples where kinases comprise critical parts of signal transduction pathways (reviewed in Hardie, 1999). Examples of plant protein kinase genes

cloned recently are the disease-resistance genes *Xa21* from rice (Song et al., 1995), and *Pto* from tomato (Martin et al., 1993), and genes involved in regulation of growth and development such as *CLAVATA1* (Clark et al., 1997) and brassinosteroid-insensitive mutant *BRI1* (Li and Chory, 1997).

Because of the possible regulatory role of *LeBR2*, a protein kinase, we decided to characterize it in more detail and cloned its full-length cDNA (Fig. 7). It shares very close similarity to two *Arabidopsis* genomic clones that have been predicted to encode serine/threonine kinase (Fig. 8). Computer analysis also predicted the theoretical size of the protein to be 55.7 kDa with an isoelectric point of 8.12. Use of TMpred, a program that predicts membrane-spanning regions, also predicted that *LeBR2* contains a single membrane-spanning region in the N-terminus (Hoffmann and Stoffel, 1993). Fig 11A shows a hydropathy plot generated using the Kyte and Doolittle algorithm (1982), that clearly shows a hydrophobic region at the N-terminus. The primary structure of the *LeBR2* protein (500 amino acids) was subjected to computer analysis using PSORT software (Nakai and Kanehisa, 1992). This revealed that the protein is most likely targeted to the mitochondrial inner membrane as a type-Ib integral membrane protein, with amino acids 26 to 42 serving as the membrane spanning domain. However, analysis of the amino acid sequence with SignalP software (Nielsen et al., 1997), which predicts the presence of signal peptides, predicted a cleavage site between amino acid position 49 and 50. Fig 11B shows a schematic form of the gene representing the main domains that were predicted based on sequence analysis. Nuclear encoded proteins targeted to mitochondria are usually

translated as precursor proteins containing N-terminal presequences that determine the mitochondrial location of the mature protein. These presequences can be cleaved yielding a mitochondrial matrix protein or retained if the protein is an integral part of the membrane (Moore et al., 1994). If *LeBR2* is truly a mitochondrial protein, we need to perform additional biochemical experiments to distinguish between these two possibilities.

The carboxy-terminus of the protein (60 amino acids) did not have any homology to known sequences when the full peptide sequence was used for BLAST analysis. Hence this region was subjected to BLAST analysis independently. This revealed that it had the most sequence identity to Vitellogenin I precursor (1912 amino acids) from amino acid residue position 1299 to 1333 (31% identity and 51% similarity). It is very interesting to note that the Vitellogenin gene is expressed in the liver in response to estrogen, a steroid hormone in animals and accumulates in the oocytes to form the yolk. This gene is transcriptionally and post-transcriptionally regulated by estrogen (Brock and Shapiro, 1983). This region of Vitellogenin I precursor codes for the phosphovitin, a phosphoglucoprotein, the uptake of which is dependent on the degree of phosphorylation of the protein (Miller et al., 1982).

In summary, *LeBR2*, is likely a novel serine/threonine protein kinase, which is predicted to be targeted to the mitochondrial inner membrane or matrix. It is a very interesting gene that warrants further experimentation. The kinase activity of the protein needs to be evaluated before embarking on any detailed

studies, and it might be useful to do yeast-mutant complementation assays to determine the possible function of this gene

## References

- Arrowsmith, D.A and de Silva, J.** (1995). Characterization of two tomato fruit-expressed cDNAs encoding xyloglucan endotransglycosylase. *Plant Mol. Biol.* 28: 391-403.
- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J.** (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.
- Bernatzky, R., and Tanksley, S.D.** (1986). Toward a saturated linkage map in tomato based on isozymes and random cDNA sequences. *Genetics.* 112: 887-898.
- Borris, R., Buettner, K., and Maentsaelae, P.** (1990). Structure of the beta-1, 3-1,4-glucanase gene of *Baccillus macerans*: Homologies to other beta-glucanases. *Mol. Gen. Genet.* 222: 278-283.
- Brock, M.L., and Shapiro, D.J.** (1983). Estrogen regulates the absolute rate of transcription of the *Xenopus laevis* Vitellogenin genes. *J. Biol. Chem.* 258: 5449-5455.
- Catala, C., Rose, J.K.C., and Bennett, A.** (1997). Auxin-regulation and spatial localization of an endo-1, 4- $\beta$ -D-glucanase and a xyloglucan endotransglycosylase in expanding tomato hypocotyls. *Plant J.* 12: 417-426.
- Cerny, R.E.** (1997). Physiological and molecular analysis of Brassinosteroid mutants and developmental genes in *Lycopersicon esculentum*. MS thesis. North Carolina State Univ.
- Clark, S.E., Williams, R.W., and Meyerowitz, E.M.** (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell* 89: 575-585.
- Clouse, S.D.** (1996). Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. *Plant J.* 10: 1-8.
- Clouse, S.D., and Sasse, J.M.** (1998). Brassinosteroids: Essential regulators of plant growth and development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49: 427-451.
- 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). Springer-Verlag, Tokyo, pp 163-190.

- Cosgrove, D.** (1997). Relaxation in a high-stress environment: the molecular basis of extensible cell walls and enlargement. *Plant Cell* 9: 1031-41.
- De Silva, J., Jarman, C.D., Arrowsmith, DA, Stronach, M.S., Chengappa, S., Sidebottom, C., and Reid, J.S.** (1993). Molecular characterization of a xyloglucan-specific *endo*-(1,4)- $\beta$ -D-glucanase (xyloglucan *endo*-transglycosylase) from nasturtium seeds. *Plant J.* 3: 701-711.
- Fujioka, S and Sakurai, A.** (1997). Biosynthesis and metabolism of brassinosteroids. *Physiologia Plantarum* 100: 710-715.
- Hanks, S.K., and Quinn, A.M.** (1991). Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Meth. Enzymol.* 200: 38-62.
- Hardie, D.G.** (1999). Plant protein serine/threonine kinases: classification and functions. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50: 97-131.
- Hirayama, T., and Oka, A.** (1992). Novel protein kinase of *Arabidopsis thaliana* (APK1) that phosphorylates tyrosine, serine and threonine. *Plant Mol. Biol.* 20: 653-662.
- Hoffmann, K., and Stoffel, W.** (1993). TM base – A database of membrane spanning protein segments. *Bio Chem. Hoppe-Seyler.* 347: 166.
- Ito, T., Takahashi, N., Shimura, Y., and Okada, K.** (1997). a serine/threonine protein kinase gene isolated by an in vivo binding procedure using the *Arabidopsis* floral homeotic gene product, AGAMOUS. *Plant Cell Physiol.* 38: 248-258.
- Kyte, J., and Doolittle, R.F.** (1982). A simple method for displaying the hydrophathic character of a protein. *J. Mol. Biol.* 157: 105-132.
- Li, J.M., and Chory, J.** (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 90: 929-938.
- Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganai, M.W., Spivey, R., Wu, T., Earle, E.D., and Tanksley, S.D.** (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science.* 262: 1432-1436.
- Mathur, J., Molnar, G., Fujioka, S., Takasuto, 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.
- Medford, J.I., Elmer, J.S., and Klee, H.J.** (1991). Molecular cloning and characterization of genes expressed in shoot apical meristems. *Plant Cell* 3: 359-370.

- Miller, M.S., Benore-Parsons, M., and White, H.B.** (1982). Dephosphorylation of chicken riboflavin-binding protein and phosphovitin decreases their uptake by oocytes. *J. Biol. Chem.* 257: 6818-6824.
- Moore, A.L., Wood, C.K., and Watts, F.Z.** (1994). Protein import into plant mitochondria. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 45: 545-575.
- Nakai, K., and Kanehisa, M.** (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* 14: 897-911.
- Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G.** (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering.* 10: 1-6.
- Oh, M.H., Romanow, W.G., Smith, R.C., Zamski, E., Sasse, J., and Clouse, S.D.** (1998). Soybean *BRU1* encodes a functional xyloglucan endotransglycosylase that is highly expressed in inner epicotyl tissues during brassinosteroid-promoted elongation. *Plant Cell Physiol.* 39: 124-130.
- Okazawa, K., Sato, Y., Nakagawa, T., Asada, K., Kato, I., Tomita, E., and Nishitani, K.** (1993). Molecular cloning and cDNA sequencing of endoxyloglucan transferase, a novel class of glycosyltransferase that mediates molecular grafting between matrix polysaccharides in plant cell walls. *J. Biol. Chem.* 268: 25364-25368.
- Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.-X., Zhu, L.-H., Fauquet, C., and Ronald, P.** (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science.* 270: 1804-1806.
- Szekeres, M., Nemeth, K., Koncz-Kalman, 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.
- Thomas, C.M., Jones, D.A., English, J.J., Carroll, B.J., Bennetzen, J.L., Harrison, K.A., Burbidge, A., Bishop, G.J., and Jones, J.D.G.** (1994). Analysis of the chromosomal distribution of transposon-carrying T-DNAs in tomato using the inverse polymerase chain reaction. *Mol. Gen. Genet.* 242: 573-585.
- Thompson, J.D., Higgins, D.G., and Gibson, T.J.** (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. *Nuc. Acid Res.* 22: 4673-4680.
- Xu, W., Purugganan, M.M., Polisensky, D.H., Antosiewicz, D.M., Fry, S.C., and Braam, J.** (1995). *Arabidopsis TCH4*, regulated by hormones and the

environment, encodes a xyloglucan endotransglycosylase. *Plant Cell*. 7: 1555-1567.

**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.

**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.

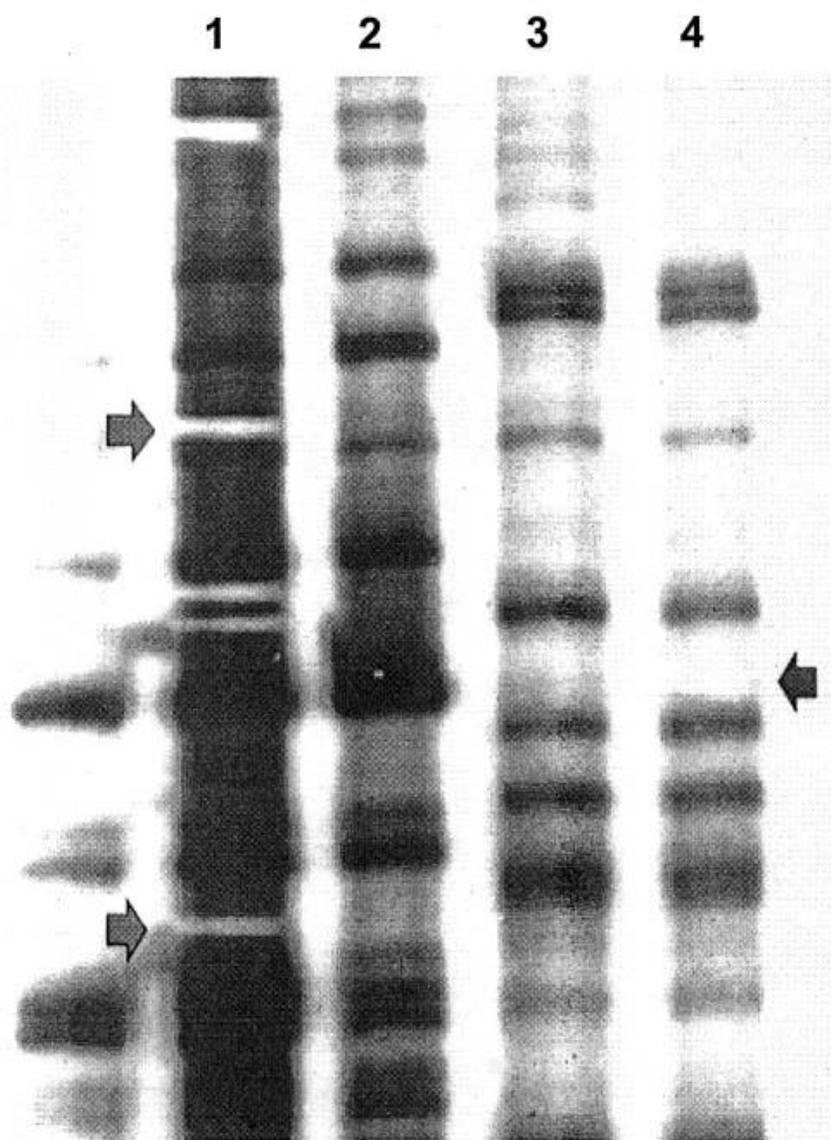
**Ye, Z.-H., Kneusel, R.E., Matern, U., and Varner, J.E.** (1994). An alternative methylation pathway in lignin biosynthesis in *Zinnia*. *Plant Cell* 6: 1427-1439.

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

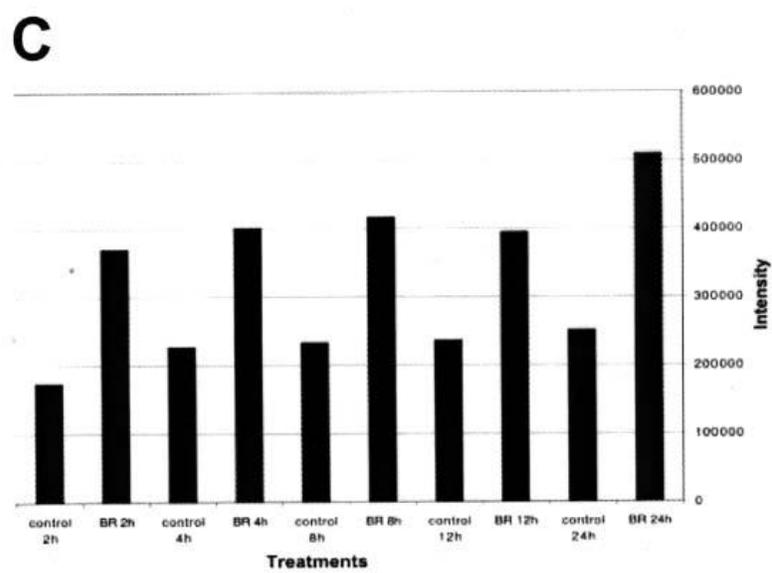
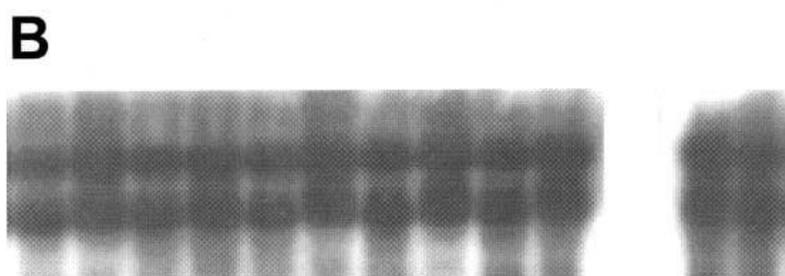
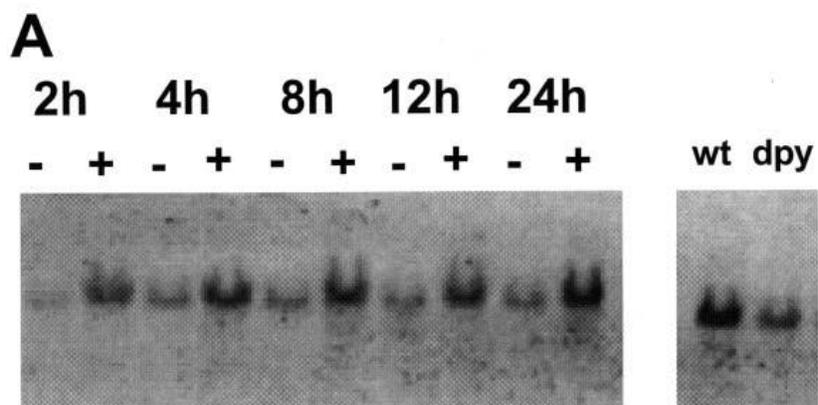
**Zurek, D.M., and Clouse, S.D.** (1994). Molecular cloning and characterization of brassinosteroid-regulated gene from elongating soybean epicotyls. *Plant Physiol.* 104: 161-170.

**Zurek, D.M., Rayle, D.L., McMorris, T.C., and Clouse, S.D.** (1994). Investigation of gene expression, growth kinetics, and wall extensibility during brassinosteroid-regulated stem elongation. *Plant Physiol.* 104: 505-513.

## Figures



**Figure 1.** Representative differential display gel. Lanes **1** and **3** are results of a run using BR-treated RNA as template. Lanes **2** and **4** are with control-treated RNA as template. The arrows indicate the differential bands that were excised from the gel for further analysis.



**Figure 2.** Northern analysis of *LeBR1*: **A)** Twenty-five  $\mu\text{g}$  total RNA was analyzed by using  $^{32}\text{P}$ -labeled *LeBR1* cDNA as the probe. RNAs are from control-treated tissues (apical meristem and first true leaf) of 21 d old *dpy* plants grown in the light and harvested at the indicated time after treatment with solvent (-) or  $10^{-7}$  M BL (+). *LeBR1* expression in untreated wildtype (wt) and *dpy* is shown on the right. **B)** The same blots as in **A** were stripped and reprobred with  $^{32}\text{P}$ -labeled rRNA as a loading control. **C)** Phosphoimager quantitation of relative *LeBR1* expression from **A**.

**TAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGTTCAAAGTTCAAGTAAAA** 60  
**AATTTTAACTACAAATGGGGTCTTTTACCCATTATGGGTTCTTGATGTTAGCACTTTTAT** 120  
**M G S F T H Y G F L M L A L L F** 16

TTAATTCTTGCATGGTTACTTATGGTGGAAATTTTTATCAAGAATTTGACTTCACTTGGG 180  
 N S C M V T Y G G N F Y Q E F D F T W G 36

GTGGCAATAGAGCCAAGATTTTCAATGGAGGTCAACTTTTATCTTTATCTTTAGACAAAG 240  
 G N R A K I F N G G Q L L S L S L D K V 56

TTTCTGGCTCTGGTTTTCAATCAAAAAAAGAACATCTCTTTGGAAGAATTGATATGCAAA 300  
 S G S G F Q S K K E H L F G R I D M Q I 76

TCAAACCTCGTTGCTGGAACTCTGCTGGCACTGTCACAACATATTACTTATCTTCTCAAG 360  
 K L V A G N S A G T V T T Y Y L S S Q G 96

GACCCACTCATGATGAAATGACTTTGAGTTCTTGGGAAATGTTACTGGTGAACCTTATA 420  
 P T H D E I D F E F L G N V T G E P Y I 116

TTCTACACACAAATATTTATGCCCAAGGCAAGGGTAACAAAGAGCAACAATTTTACCTTT 480  
 L H T N I Y A Q G K G N K E Q Q F Y L W 136

GGTTTGACCCTACAAAGAACTTCCACACCTACTCCATCATTTGGAAACCCCAACACATCA 540  
 F D P T K N F H T Y S I I W K P Q H I I 156

TATTTTGGTGGACAACACACCAATAAGAGTATACAAAATGCTGAATCAGTTGGTGTAC 600  
 F L V D N T P I R V Y K N A E S V G V P 176

CTCGGCCGCGACCACGCTGGGCGGCCGCTCGA 632  
 R P R P R W A A A R 186

**Figure 3.** Nucleotide sequence of the partial cDNA of *LeBR1* with derived amino acid sequence.

```

1 Le-BR1  TYGGNPFYQEFDFTWGGNRAKIFNNGGOLLSSLSDKVS GSGFOSKKEHLFGRIDMQLKLVAG
2 BRU1    TCASSYQDFDLTWGGDRAKIFNNGGOLLSSLSDKVS GSGFKSKEYLFGRIDMQLKLVAG
3 XTR-7   AVASNFDFEFLTWGDHRGKIFNNGGNLSSLLQVSGSGFKSKEYLFGRIDMQLKLVAG
4 tXET-B1 VWADNFYQDATVFFDQRAQIQDGRLLALSLDHISGSGFOSKNEYLFGRFDMLKLVPE
5 tXET-B2 VSADNFYQDAAVFFDQRAQIQDGRLLTLSLDHISGSGFOSKNEYLFGRFDMLKLVPE
6 TCH4    SVSADNFQDVEITWGDGRGQIKNSELLLTLSLDRSSGSGFOSKNEYLFGKVSMMKLVPE
7 LeEXT   PVDVFWKNYEPSAASHHIFLNGGTTDILLDRSSGAGFOSKKSYLFGHFSAKMRLVGE

```

\*\*\*\*\*

```

1 Le-BR1  NSAGTVTTYLSSOGPTHDEIDFEFLGNVTGEPYILHTNIYAOGKGNKEQOFYLWFDPTR
2 BRU1    NSAGTVTAYLSSOGPTHDEIDFEFLGNLSDPYILHTNIFTGKGNREOQOFYLWFDPTR
3 XTR-7   NSAGTVTAYLSSOGATHDEIDFEFLGNETGKPYVLHTNVFAOGKGDREOQOFYLWFDPTR
4 tXET-B1 NSAGTVTTYLSSOGAGHDEIDFEFLGNSSEPYTVHTNVSOGKGNKEQOFRLWFDPTS
5 tXET-B2 NSAGTVTTYLSSOGAGHDEIDFEFLGNSSEPYTVHTNVSOGKGNKEQOFRLWFDPTS
6 TCH4    NSAGTVTLYLKSPTYWDEIDFEFLGNSSEPYTLHTNVTOGKGDREOQOFKLWFDPTA
7 LeEXT   DSAGVTAFYLSSNNAEHDEIDFEFLGNREGCOPYILQTNVFTGKGNREORILWFDPTR

```

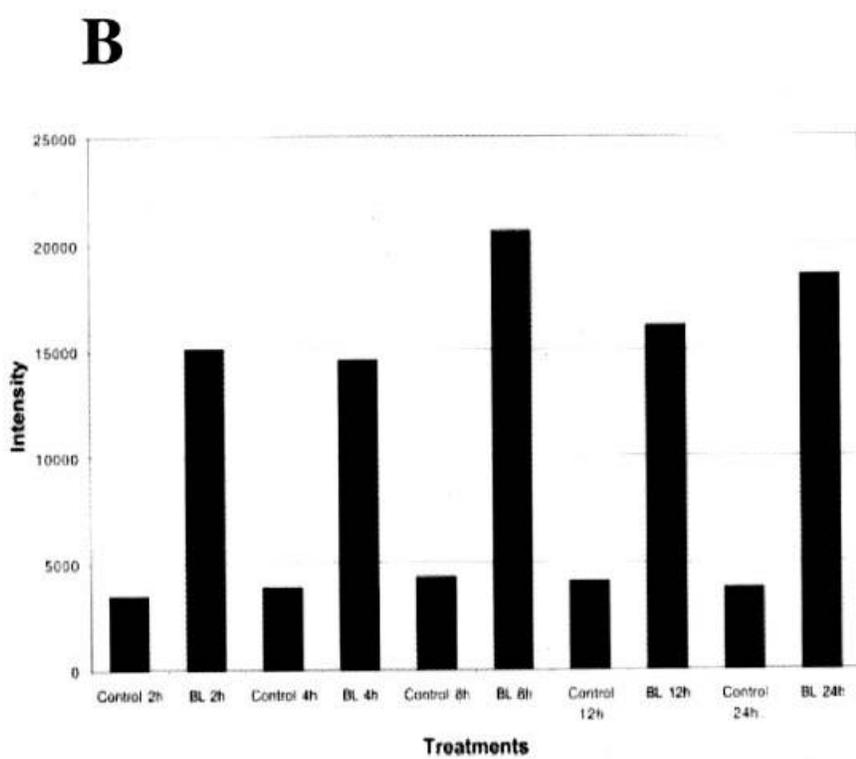
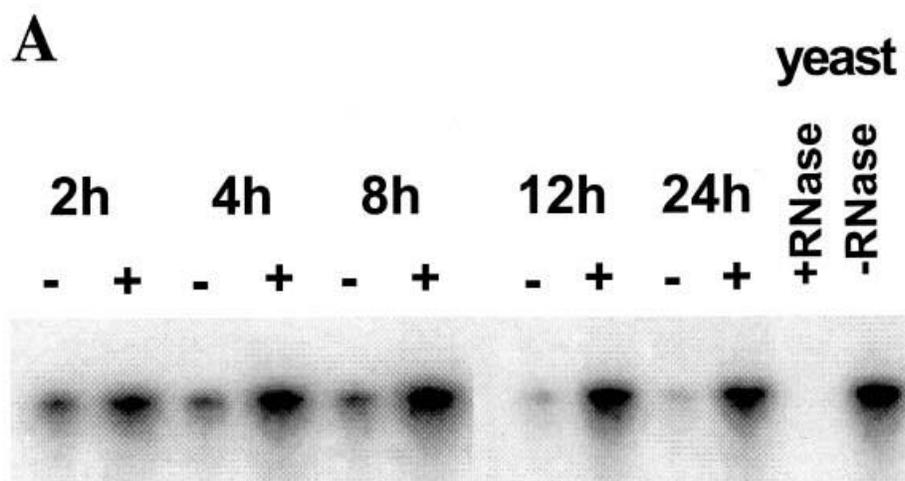
\*\*\*\*\*

```

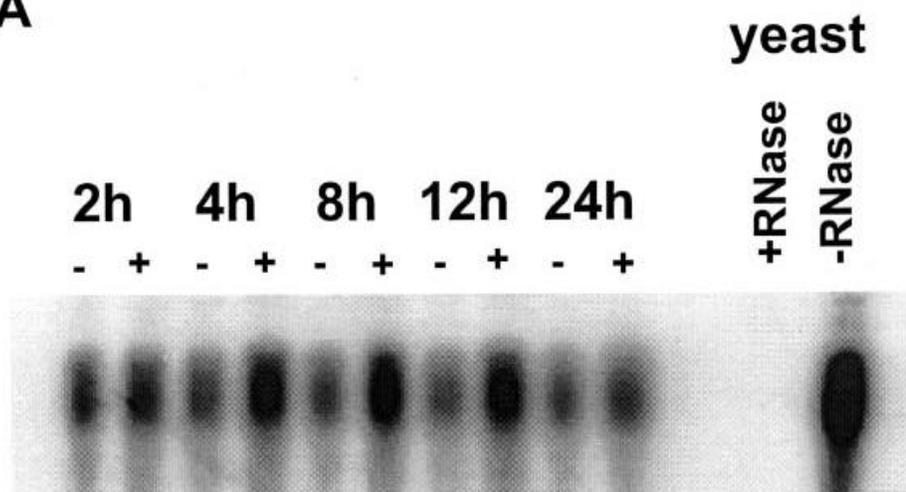
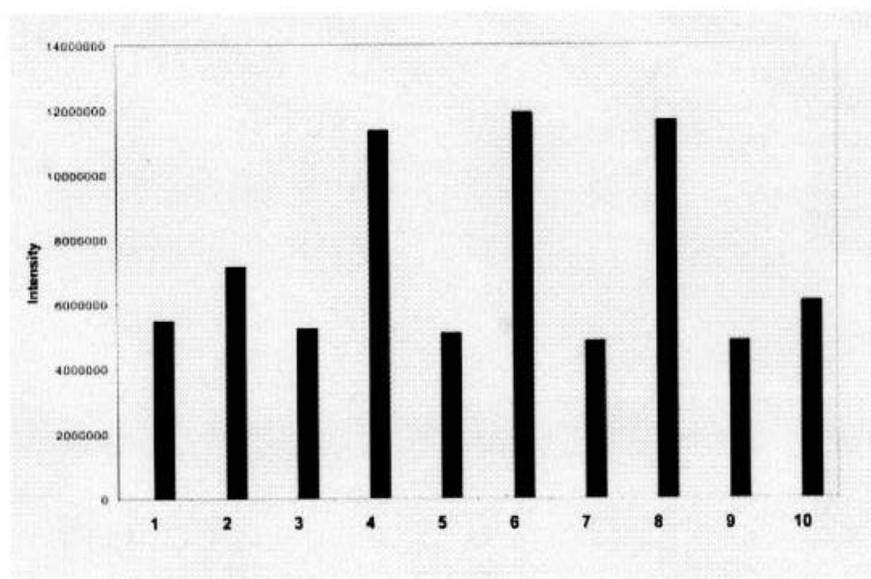
1 Le-BR1  NFHTYSIIWKPOHIIFLVDNTPIRVYKNAESVGVPRPE
2 BRU1    NFHTYSIIWKPOHIIFLVDNTPIRVFNNAEPLGVFFK
3 XTR-7   NFHTYSIVRPOHIIFLVDNLPIRVFNNAEKLGVFK
4 tXET-B1 PFHTYSIVNSCRIIFLVDNIPIRVFNNHEKLGVAFFK
5 tXET-B2 SFHTYSIVNSCRIIFLVDNIPIRVFNNHEALGVAYFK
6 TCH4    NFHTYTILNCRIIPTVEGTPIEFKNMESLTLFFK
7 LeEXT   GYSVLNTYLIVIFVDVPIRAFNSKDLGVKFF

```

**Figure 4.** Multiple sequence alignment of *LeBR1* with other XETs. ClustalW1.7 (Thompson *et al.*, 1994) was used to align *LeBR1* with other known XETs from soybean (*BRU1*), Arabidopsis (*XTR-7*, *TCH4*) and tomato (*tXET-B1*, *tXET-B2*, *LeEXT*). Amino acids identical to those in *LeBR1* are shaded in black. Asterisks indicate the sequence DEIDFEFLG, presumed to be the active site of XETs.



**Figure 5.** RNA-Protection Assay of *dpy* total RNAs with antisense *LeBR2* <sup>32</sup>P-labeled RNA. **A)** Lanes **1** to **10** contain RNAs from control-treated tissues (apical meristem and first true leaf) of 21 d old *dpy* plants grown in the light and harvested at the indicated time after treatment with solvent (-) or 10<sup>-7</sup> M BL (+). Lanes **11** and **12** are yeast RNA with (+) or without (-) RNase treatment, respectively. **B)** Phosphor-imager quantitation of relative *LeBR2* expression from **A** (yeast samples are not represented).

**A****B**

**Figure 6.** RNA-Protection Assay of wild type total RNAs with antisense *LeBR2* <sup>32</sup>P-labeled RNA probe. **A)** conditions were as in Fig. 5 except that tissues (apical meristem and first true leaf) of 21 d old wildtype were used instead of *dpy* **B)** Phosphor-imager quantitation of relative *LeBR2* expression from **A.** (yeast samples are not represented).

CACGAGTTGTACATAGCTCCTCTCTGCCCCCTTCTCTGTCTATAGTCACCGGAAAATCAAGAATCCACCGGAAAA 80  
 CCCCATTTCAAAGTTTACTACTTCTGCCGATAAACCCATTGCCCCACCATTAAAATCAAGAATCCGACCAGAATATCGC 160  
 CGGGAAACGAAACCGTACTCATTAGTATCCATTTTGTAGTTTACTCATAAAAATAGTAATACGTAGTAATAACTG 240  
 AAGTTATTGGAAATACAGATGCACTGAAGTGATTTGATTACTGAAAAAGATGTCTGTGTACGATGCGGCGTTTGTGAAC 320  
 M S V Y D A A F V N 10  
  
 AGTGAGTTATCGAAGAACACTTCGATATTTGGATTGAAAATGTGGGTGGTATTGGGATCTTTGTTGGAGCTGTATTTGT 400  
 S E L S K N T S I F G L K L W V V I G I F V G A V F V 37  
  
 TCTCATAGTCTTCTTCTATCACTCTGTATCACCGCTTCTGCCCCGCGACTACCACCCAAAAGGCAAACCTCCACCGTC 480  
 L I V F L L S L C I T A S R R R T T T Q K G K L H R P 64  
  
 CTATCAATTCTGAGCTTACCCAGTTGTCTCCAAGGAAAATCCAAGAGATTGTCCACCATGACACCGCCCAAGATCACCGC 560  
 I N S E L T P V V S K E I Q E I V H H D T A Q D H R 90  
  
 CCCATTGTCTTCAGGCTGTGCCAGAAATACAGATCAACATGGGGAAGGTGGAGCACAGAGTGGTGTTTTCAGACAAGAT 640  
 P I V L Q A V P E I Q I N M G K V E H R V V F S D K M 117  
  
 GCCTGGGGCATCAAGCGGTGAGAGCAGGGCGACTAGTGGAGCTGAAACGGGGTTCGTTAGGGAATAGTGGGCAATTGCCAG 720  
 P G A S S G E S R A T S G A E T G S L G N S G Q L P E 144  
  
 AAGTATCACATTTAGGTGGGGAAGGTGGTATACACTGAGAGAGCTGAGGCTGCCACTAATTGCTGTCTGATGAGAAT 800  
 V S H L G W G R W Y T L R E L E A A T N C L S D E N 170  
  
 GTAATTGGAGGTGGGTATGGAATTGTGTATCAAGGTGTATTGACTGATGGCACCAGAGTAGCTGTGAAAAATCTCTTGAA 880  
 V I G G G Y G I V Y Q G V L T D G T R V A V K N L L N 197  
  
 TAACAGGGGTCAAGCAGAGAAGGAGTTAAAGTGGAGGTAGAAGCTATTGGGCGTGAAGACACAAGAATCTCGTGAGGC 960  
 N R G Q A E K E F K V E V E A I G R V R H K N L V R L 224  
  
 TGCTTGGCTACTGTGTTGAAGGAGCTTACAGAATGCTGTCTATGAGTATGTAGATAATGGAACCTTGGAGCAATGGCTT 1040  
 L G Y C V E G A Y R M L V Y E Y V D N G T L E Q W L 250  
  
 CACGGAGATGTTGGGGATGTCACTCTTAAACATGGGATATTAGAATGACTATAATAGTGGGCACAGCAAAAGCTTTAGC 1120  
 H G D V G D V S P L T W D I R M T I I V G T A K A L A 277  
  
 ATATCTTCATGAGGGTCTGGAACCTAAAGTGGTTCATCGAGATGTAATAATCCAGCAACGTACTCCTCGATCGCAATGGC 1200  
 Y L H E G L E P K V V H R D V K S S N V L L D R Q W H 304  
  
 ATCCAAAGCTGTGCGATTTTGGGCTTGCTAAACCTCAATGCAGAGAGGAGCTATGTGACAACCTCGTGAATGGGAACA 1280  
 P K L S D F G L A K L L N A E R S Y V T T R V M G T 330  
  
 TTTGGTTATGTTGACCAGAAATATGCTTGTACCGCATGCTTAATGAGAAGAGTGACATTTATAGCTTTGGAATACTTAT 1360  
 F G Y V A P E Y A C T G M L N E K S D I Y S F G I L I 357  
  
 CATGGAGATAAATTAAGTGGCAGAACTCCAGTTGATTATGGTAGACCAAAAGGCGAGACTAATTTGGTGGAGTGGTTAAAAA 1440  
 M E I I T G R T P V D Y G R P K G E T N L V E W L K M 384  
  
 TGATGGTTGGTAATCGACAATCTGAGGAAGTGGTGTGCTTAAGTTGCCTGAAATGCCTTCTCAAAGGACTCAAGCGT 1520  
 M V G N R Q S E E V V D P K L P E M P S S K G L K R 410  
  
 GCTCTTTAGTTGCTCTTCGGTGTGTTGATCCTGATGCTCAAAAGAGGCGCTAAAATGGGACACATTATCCACATGCTAGA 1600  
 A L L V A L R C V D P D A Q K R P K M G H I I H M L E 437  
  
 GCGGGATGACCTGCTGGTTCGTGATGAACGTCGTATTGGTAGAGAAAAGAGAATCGTCTAATTTCCACCGTGACTACAAAC 1680  
 A D D L L V R D E R R I G R E R E S S N S H R D Y K Q 464  
  
 AAGACAACCAAGCTGGTCCCAAGTTAGCTAGAAAACAATATGGTGTAGAGCCCAAGAACTAGTGAAGGAAATAGTAGT 1760  
 D N Q A G P K L A R K Q Y G D G A P E T S E G N S S 490  
  
 AGAAACCATAAATTTGCCATCTAGTTGGAGATAAAAAGTTATAAATGAAAACATATGATCATATCAATATTTATATATCTAA 1840  
 R N H N L P S S W R 500

TGCTTATC 1849

**Figure 7.** Nucleotide sequence of the full-length cDNA of *LeBR2* with derived amino acid sequence.

```

LeBR2      VSHLGWRWYTLRELEAATNCLSDENVIG-GGVGIVYQG-----VLTQ-----GTRVAVK
ATY12776  VSHLGWRWYTLRELEAATNGLCEENVIGEGGYGIVYRG-----ILTD-----GTKVAVK
IG002N01  VSHLGWRWYTLRELEAATNGLCEENVIGEGGYGIVYSG-----ILTD-----GTKVAVK
DcSERK     EVHLGQLKRFSLRELVATDTFS--TILGRGGFGVYKQ-----RLAD-----GSLVAVK
AtAPK1A    -----KSFSAELKSATRNFRPDSVLGEGGFQGVFKGWIDEKSLTASRPGTGLVIAVK
APK2ax5    -----KAFTFNELKNATRNFRPDSLLGEGGFQGVFKGWIDGTTLTASKPGSGIVVAVK
OsPK10     QTISGAPVRFTYRELQDATSNFC--NKLGQGGFGSVYLG-----TLPD-----GSRIAVK

```

I

II

```

LeBR2      NLLNNRGQ-AEKEFKVEVEAIGRVRHKNLVRLLYGCVVEGAYRMLVVEYVDNGLEQWLHG
ATY12776  NLLNNRGQ-AEKEFKVEVEAIGRVRHKNLVRLLYGCVVEGAYRMLVYDFVDNGLEQWIHG
IG002N01  NLLNNRGQ-AEKEFRVEVEAIGRVRHKNLVRLLYGCVVEGAYRMLVYDYVDNGLEQWIHG
DcSERK     RKKEERTPGGELQEQTEVEMISMAVHRNLLRLRGFCMTPTERLVYFPMANGSVASCLRG
AtAPK1A    KLNQDGWQ-GHQEWLAEVNYLGQFSHRHLVRLLYGCVVEGAYRMLVYDFMPPRGSLENLHFR
APK2ax5    RKKTEGYQ-GHKEWLAEVNYLGQLSHPNLVRLLYGCVVEGAYRMLVYDFMPPRGSLENLHFR
OsPK10     KLEGIGQG--KKEFRSEVTTIGSIRHHHLVRLLYGCVVEGAYRMLVYDFMPPRGSLENLHFR

```

III

IV

V

```

LeBR2      DVGDVSPLTWDIRMTIIIVGTAKALAYLHEGLEPKVVHRDVKSSNILLDRQWHPKISDFGL
ATY12776  DVGDVSPLTWDIRMNIIILGMAKGLAYLHEGLEPKVVHRDKSSNILLDRQWNAKVSDFGL
IG002N01  DVGDKSPLTWDIRMNIIILGMAKGLAYLHEGLEPKVVHRDKSSNILLDRQWNAKVSDFGL
DcSERK     RQPSEPLDWPTRKRIALGSARGLSVLHDHCDPKIIHRDVKAANILLDEEFAVVGDFGL
AtAPK1A    RGLYFQPLSMKLRKVALGAAKGLAFLHS-SETRVYIRDFKASNILLSEYNAKISDFGL
APK2a     RG--AQPLTWAIRMKVAIGAAKGLTFLHD-AKSQVIRDFKAANILLDAEFNSKISDFGL
OsPK10     SKEDDHLLDWTRFNIALGTAKGLAYLHQDCDSKIVHCDIKPENVLLDNFIAKVSDFGL

```

Via

Vib

VII

```

LeBR2      AKLLNA-ERSYVTRVMGTFGYVAPEYACTGMLNEKSDIYSFGILIMEIITGRTPVDYGR
ATY12776  AKLLGS-ESSYVTRVMGTFGYVAPEYACTGMLNEKSDIYSFGILIMEIITGRNPVDYSR
IG002N01  AKLLFS-ESSYVTRVMGTFGYVAPEYACTGMLNEKSDIYSFGILIMEIITGRNPVDYSR
DcSERK     ARLMDY-KDTHVTTAVRGLGYIAPEYELSTGKSSSEKTDVFGYGMILLELITGQRAFDLAR
AtAPK1A    AKDGPIDKSHVSTRVMGTEGYAAPEYLATGHLTAKSDVYSFGVLLLELLSGRAVDKSR
APK2a     AKAGPTGDKTHVSTQVMGTEGYAAPEYVATGRLTAKSDVYSFGVLLLELLSGRAVDKSK
OsPK10     AKLMTR-EQSHVFTT-RGTRGYLAPEWLTNYAISEKSDVYSFGVLLLELLSGRKSYPDSE

```

VIII

IX

```

LeBR2      PKGE--TNLVEWLRKMVGN-ROSEEVDPKLPEPPSSKGLKRALLVVALRCVDPDANKRPK
ATY12776  PQGE--TNLVDWLKSMVGN-RRSEEVDPKIPEPSSKALKRVLVVALRCVDPDANKRPK
IG002N01  PQGE--VNLVEWLRKMVGN-RRSEEVDPKIPEPSSKALKRVLVVALRCVDPDANKRPK
DcSERK     LANDDDVMLLDVVKSLKE-KKLEMLVDPDLENNYIDTEVEQLIQVALLCTQGSFMERPK
AtAPK1A    PSGE--RNLVEWAKPYLVNKRKIFRVLDNRLQDQYSMEEACKVATLSLRCLTTEIKLRPN
APK2a     VGME--QSLVDWATPYLCDKRKLFRIMDTRLGGQYPQKGAYTAASLALQCLNPDAKLRPK
OsPK10     ISEK--AHFSPFAFKLEE-GDLQDIFDAKKYNDKDGRVETAIKVALWCIQDDFYORPS

```

X

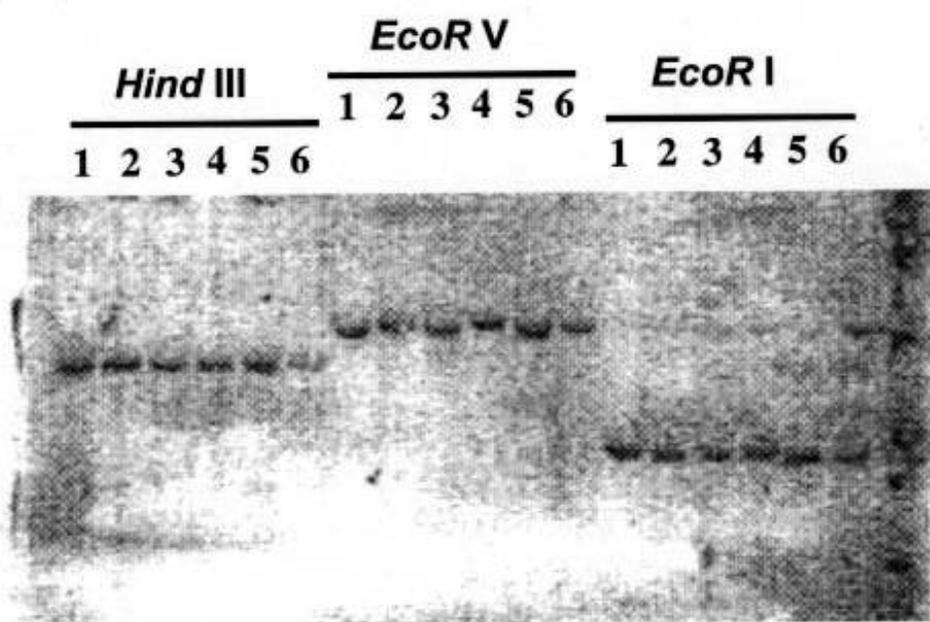
XI

```

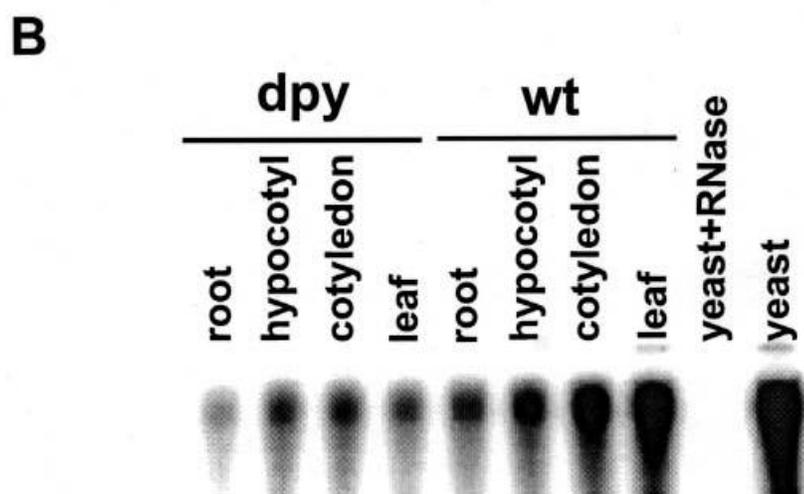
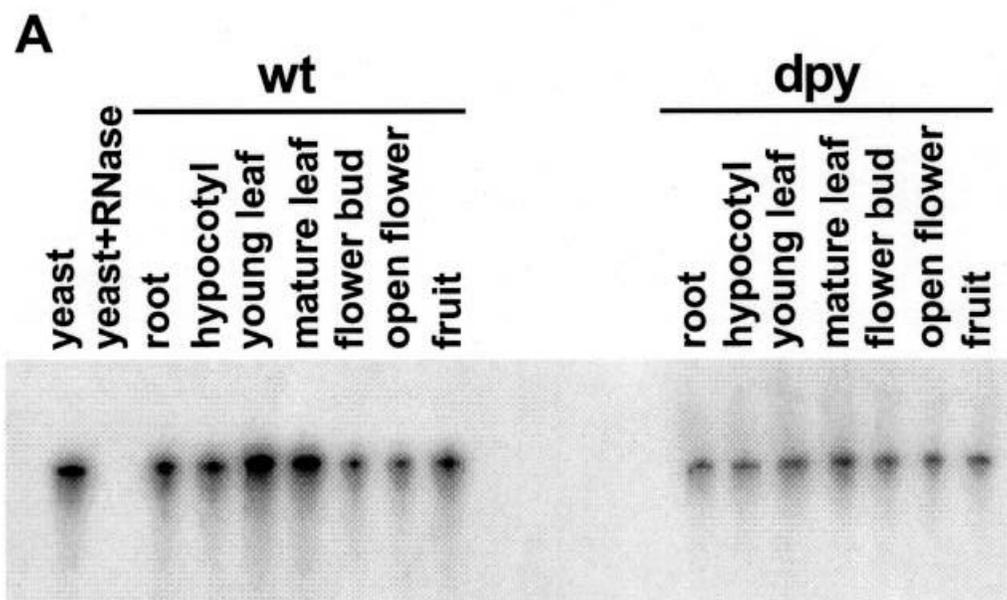
LeBR2      MGHIIHMLEADDLLVRDERRIGRERESSNSHRDYKQDNQAGPKLARKQYGDGAPETSEGN
ATY12776  MGHIIHMLEADDLLVRDERRITTRDEGSRE-----RQETAVVAAGSESGE
IG002N01  MGHIIHMLEADDLFYRDVSLIIVLMNI-----
DcSERK     MSEVVRMLEGDGLAEKWDEQKVEVIHQD-----VELAPHRTSEWILDST
AtAPK1A    MSEVVSLEHIQSLNAAIG-GNMDKTDRMR--RSDSVSKVMAGFARQTAVGSTVVA
APK2a     MSEVLAKDQLESTKPGTGVGNRQAQIDSPRG--SNGSIVQKSPRRYSYDRPLLHITPGA
OsPK10     MSKVVMLEGVCEVLQPPVSSQIGYRLAN-----AFKSSEEGTSSGMSDYNSD

```

**Figure 8.** Multiple sequence alignment of *LeBR2* with other serine/threonine kinases. ClustalW1.7 (Thompson *et al.*, 1994) was used to align *LeBR2* with other putative serine/threonine kinases from Arabidopsis (ATY12776 and IG002N01, which are predicted based on the sequence homology; and APK1A and APK2a, which are characterized biologically), carrot (SERK), and rice (PK10). Identical amino acids are shaded in black. The 11 sub-domains of the kinase domain are indicated by Roman numerals below the sequences and asterisks above the aligned sequence (Hanks and Quinn, 1991) indicate the conserved residues of the kinase domain.



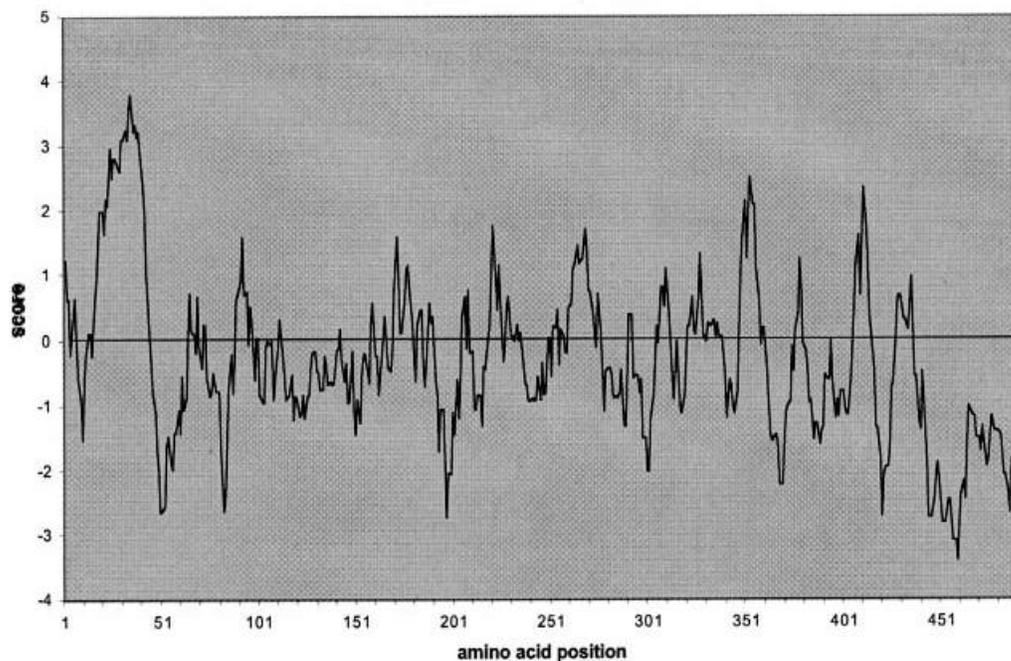
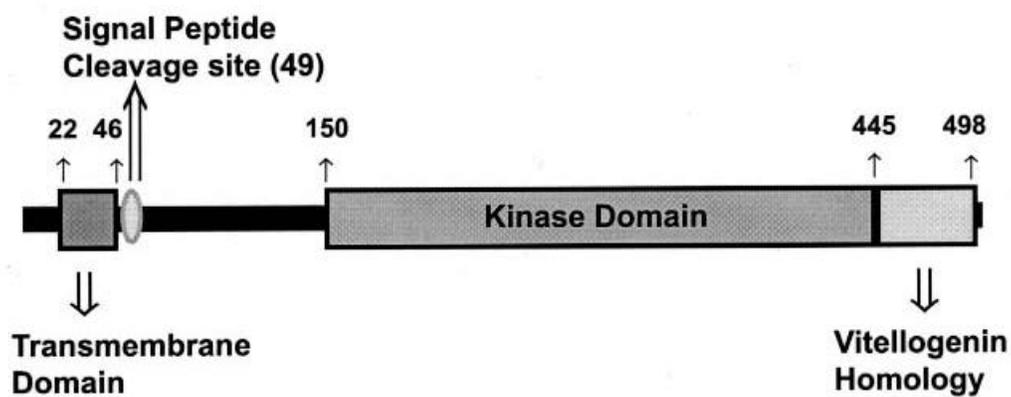
**Figure 9.** Southern analysis of *LeBR2*. Low-stringency blot probed with <sup>32</sup>P-labeled partial cDNA of *LeBR2* is shown. Lanes **1**, **2**, **3**, **4**, **5**, and **6** are *Lycopersicon esculentum* inbred tomato lines, NC EBR-6, NC EBR-5, NC 2C, *L. esculentum* cv. "Flora-Dade", *L. pennellii* LA716 and the wildtype DNA (isogenic line of *dpy*), respectively.



**Figure 10.** Tissue-specific analysis of endogenous levels of *LeBR2* mRNA using RNA-Protection Assay. **A)** Total RNA was isolated from different tissues from 8-10 week old plants grown in the greenhouse. **B)** Total RNA was isolated from different tissues from 21-d old plants grown in sterile conditions.

**A**

## Hydropathy Plot

**B**

**Figure 11. A)** Hydropathy plot of the translated *LeBR2* polypeptide based on the algorithm of Kyte and Doolittle (1982). **B)** Schematic representation of different predicted domains of the *LeBR2* polypeptide.