



**HAL**  
open science

# Auxin-Dependent Transcriptional Regulation During Fruit Development

Hua Cassan-Wang

► **To cite this version:**

Hua Cassan-Wang. Auxin-Dependent Transcriptional Regulation During Fruit Development: Functional Characterization of the DR4 Gene, an Aux/IAA Homologue in Tomato (*Lycopersicon esculentum*, Mill). *Vegetal Biology*. INP ENSAT Toulouse; INP Toulouse, 2004. English. NNT: . tel-04090093

**HAL Id: tel-04090093**

**<https://ut3-toulouseinp.hal.science/tel-04090093>**

Submitted on 5 May 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

N° d'ordre :

# **THESE**

présentée  
pour obtenir le titre de

## **DOCTEUR DE L'INSTITUT NATIONAL POLYTECHNIQUE DE TOULOUSE**

Spécialité : BIOLOGIE CELLULAIRE ET MOLECULAIRE VEGETALE

Par

**Hua WANG**

### **LA REGULATION TRANSCRIPTIONNELLE DEPENDANTE DE L'AUXINE LORS DU DEVELOPPEMENT DU FRUIT : CARACTERISATION FONCTIONNELLE DU GENE *DR4*, UN HOMOLOGUE D'*Aux/IAA* CHEZ LA TOMATE (*Lycopersicon esculentum*, Mill)**

Soutenue le 31 Mars 2004 devant le jury composé de :

M. Jean-Claude PECH	Président
M. Antonio GRANELL	Rapporteur
M. Christian CHEVALIER	Rapporteur
Mme. Catherine PERROT-RECHENMANN	Membre
M. Alain-Michel BOUDET	Membre
M. Mondher BOUZAYEN	Directeur de thèse



## *REMERCIEMENTS*

*A tous ceux qui ont contribué, tout le long ou par moments, à ce travail de recherche*

*Merci !*



## Résumé

Le développement des fruits de l'anthèse à la maturation est un processus génétiquement programmé dont le déroulement se trouve sous un contrôle multihormonal. Alors que l'éthylène joue un rôle déterminant au cours de la maturation des fruits climactériques comme la tomate, les modalités d'intervention des autres hormones ne sont toujours pas connus. Une approche de criblage différentiel a permis d'isoler au laboratoire plusieurs gènes codant pour des régulateurs transcriptionnels impliqués dans la réponse à l'auxine et montrant un profil d'expression qui varie au cours de la maturation et en réponse à l'éthylène. Le travail présenté dans cette thèse est focalisé sur la caractérisation fonctionnelle du gène *DR4* apparenté à la famille des *Aux/IAA* de la tomate dont le rôle en tant que médiateurs de la réponse des plantes à l'auxine est bien connu. Alors que de nombreux mutants "*gain de fonction*" touchant les gènes *Aux/IAA* ont été décrits chez *Arabidopsis*, de façon remarquable, aucun mutant "*perte de fonction*" n'a été caractérisé à ce jour. Le travail présenté ici montre que la sous-expression du gène *DR4* chez la tomate affecte plusieurs processus de développement dépendant de l'auxine. Ainsi, les lignées transgéniques exprimant une construction anti-sens du gène *DR4* (*DR4AS*) montrent un phénotype pléiotropique qui touche essentiellement la morphologie des feuilles et le développement du fruit. Les lignées *DR4AS* possèdent des feuilles simples et non pas composées comme chez le sauvage et le développement des fruits survient en l'absence de toute pollinisation et fécondation ce qui conduit à la production de fruits parthénocarpiques. Les plantes *DR4AS* montrent également plusieurs phénotypes associés à une réponse exacerbée à l'auxine tel que une forte élévation de l'hypocotyle, de la tige et de la racine, une réduction de la dominance apicale, des fusions d'organes et une transition d'un développement basipétal des tiges axillaires à un mode acropétal. Le croisement génétique des plantes *DR4AS* avec des lignées exprimant le gène *GUS* sous contrôle d'un promoteur répondant à l'auxine engendre une augmentation substantielle de l'expression du gène rapporteur dans les lignées hybrides indiquant que la protéine DR4 agit comme régulateur négatif des gènes de réponse à l'auxine. Les données présentées dans cette thèse constituent la première description de phénotypes associés à une sous-expression d'un gène *Aux/IAA* et révèlent pour la première fois le rôle essentiel joué par un membre de la famille des *Aux/IAA* dans la morphogénèse des feuilles et le développement du fruit.

**Mots clé :** Auxine, Aux/IAA, tomate, fruit, fructification, parthénocarpie, morphogénèse

## ABSTRACT

Fruit development is a genetically programmed process under multi-hormonal control. While the role of ethylene in triggering the ripening process is well demonstrated in the case of climacteric fruit like tomato, little is known about the involvement of other hormones. Differential screening allowed the isolation of transcriptional regulators involved in auxin responses that show ripening and ethylene-regulated expression during the late stages of fruit development. The work described here deals with the functional characterization of *DR4*, an *Aux/IAA* gene expressed in tomato fruit. *Aux/IAA* proteins are transcriptional regulators known to mediate many aspects of plant responses to the phytohormone auxin. Though a number of gain-of-function mutations affecting *Aux/IAA* genes in Arabidopsis have been characterized, strikingly, phenotypes associated with loss-of-function mutations yet have not been described. We report here on the down-regulation of *DR4*, a tomato *Aux/IAA*-like gene, which altered auxin-related responses. Under-expressing *DR4* through antisense strategy in the tomato resulted in pleiotropic phenotypes with the most dramatic changes affecting leaf morphology and fruit development. The leaves in *DR4*-inhibited were simple shaped compared to compound leaves in wild type and fruit development was triggered prior to flower fertilization giving rise to parthenocarpy. *DR4* down-regulated plants also displayed a wide range of auxin-associated responses including enhanced hypocotyl, root and stem elongation, reduced apical dominance, a multiple organ fusion and a transition from basipetal to acropetal lateral shoot development. Genetic crosses between *DR4* antisense lines and plants expressing *GUS* driven by an auxin responsive promoter resulted in massive increase of the reporter gene expression in the hybrid lines indicating that *in planta*, *DR4* acts as a negative regulator of auxin responsive genes. These data represent the first description of phenotypes associated with down-regulation of an *Aux/IAA* gene and uncover for the first time the essential role of these factors in leaf morphogenesis and fructification.

**Key words:** Auxin, *Aux/IAA*, tomato, fruit, fructification, parthenocarpy, morphogenesis

### *Un mot d'explication et d'excuse*

Malgré les progrès que j'ai accomplis depuis mon arrivée en France, j'ai le regret de constater que ma maîtrise de la langue française demeure insuffisante pour me permettre d'entreprendre la rédaction d'un document scientifique qui exige la plus grande précision dans la description des expérimentations et l'interprétation des résultats. Ceci explique mon choix de rédiger ce document en anglais alors que ma maîtrise de cette langue est également imparfaite. J'avoue que l'idée de rédiger ce document en chinois m'a traversée la tête mais je n'étais certaine que le niveau des membres du jury en chinois permette un échange suffisant sur un sujet scientifique pointu. Comme souvent, l'anglais n'est souhaité par personne mais faute de mieux accepter par tous, alors il s'impose.

Je voudrais m'en excuser auprès de vous et promettre de faire la présentation orale en français le jour de la soutenance afin de fournir la preuve de mon attachement à cette langue.

***Hua WANG***

## ABBREVIATIONS

<b>μTom</b>	Micro-Tom
<b>2, 4-D</b>	2,4-dichlorophenoxyacetic acid
<b>2,4,5-T</b>	2,4,5-trichlorophenoxyacetic acid
<b>aa</b>	Amino acid
<b>ABA</b>	Abscisic acid
<b>ABG</b>	ATP-binding cassette
<b>ABP</b>	Auxin binding protein
<b>AC</b>	Ailsa Craig
<b>ACC</b>	1-aminocyclopropane-1-carboxylic acid
<b>ACO</b>	Amino cyclopropane-1-carboxylic acid oxidase
<b>ALF</b>	Aberrant lateral root formation
<b>ARF</b>	Auxin response factors
<b>AS</b>	Antisense
<b>AuxREs</b>	Auxin response elements
<b>bp</b>	Base pair
<b>BR</b>	Brassinosteroids
<b>Br</b>	Break
<b>BSA</b>	Bovine Serum Albumin
<b>CaMV</b>	Cauliflower mosaic virus
<b>Cdc2</b>	Cyclin-dependant kinase
<b>cDNA</b>	Complementary deoxyribonucleic acid
<b>CHX</b>	Cycloheximide
<b>COP1</b>	Component of photomorphogenesis
<b>CPD</b>	2-carboxyphenyl-3-phenylpropane-1, 2-dione
<b>CTAB</b>	Cetyltrimethylammonium bromide
<b>CTD</b>	C-terminal dimerization domain
<b>ctr</b>	Constitutive triple responses
<b>Cys</b>	Cysteine
<b>DBD</b>	DNA binding domains
<b>degron</b>	Degradation region

<b>DEPC</b>	Diethyl pyrocarbonate
<b>dgt</b>	Diageotropica
<b>DNase</b>	Deoxyribonuclease
<b>dNTP</b>	Deoxyribonucleotides
<b>DR</b>	Developmental regulated
<b>E1</b>	Ubiquitin-activating enzyme1
<b>EDTA</b>	Ethylenediamine tetraacetic acid
<b>EIM</b>	Early immature green
<b>EMS</b>	Ethylmethane sulfonate
<b>eto</b>	Ethylene over-production
<b>fin219</b>	Far red insensitive 219
<b>GA</b>	Gibberlic acid or gibberellin
<b>GMO</b>	Genetically modified organism
<b>GUS</b>	B-glucuronidase
<b>hls</b>	Hookless
<b>IAA</b>	Indole-3-acetic acid
<b>IAA12/BDL</b>	Bodenlos
<b>IAA14/SLR</b>	Solitary root
<b>IAA17/AXR3</b>	Auxin resistant 3
<b>IAA19/MGS2</b>	Massugu2
<b>IAA3/SHY2</b>	Short hypocotyl or suppressor of hy2
<b>IAA6/SHY1</b>	Short hypocotyl or suppressor of hy2
<b>IAA7/AXR2</b>	Auxin resistant 2
<b>IM</b>	Immature green
<b>JA</b>	Jasmonic acid
<b>Lys</b>	Lysine
<b>MAPK</b>	Mitogen activated protein kinase
<b>MDR</b>	Multidrug resistance
<b>MG</b>	Mature green
<b>MP</b>	Monopteros
<b>MR</b>	Middle region
<b>mRNA</b>	Messenger ribonucleic acid
<b>MS</b>	Murashige and Skoog medium salts

<b>NAA</b>	$\alpha$ -Naphthalene acetic acid
<b>NLS</b>	Nuclear localization signal
<b>NPA</b>	N-(1-naphthyl) phthalamic acid
<b>NPH1</b>	Non phototropic hypocotyl
<b>OR</b>	Orange
<b>ORF</b>	Open reading Frame
<b>PBA</b>	1-pyrenoylbenzoic acid
<b>PCR</b>	Polymerase chain reaction
<b>PGRs</b>	Growth regulators
<b>PhyA</b>	Phytochrome A
<b>Pid</b>	Pinoid
<b>pin1</b>	Pin-formed 1
<b>PP2A</b>	Phosphatase 2A
<b>Pro</b>	Proline
<b>rcn</b>	Roots curl in NPA
<b>RGA</b>	Repressor of gal-3
<b>RT</b>	Reverse transcription
<b>RT-PCR</b>	Reverse transcription PCR
<b>RTY</b>	Rooty
<b>SAM</b>	S-adenosylmethionine
<b>SAUR</b>	Small auxin up-regulated RNA
<b>SCF</b>	Ubiquitin ligases (for Skp1p, Cdc53p/cullin, and F-box )
<b>SUR1</b>	Superroot 1
<b>Taq</b>	Thermus aquaticus DNA polymerase
<b>TIBA</b>	2,3,5-Triiodobenzoic acid
<b>tir1</b>	Auxin transport inhibitors resistance
<b>Tris</b>	Tris (hydroxymethyl) aminomethane
<b>Trp</b>	Tryptophan
<b>WT</b>	Wild-type
<b>x-gal</b>	5-bromo-4-cloro-3-indolil- $\beta$ -D-galactoside

## TABLE OF CONTENTS

RÉSUMÉ .....	III
ABSTRACT .....	IV
UN MOT D'EXPLICATION ET D'EXCUSE.....	IV
ABBREVIATIONS.....	V
INTRODUCTION AU SUJET DE THESE.....	1

### *CHAPTER I BIBLIOGRAPHIC REVIEW*

INTRODUCTION .....	7
A- AUXIN .....	8
A-1- AUXIN, A CRUCIAL HORMONE.....	9
A-1-1- Physiological effects of auxin.....	9
A-1-2- Synthetic auxins, anti-auxins and auxin transport inhibitors .....	17
A-1-2-1- Synthetic auxins .....	17
A-1-2-2- Anti-auxins.....	17
A-1-2-3- Auxin transport inhibitor .....	18
A-2- AUXIN METABOLISM AND TRANSPORT.....	19
A-2-1- Auxin biosynthesis.....	19
A-2-2- Auxin catabolism.....	19
A-2-3- Auxin transport .....	20
A-2-4- Auxin homeostasis.....	21
A-3- AUXIN SIGNALLING .....	23
A-3-1- Auxin signal perception.....	24
A-3-2- Signals transduction.....	24

A-3-2-1- Second messengers.....	24
A-3-2-1- Protein phosphorylation.....	25
A-3-3- Induction of auxin response genes.....	28
A-3-3-1- Auxin response genes.....	28
A-3-3-2- Promoters of auxin responsive genes.....	30
A-3-3-3- Regulators and protein degradation.....	30
A-4- AUXIN CROSS-TALK WITH OTHER HORMONES.....	31
A-4-1- Auxin and cytokinin.....	32
A-4-2- Auxin and ethylene.....	34
A-4-3- Auxin and gibberellins.....	35
A-4-4- Auxin and abscisic acid, brassinosteroids, jasmonic acid.....	38
B- AUX/IAA.....	40
B-1- WHAT IS AUX/IAA.....	40
B-1-1- Aux/IAA protein.....	40
B-1-2- <i>Aux/IAAs</i> : a large multi-gene family.....	42
B-1-3- Expression patterns of <i>Aux/IAA</i> genes.....	43
B-1-4- Auxin response patterns of <i>Aux/IAA</i> genes.....	44
B-2- DEVELOPMENTAL FUNCTIONS OF <i>AUX/IAA</i> GENES.....	45
B-3- <i>AUX/IAAs</i> INTERACT WITH ARFs.....	48
B-3-1- ARF proteins.....	48
B-3-2- Auxin responses are mediated by interaction between <i>Aux/IAA</i> and ARF.....	49
B-4- <i>AUX/IAA</i> PROTEIN DEGRADATION IS ESSENTIAL FOR NORMAL AUXIN SIGNALLING.....	50
B-4-1- SCF <sup>TIR1</sup> -dependent ubiquitination mediates the auxin response.....	50
B-4-2- <i>Aux/IAA</i> proteins are targeted to SCF <sup>TIR1</sup> .....	52
B-4-3- Regulation of the SCF <sup>TIR1</sup> - <i>Aux/IAA</i> interaction by auxin.....	52
B-4-4- <i>Aux/IAA</i> stability is critical for auxin signalling.....	53
B-5- A BASIC MODEL FOR AUXIN-INDUCED GENES EXPRESSION.....	54
C- TOMATO AS MODEL PLANT.....	55
C-1- ETHNOBOTANY OF THE TOMATO.....	56
C-2- HORTICULTURAL CHARACTERISTICS.....	57
C-3- GENETIC AND GENOMIC RESOURCES ON THE TOMATO MODEL.....	57
C-4- <i>MICRO-TOM</i> ( $\mu$ <i>TOM</i> ): A MINIATURE TOMATO WITH RAPID LIFE CYCLE.....	58
C-5- TOMATO PLANT CHARACTERISTICS.....	58
C-6- BIOLOGY OF TOMATO FRUIT.....	59
C-6-1- Tomato anatomy.....	60
C-6-2- Tomato fruit ontogeny: from flower to maturation.....	60
C-6-2-1- Flower.....	60

C-6-2-2- Fruit development.....	63
C-7- AUXIN AND TOMATO FRUIT.....	67

## *CHAPTER II MATERIALS AND METHODES*

A- PLANT MATERIAL AND CULTURE CONDITIONS.....	71
B- PHYSIOLOGICAL ANALYSIS.....	71
B-1- HORMONE TREATMENT.....	71
B-1-1- Seeds germination and seedling culture.....	71
B-1-2- Auxin response in hypocotyl elongation.....	72
B-1-3- Auxin and cytokinin interaction in cotyledon and hypocotyl explants.....	72
B-1-4- NPA, GA, Paclobutrazol treatment in seedling.....	73
B-1-5- Auxin treatment in seedling and different stage fruit for gene expression analysis.....	73
B-2- FRUIT ANALYSIS.....	73
C- GENETIC TRANSFORMATION.....	74
C-1- MOLECULAR CLONING OF <i>DR4</i> SENSE OR ANTISENS IN BINARY VECTOR.....	75
C-2- TRANSFORMATION OF AGROBACTERIUM.....	76
C-3- TOMATO COTYLEDON EXPLANTS TRANSFORMATION.....	77
D- GENE EXPRESSION ANALYSIS.....	78
D-1- RNA EXTRACTION.....	78
D-2- RT-PCR ANALYSIS OF MRNA LEVELS.....	79
D-3- NORTHERN BLOT ANALYSIS.....	80
E- TRANSIENT EXPRESSION OF GFP-FUSION PROTEIN.....	80
E-1- <i>DR4-GFP</i> CONSTRUCTION.....	80
E-2- PREPARATION OF PROTOPLASTS.....	81
E-3- TRANSIENT TRANSFORMATION AND MICROSCOPIC OBSERVATION.....	82

## *CHAPTER III GENE FUNCTION CHARACTERIZATION OF DR4*

INTRODUCTION.....	85
-------------------	----

A- <i>DR4</i> : A TOMATO <i>AUX/IAA</i> -LIKE GENE .....	86
A-1- ISOLATION .....	86
A-2- SEQUENCE ANALYSIS .....	86
A-3- <i>DR4</i> PROTEIN .....	89
A-4- SUBCELLULAR LOCALIZATION OF <i>DR4</i> PROTEIN .....	90
B- BIOLOGICAL FUNCTION OF <i>DR4</i> .....	91
B-1- REVERSE GENETIC APPROACH .....	92
B-1-1- Antisense: down-expression of <i>DR4</i> .....	92
B-1-2- Sense: gene over-expression of <i>DR4</i> .....	92
B-1-3- Ectopic over-expression of tomato <i>DR4</i> in Arabidopsis .....	94
B-2- MORPHOLOGICAL ANALYSIS .....	94
B-2-1- Phenotypes of <i>DR4</i> antisense transgenic tomato plants .....	94
B-2-1-1- Precocious fruit set and parthenocarpic fruit .....	94
B-2-1-2- Simple leaves .....	97
B-2-1-3- Organ fusion .....	98
B-2-1-4- Light-grown seedling: more lateral roots and longer hypocotyl .....	100
B-2-1-5- longer internodes .....	102
B-2-1-6- Aberrant sepals arrangements .....	102
B-2-1-7- Aberrant inflorescences .....	103
B-2-1-8- Navel-like fruits .....	104
B-2-2- Down-regulation of <i>DR4</i> expression account for the transgenic phenotypes .....	104
B-3- PHYSIOLOGICAL ANALYSIS .....	106
B-3-1- The studies on the <i>DR4AS</i> precocious fruit set and parthenocarpic fruits .....	106
B-3-1-1- <i>DR4AS</i> is able to set fruit in the absence of pollination .....	106
B-3-1-2- Both the ovule and pollen of <i>DR4AS</i> are fertile .....	107
B-3-1-3- Fully or partially parthenocarpy results in reduced seed production .....	108
B-3-2- <i>DR4AS</i> showed different hormones responses compared with WT in seedling .....	110
B-3-2-1- Auxin response .....	111
B-3-2-2- Auxin transport inhibitor response .....	115
B-3-2-3- GA and GA-inhibitor (Paclobutrazol) response .....	120
B-3-2-4- Auxin and cytokinin interaction in cotyledon and hypocotyl explants .....	123
B-3-3- <i>DR4AS</i> has reduced apical dominance .....	127
B-3-4- <i>DR4AS</i> Fruits character analysis .....	131
B-3-5- Ethylene response in <i>DR4AS</i> .....	134
B-3-6- <i>DR4AS</i> has normal photo-/ gravi- tropism .....	137
B-4- GENES EXPRESSION ANALYSIS .....	137
B-4-1- <i>DR4</i> expression pattern .....	137
B-4-2- <i>DR4</i> mRNA transcripts levels are slightly and slowly induced by auxin .....	138
B-4-3- Comparison of genes expression in different tissue of WT and AS .....	139
B-4-3-1- Auxin-inducible gene <i>DR3</i> is up-regulated in <i>DR4AS</i> seedling .....	141
B-4-3-2- Down regulation of <i>DR4</i> does not alter examined genes expression levels .....	141

B-4-4- Compare genes expression in WT and AS in response to auxin.....	144
B-5- ANATOMICAL ANALYSIS .....	146
C- THREE <i>DR4</i> SEQUENCES.....	149

## *CHAPTER IV GENERAL DISCUSSION AND PERSPECTIVES*

A- GENERAL DISCUSSION .....	155
A-1- THE FIRST PHENOTYPE ASSOCIATED WITH <i>AUX/IAA</i> LOSS-OF-FUNCTION MUTANT.....	155
A-2- <i>DR4</i> : A KEY REGULATORY GENE IN FRUIT SET AND DEVELOPMENT .....	156
A-3- <i>DR4</i> : A CRUCIAL GENE IN LEAF MORPHOGENESIS.....	159
A-4- DOWN-REGULATION OF <i>DR4</i> REVEALS OVERLAPPING AND DISTINCT ROLES FOR <i>AUX/IAA</i> GENES.....	160
A-5- <i>DR4</i> , A NEGATIVE REGULATOR OF AUXIN RESPONSE .....	162
B- OUTCOME OF THE STUDY AND PERSPECTIVES .....	164
REFERENCE .....	169



# *Introduction au sujet de thèse*

---

---



## ***INTRODUCTION AU SUJET DE THESE***

### *Objectifs des travaux et principales acquisitions*

#### ***La problématique générale***

Comme l'ensemble des processus de développement des plantes, le développement des fruits correspond à un processus génétiquement programmé dont l'avènement et le déroulement sont orchestrés par un jeu complexe d'hormones. Ainsi par exemple, le rôle déterminant de l'éthylène au cours de la maturation des fruits de type climactérique comme la tomate, est maintenant bien démontré. Il reste cependant à élucider les mécanismes de l'acquisition de la compétence à mûrir qui, à partir d'un stade déterminé du développement, confère au fruit la capacité de répondre de façon spécifique à l'éthylène en déclenchant le programme de maturation et en activant tous les gènes associés aux voies métaboliques qui accompagnent ce processus. Pour expliquer ce phénomène, les physiologistes ont avancé depuis longtemps la notion de changement de la balance hormonale. Cette hypothèse est appuyée par le fait que le niveau de plusieurs phytohormones dans le fruit subit des variations importantes tout au long du développement de cet organe, de l'anthèse à la maturation. Ces données suggèrent l'existence d'un contrôle multi-hormonal présidant à ce processus de développement et notre hypothèse de travail postule que le changement du statut hormonal du fruit serait responsable de la réponse différenciée à l'éthylène. Le projet de thèse s'inscrit dans un champ de recherche plus vaste qui vise à identifier les signaux hormonaux qui agissent de concert avec l'éthylène pour contrôler le développement et la maturation du fruit et à déterminer, au niveau moléculaire, leurs modalités d'intervention. Alors même que les hormones exercent des effets pléiotropiques, les mécanismes qui conduisent à une réponse ciblée selon le tissu et le stade de développement demeurent peu connus.

***Le modèle végétal***

La tomate (*Lycopersicon esculentum*) est considérée par l'ensemble de la communauté scientifique comme la plante modèle pour l'étude des fruits charnus. Le choix de ce modèle tomate est justifié par la disponibilité de nombreuses ressources génétique et de génomique sur cette espèce. L'avantage de cette espèce en tant que modèle expérimental réside également dans sa facilité de culture (3 à 4 générations par an) et de transformation génétique. Bien que les études de génétique moléculaire sur la plante modèle *Arabidopsis thaliana* aient permis de décrypter les voies de transduction de plusieurs hormones, les mécanismes qui canalisent ces voies de signalisations vers l'activation du processus de maturation des fruits restent totalement inconnus. De plus, *Arabidopsis* n'est pas un modèle adapté à l'étude des fruits charnus qui constituent la presque totalité de la production fruitière mondiale. La tomate est depuis longtemps le modèle pour l'étude des fruits dits climactériques dont la maturation s'accompagne d'une crise respiratoire et d'une synthèse autocatalytique d'éthylène. Cependant, la plupart des mécanismes physiologiques ou moléculaires élucidés chez la tomate ont été jusqu'à présent validés chez l'ensemble des fruits qu'ils soient de type climactérique ou non.

***La régulation transcriptionnelle associée à l'auxine***

Il est admis que les voies de signalisation hormonales mobilisent en aval des facteurs de transcriptions spécifiques qui vont activer des processus strictement régulés au niveau spatial et temporel. De ce fait, les facteurs de transcription apparaissent comme les médiateurs principaux de la modulation de l'expression des gènes spécifiques de la réponse hormonale. Des travaux antérieurs au début de ma thèse, des approches de criblage différentiel ont permis d'isoler à partir des fruits de tomate plusieurs gènes codant pour des régulateurs transcriptionnels impliqués dans la réponse à l'auxine présentant un profil d'expression qui varie au cours de la maturation et en réponse à l'éthylène. Ces études révèlent pour la première fois que certains facteurs de transcription de l'auxine sont différentiellement régulés par l'éthylène dans le fruit et suggèrent de ce fait l'existence d'un dialogue entre les voies de signalisation de l'auxine et de l'éthylène. Dans le but de mieux comprendre le rôle de l'auxine au cours du développement du fruit nous avons entrepris la caractérisation fonctionnelle de plusieurs gènes codants pour des facteurs potentiellement impliqués dans la régulation transcriptionnelle associée à cette hormone. Parmi les clones isolés, les études d'homologie de

séquences ont permis d'identifier des gènes appartenant aux deux familles connues de facteurs de transcription de l'auxine. Le clone *DR12* est homologue aux membres de la famille des *ARF* (*Auxin Response Factor*) alors que plusieurs autres (*DR1*, *DR3*, *DR4*, *DR8*) sont apparentés aux *Aux/IAA* également impliqués dans la réponse à l'auxine. L'exploration fonctionnelle de ces gènes a été mise en oeuvre par des approches de biologie moléculaire et cellulaire et des stratégies de génétique inverse consistant à créer des lignées transgéniques altérées dans l'expression de ces gènes. Les premières caractérisations des lignées transgéniques ont orienté les études de façon prioritaire vers celles (*DR12* et *DR4*) qui montrent des phénotypes associés au développement du fruit.

### ***Le gène DR4, un membre de la famille des Aux/IAA impliqué dans les processus de développement et de croissance dépendant de l'auxine***

Le projet de recherche réalisé au cours de cette thèse est focalisé sur la caractérisation fonctionnelle du gène *DR4* apparenté à la famille des *Aux/IAA* de la tomate. Au cours des dernières années, plusieurs travaux basés soit sur l'analyse des mutants de la plante modèle *Arabidopsis* soit sur des études d'interactions fonctionnelles entre les protéines *Aux/IAA* et des promoteurs cibles utilisant ont montré le rôle de certains membres de la famille des *Aux/IAA* en tant que médiateurs de la réponse à l'auxine. Toutefois, alors que de nombreux mutants "gain de fonction" touchant les gènes *Aux/IAA* ont été décrits chez *Arabidopsis*, de façon remarquable, aucun mutant "perte de fonction" n'a été caractérisé à ce jour. L'objectif premier de la thèse est d'explorer le rôle potentiel du gène *DR4* dans le processus de développement du fruit. Une approche de génétique inverse a été adoptée qui vise à modifier l'expression endogène du gène *DR4* dans les tomates. La stratégie des ARNs antisens utilisée consiste à exprimer de façon stable une construction antisens de ce gène sous le contrôle du promoteur constitutif 35S.

Il est montré dans ce travail de thèse qu'une sous-expression du gène *DR4* chez la tomate entraîne l'altération de plusieurs processus de croissance et de développement dépendant de l'auxine. Ainsi, les lignées transgéniques exprimant une construction anti-sens du gène *DR4* (*DR4AS*) montrent un phénotype pléiotropique affectant en particulier la morphologie des feuilles et le développement du fruit. Les lignées *DR4AS* présentent une altération de la morphogenèse des feuilles qui passent d'une forme composée à la forme. Comparé à celui des plantes non-transformées, le développement des fruits est accéléré chez

les transgéniques et survient en l'absence de toute pollinisation, ce qui conduit à la production de fruits parthénocarpiques. Les plantes DR4AS montrent de nombreux autres phénotypes qui peuvent être attribués à une réponse exacerbée à l'auxine telle que une forte élévation de l'hypocotyle, de la tige et de la racine, une réduction de la dominance apicale ou des fusions d'organes. Également, le développement des tiges axillaires qui se produit normalement sur un mode basipétal se présente chez les lignées sous-exprimant le gène *DR4* sous un mode acropétal. Des croisements génétiques entre les plantes DR4AS et des lignées exprimant le gène *GUS* sous la dépendance d'un promoteur d'un gène de réponse à l'auxine révèle une augmentation substantielle de l'activité de ce promoteur lorsqu'il se trouve dans un fond génétique qui exprime faiblement *DR4*. Ces données suggèrent fortement que la protéine DR4 agit comme un répresseur des gènes de réponse à l'auxine.

Les travaux réalisés au cours de ce projet scientifique de thèse apportent des connaissances nouvelles sur l'implication de l'auxine dans des processus spécifiques de développement des plantes et fournissent des informations originales sur le mode d'action de cette hormone. Il s'agit en particulier des premières descriptions de phénotypes associés à une sous-expression d'un membre de la famille des *Aux/IAA* qui révèlent pour la première fois le rôle essentiel et spécifique joué par ce type de gènes dans la morphogenèse des feuilles et dans le développement du fruit.

Si on replace la caractérisation du gène *DR4* dans le contexte de la maturation des fruits, il est important de rappeler que les effets antagonistes de l'auxine et de l'éthylène ont été souvent évoqués dans la littérature. Toutefois, on constate dans le cadre de notre étude que le phénotype d'hypersensibilité à l'auxine n'engendre pas de perturbation notable du déroulement de la maturation chez les lignées transgéniques à l'exception d'une accumulation plus importante de sucre dans les fruits DR4AS. Ces résultats n'excluent pas définitivement l'éventualité d'un rôle de l'auxine dans le processus de maturation qui passerait par une voie indépendante du gène *DR4*. Il est également possible que l'absence de phénotype visible sur la maturation soit le résultat d'une redondance fonctionnelle au sein de la famille des *Aux/IAA*. La création de doubles et triples mutants par croisements génétiques entre lignées transgéniques affectées dans différents membres de la famille des *Aux/IAA* est maintenant envisagée pour essayer d'éviter une éventuelle redondance fonctionnelle.

*CHAPTER I*

*Bibliographic Review*

---

---



## Introduction

The plant hormone auxin as the first identified plant hormone was discovered more than 100 years ago (Darwin, 1880), now it is well known that the auxins regulate numerous cellular and developmental responses including apical dominance, tropism, vascular patterning, embryos patterning and fruit set. This multiplicity of regulatory activities has spurred considerable interest in mechanisms of auxin signalling and response. Based on the introduction of modern molecular techniques in combination with plant genetics and single-cell biophysics within the past decade the auxin field has advanced enormously. These studies have led to the identification of auxin-response mutants, the characterization of auxin-responsive genes and recently the isolation of auxin uptake and efflux carriers (Bennett et al., 1996; Galweiler et al., 1998; Walker and Estelle, 1998; Palme and Galweiler, 1999). A dominant role for auxin in the control of plant growth and morphogenesis has also been elaborated and preliminary models are emerging to describe the complex molecular relation between auxin signalling and that of other hormones, such as ethylene and cytokinin (Burg and Burg, 1966; Hansen and Grossmann, 2000; Merritt et al., 2001; Rahman et al., 2001).

The *Aux/IAA* genes are a large gene family, many of which are induced by auxin, and can regulate development and auxin-induced gene expression; furthermore, auxin responses require Aux/IAA protein degradation. Being one of the world's most popular vegetable and an important crop, tomato (*Lycopersicon esculentum*, Mill) has long served as the primary model for climacteric fruit ripening research. It has also been a popular organism for genetic and reverse genetic studies.

This chapter describes auxin and auxin action in the first section (A), including its physiological effects, metabolism and signalling, as well as the cross-talk with other hormones. The second section (B) focuses on the *Aux/IAA* genes and their roles in auxin signalling. The last section (C) is the general review on the model plant tomato including fruit development and ripening.

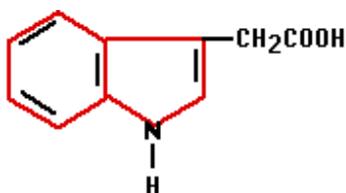
## A- Auxin

*Hormone:* Substance secreted by cells in one part of the body which, after being transported by body fluids, exerts an effect on the activities of cells elsewhere in the body.

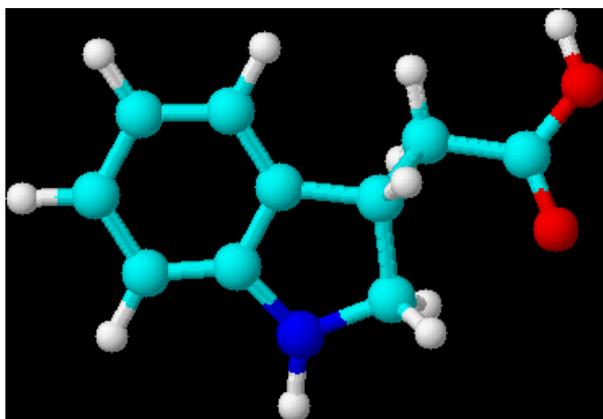
*Plant hormones:* a group of naturally occurring, organic compounds which influence physiological processes at low concentrations ( $<10\mu\text{M}$ ) in plant. Plant hormones are key players in regulating plant growth and development. They control the rate of growth and differentiation of the individual organ systems as they develop to produce the form that we recognize as a mature plant (Davis, 1995).

Auxins are plant hormones.

The natural auxin produced by plants is: indole-3-acetic acid



Indole-3-Acetic Acid (IAA)



**Figure I- 1.** 3D structure of indole-3-acetic acid (IAA)

(<http://www.biochem.arizona.edu/classes/cioc462>)

## ***A-1- Auxin, a crucial hormone***

### **A-1-1- Physiological effects of auxin**

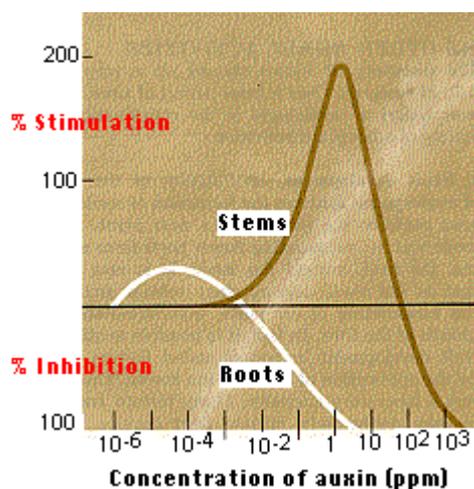
A plant's life cycle from germination to reproduction is under the control of phytohormones, among the growth hormones, auxin was the first plant hormone identified. Based on the phenomenon plants will grow toward a source of light (phototropism), Charles & Francis Darwin found in 1880 that it was the tip of the coleoptile of oat seedlings that sensed the light, but the curvature growth occurred below the tip, so they supposed that the tip sent a signal to the elongation region; in 1913 Boysen-Jensen showed the coleoptile no longer responded to light if water movement was prevented then he concluded that that growth stimulating substance was a water soluble compound; in 1926 F.Went extracted the substance by placing coleoptile tips on agar for an hour, then discarding the tips and using agar to produce growth in the decapitated coleoptiles, and he also named the substance "AUXIN" which comes from the Greek word for growth. Kenneth Thimann later purified and characterized auxin.

The plant hormone auxin plays a crucial role in the control of plant growth and development. Alone or in concert with the other phytohormones, auxin regulates many plant activities, including:

- Cell elongation and division
- Fruit setting, growth and ripening
- Root initiation
- Apical dominance
- Phototropism
- Gravitropism
- Abscission
- Embryo and vascular pattern

➤ *Cell elongation, division and differentiation*

At appropriate concentration auxins can induce cell elongation in coleoptiles (which lead to the discovery of auxin) hypocotyls, stems and intact roots. Division and growth of most types of in vitro-cultured plant cells require an external source of auxin (Petrasek et al., 2002; Zhao et al., 2002). Based on this phenomenon, the “acid-growth hypothesis” states that auxin-induced cell elongation is due to stimulation of a proton pump which acidifies the cell wall by auxin. Acidification causes the crosslinks between the cellulose microfibrils of the cell walls to break, and this loosens the wall, allowing water uptake which results in elongation of the cell (Cleland et al., 1991; Rayle and Cleland, 1992; Schopfer, 1993). In the whole plant body, different parts of cells or tissue have different sensitivity to auxin. The works of Thimann (Thimann, 1963) showed the different effects of different auxin concentrations on root and stem growth (Figure I-2). For example, auxin in a relatively high concentration stimulates stem growth but inhibits root growth (Evans, 1985; Zhao et al., 2002).



**Figure I-2.** *Different auxin dose response to root and shoot*

(<http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/T/Tropisms>)

The function of auxin, as a growth promoter or not, is dependent on its dose. Too much auxin even “kill” the plants. Since the 1940s, synthetic auxins have been used widely as weed killers. These include 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). 2,4-D and its many variants are popular because they are inexpensive and relatively non-toxic to humans. More than that, they are selective herbicides, killing broad-leaved plants but not grasses (till now no one knows the basis of this selectivity).

➤ *Fruit set, growth and ripening*

The onset of ovary development into fruit (fruit set) and fruit development are usually triggered by signals from pollination and fertilization. Fertilization-independent fruit set can also occur either naturally in parthenocarpic fruits (genetic parthenocarpy) or by induction via exogenous application of plant growth regulators (PGRs) such as auxin or gibberellins to flower pistils. Auxins are produced by the pollen tube as it grows through the style, and also by the embryo and endosperm in the developing seeds, fruit set and growth depends on these sources of auxin (Levetin and McMahon, 1998).

Artificial induction via auxin has long been used to study parthenocarpy in tomato (Gustafson, 1937). Application of auxin transport inhibitors that block export of auxins from the ovary also stimulates the development of parthenocarpic fruits (Beyer and Quebedeaux, 1974), which is consistent with reports of higher levels of auxins in ovaries of parthenocarpic tomato fruits (Mapelli et al., 1978; Mapelli and Mombardi, 1982). Using flower bud explants *in vitro* tomato fruit culture demonstrated, among the different phytohormones applied such as cytokinins, only the IAA treated flowers were able to produce fruits, and these fruits didn't contain any seeds (Cohen, 1996). Parthenocarpy was engineered in tobacco, eggplant and different genotypes of tomato using *DefH9-iaaM* chimeric gene. The *iaaM* gene codes for tryptophan monooxygenase, who confers auxin synthesis from the *Pseudomonas syringae* pv. *Savastanoi*, while the *DefH9* controlling regions drive expression of the gene specifically in the ovules and placentas from *Anthirrhinum majus*. Seedless parthenocarpic fruits were obtained from emasculated flowers of transgenic plants, and fruits with seeds from pollinated flower (Ficcadenti et al., 1999).

Auxin not only play a role in cell division and cell expansion during fruit development (Abel and Theologis, 1996), it is also involved in the fruit ripening process as exogenous applications of auxins delay fruit ripening in many crop species (Vendrell, 1985; Cohen, 1996). Moreover, the recent studies showed that down-regulated *DR12* (Developmental Regulated), an auxin-response-factor in tomato, results in dark green and blotchy ripening fruit (Jones et al., 2002). The early fruit development is dramatically altered in *dgt* (*diageotropica*) one auxin-resistant mutant of tomato: the fruit weight, fruit set, and numbers of locules and seeds are reduced in *dgt*, time to flowering and time from anthesis to the onset of fruit ripening are increased, whereas ripening is normal (Balbi and Lomax, 2003).

It was concluded that a number of independent and possibly redundant pathways can direct hormone-induced parthenocarpy (Vivian-Smith et al., 2001). In the absence of

fertilization, *Arabidopsis* pistils treated with auxin, GA and cytokinin produced parthenocarpic siliques that varied in length and morphology depending on the ecotype and the growth regulator applied. The ability to induce silique growth with different classes of PGR indicated that *Arabidopsis* pistils are receptive to a variety of hormonal signals that can subsequently promote parthenocarpic growth and thus uncouple silique growth from the normally linked process of seed development. Structural differences between fertilization-induced fruit and PGR-induced parthenocarpic fruit have also been observed in tomato, blueberry, citrus, rape, watermelon, and pea (Vivian-Smith et al., 2001).

➤ *Root initiation*

Exogenous auxin promotes cell division in the root pericycle, resulting in the production of many lateral roots (Laskowski et al., 1995). In addition, inhibitors of auxin transport suppress lateral root initiation, indicating that normal polar auxin transport is required for lateral root formation (Reed et al., 1998; Casimiro et al., 2001). Mutation analyses of *Arabidopsis* have also shown the importance of auxin in lateral root formation. The *alf1* (*aberrant lateral root formation*) / *sur1* (*superroot1*) / *rty* (*rooty*) mutants, which have elevated levels of endogenous auxin, produce many lateral root, consistent with the observation that auxin promotes lateral root formation (King et al., 1995; Fukaki et al., 2002). In contrast, several mutants defective in auxin transport or auxin sensitivity have reduced numbers of lateral roots, *aux1*, mutant of auxin influx carrier results in reduced lateral root formation (Hobbie and Estelle, 1995; Bennett et al., 1996). Two gain-of-function mutants in *Aux/IAA* gene family *slr* (*solitary-root/IAA14*) and *IAA28* also have greatly reduced lateral roots (Rogg et al., 2001; Fukaki et al., 2002). In addition, the auxin-resistant mutants *axr4* (*auxin resistant4*) and *tir1* (*transport inhibitor response1*) also have reduced numbers of lateral roots (Hobbie and Estelle, 1995; Ruegger et al., 1998; Gray et al., 1999). These studies show that the establishment of an appropriate auxin transport and response system is important for lateral root formation.

Auxin-induced root initiation is widely used to propagate plants. Horticulturists may propagate desirable plants by cutting pieces of stem and placing them base down in the moist soil, eventually when the cutting dipped or coated with small amounts of auxin, the adventitious roots will develop more quickly and in higher number. Most commercially available rooting powders take advantage of this effect.

➤ *Apical dominance*

Growth of the shoot apex (terminal shoot) usually inhibits the development of the lateral buds on the stem beneath. This phenomenon is termed apical dominance. If the terminal shoot of a plant is removed, the inhibition will be lifted, and lateral buds begin growth. Apical dominance seems to result from the downward transport of auxin produced in the apical meristem. In fact, if the apical meristem is removed and IAA applied to the stump, inhibition of the lateral buds is maintained. Further researches demonstrated that auxin derived from apical buds transports basipetally in the stem and does not transport into axillary buds, implying that auxin acts in the stem or nodes, not in axillary buds, and auxin cannot suppress axillary bud growth once it has begun. However, the molecular mechanism underlying regulation of shoot branching is still poorly understood. Gardeners exploit this principle by pruning the terminal shoots of ornamental shrubs, flowers, and to control branching habit in fruit trees. The releasing of apical dominance enable branches to develop and the plant become bushier. The process must be repeated, because one or two lateral will eventually outstrip the others and re-impose apical dominance. “Chemical pinching” in flowering crops to get “bushier” plants with more flowers, and “disbudding” to get single stem with large flower.

➤ *Phototropism*

Phototropism is a plant response to light. Plant shoots display positive phototropism when illuminated from one direction, the shoot proceeds to grow in that direction, roots are negatively phototropic. The direction of light is detected at the tip of the shoot (blue light is most effective): absorbed by a flavoprotein called phototropin, auxin is synthesized at the tip, translocated down in the vascular tissue, and pumped out of cell by efflux transporters (PIN proteins, which are inserted in the plasma membrane of cells on the shady side of the shoot), then stimulates elongation of the cells on the shady side causing the shoot to bend toward the light. (Shen-Miller et al., 1967; Muday, 2001; Stowe-Evans et al., 2001)

➤ *Gravitropism*

Gravitropism is a plant growth response to gravity. Plant roots display positive gravitropism, they grow down in the soil; while the plant shoots display negative

gravitropism: when placed on its side, a plant shoot will grow up. Gravitropism was well documented mediated by auxin. Recent research works showed the possible mechanism of gravitropism. When a root is placed on its side, amyloplasts settle by gravity to the bottom of cells in the root tip, these amyloplasts may be attached to actin filaments which are also attached to vesicles containing PIN proteins, tethered to amyloplasts, PIN proteins are inserted in the plasma membrane on the underside of pericycle cells causing auxin to accumulate on the under side of the root, this inhibits root cell elongation (much higher concentration), so the cells at the top surface of the root elongate, causing the root to grow down. The auxin sent down from the shoot arrives in the central tissues of the root tip. (Chen et al., 1999; Muday, 2001; Boonsirichai et al., 2002; Chen et al., 2002; Yamamoto and Tatematsu, 2002; Ottenschlager et al., 2003)

➤ *Abscission*

In concert with the other phytohormones, auxins regulate the senescence and abscission in fruits and leaves. Young leaves and fruits produce auxin and so long as they do so, they remain attached to the stem, but stresses such as drought, cold, or nutrient deficiencies cause the production of auxin to decrease. Auxin decrease make cells in the abscission layer more sensitive to ethylene, cells then produce more ethylene, which induces synthesis of enzymes that digest the polysaccharides in the cell walls, further weakening the abscission layer. Wind and/or weight make the leaf or fruit to fall by causing a separation in the abscission layer. Fruit growers often apply low level auxin sprays to cut down the loss of fruit from premature dropping, such as apples, oranges and grapefruits. This topical application tricks the plant, activating signals maintain growth. However, high doses of auxins promote abscission by increase ethylene synthesis, then cause fruits or flowers drop. Synthetic auxins have been used commercially in the plant industry for years, heavy applications of synthetic auxins are used to promote a coordinated abscission of various fruits to facilitate harvesting (Levetin and McMahan, 1998).

➤ *Embryo and vascular pattern*

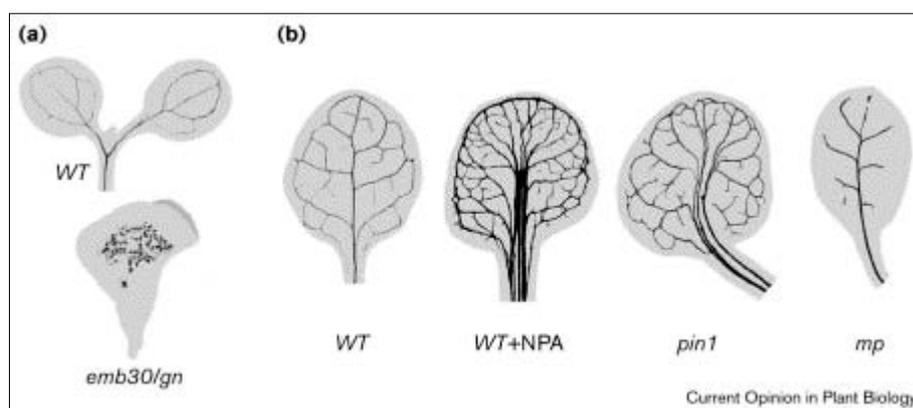
Vascular tissues are conduits for water and nutrients throughout the plant body. They are generated during embryogenesis and organogenesis, expanding along the growth axis of the organ. Vascular development begins with the differentiation of provascular tissue (the

procambium) through periclinal cell division, elongation and alignment (Avsian-Kretchmer et al., 2002). Auxin distribution plays a key role in embryonic pattern formation and the respective vascular pattern (Fischer et al., 1997; Fischer-Iglesias et al., 2001), inhibitors of auxin polar transport disrupt normal embryogenesis (Figure I-3). *In vitro* manipulation of dicot somatic and isolated zygotic embryos provided first evidence that auxin and its polar transport are involved in early pattern formation (Schiavone and Cooke, 1987; Liu et al., 1993; Hadfi et al., 1998). Several *Arabidopsis* mutants well demonstrated that the key role of auxin in embryo and vascular pattern. *monopteros (mp) /auxin response factor 5(arf5)*, *auxin resistant 6 (axr6)*, and *bodenlos (bdl)/iaa12*, three auxin mutants are associated with incomplete vascular systems and defects in the formation of the embryo axis and of the embryonic root (Przemeck et al., 1996; Hamann et al., 1999; Berleth and Mattsson, 2000; Hobbie et al., 2000; Mattsson et al., 2003). *PINOID*, which encodes a serine-threonine protein kinase is first expressed at the globular stage and is proposed to regulate negatively auxin signaling (Christensen et al., 2000).

**Table I-1.** *Arabidopsis* genes essential for vascular strand formation (Berleth et al., 2000)

Gene	Auxin physiology	Vascular anatomy	Seedling & adult morphology
<i>PIN1</i>	Reduced auxin transport: working as auxin efflux carrier membrane protein	Excess vascularization in stem and leaves	Fused cotyledons, defective phyllotaxy, pin-shaped inflorescences
<i>MP</i>	Reduced auxin perception and transport	Reduced vascularization	Missing hypocotyls and primary root, fused cotyledons, pin-shaped inflorescence
<i>AXR6</i>	Reduced auxin perception in gain-of-function mutant	Reduced vascularization	Missing hypocotyls and primary root, fused cotyledons
<i>BDL</i>	Reduce auxin perception	Reduced vascularization	Reduced hypocotyls, no primary root
<i>LOP1</i>	Reduced auxin transport	Reduced vascularization midvein bifurcation	Various organ shape defects
<i>EMB30 /GN</i>	Working as Guanine exchange factor, thought to be essential for polar localization of auxin efflux carriers	Disconnected, randomly oriented vascular cells	Defective seedling polarity, no further organized development
<i>CVPI</i>	Normal auxin content, transport and perception	Reduced cotyledon vascularization, excess stem vascularization	Reduced inflorescence internode length
<i>CVP2</i>	Normal auxin content, transport and perception	Altered cotyledon and leaf vascularization	Normal
<i>HVE</i>	Unknown	Reduced vascularization	Normal
<i>REV/IFLI</i>	Unknown	Missing interfascicular fibers, reduced stem vasculature	Defects in shoot meristem, reduced apical dominance in <i>tev</i> alleles

Auxin is implicated in vascular development, although the molecular details of patterned vascular differentiation have remained elusive. Auxin (supported by cytokinins) can induce xylem tracheary element differentiation in suspension culture cells of suitable species (Fukuda, 1997); auxin-overproducing transgenic plants have increased amounts of vascular tissues (Klee et al., 1987); IAA application can replace leaf primordia in inducing vascular connections in stem; and local auxin sources can induce the formation of new vascular strands from parenchymatic cells (Sachs, 1986). Vascular differentiation in response to IAA application does not occur in all genotypes, suggesting that further factors are often required, including the other hormones. Research in the past years has identified new genes that control vascular patterning, and auxin transport and perception (Table I-1) (Berleth et al., 2000).



**Figure I-3.** Vascular abnormalities resulting from presumed defects in polarity and the intensity of auxin transport, and in auxin sensitivity (Berleth and Mattsson, 2000). (a) Disrupted vascular strands in the *emb30/gn* mutant seedling as opposed to the Arabidopsis wild-type (WT) seedling. *EMB30/GN* has been implicated in the proper polar positioning of auxin efflux carriers and mutations in *emb30/gn* interfere with vascular-strand formation but not with vascular differentiation per se. (b) Arabidopsis leaf-venation patterns are altered (i.e. vascular differentiation close to the leaf margin is enhanced) in leaves treated with the auxin-efflux inhibitor naphthylphthalamic acid (NPA) and in leaves defective for the presumed auxin efflux carrier PIN1. Leaf vascular patterns are characteristically reduced in a class of presumed auxin-insensitive mutants including *mp*.

➤ *How does auxin achieve its many different effects in the plant?*

Auxin effects may be mediated by two different pathways: i) immediate, direct effects on the cell. The arrival of auxin at the surface of the cell initiates such immediate responses as: changes in movement of ions in and out of the cell through the plasma membrane, extension of the cell wall causing elongation of the cell. ii) Turning on of new pattern of genes expression, which in turn to carry out the required responses. It has been known for

decades that auxin can regulate gene expression. Several families of auxin-regulated genes (e.g., the *Aux/IAA*, *GH3* and *SAUR* families) have been identified. Based on the introduction of modern molecular techniques in combination with plant genetics within the past decades the auxin field has advanced enormously, these studies have led to identification auxin-response mutants, characterization of auxin-response genes and isolation of auxin influx and efflux carriers (Bennett et al., 1996; Galweiler et al., 1998; Walker and Estelle, 1998; Palme and Galweiler, 1999). A dominant role for auxin in the control of plant growth and morphogenesis has been elaborated and preliminary models are emerging to describe the complex molecular relation between auxin signalling and that of other hormones such as ethylene, cytokinin. However, only very recently insights into the molecular mechanisms of these auxin-regulated processes have been gained.

### **A-1-2- Synthetic auxins, anti-auxins and auxin transport inhibitors**

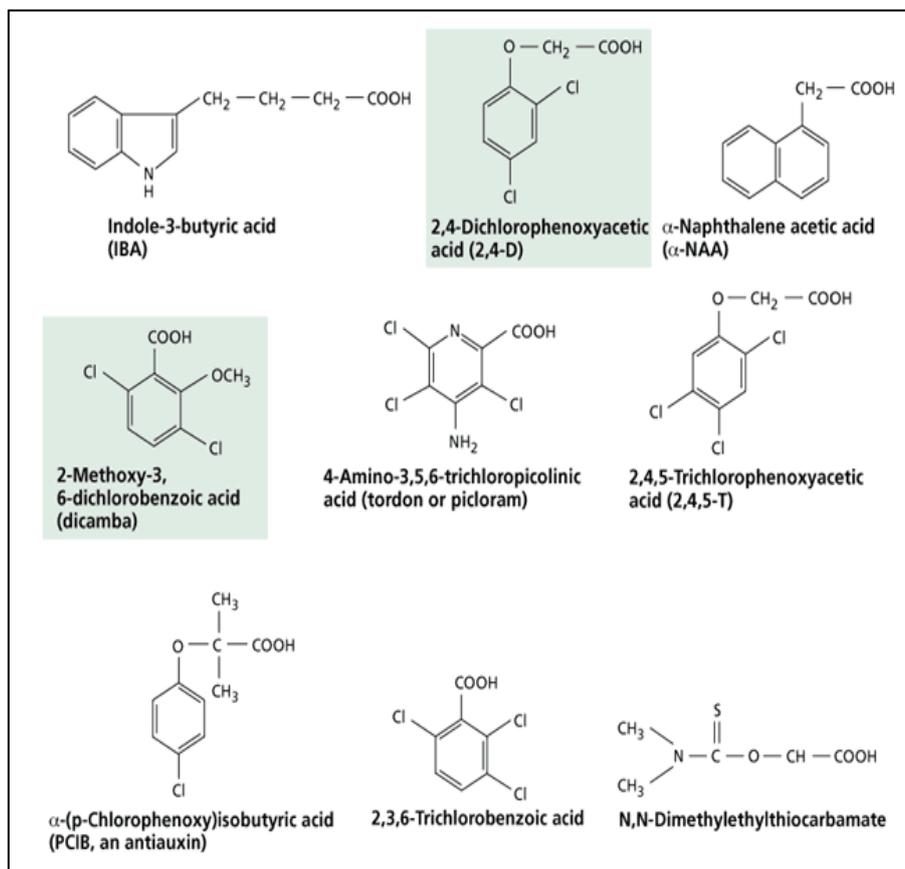
#### ***A-1-2-1- Synthetic auxins***

The natural auxin found in plants is IAA, synthetic auxins (Figure I-4) is a diverse group of chemicals who show IAA-like activities in plants such as be able to stimulate coleoptile elongation, promote lateral roots initiation, etc. Most of synthetic auxins are used as herbicides or plant growth regulators in horticulture and agriculture. The most widely used are probably dicamba and 2,4-D as herbicides, which are not subject to breakdown by the plant and are very stable. A comparison of the compounds that possess auxin activity reveals that at neutral pH they all have a strong negative charge on the carboxyl group of the side chain that is separated from a weaker positive charge on the ring structure by a distance of about 0,5 nm. This charge separation may be an essential structural requirement for auxin activity. The main part of the molecule, the indole ring, is not essential for activity, although an aromatic or fused aromatic ring of a certain size range is required.

#### ***A-1-2-2- Anti-auxins***

Anti-auxins are another class of synthetic auxin analogs. These compounds such as *p*-chlorophenoxy isobutyric acid, or PCIB (*p*-chlorophenoxyisobutyric acid), have little or no auxin activity but specifically inhibit the effects of auxin. When applied to plants, anti-auxins may compete with IAA for specific receptors without triggering an auxin response,

thus inhibiting normal auxin action. It is possible to overcome the inhibition of an anti-auxin by adding excess IAA.



**Figure I-4.** Structure of some synthetic auxins (<http://www.plantphy.net/chapter.php?ch=19>)

### A-1-2-3- Auxin transport inhibitor

Auxin transport inhibitors can block auxin polar transport, which is essential for plant development of leaves, vascular bundles and roots, embryo formation etc. These compounds also termed phytotropins, including:

- NPA: *N- (1-naphthyl) phthalamic acid*
- CPD: *2-carboxyphenyl-3-phenylpropane-1, 2-dione*
- TIBA: *2,3,5-triiodobenzoic acid*
- PBA: *1-pyrenoylbenzoic acid*
- Morphactin: *chlorflurecol-methylester*
- Quercetin dihydrate: *3, 3',4', 5, 7-pentahydroxyflavone\**
- Apigenin: *4', 5, 7-trihydroxyflavone\**

\*Quercetin dihydrate and Apigenin, they are naturally occurring flavonoid with polar auxin transport inhibiting activity.

## **A-2- Auxin metabolism and transport**

### **A-2-1- Auxin biosynthesis**

The natural auxin IAA, is thought to be synthesized in plants locally, in young leaves (the most important IAA source), stem apical meristem, root tips, cotyledon, and seeds in the developing fruits; yet very little research has been performed to verify this “hypothesis” by actually measuring IAA level and IAA biosynthetic rates in different tissues (Ljung et al., 2001; Ljung et al., 2002). Although the physiological role of IAA is well documented, the precise routes of its biosynthesis remain elusive. In 1930, Thimann showed that IAA could be synthesized from the aromatic ring of the amino acid tryptophan. For many years, it was believed that IAA was a product of tryptophan, but later a tryptophan independent way was observed (Baldi et al., 1991). Only with the past twenty years precise details of the IAA biosynthetic pathways begun to emerge, thanks to the stable isotope labelling technique and cloning of genes encoding IAA biosynthetic enzymes. Tryptophan is thought to be the primary precursor for auxin biosynthesis *in vivo*. It was demonstrated (Hull et al., 2000) that the two most commonly accepted routes for IAA biosynthesis *in vivo* are tryptophan, indole-3-acetamide, IAA; or tryptophan, indole-3-acetaldoxime, IAA. While a third route, which converts tryptophan to tryptamines then IAA is still need to demonstrate its function *in vivo* due to lack of experimental data. The Try-independent pathway came from labelling studies of the aquatic plant: *Lemna gibba*, which grows floating on water and readily takes up [<sup>15</sup>N]Trp from medium. Baldi et al (Baldi et al., 1991) showed that when the Trp pool was labelled to 98% with [<sup>15</sup>N]Trp very little <sup>15</sup>N was incorporated into IAA. Later in other plants such as maize, bean, Arabidopsis, Orange, Scots pine were found this Try-independent pathway (Jensen and Bandurski, 1994; Ostin et al., 1999; Ljung et al., 2002). Recent studies have shown that the Trp-dependent and Trp-independent IAA biosynthetic pathways have distinct developmental roles and differ in their environmental responses.

### **A-2-2- Auxin catabolism**

The general model for auxin metabolism includes the synthesis, degradation, and conjugation of IAA. The IAA catabolism describes oxidation and conjugation processes that modify the indole ring or auxins side-chain, and thus cause loss of biological activity. Plant

peroxidases can catalyse oxidation both the side-chain (decarboxylative metabolism) and the indole ring (non-decarboxylative metabolism of IAA), and the later is the major IAA catabolic pathway in *Arabidopsis* (Jensen and Bandurski, 1994; Ostin et al., 1998; Ljung et al., 2002). Conjugation can be considered to be a catabolic process because some conjugates cannot be hydrolysed back to the free active IAA. During conjugation the side-chain of the active hormone is modified, and two distinct groups of conjugates have been described in a variety of plant species: (1) ester-type conjugates, in which the carboxyl group of IAA is linked via the oxygen bridge to sugars (for example glucose) or cyclic poly-ols (like inositol), and (2) amide-type conjugates in which the carboxyl group forms an amide (eg. peptide) bond amino acids or polypeptides (Bandurski et al., 1992; Jensen and Bandurski, 1994; Normanly et al., 1997; Normanly and Bartel, 1999; Ljung et al., 2002).

### **A-2-3- Auxin transport**

Polar auxin transport controls multiple aspects of plant development including differential growth, embryo and root patterning, and vascular tissue differentiation. The disruption of auxin transport interferes with almost all auxin-related developmental processes (Muday and DeLong, 2001; Friml and Palme, 2002; Friml, 2003). It was shown that auxin can be distributed via the phloem, or by directional cell-to-cell polar transport system (Figure I-5) (Firmin and Palme, 2002). The large amount of physiological and biochemical data on polar auxin transport has been integrated into the 'chemiosmotic hypothesis' (Figure I-6). This classical model explains the cell-to-cell movement of auxin by the action of specific auxin-influx and auxin-efflux carriers. The asymmetric positioning of the latter at a particular side of the cell was proposed to determine the direction of auxin flux (Rubery and Shelldrake, 1974; Raven, 1975). This model was reinforced by the identification and characterization of candidate proteins for auxin influx (AUXIN1 [AUX1]/ LIKE-AUX1 [LAX] family) and efflux carriers (PIN family) (Bennett et al., 1996; Galweiler et al., 1998; Luschnig et al., 1998). Numerous pieces of circumstantial evidence demonstrate the role of these proteins in auxin transport despite the lack of rigorous proof for their function as carriers (Friml and Palme, 2002). PIN protein are asymmetrically localized in different cells, and this localization impressively coincides with the known directions of auxin flux (Galweiler et al., 1998; Muller et al., 1998; Friml et al., 2002b; Friml et al., 2002a). The AUX1 protein also localizes asymmetrically in root protophloem cells at the opposite cell side from PIN1 (Friml et al., 2002b). Recently, members of another protein family, Multidrug resistance (MDR)-type

ATP-binding cassette (ABC) proteins were demonstrated to be implicated in auxin transport. Auxin polar transport can be blocked by several compounds such as auxin transport inhibitors or phytoalexins. The *pin* mutant mimicked the phenotype of NPA-treated plants and had reduced auxin transport (Liu et al., 1993).

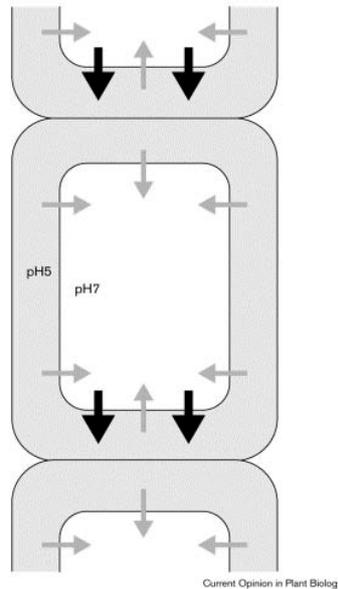


**Figure I-5.** Auxin response and transport in a gravi-stimulated *Arabidopsis* hypocotyl (Friml, 2003). Auxin transport throughout the plant involves both non-polar transport in phloem (dashed line) and an active, cell-to-cell polar transport (red arrows), which can transport auxin either basipetally (from the apex to the base) or laterally. During gravitropic or phototropic bending, increased auxin response (as indicated by *DR5::GUS*, displayed as blue staining) corresponding to higher auxin levels is found in the more elongated, outer side of bending hypocotyl. This asymmetric lateral auxin distribution appears to be established by lateral auxin transport and to trigger asymmetric growth.

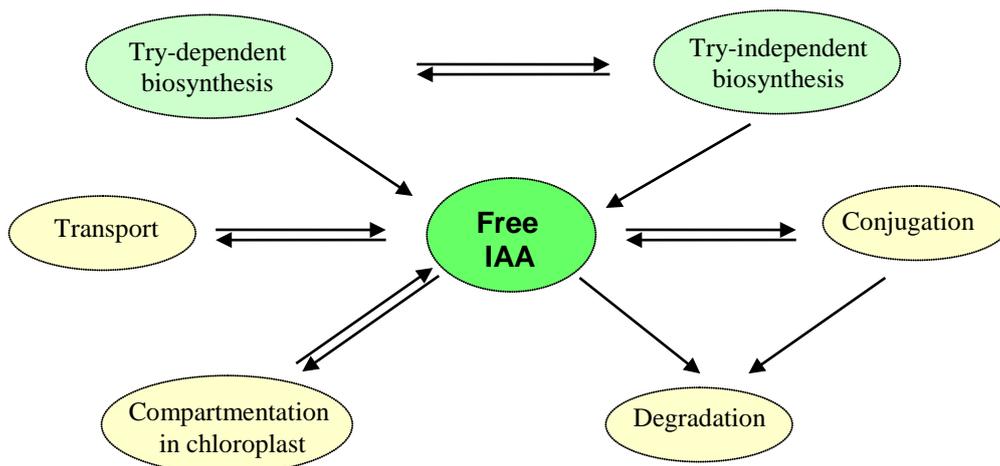
#### A-2-4- Auxin homeostasis

The quantitative temporal and spatial distributions of IAA play important roles in the development of a plant throughout its life cycle. The IAA homeostasis in a specific tissue is dependent on the combined effects of all the processes: biosynthesis, conjugation, degradation, transport and compartmentation in chloroplast (Figure I-7). IAA homeostasis plays a crucial role in plant which maintains the active IAA pool at a level that is optimal for growth and development. For example, in tomato over producing IAA transgenic plants were generated by over-express *iaaM* gene, which confer auxin synthesis, it was shown that the total IAA levels in the leaves of transgenic plants were 50-150 times higher than those in wild

type ones, while free IAA was only 2-3 times higher, indicating a strong homeostatic control of the levels of IAA.



**Figure I-6.** `Chemi-osmotic' model for polar auxin transport (Berleth *et al.*, 2000). The polarity of the overall flow of the auxin IAA is attributed to the polar (selectively basal) localization of auxin efflux carriers in the plasma membrane (black outward arrows). Auxin influx into the cell occurs either directly through the plasma membrane (as IAAH) or is mediated by proton-symport carriers (acting on IAAH plus  $H^+$ ; gray inward arrows). IAAH dissociates at intracellular pH and efflux of  $IAA^-$  is strictly dependent on basally localized efflux carriers.

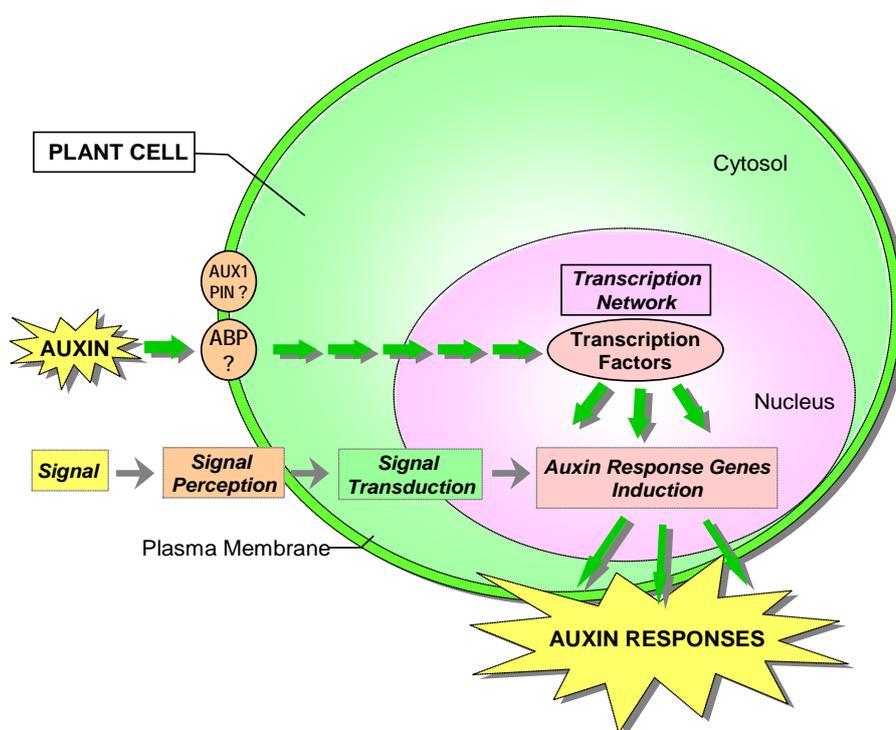


**Figure I-7.** Homeostasis of IAA in plant cell (After Normanly *et al.* 1995). Biosynthesis by tryptophan-dependent and tryptophan-independent pathways can lead only to an increase in the concentration of free IAA. Degradation (either by non-decarboxylative oxidation or by decarboxylation) leads only to decrease in IAA concentration, while a part of conjugation is reversible and can therefore lead to either an increase or a decrease. Both transport and compartmentation can

cause either an increase or a decrease in the cytosolic free IAA concentration, depending on the direction of hormone movement.

### A-3- Auxin Signalling

Signalling pathways mediate the responses of plant cells to environmental and developmental stimuli. In a common view of signal transduction, three steps are involved in each pathway: perception, transduction and induction.



**Figure I-8.** *Auxin signalling and auxin response.* The auxin signal is perceived by plant cells and activates a signalling transduction cascade, which finally recruits specific transcription factors to activate or repress the downstream executor genes, which in turn carry out the required responses in growth and development.

Auxins are small non-protein molecules. The auxin signal is perceived by plant cells and activates a signalling transduction cascade, which finally recruits specific transcription factors, these transcription factors activate the downstream executor genes, which in turn carry out the required responses in growth and development. In such a scheme (Figure I-8), specificity is provided primarily by the transcription factors, which usually have highly spatial and temporal specificity dependent on the different cells and tissues.

### **A-3-1- Auxin signal perception**

Auxin perception is the detection of auxin by plant cells, which only occurs in the target cell that possesses the specific protein receptor. The mechanism of auxin perception is still poorly understood. Several lines of evidence suggest the possibility of several models of perception. Naturally, the search for auxin receptors has focused on proteins that are able to bind auxin. The best candidate auxin receptor is ABP1 (Auxin Binding Protein 1), which was first identified from maize for its ability to bind auxin specifically and with physiologically relevant affinity (Napier et al., 2002). This protein is essential for embryogenesis and manipulation of its levels in transgenic tobacco plants results in changes in auxin sensitivity, including cell division and guard-cell potassium currents (Jones et al., 1998; Leblanc et al., 1999; Baulry et al., 2000; David et al., 2001). However the biology of ABP1 is very curious: although it is clear that it can act at the cell surface, the majority of ABP1 in the cell is retained in the endoplasmic reticulum (Kepinski and Leyser, 2002). Furthermore little is known of the events downstream of ABP1 and the relationship between ABP1 and the other components of auxin signaling is entire opaque.

There is considerable evidence for multiple sites of auxin perception and there is no reason to suppose that ABP1 is the only auxin receptor (Leyser, 2001). In addition to the possible role of auxin binding proteins, it has been proposed that proteins involved in auxin transport might be able to perceive auxin level by monitoring the flux of auxin through transporters (Palme and Galweiler, 1999). In Arabidopsis, there are four members in the auxin influx carrier family, typified by the amino acid permease-like AUX1 (Bennett et al., 1996), and eight members in the auxin efflux carrier family, including PIN1 and PIN2/EIR1/AGR1/WAV6 (review by (Palme and Galweiler, 1999). It is possible that some of these auxin influx/efflux carriers might possess a specialized receptor function in addition to, or instead of, their transport activity (Kepinski and Leyser, 2002).

### **A-3-2- Signals transduction**

#### ***A-3-2-1- Second messengers***

Regulation of pH may both act as a cytosolic second messenger and act in the regulation of cell wall pH described in 'acid growth theory'. In the case of low concentration of auxin, a rapid cytosolic acidification was found, whereas alkalization occurred in the case of very high auxin or of physiological abscisic acid concentrations (Blatt and Thiel, 1994). A

change of the cytosolic pH clearly might activate enzymes inducing the next step(s) (Tena and Renaudin, 1998). Auxin may promote proton extrusion in part by activation of preexisting H<sup>+</sup>-ATPases and in part by causing the de novo synthesis of the H<sup>+</sup>-ATPases. The H<sup>+</sup>-ATPases are activated post-translationally. Enhanced proton pumping hyperpolarizes the membrane, acidifies the apoplast and thereby activates voltage- and proton-activated K<sup>+</sup><sub>in</sub> channels such as KAT1 and KST1 (guard cells) or ZMK1 (coleoptiles) (Becker and Hedrich, 2002). However the auxin-induced proton extrusion has a lag time of about 10 minutes and is inhibited by cycloheximide indicating an indirect mechanism of activation. In conclusion, at least one branch of auxin signal transduction leads directly to activation changes of proteins (ion channels or the proton pump or both) and does not need gene regulation. The resulting change in cytosolic pH may be part of the mechanism of auxin signalling.

Where as or not cytosolic calcium participates in auxin signal transduction is still an enigma, while several papers provided indirect evidence that calcium could be involved in auxin signalling. Induction of a calmodulin-related gene by auxin may be support for this idea but gene regulation is a downstream consequence of signal transduction so that any calmodulin-dependent function late in auxin action would explain its induction by auxin (Becker and Hedrich, 2002).

### ***A-3-2-1- Protein phosphorylation***

Reversible protein phosphorylation is a fundamental post-translational regulatory mechanism, and is involved in controlling vast cellular events and processes. Genetic screens have identified crucial kinase and phosphatase activities that are required for perception and transduction of ethylene, cytokinin, abscisic acid and brassinosteroid signals, whereas, genetic screens for auxin sensitivity mutants have identified no protein kinase- or phosphatase-encoding genes. Several factors may explain this disparity. Kinases and phosphatases crucial to auxin action may play essential roles or may not be 'dedicated' to auxin-related functions, leading to lethal or pleiotropic mutant phenotypes that obscure specific roles in auxin biology. Alternatively, partial functional redundancy of members of a gene family may impede isolation of loss-of-function mutants with dramatic alterations in hormone sensitivity. Finally protein phosphorylation enzymes may provide rheostatic regulation, modulating auxin action rather than functioning as on/off switches (DeLong et al., 2002).

Nonetheless, recent work suggests that protein phosphorylation does play important regulatory roles in both the delivery of and response to auxin signal, and polar auxin

transport. PINOID (PID), a plant-specific serine/threonine protein kinase, is required for the transmission of propagation of auxin signals in the aerial parts of the plant. Changes in *PINOID* expression results in auxin-related defects. Mutants *pid* exhibit indeterminate inflorescence growth and produce floral meristems, characteristic defects in floral organ number and patterning, also a dramatic loss of lateral primordium formation, resulting in a *pin-like* inflorescence, the precise spiral phyllotaxy of wild-type plants is replaced by an apparently random distribution of lateral structures (Bennett et al., 1995; Christensen et al., 2000). These floral defects are accompanied by a characteristic disruption of the underlying vascular elements similar to that found in *ett/arf3* mutants (Christensen et al., 2000). Finally, *pid* mutants are also deficient in the ability to establish bilateral symmetry during late embryogenesis (Bennett et al., 1995). The *pid* phenotypes are similar to but not identical with those associated with mutations of *PINFORMED1* (*PIN*) and *MONOPTEROS* (*MP*) (Okada et al., 1991; Przemeck et al., 1996; DeLong et al., 2002). *PIN* and *MP* are now cloned and encode a putative auxin efflux transport protein and a member of the *ARF* family of transcriptional regulators respectively (Galweiler et al., 1998; Hardtke and Berleth, 1998). These similarity phenotypes suggest that PID also regulates steps in either the transport of or response to auxin. Further more the *PID* promoter contains a consensus auxin response element, TGTCTC, 240 bp upstream of the transcription start site (Ulmasov et al., 1997a), indicating that the induction or de-repression of *PID* expression may involve ARF binding (Ulmasov et al., 1997a).

Although the experimental evidence that auxin signalling plays fundamental role in plant light-response, the mechanism by which auxin potentates light-signalling events, and the point of convergence between the light- and auxin signalling pathways has remained largely speculative. However recent studies indicate that one role of photoreceptor serine/threonine protein kinases involves the auxin- and light-signalling pathway. Semi-dominant mutations in the *SHY/IAA3* gene, a member of the auxin-up-regulated *Aux/IAA* transcription factor family, result in photomorphogenic development and the expression of light-inducible genes in dark-grown *Arabidopsis* seedlings (Tian and Reed, 1999). Analogous gain-of-function mutations in *AXR2/IAA7* and *AXR3/IAA17* also result in similar seedling phenotypes (Nagpal et al., 2000). These observations suggest that for certain photomorphogenic responses, the phytochromes function, directly or indirectly, through the regulation of *Aux/IAA* activity. It was demonstrated that a number of *Aux/IAA* protein are also substrates for PhyA (Phytochrome A) phosphorylation *in vitro* (Colon-Carmona et al., 2000). The biological relevance of phosphorylation to *Aux/IAA* activity is unclear, but it is tempting to speculate that the

proposed interaction between the light- and auxin-regulated signalling pathways may affect the post-translational regulation of the *Aux/IAA* family. However the precise intermolecular interactions that define the convergence of the light- and auxin- signalling pathways are not yet firmly established.

The recognition that the ubiquitin/RUB pathway is a key component of the auxin signalling apparatus, which has identified additional steps in which the regulation of protein stability by phosphorylation is likely to be important. It has been suggested that the predicted repressor of the early response genes, or the short-lived *Aux/IAA* proteins themselves, may be targeted for ubiquitin-mediated degradation. Furthermore, the recruitment of these proteins into the ubiquitin ligase complex, a step known to require substrate phosphorylation, may be controlled by an auxin up-regulated kinase (Gray and Estelle, 2000).

In *Arabidopsis* an important receptor for blue light-stimulated stem elongation is encoded by the *non-phototropic hypocotyl 1 (NPH1)* locus. The *NPH1* gene has been shown to encode a membrane-associated photoreceptor serine/threonine protein kinase with properties distinct from those of the phytochromes (Liscum and Briggs, 1995). Although *NPH1* undergoes light-dependent autophosphorylation, no other substrates for *NPH1* kinase activity have as yet been identified. However biochemical analyses have shown that *NPH1* interacts with the protein encoded by a second phototropic gene, *NPH3*. Significantly, *NPH3* co-localized with *NPH1* to the plasma membrane and appears to undergo post-translational modification in response to blue light irradiation. Liscum and colleagues have suggested that *NPH3* may function as a scaffolding protein that anchors an *NPH1*-activated phosphorelay complex (Motchoulski and Liscum, 1999). A third phototropic mutant, *nph4*, that is conditionally required for phototropic responses, encodes a member of the auxin response factor protein (ARF) family (Harper et al., 2000). While *NPH4* activity has not been molecularly linked to *NPH1* or *NPH3*, it is likely that *NPH4* acts downstream of the proposed membrane-associated phosphorylation complex.

It was suggested that protein phosphorylation regulates auxin efflux. Several endogenous flavonoid compounds that have polar auxin transport inhibiting activity, was identified as possible endogenous regulators inhibit the activity of protein tyrosine kinases, Bernasconi (1996) hypothesized that NPA-binding protein may have tyrosine kinase activity. The analysis of the *Arabidopsis rcn1* (Roots Curl in NPA) mutant indicates a role for protein phosphatase 2A (PP2A) activity in the regulation of polar auxin transport (Garbers et al., 1996). Signals for numerous growth and developmental responses in eukaryotes are communicated through sequential protein phosphorylation within mitogen-activated protein

kinase (MAPK) pathways. Recently MAPK activity was shown to be rapidly activated by auxin and correlated to auxin response in roots. It was also suggested that MAPK pathway involved in auxin-influenced cell division (DeLong et al., 2002). Although mutations in auxin-activated MAPK components are not yet available, the relevance of this cascade to auxin effects has been demonstrated using an auxin response mutant and an auxin-responsive reporter gene (Mockaitis and Howell, 2000).

### **A-3-3- Induction of auxin response genes**

#### ***A-3-3-1- Auxin response genes***

Over the past 20 years, it has been clearly demonstrated that auxins can also exert rapid and specific effects on genes expression at the molecular level. Numerous sequences that are up-regulated or down-regulated by auxin have been described. The genes that have been most extensively studied are those that are specifically induced by active auxins within minutes of exposure to the hormone, and are induced by auxin in the absence of protein synthesis. These genes are referred to as early (or primary) auxin response genes, and fall into three major classes: *Aux/IAAs*, *SAURs* and *GH3s*.

#### ➤ *Aux/IAA genes*

*Aux/IAA* genes have been identified as rapidly induced auxin response genes in several labs (Hagen and Guilfoyle, 1985; Theologis et al., 1985). The original *Aux/IAA* genes to be described (soybean *GmAux22*, *GmAux28*, *GHI* and pea *PS-IAA4/5* and *PS-IAA6*) were expressed to moderate levels in elongating regions of etiolated hypocotyls or epicotyls. When these elongating regions are excised and incubated in auxin-free medium, the *Aux/IAA* mRNAs are rapidly depleted, and can be rapidly induced by addition of auxin to the medium. Besides auxins, protein synthesis inhibitors, such as cycloheximide (CHX), also induce the accumulation of *Aux/IAA* transcripts (Guilfoyle et al., 1998). Review of *Aux/IAA* genes will be present later in this chapter.

#### ➤ *SAURs genes*

*SAURs*, small auxin up-regulated RNAs, were identified in a differential hybridization screen of clones from auxin-treated soybean elongating hypocotyl sections (McClure et al.,

1989). These RNAs are induced within 2-5 min of exposure to exogenous auxin. *SAURs* are moderately abundant in the zone of cell elongation in soybean hypocotyls, and last strongly expressed in epidermal and cortical cells; induction by auxin results in an elevation of transcripts within the same cell types (Gee et al., 1991). Auxin specifically and transcriptionally regulated soybean *SAURs* (McClure et al., 1989). Auxin-inducible *SAURs* have also been described from mung bean (Yamamoto, 1994), pea (Guilfoyle et al., 1992), *Arabidopsis* (Gil et al., 1994), radish (Anai et al., 1998), and *Zea mays* (Yang and Poovaiah, 2000). In addition to auxin, some of these *SAURs* mRNAs are induced by CHX, but the CHX does not enhance or inhibit auxin-induced transcriptional activation of *SAURs* mRNAs. Many of the *SAUR* genes in *Arabidopsis* are found in clusters, and contain no introns, like those originally identified in soybean. There are evidence that *SAUR* genes are regulated both transcriptionally (by auxin) and post-transcriptionally (*SAURs* are unstable mRNA). *SAUR* proteins may also be regulated post-translationally. The *SAUR* protein abundance is low, suggesting that *SAUR* protein half-life may be very short.

The function of *SAUR* proteins is still unknown; however they may play some role in an auxin signal transduction pathway that involves calcium and calmodulin, suggesting by the experiments that demonstrate in vitro binding of calmodulin to an amino terminal domain in several *SAUR* proteins (Yang and Poovaiah, 2000).

#### ➤ *GH3* genes

The *GH3* mRNA is one of several sequences that were recovered in a differential hybridization screen of auxin-induced cDNA sequences derived from auxin-treated, etiolated soybean seedlings. *GH3* mRNA is transcriptionally induced within 5 minutes of auxin treatment, and is specifically induced only by active auxins and unaffected by treatment with protein synthesis inhibitors (Hagen and Guilfoyle, 1985). The *GH3* gene encodes a 70 kDa protein of unknown function, and is part of a small, multigene family in soybean (Hagen et al., 1991). The protein is localized to the cytoplasm and appears to be stable (Hagen and Guilfoyle, 2002). A family of *GH3* genes exists in *Arabidopsis*, consisting of 19 members; an additional partial gene contains only the amino-terminal third of protein.

Recent studies of two mutants involving members of *Arabidopsis GH3* gene family suggest that at least some *GH3* proteins in photomorphogenesis, and strengthens the potential link between phytochrome signaling and auxin responses (Tian and Reed, 1999). The *FIN219* (far red insensitive 219) gene is auxin-inducible and encodes a cytoplasmically-localized

protein with homology to soybean GH3 (designated *AtGH3-11*). The *fin219* mutant was isolated as a suppressor of a mutation in a key regulatory component of photomorphogenesis, *COP1* (Hsieh et al., 2000). COP1 is a repressor of photomorphogenic development, and its activity is negatively regulated by light. It suggests that FIN219 defines a new component of the phytochrome A (phyA) pathway, and may play a specific role in the phyA-mediated far-red inactivation of COP1 (Hsieh et al., 2000). The *DFLI-D* gene encodes another GH3 family member (*GH3-6*), and also is auxin-inducible. In the light both hypocotyl cell elongation and lateral root formation are inhibited in the *dfli-D* mutant in whom *DFLI-D* transcript is highly expressed. Also, over expressing soybean GH3 in Arabidopsis plants results in an exaggerated dwarf phenotype and show a deetiolated phenotype when germinated and grown in the dark (Hagen and Guilfoyle, 2002).

### ***A-3-3-2- Promoters of auxin responsive genes***

The promoters of several auxin-responsive genes have been analyzed in some detail, using a variety of methods (e.g. deletion analysis, linker-scanning, site directed mutagenesis, gain of function analysis). The smallest element to be identified as auxin-responsive is a six-base pair sequence, TGTCTC (Ulmasov et al., 1997a; Ulmasov et al., 1997b). This element has been shown to function in both composite and simple auxin-response elements (AuxREs). In composite AuxREs, such as those found in the *GH3* promoter fragment D1 and D4, the TGTCTC element is only functional if combined with a coupling or constitutive element (Figure I-9). Simple AuxREs, derived from the alteration of naturally occurring AuxREs, may function in the absence of a coupling element if the TGTCTC elements occur as direct or palindromic repeats that are appropriately spaced (Figure I-9), (Guilfoyle et al., 1998). Natural and synthetic AuxREs promoter-reporter constructs have been used to study organ and tissue expression patterns of auxin-responsive genes, these constructs have been valuable tools to follow gene expression events during growth responses associated with changes in auxin gradients or sensitivities.

### ***A-3-3-3- Regulators and protein degradation***

To identify proteins that bind to the TGTCTC element, the synthetic AuxRE P3 (4X) was used as bait in a yeast one-hybrid screen of an Arabidopsis cDNA expression library. A novel transcription factor, referred to as auxin response factor1 or ARF1, was identified and

shown to bind with specificity to TGTCTC AuxREs. Genetic and molecular/biochemical approaches have provided a large body to preliminary evidence that both ARFs and Aux/IAA proteins function as transcription factors on primary auxin-response genes. The possible mechanisms involved in auxin-responsive gene expression regulators and protein degradation will be reviewed in detail later (Section B) in this chapter.

### Simple AuxREs



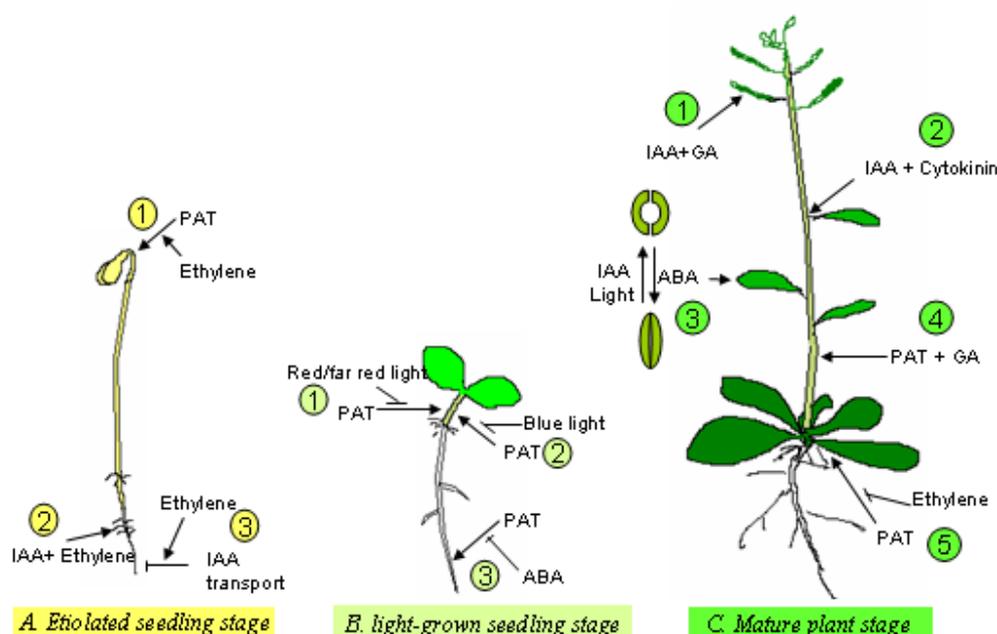
### Composite AuxREs



**Figure I-9.** Simple and composite AuxREs. The TGTCTC elements or inverted GAGACA elements are indicated by arrows. The coupling or constitutive elements in the D1 and D4 promoter fragments of the *GH3* gene are indicated by the underlines. P3 (4X) contains an everted repeat separated by 7 bp, which is identical to ER7, and an inverted repeat separated by 3 bp. ARF1 protein was shown to bind to the ER in a P3 (4X) probe (Ulmasov et al., 1997a). DR5 is a direct repeat, and DR5R is the inverse of the DR5 repeat; both are active AuxREs, indicating that the orientation of the DR has no effect on activity.

#### A-4- Auxin cross-talk with other hormones

Plant sense and respond to endogenous signals and environmental cues to ensure optimal growth and development. Plant cells must integrate the myriad transduction events into a comprehensive network of signalling pathways and response. Auxin occupies a central place within this transduction network, frequently acting in conjunction with each other signals, to coordinately regulate cellular processes such as division, elongation and differentiation (Figure I-10). As a non-cell autonomous signal, auxin also interacts with other signalling pathway to regulate inter-cellular developmental processes.

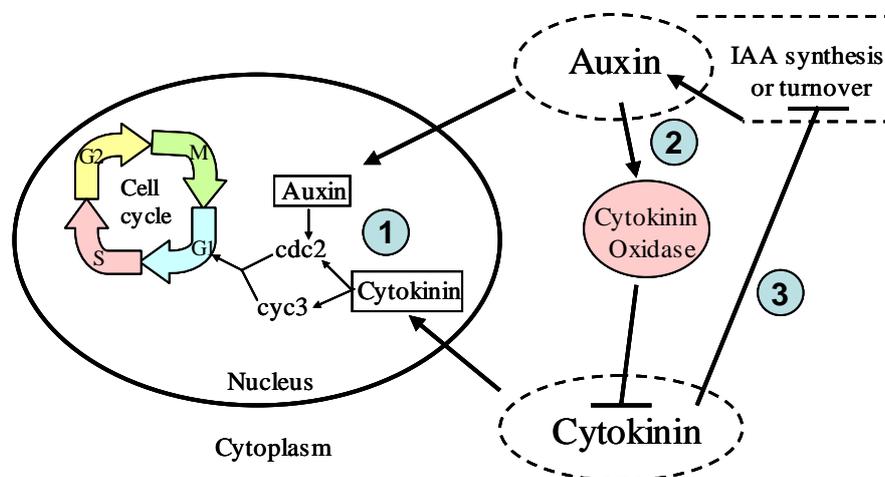


**Figure I-9.** Auxin interacts with other signals to regulate plant development (Swarup et al., 2002b). **A. Etiolated seedling stage;** ❶, ethylene promotes apical hook formation via polar auxin transport (PAT) (Raz and Ecker, 1999); ❷, root hair differentiation and elongation are regulated by ethylene and auxin (Masucci and Schiefelbein, 1996; Pitts et al., 1998); ❸, root growth inhibition by ethylene requires auxin transport (Rahman et al., 2001). **B. Light-grown seedling;** ❶, PAT represents a target for red and far-red light to inhibit hypocotyl elongation (Jensen et al., 1998); ❷, blue light induced phototropic curvature requires PAT-mediated auxin distribution (Briggs and Huala, 1999); ❸, lateral root initiation requires PAT but is repressed by ABA (Casimiro et al., 2001). **C. Mature plant stage;** ❶, both auxin and GA signals promote parthenocarpic fruit development (Vivian-Smith and Koltunow, 1999); ❷, PAT is required for apical dominance, while auxin and cytokinin balance control lateral bud development; ❸, ABA and auxin/light interact antagonistically to regulate stomata aperture (Eckert and Kaldenhoff, 2000); ❹, stem elongation requires both auxin and GA to positively regulate each others biosynthesis (Ross et al., 2000); ❺, ethylene-promoted leaf abscission correlates with reduced polar auxin transport (Beyer and Quebedeaux, 1974).

#### A-4-1- Auxin and cytokinin

Classic experiments by Skoog et al. originally demonstrated that auxin and cytokinin synergistically regulated tobacco pith cell proliferation (Skoog and Miller, 1957). Recent studies conclude that auxin and cytokinin control tobacco cell proliferation by regulation the expression of inter-acting cell cycle components (Figure I-11); the cell cycle in plants is controlled by serine-threonine kinases whose function is controlled by their association with regulatory cyclin subunits; the expression of proteins related to the class of *cdc2* (cyclin-dependent kinases) in tobacco pith explants is up-regulated by auxin. Although the expression of these genes is elevated by auxin, the catalytic activity of the *cdc2* kinase is only increased when tobacco explants are also treated with cytokinin (John et al., 1990). The *cycD3*

transcript has been observed to increase in *Arabidopsis* mutants containing elevated cytokinin levels, and it could be induced by the application of exogenous cytokinin (Nogue et al., 2000).



**Figure I-11.** Auxin–cytokinin cross-talk in plant cell (Swarup et al., 2002b). Auxin and cytokinin regulate plant cell proliferation by ❶ controlling the expression of the cell cycle components *cdc2* and *cycD3* (John et al., 1990; Nogue et al., 2000), whilst the size of cellular pools of auxin and cytokinin are controlled via hormone-regulated enzyme such as cytokinin oxidase ❷ (Zhang et al., 1995), and *Nicotiana tabacum* plants expressing the *ipt* gene have lower levels of IAA due to reduced rates of IAA synthesis and turnover ❸ (Eklof et al., 2000).

It is well established that exposing callus cultures to a high auxin-to-cytokinin ratio results in root formation, while a low ratio of these hormones promotes shoot development (Skoog and Miller, 1957). The ratio hypothesis has been extended to the intact plant to explain other hormone-regulated developmental processes such as apical dominance. Classical experiments have demonstrated that auxin is required for apical dominance (Figure 9), whilst cytokinin promotes lateral bud formation (Wickson and Thimann, 1958). The altered apical dominance phenotype of auxin and cytokinin mutants supports this model. Moreover, the decapitation of the apical bud in *Phaseolus vulgaris* reduced polar auxin transport and increased cytokinin transport from the root by 4000% (Bangerth, 1994).

Auxin and cytokinin frequently control plant development by regulation each other's abundance (Figure I-11), *Nicotiana tabacum* plants expressing the *ipt* gene had lower levels of IAA due to reduced rates of IAA synthesis. Conversely, over-production of IAA when using the *Agrobacterium iaaM/iaaH* genes resulted in a reduced pool size of cytokinins (Eklof et al., 2000). It was demonstrated that cytokinin inhibited the formation of the inactive conjugated IAA-aspartate in mungbean hypocotyl tissues. Cytokinin levels can be reduced in

two ways, through oxidative breakdown and by glucosylation. The enzyme responsible for cytokinin oxidative breakdown is cytokinin oxidase and its activity is increased by auxin (Zhang et al., 1995). However little is known about auxin and cytokinin interact at the signal transduction level, although that is indicated indeed occurring from the cytokinin-response phenotype of auxin mutants such *axr1* (Hobbie and Estelle, 1994) and *axr2* (Wilson et al., 1990).

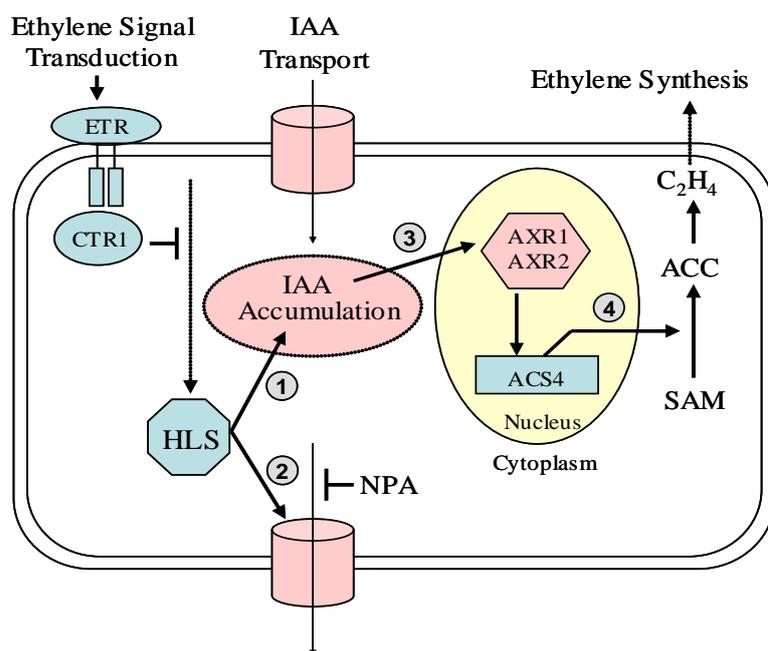
#### A-4-2- Auxin and ethylene

Auxin and ethylene coordinately regulate several developmental programs in plants (Figure I-10, 12), such as apical hook formation (Lehman et al., 1996; Raz and Ecker, 1999), root hair differentiation (Masucci and Schiefelbein, 1994), root hair elongation (Pitts et al., 1998), root growth (Rahman et al., 2001), hypocotyl phototropism (Harper et al., 2000), and leaf abscission (Beyer and Morgan, 1971). It was demonstrated that auxin induce ethylene synthesis in young vegetative tissue. Ethylene synthesis requires the conversion of the precursor S-adenosylmethionine (SAM) by ACC synthase (ACS) and ACC oxidase (ACO). Both *ACS* and *ACO* expression were enhanced by auxin in different plants such as tomato (Yip et al., 1992), *Arabidopsis* (Abel et al., 1995b), and mungbean (Yu et al., 1998).

The induction of apical hook formation in *Arabidopsis* represents one of the best described examples of auxin-ethylene cross-talk in plants (Lehman et al., 1996; Raz and Ecker, 1999). The hypocotyl tissues of etiolated seedling proximal to the shoot apical meristem transiently form a hook. *Arabidopsis* seedling when germinated in the presence of ethylene exhibit exaggerated hook curvature; similarly, ethylene over-producing mutants *eto* and the constitutive ethylene-response mutant *ctr1* feature exaggerated apical hooks in the absence of exogenous ethylene (Kieber, 1997). Auxin has also been demonstrated to regulate hook development. Wild-type *Arabidopsis* seedling germinated in the presence of auxin or the auxin transport inhibitor NPA exhibit no apical hook (Lehman et al., 1996). Likewise, the auxin-over-producing mutant alleles of *superroot1*, *sur1/alf1/hls3*, or auxin transport mutant *aux1* disrupt hook formation (Boerjan et al., 1995; Celenza et al., 1995; Lehman et al., 1996).

Since the auxin transport inhibitor NPA is able to disrupt the hook formation in *eto* and *ctr1* plants, auxin appears to act downstream of ethylene to control hook development (Lehman et al., 1996). The auxin up-regulated gene *AtAux2-11* and *SAUR-AC1* are non-symmetrically expressed in hook tissue, suggesting that differential growth results from either an asymmetric pattern of auxin sensitivity or auxin redistribution in hook tissues. But this

pattern of *AtAux2-11* and *SAUR-AC1* expression in the hook, together with ethylene-induced hook formation, is disrupted by mutations in the *hookless1* (*HLS1*) gene. Ethylene appears to regulate hook formation via *HLS1* since *HLS1* expression is up-regulated by ethylene and *HLS1* over-expressing plants exhibit an exaggerated hook phenotype (Lehman et al., 1996). However, it was also reported that the ACC oxidase gene, *AtACO2*, is differentially expressed in outer versus inner hook tissues, hence the characteristic pattern of differential hook growth may result from asymmetric ethylene biosynthesis.

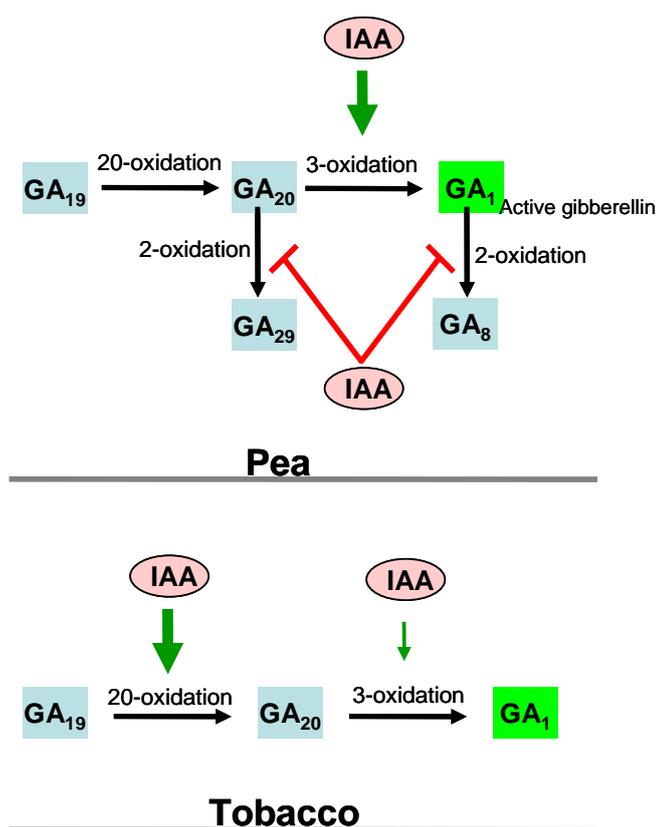


**Figure I-12.** Auxin-ethylene cross-talk in plant cell (Swarup et al., 2002b). Ethylene up-regulates *HOOKLESS1* (*HLS*) gene expression to facilitate apical hook formation by regulation either ① IAA levels or ② auxin transport activity (Kieber, 1997). Auxin up-regulates ethylene biosynthesis via ③ *AXR1*- and *AXR2*- dependent expression of the ④ ACC synthase 4 (*ACS4*) gene (Abel et al., 1995b).

### A-4-3- Auxin and gibberellins

Auxin and gibberellins have been reported to coordinately regulate several developmental programs in plants (Figure I-10) including stem elongation (Ross et al., 2000), root elongation (Fu and Harberd, 2003), and parthenocarpy (Vivian-Smith and Koltunow, 1999). Recently it was discovered that auxin promotes gibberellins biosynthesis in decapitated stems of pea (*pisum sativum* L.) and tobacco (*Nicotiana tabacum* L.) (Ross et al., 2001). Early research demonstrated the Mendel's *le-1* dwarf mutant is gibberellin-deficient, and applying

GA<sub>3</sub> (Gibberellin Acid) to intact plants can restore the stem elongation; in the 1980s it was shown that *le-1* blocks the crucial activation step GA<sub>20</sub> to GA<sub>1</sub> (Figure I-13), reducing the content of bioactive GA<sub>1</sub> and thereby resulting in dwarfism. More recently *LE* was cloned and shown to encode the enzyme for the step GA<sub>20</sub> to GA<sub>1</sub> (Martin et al., 1997).



**Figure I-13.** Relevant parts of the GA pathway in pea and tobacco (Ross et al., 2001). Green arrows indicate the activation by IAA and red bar indicate deactivation by IAA, on the basis of metabolism experiments. The stronger the effect, the arrow is broader.

In pea shoots the main auxin is IAA, which is transported from the apical bud into the elongation internodes. Decapitation (removing the apical bud) reduces the IAA content of the remaining stem tissue (Beveridge et al., 1994; Ross et al., 2000). In decapitated stem, *PsGA3ox1* transcript levels, and consequently GA<sub>1</sub> biosynthesis, are also dramatically reduced. On the contrary the application of IAA (in lanolin paste) to the ‘stump’ of decapitated peas restores both *PsGA3ox1* transcript level and GA<sub>1</sub> biosynthesis. In contrast, expression of the GA deactivation gene *SLN* (*PsGA2ox1*), which encodes the enzyme for the steps GA<sub>20</sub> to GA<sub>29</sub> and (at least *in vitro*) GA<sub>1</sub> to GA<sub>8</sub>, is increased by decapitation; while IAA

application can down-regulate *PsGA2ox1* transcript levels. Similarly, in tobacco, decapitation again inhibited the step  $GA_{20}$  to  $GA_1$ , and this was reversed by IAA, but the effects were less dramatic than in pea (Wolbang and Ross, 2001), but the most intriguing outcome was that the conversion of  $GA_{19}$  to  $GA_{20}$  was strongly inhibited by decapitation (Figure I-13). Once again, IAA counteracted the effect of decapitation, increasing  $GA_{20}$  levels to three times that observed in intact plants. Hence it was concluded that in tobacco, as in pea, IAA from the apical bud is required for GA biosynthesis in stem. However, the main steps promoted appear to differ between the two species: 20-oxidation in tobacco, and 3-oxidation in pea (Figure I-13).

Recently, it was demonstrated that auxin promotes Arabidopsis root growth by modulating gibberellin response (Fu and Harberd, 2003). GA promotes the growth of plants by opposing the effects of nuclear DELLA protein growth repressors, one of which is Arabidopsis RGA (Repressor of *gal-3*). GA opposes the action of several DELLA proteins by destabilizing them, reducing both the concentration of detectable DELLA proteins and their growth-restraining effects. It was shown that auxin was necessary for GA-mediated control of root growth, and that attenuation of auxin transport or signalling delays the GA-induced disappearance of RGA from root cell nuclei. Removal of the shoot apex inhibited the growth of primary roots of wild-type Arabidopsis seedlings; application of auxin to the site from which the shoot apex had been removed restored the growth of the roots of decapitated seedlings to normal. Thus, shoot apex-derived auxin passes to the root and promotes root growth; On the other hand primary roots of the GA-deficient Arabidopsis *gal-3* mutant are shorter than those of the wild type, whereas GA-treated *gal-3* roots are of a similar length to those of the wild type, indicating that GA regulates root growth; Furthermore the roots of decapitated *gal-3* plants were much less responsive to GA, application of IAA to the 'removed shoot apex' site resulted in recovery of the response of roots of *gal-3* seedling to GA; Thus, shoot apex-derived auxin promotes root growth by facilitating the response of root cells to GA. Normally the RGA proteins disappear rapidly from root nuclei by GA treatment; however in the decapitated seedling the RGA proteins remain detectable in the roots, while application of IAA to the removed-shoot-apex site can restore the GA-induced rapid disappearance of RGA proteins, indicating that GA-induced disappearance of RGA is delayed in the absence of shoot apex-derived auxin. Auxin transport inhibitor NPA also causes RGA proteins to be relatively resistant to GA-mediated destabilization. The same, it was demonstrated that auxin efflux carrier AtPin1 promotes the GA-mediated destabilization of RGA (Fu and Harberd, 2003). Mutations at the AXR1 locus reduce auxin response through

attenuation of the function of the SCF<sup>tir1</sup> E3 polyubiquitin ligase. In *axr1-12* mutant the GA-induced disappearance of RGA is also relatively restrained, indicating that a mutation affecting auxin signalling compromised the GA-dependent reduction in RGA level (Fu and Harberd, 2003).

In the absence of fertilization, *Arabidopsis* pistils treated with GA or auxin produced parthenocarpic siliques that varied in length and morphology: GA treatment caused development similar to that in pollinated pistils, while auxin treatment produced shorter siliques. GA is an essential component for silique development in *Arabidopsis*, because both seed and fruit development in the *gal* GA-biosynthetic mutant were dependent on the application of exogenous GA<sub>3</sub> following pollination. While emasculate *gai* gibberellin perception mutants did not produce parthenocarpic siliques following GA<sub>3</sub> application, but siliques development occurred following pollination or application of auxin and cytokinin. Pollinated *gai* siliques had decreased cell numbers and morphologically resembled auxin-induced parthenocarpic siliques. It was suggested that a number of independent and possibly redundant pathways can direct hormone-induced parthenocarpy, and that endogenous GA play a role in regulation cell expansion and promoting cell division in carpels.

Both auxin and GA can regulate seedling hypocotyl elongation. The GA synthesis mutant *gal* has shortened hypocotyl, whilst the constitutive GA-response mutant *spy* has a longer hypocotyl (Swarup et al., 2002a). Transgenic *Arabidopsis* plants *iaaL* having lower IAA level present reduced hypocotyl length, while those have elevated IAA level (*iaaM*) present longer hypocotyl (Romano et al., 1991). Similarly auxin over-producing mutant *sur1* has longer hypocotyls, whilst the auxin-response mutant *axr1* and *axr3* have shorter hypocotyls (Lincoln et al., 1990; Leyser et al., 1993; Rouse et al., 1998). Despite dual control of a common growth process, current evidence suggests that auxin and GA independently regulate *Arabidopsis* hypocotyl elongation (Collett et al., 2000). For example, the hypocotyl of the auxin response mutant *axr1* shows a normal GA response; likewise the *spy* mutant exhibits a wild-type auxin response (Collett et al., 2000).

#### **A-4-4- Auxin and abscisic acid, brassinosteroids, jasmonic acid**

Abscisic acid (ABA) and auxin have been observed to interact antagonistically to regulate stomatal aperture. Auxin causes a reduction in turgor within guard cells which concomitantly serves to open the stomatal pore. Conversely, ABA serves to increase turgor within guard cells thus closing the stomatal pore and reducing water loss via transpiration

(Eckert and Kaldenhoff, 2000). The antagonistic nature of this interaction requires the precise co-ordination of ion channel activity within guard cells. These channels allow the flow of ions that decrease auxin or increase ABA the cytosolic pH and therefore affect the turgor of the guard cells. The other genetic evidence from *Arabidopsis* indicates auxin and ABA may interact to influence root growth and seed germination. Dominant mutations in the auxin-response gene *AXR2/IAA7* confer an ABA-insensitive phenotype to roots, both *axr1* and *axr2* have weak ABA-insensitive phenotypes as measured by seed germination (Wilson et al., 1990; Timpte et al., 1994; Nagpal et al., 2000). Furthermore, *abi3* mutants, originally identified as highly insensitive to ABA, are insensitive to NPA at the level of lateral root growth. Interestingly, in the root primordium, ABA inhibition coincides with a suppression of auxin response and the down-regulation of the putative auxin influx and efflux carriers.

Recent studies indicated that auxin and BR (brassinosteroids) coordinately regulate several plant developmental processes. The gravitropic curvature is increased in maize by either BR alone and in the presence of IAA and BA, but it can be abolished in the presence of auxin transport inhibitor TIBA showing that BR is acting via an auxin-mediated process (Kim et al., 2000). Auxin and BR have recently been shown to synergistically and antagonistically regulate expression of the *ACS* gene family in mung bean hypocotyl tissues (Yi et al., 1999). Upon the characterization of the BR biosynthesis *sax1* mutant, a molecular link has been described between the auxin-response pathway and BR synthesis (Ephritikhine et al., 1999).

Auxin and jasmonic acid (JA) were reported interact antagonistically in the majority of those involved paper. For example, JA inhibits auxin-regulated elongation in etiolated oat coleoptiles (Ephritikhine et al., 1999). Two vacuolar glycoprotein acid phosphatases (*VspA* and *VspB*) from soybean are differentially regulated by JA and auxin during early stages of seedling growth. A promoter dissection study of the *VspA* and *VspB* sequences has revealed specific motifs that positively respond to JA whilst some are negatively regulated by auxin (Tang et al., 2001). Interestingly, *COII*, JA-response gene, is homolog to *TIR1*, and is able to interact with other components of the plant SCF complex which is essential to auxin response (del Pozo and Estelle, 1999b; Kepinski and Leyser, 2002). That raises the intriguing possibility that the JA response, like auxin, is regulated by ubiquitin-mediated protein degradation (Gray and Estelle, 2000). Further research is needed to determine whether any integration of auxin- and JA-signalling pathways is achieved through degradation of common target regulatory proteins.

## **B- Aux/IAA**

### ***B-1- What is Aux/IAA***

Aux/IAAs are auxin-modulated transcription factors; they can regulate development and auxin-induced gene expression. Moreover, the degradation of Aux/IAA proteins is essential for normal auxin response.

#### *➤ Identification of Aux/IAA genes*

*Aux/IAA* genes encoding the Aux/IAA proteins are themselves regulated by auxin, and were discovered and named for this property. *Aux/IAA* genes have been identified in several laboratories as rapidly induced auxin response genes, using differential hybridization with probes from untreated and auxin-treated hypocotyls or epicotyls. The original *Aux/IAA* genes to be described (soybean *GmAUX22*, *GmAUX28*, *GHI* and pea *PS-IAA4/5* and *PS-IAA6*) were expressed to moderate levels in epicotyls. When these elongating regions are excised and incubated in auxin-free medium, the *Aux/IAA* mRNAs are rapidly depleted, and can be rapidly induced by addition of auxin to the medium. Additional *Aux/IAA* genes were subsequently found based on sequence similarity to known *Aux/IAA* genes or in yeast two-hybrid assays with Aux/IAA proteins (Hagen and Guilfoyle, 2002).

#### *➤ Existence*

*Aux/IAA* genes have been found in dicots (pea, soybean, *Medicago truncatula*, Arabidopsis, tomato, tobacco, potato, and cotton), monocots (maize, rice) and gymnosperms pine trees. They have not been found in bacterial, animal or fungal genomes and therefore probably unique to plants (Reed, 2001; Hagen and Guilfoyle, 2002).

### **B-1-1- Aux/IAA protein**

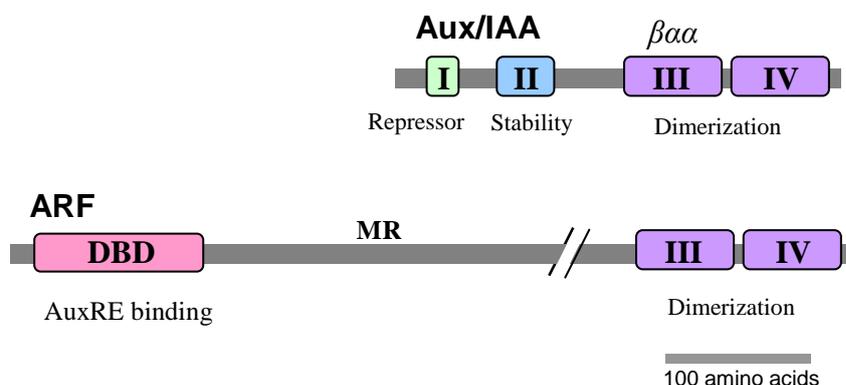
Aux/IAA proteins generally range in size from 20 to 35 kDa. They are short-lived, having half-lives as short as 6-8 min, thereby rare in plants (Abel et al., 1994; Abel et al., 1995a). Function as transcription factors, they are nucleus-localized.

Canonical Aux/IAA proteins share four conserved amino acid sequence motifs (Figure I-14) called domains I, II, III, and IV (Ainley et al., 1988; Abel et al., 1995a), although several predicted proteins lacking one or more of these domains are also included in the family. On the basis of domains I-IV are conserved in multiple Aux/IAA proteins and in different plants families, they presumably have important structural or regulatory functions. Domain I is the smallest one (about nine amino acids long) and is at least strictly conserved. It has not been ascribed a specific biochemical activity, but recent experiments suggest that it may play a role in their repression (Tiwari et al., 2004) and homodimerization of Aux/IAA proteins (Ouellet et al., 2001). Domain II (~ 15 amino acids long) is highly conserved and mutations in this domain increase activity of the corresponding proteins by stabilizing them. Domain II therefore play a role in destabilizing Aux/IAA proteins, and may be a target for ubiquitination (Colon-Carmona et al., 2000; Worley et al., 2000; Ouellet et al., 2001). In a transient assay measuring protein accumulation, 13-amino acid consensus sequence in domain II was sufficient to garget firefly luciferase for low protein accumulation, single amino acid substitutions on these 13 amino acid, corresponding to known auxin response mutants, resulted in a six-fold to 20-fold increase in protein accumulation (Ramos et al., 2001).

Domain III (~40 amino acids long) and IV (~45 amino acids long) have been showed that they can mediate homo- and hetero-dimerization between Aux/IAA proteins and ARF proteins (which share these domains) by yeast two-hybrid assays. Domain III mutant versions of *iaa17/axr3* do not dimerize, further confirming that domain III is required for dimerization (Kim et al., 1997; Ulmasov et al., 1997a; Morgan et al., 1999; Ouellet et al., 2001). Domain III has sequence similarity to the prokaryotic DNA-binding repressor Arc and MetJ (Abel et al., 1994), which folds into an amphipathic  $\beta\alpha\alpha$  structure, and a synthetic peptide containing domain III (but not domain IV) can fold and dimerize *in vitro* (Morgan et al., 1999; Ouellet et al., 2001). However, a role for this motif in DNA binding has not been demonstrated. Thus domain III probably forms a true domain in the protein structural sense and might be sufficient by itself for dimerization. However Domain IV's function is not clear, might also contribute to dimerization, and it has a functional nuclear localization signal (Reed, 2001; Hagen and Guilfoyle, 2002).

The divergent amino acids between these conserved domains might simply be 'filler' loops that enable the conserved domains to adopt the correct tertiary structure, or they might mediate interactions with other proteins, thereby conferring functional specificity on different members of the family. Such functions could include specific regulatory inputs from different

endogenous or environmental signals, or different interactions with transcription factors. If the divergent inter-domain loops indeed confer specific functional attributes on particular Aux/IAA proteins then the Aux/IAA proteins that lack such loops might have more limited and general functions than those with large loops (Reed, 2001).



**Figure I-14.** Diagram of Aux/IAA protein and of ARF protein with conserved domains. In Aux/IAA proteins, domain I acts as a repressor (Tiwari et al., 2004). Domain II plays a role in destabilizing Aux/IAA proteins (Colon-Carmona et al., 2000; Worley et al., 2000; Ouellet et al., 2001). Domain III and IV function as dimerization domains, facilitating interactions among Aux/IAA and ARF proteins (Kim et al., 1997; Ulmasov et al., 1997a; Morgan et al., 1999; Ouellet et al., 2001). Domain III contains a predicted  $\beta\alpha$  fold, which has been characterized in DNA-binding domains of Arc and MetJ repressor proteins (Abel et al., 1994). Sequences between the conserved domains vary in size. ARF contains conserved DNA-binding domain (DBD) that binds to TGTCTC AuxREs of auxin-regulated genes, and shares carboxyl-terminal domain III and IV with Aux/IAA proteins, the divergent regions vary in size. The variable size of the ARF middle region (MR), which confers the ability to activate (Q- rich) or repress transcription, is represented by the break. The relative size represented by the scale. Some Aux/IAA and ARF proteins lack one or more of the conserved domains. For example, IAA20 lacks of domain II and ARF3/ETTIN lacks domain III and IV.

### B-1-2- Aux/IAAs: a large multi-gene family

Aux/IAA genes are present as multigene families in soybean (Ainley et al., 1988), pea (Oeller et al., 1993), mung bean (Yamamoto, 1994), tobacco (Dargeviciute et al., 1998), tomato (Nebenfuhr et al., 2000), and cucumber (Fujii et al., 2000). Arabidopsis may contain as many as 29 Aux/IAA genes, provided by the complete Arabidopsis genome sequence (Table I-2); where 25 deduced genes encoding Aux/IAA proteins that have all four of the conserved domains that define the family, in addition, at least four predicted sequences bear similarity to portions of Aux/IAA proteins but lack one or two of the conserved domains.

**Table I-2. Aux/IAA genes of Arabidopsis.** Aux/IAA genes listed here have been identified based on PCR and hybridization analyses with DNA sequences encoding conserved domains of the proteins, yeast two-hybrid screens with other Aux/IAA proteins as baits, and conceptual translation of the complete genome sequence (Arabidopsis Genome Initiative, 2000). Detailed information (e.g. cDNA accession number, protein properties, gene structure) about each gene/protein can be obtained at the Munich Information Center for Protein Sequences Arabidopsis thaliana Database (MATDB) (<http://mips.gsf.de/proj/thal/db/index.html>) using the MIPS protein code. The position of each gene within the Arabidopsis genome is given by chromosome number and approximate location on that chromosome in megabases (in parenthesis) as determined with the Arabidopsis Information Resource Map viewer (<http://www.arabidopsis.org/servlets/mapper>). Clones on which each Aux/IAA gene resides can also be found at MATDB.

Gene	MIPS protein code	Genome position
IAA1	At4g14560	4 (7.4)
IAA2	At3g23030	3 (9.2)
IAA3/SHY2	At1g04240	1 (1.8)
IAA4	At5g43700	5 (24.3)
IAA5	At1g15580	1 (6.0)
IAA6/SHY1	At1g52830	1 (19.7)
IAA7/AXR2	At3g23050	3 (9.2)
IAA8	At2g22670	2 (10.8)
IAA9	At5g65670	5 (26.4)
IAA10	At1g04100	1 (1.7)
IAA11	At4g28640	4 (19.0)
IAA12/BDL	At1g04550	1 (1.9)
IAA13	At2g33310	2 (15.2)
IAA14/SLR	At4g14550	4 (7.4)
IAA15	At1g80390	1 (32.6)
IAA16	At3g04370	3 (2.0)
IAA17/AXR3	At1g04250	1 (1.8)
IAA18	At1g51950	1 (22.7)
IAA19/MSG2	At3g15540	3 (6.2)
IAA20	At2g46990	2 (20.4)
IAA26/PAP1	At3g16500	3 (6.6)
IAA27/PAP2	At4g29080	4 (19.2)
IAA28	At5g25890	5 (9.1)
IAA29	At4g32280	4 (14.8)
IAA30	At3g62100	3 (23.7)
IAA31	At3g17600	3 (6.0)
IAA32	At2g01200	2 (0.9)
IAA33	At5g57420	5 (23.4)
IAA34	At1g15050	1 (5.8)

Some of the deduced sequences might be pseudogenes, because no information about expression is available for several of them. Arabidopsis Aux/IAA genes have been given *IAAx* designation (such as *IAA1* and *IAA2*) and several of them have compound names (e.g. *IAA17/AXR3*) that reflect the phenotypes of mutants in the corresponding genes. Arabidopsis Aux/IAA genes Accession numbers, genomic locations are as following table I-2, which were compiled in a recent review (Liscum and Reed, 2002).

### B-1-3- Expression patterns of Aux/IAA genes

Expression analysis in seedling or mature *Arabidopsis* plants reveals organ-preferential accumulation of *Aux/IAA* transcripts (Abel et al., 1995a). Some of these genes are expressed in distinct spatial and temporal patterns, thus underscoring the diversity of auxin responses in different plant tissues and organs (Abel and Theologis, 1996; Guilfoyle et al., 1998). This view is corroborated by a study in soybean demonstrating that two classes of early auxin-responsive transcripts, *GH3* and *SAUR*, display both unique and overlapping patterns of tissue-specific gene expression (Gee et al., 1991). More detailed information on cell and tissue-specific expression has been obtained for *AtIAA4*, that is expressed in specific spatial and temporal patterns that correlate with sites and physiological progresses known to be modulated by auxin (Wyatt et al., 1993). A similar and comparative analysis has been performed in tobacco seedling transgenic for promoter-GUS fusions of *Ps-IAA4/5* and *Ps-IAA6* from pea, the data support the observations reported by Wyatt *et al.* (1993) and reveal, in addition that both genes exhibit distinct and common patterns of restricted expression.

#### **B-1-4- Auxin response patterns of *Aux/IAA* genes**

In general *Aux/IAA* genes induced by auxin, and show a range of induction kinetics. Most of them induced very quickly, first detectable increases in steady-state mRNA occur between 4-30 min, and insensitive to CHX, that means their expression is independent of the *de novo* protein synthesis, qualifying them as primary/early auxin response genes. However some others (e.g. *IAA7*, *IAA8*, *IAA9*) mRNA accumulation start to increase only modestly between one or two hours of auxin exposure, and whose induced mRNAs were decreased when administrated together auxin and CHX, that qualifies those genes as late genes (or secondly response genes) whose expression appear to be dependent on *de novo* protein synthesis. Secondly some of them (e.g. *IAA3*, *IAA6*) showed transient response of genes expression to auxin, whose mRNA accumulation are transiently stimulated by IAA, and transcripts levels are adjusted to low basal level after 4 hours in the presence of auxin. However auxin does not only induce, but also can repress other *Aux/IAA* genes (Reed, 2001; Rogg and Bartel, 2001), even some of them such as *IAA28* is found not responsive to exogenous auxin (Rogg et al., 2001).

*Arabidopsis Aux/IAA* genes show a characteristic bimodal shape to auxin dose response and the optimal is 10  $\mu$ M IAA (Abel et al., 1995a). Higher concentrations of IAA are suboptimal, and IAA at 500  $\mu$ M completely inhibits steady-state *Aux/IAA* mRNA accumulation. Simultaneously, at IAA concentrations below 10  $\mu$ M, individual members of

*Aux/IAA* gene family respond in a differential fashion. Some of them start to increase at an IAA concentration as low as 10 nM, and continue to accumulate over a concentration range of 3 orders of magnitude. Some of them are gradually less sensitive; begin to respond only as high IAA concentration as 5  $\mu$ M (as *IAA3*, *IAA7*, *IAA8*) (Abel et al., 1995a). Interestingly, this biphasic dose response to IAA is paralleled by similar bimodal characteristics of other auxin-elicited response in plants, such as stem or hypocotyl elongation, apical dominance, stomatal opening, or auxin-modulation of the inward-rectifying  $K^+$  channel,  $I_{k,in}$  in stomatal guard cells (Blatt and Thiel, 1994).

### ***B-2- Developmental functions of Aux/IAA genes***

Mutation in *Aux/IAA* genes have been detected in several distinct screens for plants with altered auxin response or morphology (Table I-3), and characterizations of these mutants have begun to reveal developmental roles of the corresponding genes. So far, all of those *Aux/IAA* mutations are occurred in the strictly highly conserved domain II, and genetic and molecular studies indicate that they cause gain of function of the corresponding genes, because (1) those mutations confer their corresponding protein more steady, (2) they are each dominant or semi-dominant for some phenotypes, (3) several of the mutant genes (*iaa3/shy2-2*, *iaa7axr2-1*, *iaa12/bdl*, *iaa14/slr1-1*, *iaa17/axr3-1*, and *iaa28-1*) confer phenotypes when introduced into a wild-type background (Tian and Reed, 1999; Worley et al., 2000; Rogg et al., 2001; Liscum and Reed, 2002). All those mutations indicate that domain II play a key role in the function of multiple *Aux/IAA* proteins, and that domain II destabilizes the corresponding protein. Mutations in domain II increase steady-state levels of *IAA3/SHY2* and *IAA17/AXR3* (increased about 7-fold) (Colon-Carmona et al., 2000; Ouellet et al., 2001).

The *aux/iaa* gain-of-function mutations cause auxin-related developmental phenotypes. Interpreting these phenotypes is somewhat problematic because the stabilization caused by the domain II mutations may not mimic regulatory events actually occurring in wild-type plants. It is possible that the stabilized *Aux/IAA* proteins in the mutants are present at concentrations high enough to interact with ARF proteins that they would not normally contact. If this is true, then the phenotypes of the gain-of-function mutants may reflect the expression patterns of the mutated genes more than their 'real' function. In this case, we should compare these results with phenotypes of loss-of-function mutants in the same gene. Unfortunately, the putative *Aux/IAA* gene null mutants that have been characterized have

subtle phenotypes, probably because of redundant activities or feedback regulatory loops that enable the mutant plants to compensate for absence of a particular Aux/IAA protein.

**Table I-3. Auxin signaling mutants**

Gene	Mutant	Protein	Mutant phenotype	Reference
<i>IAA3</i>	<i>shy2-2, -1,</i> <i>shy2-3, -6</i>	Aux/IAA	Reduced stature, short hypocotyl, leaves in dark, up-curved leaves, long root hair	(Reed et al., 1998; Tian and Reed, 1999)
<i>IAA6</i>	<i>shy1-1</i>	Aux/IAA	Reduced stature, short hypocotyl, up-curved leaves	(Kim et al., 1996)
<i>IAA7</i>	<i>axr2-1</i>	Aux/IAA	Reduced stature, short hypocotyl, leaves in dark, wavy leaves, no root hair, agravitropic root and inflorescence stems, reduced adventitious rooting	(Wilson et al., 1990; Timpte et al., 1994; Nagpal et al., 2000)
<i>IAA12</i>	<i>bdl</i>	Aux/IAA	Reduced stature, no embryonic root, reduced shoot apical dominance, curled leaves	(Hamann et al., 1999)
<i>IAA14</i>	<i>slr1-1</i>	Aux/IAA	No lateral roots, agravitropic hypocotyl and root, reduced root hair	(Fukaki et al., 2002)
<i>IAA17</i>	<i>axr3-1, -3</i>	Aux/IAA	Reduced stature, short hypocotyl, leaves in dark, up-curved leaves, increased shoot apical dominance, no root hair, agravitropic root, increased adventitious rooting	(Leyser et al., 1996; Rouse et al., 1998)
<i>IAA18</i>	<i>iaa18-1</i>	Aux/IAA	Long hypocotyl, fused cotyledons, short root, up-curved leaves	(Reed, 2001)
<i>IAA19</i>	<i>msg2-1, -2</i> <i>msg2-3, -4</i>	Aux/IAA	Agravitropic and Aphototropic hypocotyl, reduced lateral rooting	(Watahiki and Yamamoto, 1997)
<i>IAA28</i>	<i>iaa28-1</i>	Aux/IAA	Reduced stature, decreased apical dominance, reduced lateral rooting	(Rogg et al., 2001)
<i>ARF3</i>	<i>ett</i>	ARF	Abnormal apical-basal gynoecium development	(Sessions et al., 1997)
<i>ARF5</i>	<i>mp</i>	ARF	Severely reduced leaf vasculature (both numbers and connections); defects in root meristem initiation in the early embryo, reduced auxin transport	(Hardtke and Berleth, 1998; Mattsson et al., 1999)
<i>ARF7</i>	<i>nph4, tir5,</i> <i>msg1</i>	ARF	Aphototropic hypocotyl with reduced gravitropic response, reduced auxin-inducible gene expression	(Liscum and Briggs, 1995; Ruegger et al., 1998; Watahiki et al., 1999)
<i>AXR1</i>	<i>axr1-12</i> <i>axr1-3</i>	E1 RUB-activating enzyme	Auxin resistant, reduced stature, reduced shoot apical dominance, crinkled leaves, abnormal flowers, reduced lateral rooting, reduced auxin-inducible gene expression	(Lincoln et al., 1990; Timpte et al., 1995)
<i>TIR1</i>	<i>tir1-1</i> <i>tir1-3</i>	F-box	Auxin resistant, reduced auxin-induced hypocotyl elongation, reduced lateral rooting	(Ruegger et al., 1998)
<i>ASK1</i>	<i>ask1-1</i>	SKP	Auxin resistant, abnormal flowers, reduced lateral rooting	(Gray et al., 1999)
<i>PINOID</i>	<i>pid</i>	kinase	Loss of lateral primordium formation, lose of spiral phyllotaxy, defects in floral organ number and patterning	(Bennett et al., 1995)

Despite these caveats, the gain-of-function phenotypes do provide useful insight into the developmental processes that Aux/IAA proteins can potentially regulate. These domain II mutations in different *Aux/IAA* genes affect various tissues and developmental responses, including root or shoot gravitropism, lateral root formation, shoot apical dominance, stem elongation, leaf expansion and leaf formation in the dark (Table I-3). In general, the phenotypes can be explained in terms of reduced auxin response, and some auxin-induced genes are expressed at lower levels in *axr2-1* and *shy2-2* mutants (Reed, 2001). However, *axr3-1* plants have some phenotypes consistent with hypersensitivity to auxin, such as ectopic expression of an auxin-regulated gene and formation of adventitious roots (Leyser et al., 1996). Therefore, Aux/IAA proteins might both activate and inhibit auxin responses, depending on the particular Aux/IAA protein and /or the tissue.

Loss-of-function *aux/iaa* mutations have been isolated by screening for intragenic suppressors of gain-of-function alleles (Timpote et al., 1994; Rouse et al., 1998; Tian et al., 2002), or for insertions among T-DNA-insertional mutant populations. Several of the intragenic suppressors create premature stop codons or shift the reading frame, and are therefore probably null mutations. Other affect single amino acids in one of the other conserved domains. The T-DNA insertion mutation that has been described in *AXR2/IAA7* lacks the corresponding transcript and is probably a null allele (Nagpal et al., 2000). However, in contrast to the gain-of-function counterparts, all of the examined loss-of-function mutants have relative minor effects on growth and development. The subtlety of these phenotypes suggests that other Aux/IAA proteins may compensate for absence of one of them in these mutants. This could occur if the genes are redundant, or if feedback regulatory mechanisms buffer the effects of loss of a single protein function. Efforts are underway in several laboratories to find insertion mutations in additional *Aux/IAA* genes, and double or more mutants (Liscum and Reed, 2002). Analysis of these mutants should reveal other functions of *Aux/IAA* genes.

Protoplast transfection assays also demonstrated that Aux/IAA proteins function as active repressors by dimerizing with ARFs bound to auxin response elements (Tiwari et al., 2001), and degradation of Aux/IAA protein is essential for normal auxin signalling (Worley et al., 2000; Ramos et al., 2001; Zenser et al., 2001). Mutations in domain II resulted in increased repression, whereas mutations in domain I and III partially relieved repression. Aux/IAA proteins fused to heterologous DNA binding domain were targeted to promoters of constitutively expressed reporter genes and actively repressed transcription in an auxin responsive and dose-dependent manner. In comparison with an unfused luciferase protein,

luciferase fused to Aux/IAA proteins displayed less activity, which further decreased in the presence of auxin in transfected protoplasts. Domain II mutations increased and domain I mutations decreased luciferase activity with the fusion proteins (Tiwari et al., 2001).

### ***B-3- Aux/IAAs interact with ARFs***

#### **B-3-1- ARF proteins**

Studies suggest that Aux/IAAs may interact with ARFs to regulate auxin responses. ARFs encode auxin response factors; they are transcriptional activators and repressors that bind with specificity to TGTCTC auxin response elements (AuxREs) (Figure I-14) in promoters of primary/early auxin response genes. ARFs contain an N-terminal DNA binding domains (DBD) which bind to TGTCTC with specificity and in most cases a conserved C-terminal dimerization domain (CTD). The ARF CTD is related in amino acid sequence to motifs III and IV found in Aux/IAA proteins. Just C-terminal to the DBD, ARFs contain a non-conserved region referred to as the middle region (MR), which has been proposed to function as a transcriptional repression or activation domain. Results with transfected protoplasts reported showed that ARFs with Q-rich MRs function as activators, whereas most, if not all other ARFs, function as repressors (Ulmasov et al., 1997a; Ulmasov et al., 1997b; Ulmasov et al., 1999a, b; Tiwari et al., 2003). ARF DBDs alone are sufficient to recruit ARFs to their DNA target sites, and auxin does not influence this recruitment. ARF MRs alone functions as activation or repression domains when targeted to reporter genes via yeast Gal4 DBD, and auxin does not influence the potency of activation or repression. ARF CTDs, along with Q-rich MR, are required for an auxin response whether the MR plus CTDs are recruited to a promoter by an ARF DBD or by Gal4 DBD (Tiwari et al., 2003).

*ARFs* are found in dicots, monocots, gymnosperms and ferns (GenBank EST database), but are not found outside of the plant kingdom. There are 22 *ARF* genes and 1 or more pseudo genes in *Arabidopsis* (Liscum and Reed, 2002). Analysis of *AtARF1- AtARF10* has shown that these *ARF* mRNAs are ubiquitously expressed in most major organs, as well as suspension culture cells. Presently, it is not known if *ARF* mRNAs are expressed in a tissue-specific manner within major organs. Of the *ARFs* been tested, exogenous auxin treatment (50  $\mu$ M NAA 5 h) did not alter *ARF* mRNA levels (Liscum and Reed, 2002). In contrast to loss-of-function mutations in the *Aux/IAA* genes, loss-of-function mutations in *ARF* genes have quite distinct phenotypes (Table I-3). Mutations in three different *ARF* genes

(*ARF3*, *ARF5*, and *ARF7*) have been identified through morphological screens, each of these mutations control separate morphological and developmental processes and have little functional overlap. Mutations in several other *ARF* genes have been identified in reverse-genetic screens but do not confer such dramatic phenotypes, and it is proposed that they may act redundantly with each other or have less essential roles (Liscum and Reed, 2002).

### **B-3-2- Auxin responses are mediated by interaction between Aux/IAA and ARF**

All but two *ARFs* in Arabidopsis (*ARF3/ETTIN* and *ARF17*) contain a C-terminal dimerization domain (Figure I-14), which is related in amino acid sequence to motif III and IV found in Aux/IAA proteins. Yeast two-hybrid experiments suggest that ARF CTDs and Aux/IAA proteins can homodimerize and heterodimerize with some selectivity (Kim et al., 1997; Ulmasov et al., 1997b; Ouellet et al., 2001). In contrast DNA-binding capacity of the Aux/IAA proteins has yet to be conclusively demonstrated. It has therefore been hypothesized that Aux/IAA proteins regulate transcription by modifying ARF activity. The number of interactions within and between Aux/IAAs and ARFs is potentially vast. Both the Aux/IAAs and ARFs constitute large families in Arabidopsis, with 29 Aux/IAAs and 23 ARFs. Tissue-specific and developmental variations in the expression suggest that some of these proteins, although capable of dimerizing, will be separated spatially and temporally in the growing plant. Nevertheless, the interactions are likely to be complex, reflecting the need for fine control of auxin induction of numerous genes (Kepinski and Leyser, 2002).

ARF DBDs alone are sufficient to recruit ARFs to their DNA target sites, and auxin does not influence this recruitment. ARF MRs alone function as activation or repression domains, auxin does not influence their function. However, ARF CTDs, along with Q-rich MR, are required for an auxin response whether the MR plus CTDs are recruited to a promoter by an ARF DBD or by Gal4 DBD (Tiwari et al., 2003). Further more auxin response requires Aux/IAA proteins turn over. Therefore, it was proposed that Aux/IAA proteins are repressors that do not bind to TGTCTC AuxREs directly but are targeted to AuxREs by dimerizing with ARF transcriptional activators when auxin concentrations are low, resulting in the repression of primary auxin response genes. When auxin concentrations are increased, the auxin response genes are postulated to be de-repressed after the dissociation (and proteolysis) of the Aux/IAAs from the ARF activators (Tiwari et al., 2003).

In light of this model, functions of ARF proteins are likely to provide insight into functions of Aux/IAA proteins that might interact with them. Mutations in genes encoding

three ARF proteins indicate that they mediate auxin responses. The *ETTIN/ARF3* mutations affect floral patterning; *MONOPTEROS/ARF5* mutations affect formation of vasculature and embryonic phyllotaxy, and *MSG/NPH4/ARF7* mutations decrease auxin sensitivity in the hypocotyl and leaf, and cause defective tropic responses and auxin-regulated gene expression. All three of these developmental phenotypes reflect processes that depend on spatial gradients of auxin concentration, suggesting that these three ARF proteins might control cell fate decisions in response to local auxin concentrations. *ETTIN/ARF3* probably does not interact physically with Aux/IAA proteins because lacks domains III and IV. However, competition among ARFs for auxin-binding promoter elements might provide an indirect means for interactions with Aux/IAA proteins (Reed, 2001).

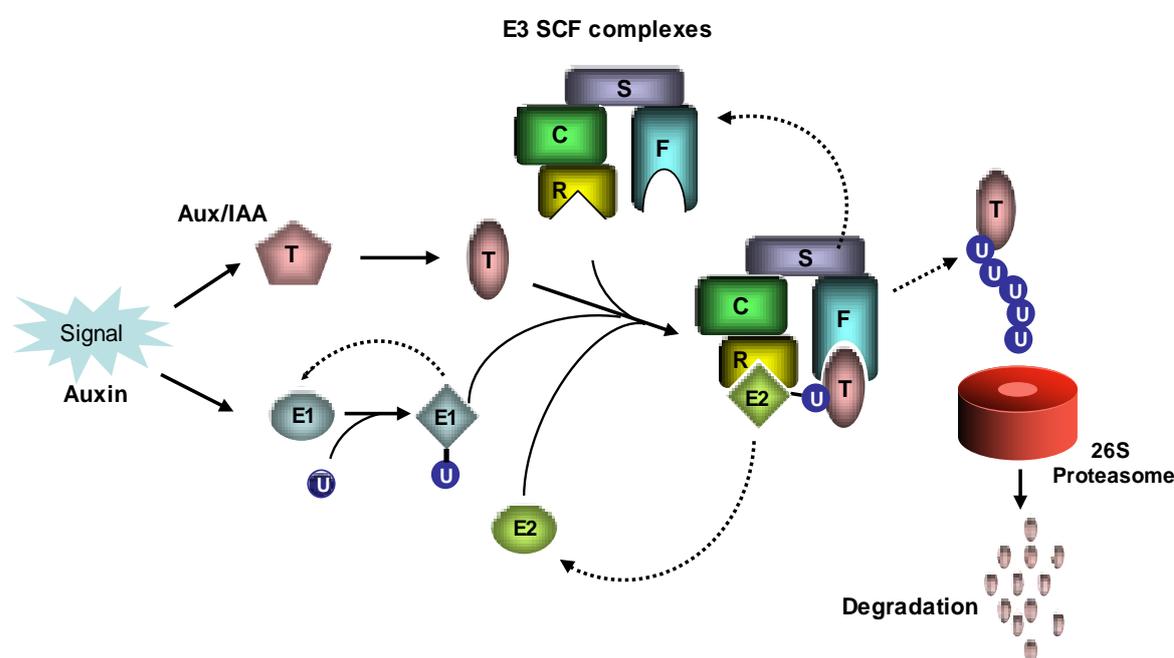
#### ***B-4- Aux/IAA protein degradation is essential for normal auxin signalling***

Recently it was demonstrated that auxin rapidly modulated the degradation rate of Aux/IAA proteins, and SCF<sup>TIR1</sup>-dependent degradation of Aux/IAA proteins is essential for normal auxin signalling (Worley et al., 2000; Ramos et al., 2001; Tiwari et al., 2001; Zenser et al., 2001; Tiwari et al., 2003).

##### **B-4-1- SCF<sup>TIR1</sup>-dependent ubiquitination mediates the auxin response**

Ubiquitin-mediated proteolysis has emerged as being as fundamentally important as phosphorylation in terms of its involvement in diverse cellular events. Target proteins are condemned to degradation in the 26S proteasome by the addition of a polyubiquitin chain in what is essentially a three-step process (Figure I-15). First, ubiquitin is activated by the ATP-dependent formation of a thiolester bond with a conserved Cys residue of an ubiquitin-activating enzyme, E1. Activated ubiquitin then is passed to one of a family of E2 ubiquitin-conjugating enzymes, from which it is conjugated to a Lys residue of the target protein, often with the assistance of an E3 enzyme, ubiquitin protein ligase. This process is reiterated such that one or two additional ubiquitin are polymerized upon the first ubiquitin molecule. Target proteins marked with less than four ubiquitins are poor substrates of the 26S proteasome and often escape degradation. It is E3 enzyme that recruits the substrate target protein. Hundreds of E3s of various classes are sequenced to date (Deshaies, 1999; Xiao and Jang, 2000). Prominent among these are SCF ubiquitin ligases (for Skp1p, Cdc53p/cullin, and F-box

protein in yeast and mammals) (Deshaies, 1999; Xiao and Jang, 2000). SCF complexes are multimeric enzymes of at least four subunits built around a member of the cullin family of proteins. In conjunction with a second subunit, RBX1, the cullin catalyzes polyubiquitin chain formation by interacting with the E2 enzyme. The cullin also binds to a member of the SKP1 protein family, which, in turn, binds to an F-box protein, so called because it contains an N-terminal F-box motif required for its interaction with SKP1. The C-terminus of the F-box protein consists of protein-protein interaction domains that bind the relevant target protein, making it accessible to the E2-SCF complex (Kepinski and Leysner, 2002).



**Figure I-15.** Auxin regulates ubiquitination of target proteins, leading to their degradation by 26S proteasome. Key components of this pathway are shown. E3 SCF complexes are multimeric enzymes, in which at least four subunits were built around a member of the cullin (C) proteins. In conjunction with a second subunit, RBX1 (R), the cullin catalyzes polyubiquitin chain formation by interacting with the E2 enzyme. The cullin also binds to a member of the SKP1 (S) protein family, which, in turn, binds to an F-box protein (F). The C-terminus of the F-box protein consists of protein-protein interaction domains that confer its ability to bind the relevant target protein (T). Under the auxin inducement, target proteins are recruited to the E3 SCF ligase by the F-box protein. Auxin-regulated modification of targets, which include the Aux/IAA proteins, is likely to be required before recognition by the F-box protein. Ubiquitin (U) is activated (via an E1 enzyme) and conjugated to target (via E2-E3 interaction), a process that is reiterated to form a polyubiquitin chain. The ubiquitination activity of the SCF is enhanced by modification of the cullin subunit. Then the polyubiquitinated-target proteins are sent to the 26S proteasome to degradation.

The crucial evidence for the involvement of SCF-dependent protein degradation in auxin signalling came through the characterization of the *tir1* mutants of Arabidopsis (Table I-3) (Ruegger et al., 1998). Although the *tir1* alleles were identified in a screen for resistance to inhibitors of auxin transport, the mutants were more markedly resistant to inhibitory concentrations of auxin, suggesting a defect in auxin response rather than auxin transport. *tir1* was found to encode an F-box protein (Ruegger et al., 1998), and subsequently was shown to interact with either of the Arabidopsis SKP1-like proteins, ASK1 or ASK2, and the cullin AtCUL1 to form the functional SCF<sup>TIR1</sup> (Gray et al., 1999; Gray et al., 2001). The idea that regulated protein degradation via SCF<sup>TIR1</sup> is required for proper auxin signalling was strengthened by the studies of mutation in *ASK1* (Table I-3). *ask1-1* shows reduced auxin response, being slightly more auxin resistant than *tir1-1*. This may reflect the fact that *TIR1* has several close homologs in Arabidopsis, indicating the potential for redundancy in *TIR1* function (Gray et al., 1999). The C-terminus of TIR1 and its homologs consists of leu-rich repeats, which presumably are involved in target selection (Ruegger et al., 1998). What, then, are the targets for SCF<sup>TIR1</sup>? In the search for candidates, the Aux/IAAs are likely suspects.

#### **B-4-2- Aux/IAA proteins are targeted to SCF<sup>TIR1</sup>**

The evidence of the involvement of SCF<sup>TIR1</sup> in Aux/IAA proteins degradation came from the analysis of *AXR3/IAA17* reporter fusion proteins in the *tir1* mutant background. This fusion protein consists of N-terminus (including domain I and II) of *AXR3*, fused to *GUS* and expressed under the control of a heat-inducible promoter in transgenic Arabidopsis. After heat induction in a wild-type background, the fusion protein accumulates to relatively low levels and is depleted rapidly over time compared with controls. However, the stability of the same fusion protein was increased significantly when the construct was crossed into the *tir1* background, indicating that TIR1 was required for the degradation of *AXR3 in planta*. That TIR1 activity ultimately might result in *AXR3* degradation in the 26S proteasome was supported by the finding that specific proteasome inhibitors stabilise *AXR2-*, *AXR3-*, and other Aux/IAA-reporter fusion proteins (Gray et al., 2001). It was also demonstrated that in plant extracts the direct interaction between SCF<sup>TIR1</sup> and *AXR2* or *AXR3* (Gray et al., 2001).

#### **B-4-3- Regulation of the SCF<sup>TIR1</sup>-Aux/IAA interaction by auxin**

The mechanism by which auxin influence Aux/IAA proteins target to SCF<sup>TIR1</sup>, in turn results in Aux/IAs turnover, is not understood. However, clues to what might be happening may be gleaned from the many characterized examples of SCF-mediated signalling pathways in yeast and mammals. SCF-mediated ubiquitination usually is preceded by substrate modification. These changes turn stable proteins into susceptible targets of the SCF, and by far the most common modification is phosphorylation (Deshaies, 1999). Although there is certainly an increasing body of biochemical and genetic evidence supporting a role for kinases in auxin signalling, their potential role in regulating the stability of Aux/IAs is far from certain. This is because the 13-amino acid degron (for degradation region) of domain II that is sufficient to confer auxin-inducible destabilization on a reporter protein does not contain any essential phosphorylation sites (Ramos et al., 2001). This means that regulation of Aux/IAA stability is unorthodox, and if phosphorylation is a pre-requisite of interaction with SCF<sup>TIR1</sup>, it must involve some kind of adaptor protein. On the other hand, examples of non-phosphorylation-dependent regulation of substrate-SCF interactions are beginning to emerge. For example through the hydroxylation of pro residue, which is the most striking feature of the consensus domain II degron (two highly conserved tandem pro residues).

Another point at which the regulation of Aux/IAA ubiquitination could conceivably take place is at the level of SCF complex abundance, assembly, or activity. The abundance of TIR1 might be important for normal auxin sensitivity, because over expression of *TIR1* in transgenic plants results in increased auxin response. Another important event is affecting the activity of SCF complex. It involves the modification of the cullin subunit of the SCF by its conjugation with an ubiquitin-like protein called RUB1 that is important for optimal SCF function (del Pozo and Estelle, 1999a). This modification with RUB1 (rubinylation) requires AXR1, one of the first mutationally defined auxin response genes. The *axr1* mutants exhibit pleiotropic auxin-related phenotypes indicative of wide-ranging auxin resistance, even more severe than that displayed by the *tir1* alleles. Importantly, Aux/IAA-reporter protein fusions are stabilized in an *axr1* background (Gray et al., 2001). And *axr1 tir1* double mutants have a synergistic phenotype, indicating they act in an overlapping pathway to destabilize Aux/IAA proteins (Ruegger et al., 1998). *AXR1* encodes a subunit of enzyme RUB-1 activating enzyme, which is structurally and functionally homologous with the E1 ubiquitin-activating enzyme (Leyser et al., 1993; del Pozo and Estelle, 1999a; del Pozo et al., 2002).

#### **B-4-4- Aux/IAA stability is critical for auxin signalling**

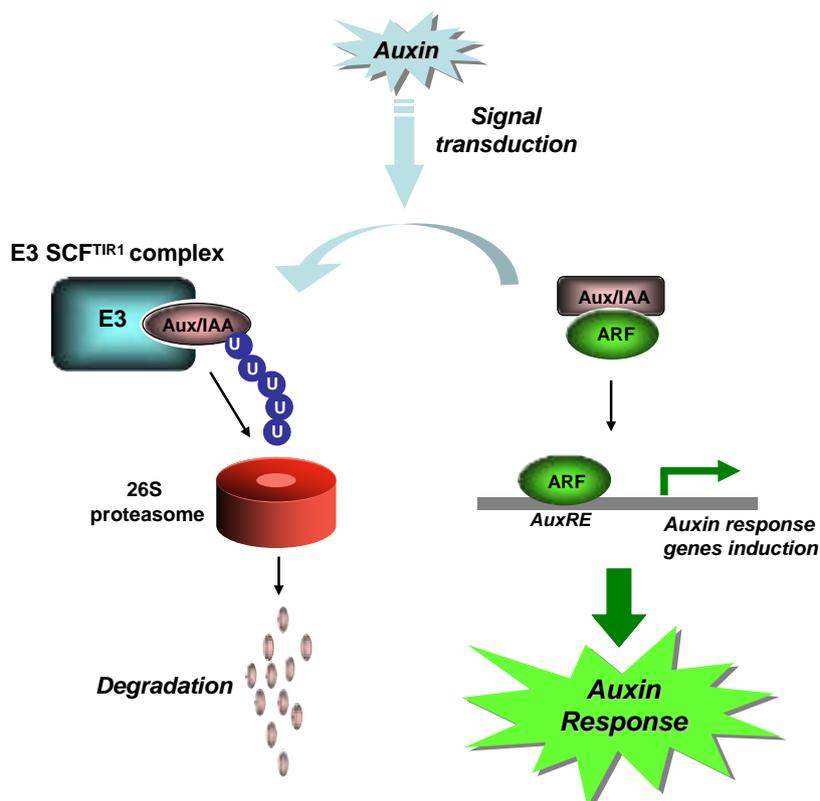
Aux/IAAs are short-lived nuclear proteins, and rapidly degrade with auxin inducement. The importance of the Aux/IAAs destabilisation is manifest in the severe auxin-related *aux/iaa* mutants (*iaa3/shy2-2*, *iaa7axr2-1*, *iaa12/bdl*, *iaa14/slr1-1*, *iaa17/axr3-1*, and *iaa28-1*) (Table I-3). Each of these mutations change an amino acid in domain II, the further studies shown that those mutations confer the corresponding protein more steady, plus the other genetic and molecular studies indicate that they cause gain of function. The spectacular auxin-related phenotypic effects of these single-amino acid substitutions illustrate the critical importance of domain II to auxin signalling. Domain II is the site of the destabilization that confers instability on Aux/IAA members. Translational fusions of Aux/IAA N-terminus containing domain II with reporter proteins such as  $\beta$ -glucuronidase (GUS) or luciferase have shown that this liability can be conferred on otherwise stable proteins (Worley et al., 2000; Gray et al., 2001). The transferable destabilization signal within Aux/IAAs has been finely mapped to a 13-amino acid region around the core GWPPV residues. Together, these data stress the importance of Aux/IAAs and their instability to auxin response.

### ***B-5- A basic model for auxin-induced genes expression***

Summing up all of those data have led to a basic model for auxin-induced genes expression (Figure I-16). At basal levels of auxin, Aux/IAAs are relatively stable and dimerize with ARF proteins on AuxREs, reducing transcription, or preventing the recruitment of activating ARFs. With auxin inducement, an increasing proportion of Aux/IAAs to be degraded by 26S proteasome via SCF complex, allowing the formation of a greater number of ARF-ARF dimers on AuxREs, and hence higher levels of transcription of auxin-responsive genes, which in turn, carry out the required auxin responses. However this model lacks details, such as the level of multi-merization of the interacting Aux/IAAs and ARFs.

Recent progress toward discovering the functions of *Aux/IAA* genes has come from the fortuitous discovery of gain-of-function mutations in several of these genes. However, we do not know the precise developmental functions of each of these genes and analyses of loss-of-function single and multiple mutants might lead to the next advances in understanding *Aux/IAA* gene function. We would also like to know the extent to which Aux/IAA proteins function as antennae for the myriad hormonal and environmental signals that influence the basic mechanisms of cell growth, division and differentiation, and the extent to which Aux/IAA proteins can integrate multiple signals. In vivo biochemical studies will be essential

to understand whether (and how) auxin and other signals regulate the activity of the Aux/IAA and/or ARF proteins, and might provide important insight into how auxin is perceived. Finally, global gene expression studies of both gain- and loss-of-function mutants might reveal the regulatory targets of Aux/IAA and ARF proteins, thereby providing a clearer view of how auxin-regulated developmental processes unfold.



**Figure I-16.** A basic model for the auxin-induced gene expression. In this model, at basal levels of auxin, Aux/IAA proteins remain bound to ARFs and prevent their function. Auxin may induce the ubiquitin proteasome pathway-mediated degradation of Aux/IAA proteins, releasing ARFs then bind to AuxREs in auxin-inducible gene promoters, which in turn, give auxin response. Gene activation might be enhanced further by the dimerization of ARF transcriptional activators to ARFs that are bound to AuxRE target sites.

## C- Tomato as model plant

Development, maturation, ripening and associated quality characteristics of fleshy fruit have been studied in a variety of plant species and tomato (*Lycopersicon esculentum*, Mill) has emerged as a model system from which the majority of available information

regarding the molecular regulation of development and ripening has been derived. However, the plant model *Arabidopsis* has also served as a useful model to unravel the molecular mechanism of ethylene signalling which positively impacted the study of climacteric fruit such as the tomato. Strawberry served as a model for non-climacteric fruit in a number of studies though in a much lesser extent than the tomato. Noteworthy, direct assessment of gene function via stable integration of transgenes can be routinely carried out with all three species.

In large, partly due to its importance as a crop species, tomato has long served as the primary model for climacteric fruit ripening and offers a good model for other crop species whose fruit is also a fleshy berry. It has also been a popular organism for genetic studies and within the last two decades has become the model system for fundamental studies on fruit ripening, partially because of its importance combined with diploid inheritance, ease of seed and clonal propagation, efficient sexual hybridization, a relatively short generation period, and year-round growth potential (in greenhouse) has fostered tomato as the primary model for ripening research. The tomato is an excellent species for genetic and reverse genetic studies. It is a simple diploid consisting of twelve chromosome pairs (Rick and Yoder, 1988). Compared to many species, it has a relatively small genome and a high density molecular map containing >1000 markers have been established (Rick and Yoder, 1988). Approximately 978 monogenic mutants are available, affecting all aspects of plant development. Tomato cells are easily cultured and whole plants readily regenerated from cells and callus tissue. Furthermore, the tomato is also susceptible to *Agrobacterium tumefaciens* infection, allowing ready use of *Agrobacterium*-mediated transformation techniques.

### ***C-1- Ethnobotany of the tomato***

The origin of the cultivated tomato is somewhat unclear. Ethnobotanists and geneticists however have attempted to track down the centre of domestication. The genus *Lycopersicon*, the botanical group to which the tomato belongs, is native to western South America, and only *Lycopersicon lycopersicum* var. *cerasiforme*, the wild cherry form of the cultivated species, has spread throughout the world. Mexico, particularly the Vera Cruz-Puebla region, appears to have been the site of domestication and the source of the earliest introductions. When the tomato finally made its way to Europe in the 16<sup>th</sup> century, the public responded with fear for several probable reasons. The tomatoes belong to the family *Solanaceae*, which includes the deadly poisonous species *Datura* and *Belladonna*, the

assumption was that tomatoes must be poisonous as well. In Germany, the tomato plant was rejected because of its terrible smell, and tomato acquired names like “Devil’s wolf apple”. Today the toxicity of the *Solanaceae* family has been studied extensively, and it has been found that most of the species are poisonous, potato plant is also quite toxic, and *Lycopersicon* spp., which are less toxic than the other members of the family, contain tomatine, a toxic glycoalkaloid. Today it is almost impossible to imagine the cuisines without the tomato in Europe, the Italians called it “*pomodoro*” (golden apple), while the French originally called it “*pomme d’amour*” (apple of love). The name tomato, however, is actually derived from the Aztec name “*tomatl*”

### ***C-2- Horticultural characteristics***

It is one of the world’s most popular vegetable and an important crop in the fresh vegetable market as well as in the food processing industry (Rick and Yoder, 1988). It is grown in practically all over of the world in outdoor fields, greenhouse and net-house. The principal producers for the world market in the FAS list are the United States, Italy, Turkey, Greece and Spain. The tomato plant is very versatile and the crop can be divided into two categories: fresh market tomatoes and processing tomatoes, which are grown only outdoors for the canning industry and mechanically harvested. In both cases world production and consumption has grown quite rapidly over the past 25 years. In developing countries the tomato is becoming more and more important part of the food basket. Tomato fruit is rich in vitamin C and A, a fact which is becoming more important in modern diets, and contains an antioxidant lycopene; the average tomato is more than 90% water, high in vitamin C and can be incorporated into almost every food preparation.

### ***C-3- Genetic and genomic resources on the tomato model***

Mutants of tomato have been collected over several decades, and today 978 monogenic stocks (at 607 loci) are described and deposited in the CM Rick Tomato Genetic Resource Center at the University of California, Davis (<http://tgrc.ucdavis.edu>). These stocks are from several sources: spontaneous and induced mutants, natural variants from the edible tomato and wild relatives. The number of sequenced ESTs is 141734 as of November 30, 2001 ([http://www.ncbi.nlm.nih.gov/dbEST/dbEST\\_summary.html](http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html)), corresponding to 29 210

unique sequences in the most recent EST assembly (<http://www.tigr.org/tdb/lgi>). The link between mutants and DNA sequences is known for only a few dozen tomato genes. Ideally, knockout mutants should be available for each gene, and this is one of the goals of the Arabidopsis 2010 genome project and of maize genome projects. With current rough estimates of the number of genes in tomato ranging from 30 000 to 40 000, much work remains to be done. The population size required for saturated mutagenesis in plants is in the  $10^3$  range for EMS (ethylmethane sulfonate) or fast-neutron irradiation, and in the  $10^5$  range for low-copy insertional mutagenesis (Emmanuel and Levy, 2002).

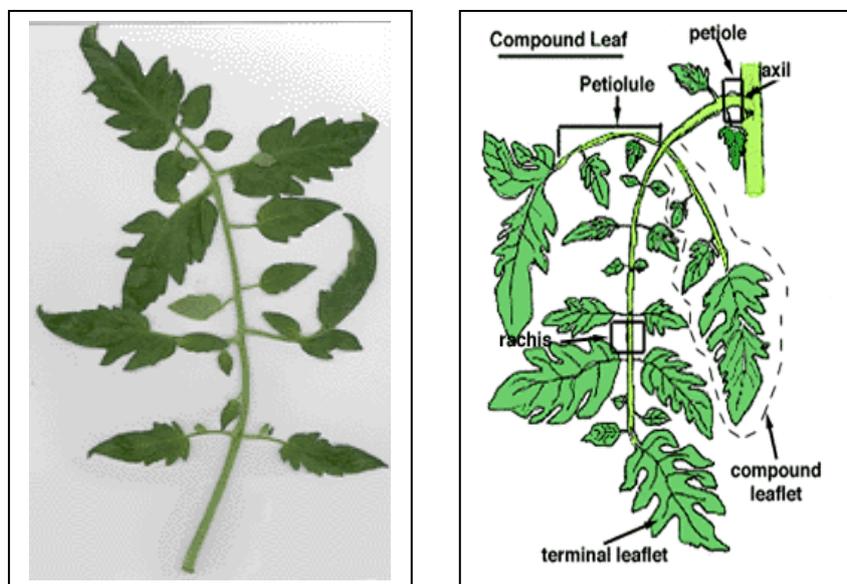
#### ***C-4- Micro-Tom ( $\mu$ Tom): a miniature tomato with rapid life cycle***

The determinate miniature *L. esculentum* cultivar *Micro-Tom* is a new good system for tomato genetics research. It has small size (10-20cm tall) and rapid life cycle (70-90 days from sowing to fruit ripening). It grows at high density; thousands of plants can be grown in three or four generations per year in confined environments. It is also easily transformed at frequencies up to 80% through *Agrobacterium*-mediated transformation of cotyledons.  $\mu$ Tom offers the possibility of conducting mutagenesis experiments on a routine basis, for example, to find second-site mutants or in the background of a transgenic plant; with heterologous transposons; for T-DNA tagging; for activation tagging and so on. The small size of  $\mu$ Tom allows experiments to take place in a confined and controlled environment that complies with genetically modified organism (GMO) safety regulations and protects the experiments from vandalism. Moreover,  $\mu$ Tom differs from standard tomato cultivars by only two major genes. Therefore, any mutation or transgene can be conveniently studied in  $\mu$ Tom background and, when needed, transferred into a standard background by standard cross. One of the shortcomings of  $\mu$ Tom is that it produces only a small number of seeds per fruit and just a handful of fruit when grown in dense conditions. The utility of  $\mu$ Tom mutagenesis in functional genomics is starting to emerge (Meissner et al., 1997; Emmanuel and Levy, 2002).

#### ***C-5- Tomato plant characteristics***

Tomatoes are short-lived perennials cropped as annuals. Cultivated tomato is divided into two types, indeterminate and determinate, the former being vine type and the latter terminating in a flower cluster with shoot elongation stopping. Tomato plants carry compound

unipinnate leaves that exhibit a basipetal order of leaflet initiation and maturation (Figure I-17). Upon maturation each leaf carries major and minor leaflets, most of which are lobed to various degrees, which exhibit plagiotropic growth. The tomato phyllotaxy is spiral, only one leaf is present at each node and each successive leaf is displaced approximately 137,5 degrees from the last. Many types of trichomes are found on the stem of the tomato plant. Among the most interesting are the multi-cellular glandular trichomes which often contain crystals composed of calcium oxalate and cadmium oxalate. Essential oils are often stored in the bulbous region of these structures as well, giving the plant its characteristic odor and sticky texture. Both crystals and essential oils may serve as defenses against herbivory.



**Figure I-17.** Unipinnate compound leaf of tomato. The tomato plant has compound leaves. It is made up of leaflets which are distributed along the leaf rachis. While the entire leaf is connected to the stem by the petiole, the leaflets are connected to the rachis of the leaf by the petiolule. Some of the leaflets on this leaf are compound as well.

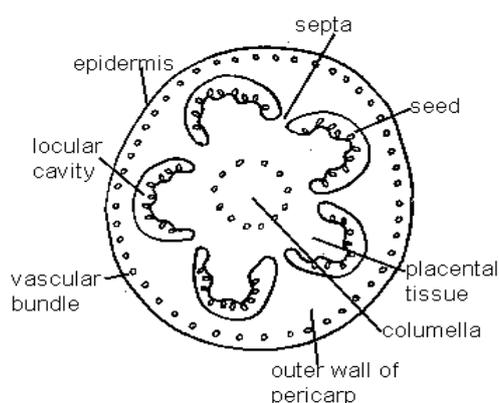
### **C-6- Biology of tomato fruit**

The fruit is a specialized organ that evolved to provide a suitable environment for seed maturation and, in many cases, as a means for seed dissemination. Of the incredible diversity of fruit that exists in nature, humanity has chosen relatively few to form the basis of our diet. The majority of these have been subjected to centuries of intensive genetic selection for size and organoleptic traits. Despite this, there remains great potential for improving fruit quality in even the most thoroughly modified species.

Fruits can be divided into two broad categories, climacteric and non-climacteric, depending on whether or not they exhibit a peak in respiration and ethylene production during ripening. Common climacteric species include the tomato, melon, apples, bananas and avocado. Species that ripen non-climacterically include cherries, grapes, strawberries, pineapple, and citrus fruit. Owing largely to the economic importance of ripening, a great deal of research has centered on unraveling the mechanisms involved in this process. With the exception of the ripening process itself, however, our understanding of the underlying mechanisms involved in fruit development remains, at best, rudimentary.

### C-6-1- Tomato anatomy

Tomato fruit is a fleshy berry consisting of a pericarp, derived from the ovarian walls, which surrounds the placental tissue and the seeds (Figure I-18).



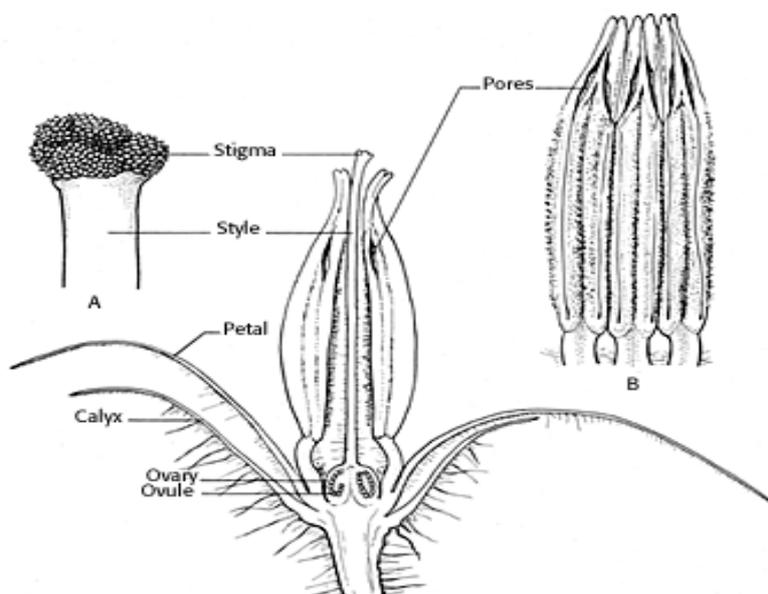
**Figure I-18.** Transverse section of five locule tomato fruit. Tomatoes can be either bilocular or multilocular. Most cultivated varieties except cherry tomatoes have four or five locules. The locules are surrounded by the pericarp. The pericarp includes the inner wall, columella; the radial wall, septa; and the outer wall. The pericarp and the placenta comprise the fleshy tissue of the tomato. The seeds are located inside of the locular cavities and are enclosed in gelatinous membranes. There are vascular bundles throughout the outer wall of the pericarp and traveling from the stem to the center of the tomato and from there radiating to each seed.

### C-6-2- Tomato fruit ontogeny: from flower to maturation

#### C-6-2-1- Flower

Tomato plant is day neutral, flowering under condition of either short or long days; the inflorescence is a monochasial cyme of 4 to 12 perfect hermaphrodite and hypogynous flowers (Figure I-19). Primitive tomatoes have the *solanaceous* trait of five flower parts and

green sepals, but modern tomato varieties often have more than five yellow petals. The five anthers are joined to form a yellow cylinder that around the pistil in *Lycopersicon*, one of the

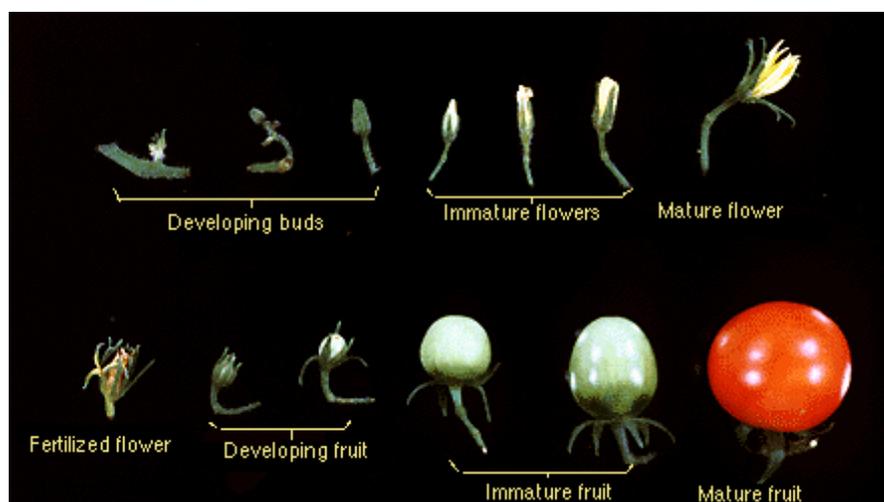


**Figure I-19.** *Tomato carries perfect hermaphrodite and hypogynous flowers.* The outermost whorl of tomato flowers consists of the five to six green hairy sepals. Collectively, the sepals are called the calyx; the next whorl, the bright yellow petals, serves to attract pollinators, together, the petals are called the corolla. In the full of bloom both sepals and petals curved backward. Sitting inside of petals, stamens are fused and form a cylindrical cone surrounding the carpel. A single tomato stamen consists of two elongated compartments. The tomato carpels are green; consist of stigma, style, and ovary which will develop into tomato fruit. The ovules which will develop into seeds are protected in the carpel.

key distinctions from the closely related *Solanum* genus. Wild *Lycopersicon* species, which are self-incompatible and therefore are obligate cross pollinators, have their style exerted beyond the anther cone. Cultivated tomatoes are self-fertile, and their style length is similar to the anther length, a characteristic that favors self-pollination. The style elongates about the time the anthers begin to split at the terminal end and release their pollen into the style tube.

The stigma is receptive to its own or other pollen 1 or 2 days before anther dehiscence, which favors cross-pollination. The stigma remains receptive for 4 to 8 days. The construction of the anthers, delicately united with the filament, permits them to vibrate at the slightest touch and send a rain of pollen down the cone outlet and around the stigma. The tomato carpels are green, they vary in number from cultivar to cultivar, but they are invariably fused together into a single bulb-like structure. The number of carpels in the tomato flower corresponds to the number of locules found in the fruits. The ovules which will develop into seeds are protected in the carpel.

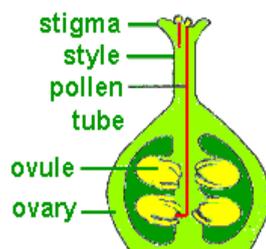
The tomato flower goes through many stages as it develops into fruit (Figure I-20). When the bud first forms the petals are protected by the sepals. Some buds are aborted before they can mature into flowers. As the bud matures the sepals separate to expose the pale yellow-green petals. The petals later turn a deeper yellow. Once the flower is mature it will either be fertilized to develop into a fruit, or it will be abscised. If the flower is not fertilized, it separated from the plant at the abscission zone, this causes the flower to wither and die. If the flower is fertilized it will undergo senescence, during senescence the flower's petals, style, and stigma are shed.



**Figure I-20.** *The tomato flower goes through many stages as it develops into fruit.*

The flowers on a given inflorescence develop at different times. A single tomato inflorescence may have developing buds, mature flowers and developing fruit at one time. The ovule is preceded with double fertilization (Figure I-21); it produces a diploid zygote and a triploid endosperm. The fertilized ovule will become one of the seeds of the tomato fruit.

The seed contains the embryo and the endosperm and is covered by a strong seed coat called the testa. The testa is unique to tomatoes.



**Figure I-21.** *The important event in the life of tomato: fertilization.* Once the pollen grain has landed on the stigma the tube cell of the pollen grain elongates to form a tube. This tube reaches down the style all the way to one of the ovules in the ovary of the flower. Once the tube is formed the pollen grain's two sperm cells then travel down the tube into the embryo sac inside the ovule. The ovule is preceded with double fertilization; it produces a diploid zygote and a triploid endosperm which store food for the embryo. The fertilized ovule will become one of the seeds of the tomato fruit.

#### ***C-6-2-2- Fruit development***

In the tomato and many other fruit, development can be divided into four phases: phase I, fruit set; phase II, cell division; phase III, cell expansion; and phase IV, ripening and senescence.

##### ***➤ Phase I: Fruit set and parthenocarpy***

Phase I is generally referred to as fruit set and involves the initial development of the ovary and the decision to abort or to proceed with fruit development. In normal development, fruit set is dependent on successful fertilization. Fertilization, in turn, is the result of successful pollen germination and pollen tube penetration and growth in the styler tissue towards the ovule. Upon reaching the embryo sac, the pollen tube fuses with the egg cell and fertilization occurs. In general, it is the presence of fertilized ovules that triggers fruit set and the consequent development of the ovary into a fruit. In contrast to the genetic analysis of flower development, where detailed studies have resulted in an in-depth understanding of the action of homeotic genes involved in flower organ determination, little is known of the molecular controls involved in early fruit development.

In parthenocarpic or seedless fruit, development proceeds in the absence of fertilization. In addition, unfertilized, non-parthenocarpic fruit can be induced to set by the application of auxins (Vivian-Smith and Koltunow, 1999), auxin transport inhibitors or gibberellins (Rotino et al., 1997; Fos et al., 2001); This suggests that an altered response to, or metabolism of, auxin and/or GAs may be critical in the ability of parthenocarpic fruit to proceed with development. It further indicates these phytohormones play a role in normal fruit set and early development. Further evidence for a role for auxin in fruit set comes from the work of Rotino *et al.* (Rotino et al., 1997) who showed that parthenocarpy can be induced in transgenic tobacco, tomato and eggplant fruit transformed with the *iaaM* gene from *Pseudomonas syringae*. The product of the *iaaM* gene, tryptophan mono-oxygenase, synthesises indolacetamide that is converted to IAA in the plant. Expression of *iaaM* therefore induces auxin over-biosynthesis in transgenic tissues and organs. A chimeric gene, *DefH9-iaaM*, allowing ovule-specific expression was introduced into plants. Expression of the transgene was observed during flower development in eggplant, tomato and tobacco. Seedless fruit, of a comparable size to the wild-type, were produced by emasculated flowers in the transgenic plants. No fruit set took place in emasculated wild-type flowers, providing clear evidence that high levels of auxin in the ovule can substitute for fertilization and subsequent fruit set (Rotino et al., 1997). In the tomato, natural parthenocarpic fruit contain more GA-like substances than their non-parthenocarpic near-isogenic lines early after anthesis (Mapelli et al., 1978). Also, parthenocarpic tomato fruit induced by the auxin, 4-chlorophenoxyacetic acid, contain higher endogenous levels of GAs, suggesting that seedless fruit development requires modification in the levels or response to both hormones (Mapelli et al., 1978).

➤ *Phase II: Cell division, seed formation and early embryo development*

Cell division is activated in the ovary following successful fruit set. For the next 4 to 8 days it is the primary means of ovary enlargement. The dividing cells in the developing fruit are small and compressed and contain small vacuoles. In early phase II, high mitotic activity is seen primarily in the outer and inner pericarp, in the columellar and placental tissue and in the peripheral integument layers of the developing seeds. Mid phase II, at around four to six days after anthesis, mitotic activity is mostly confined to the vascular tissues, the developing seeds, in the outer layer of the pericarp and in the cell layers peripheral to the seeds (Gillaspy et al., 1989). Towards the end of phase II and in phase III, mitotic activity becomes restricted to the cells in the outer pericarp, the outer placenta (from which the cells in the locule are

derived), and to the vascular tissue. At this stage, mitotic activity can also be detected in the developing embryo. In normal fruit, a positive correlation exists between the number of seeds and sustained fruit growth. Lopsided or malformed fruit form if seeds do not develop in part of a fruit, with retarded development corresponding to the absence of seeds (Nitsch et al., 1960). This indicates that in normal fruit, fertilized seeds are important for continued growth. Final fruit size is dependent on a combination of the number of cell divisions and the extent of cell expansion. The final number of cells results from a genetically predetermined number of pre- and post-fertilization divisions. The extent of post-fertilization cell division is generally thought to be dependent on the number of successful fertilizations. The seedless fruit were of a comparable size to normal, seeded fruit in *DefH9-iaaM* transgenic plants (Rotino et al., 1997), suggesting that the effect of the transgene continued throughout development or alternatively, in the case of tobacco and eggplant, final fruit size is genetically determined early in phase I and is not dependent on the presence of fertilization. Generally, final fruit size is the product of the number of cells within the ovary prior to fertilization, the number of successful fertilizations, and the extent of cell expansion (Bohnert and Bangerth, 1988).

➤ *Phase III: Cell expansion and embryo maturation*

As the short cell division phase ends the cell expansion phase begins. At the cellular level this phase is characterized by a thinning of the primary cell wall and a large increase in the proportion of the cell occupied by the expanding vacuole. Although the number and timing of cell divisions contributes to the determination of final fruit size, cell expansion makes, by far, the greatest contribution. In the tomato, cells comprising the placenta, locular tissue, and mesocarp can increase by more than ten-fold during this phase (Gillaspy et al., 1989). Even though auxin levels in the pericarp are consistently low during this period, it is generally accepted that they are primarily responsible for cell expansion in fruit tissue (Rayle and Cleland, 1992). Seed auxin levels are higher than pericarp levels throughout this phase and it is possible that a seed-derived signal is responsible for regulating cell expansion in the pericarp. Catala *et al.* (2000) recently isolated an auxin-inducible tomato expansin gene (*LeExp2*), presumably involved in tomato fruit expansion, which is expressed in pericarp tissue throughout this period. The fact, that lopsided fruit can develop when cell expansion is greatly reduced in locules where seeds have been aborted or are absent, points to a role for a seed-derived signal. Similarly, cell expansion is generally reduced in parthenocarpic fruit (Gillaspy et al., 1989). While auxin is likely to be important in the process, it is unlikely that

seed-produced auxins are solely responsible for fruit cell expansion. In the absence of seeds, exogenous auxins are unable to sustain fruit cell expansion *in vitro* (Asahira and Hosoki, 1977). However, it has been suggested that seed-derived auxins could work in concert with other seed or embryo-produced molecules in directing pericarp sink activity, volume increase, and development (Gillaspy et al., 1989).

➤ *Phase IV: Ripening*

In the tomato, the cell expansion phase continues for 4 to 7 weeks, depending on the variety. Once expansion is complete, fruit develop a maturity, or a capacity to undergo the dramatic developmental process of ripening. Ripening is a process unique to fruit and comprises a series of physiological, biochemical and structural changes occurring concomitant with, or following, maturation of the seeds. From an evolutionary standpoint, ripening can be seen as an optimization of the attractiveness of the fruit, in order to encourage its consumption and hence the dispersal of mature seed. As a result of concerted efforts, the ripening process is better understood than any other fruit developmental process.

As previously outlined, fruit can be either climacteric or non-climacteric. In climacteric fruit the majority of processes, which in total constitute what we call ripening, depend on the plant hormone ethylene for their onset and maintenance. In contrast, in non-climacteric fruit the majority of ripening-associated processes are ethylene-independent. Recent results have clearly shown the presence of ethylene-independent or non-climacteric-like processes during ripening of climacteric melon fruit (Lelievre et al., 1997). Similarly, ethylene-dependent, or climacteric processes have been identified in citrus fruit. Clearly, both climacteric and non-climacteric fruit ripening are the culmination of a broad range of developmentally-regulated processes.

In the tomato, the changes associated with ripening comprise processes such as degradation of chlorophyll and an increase in  $\beta$ -carotenes and lycopenes which are responsible for the orange and red colors in ripe fruit (Gray et al., 1992). Tomato fruit ripening is also accompanied by an accumulation of the monomeric sugars, glucose and fructose, of organic acids in the vacuoles, and the production of aroma volatiles giving the tomato its characteristic aroma and taste. Finally, there are also substantial changes in the texture of the fruit, due to changes in the major cell wall constituents, cellulose, hemi-

cellulose and pectin. The majority of these processes are initiated and moderated by the phytohormone ethylene (Lelievre et al., 1997).

### ***C-7- Auxin and tomato fruit***

As previously outlined, auxin levels alter during tomato fruit development and ripening, with two peaks, the first at the beginning of the cell expansion phase and the second corresponding with the final phase in embryo development (Gillaspy et al., 1989). Levels in the pericarp fall rapidly after an initial high, coinciding with the initiation of cell expansion. A second peak, attributable to high levels of the hormone in the seed, occurs late in fruit development and coincides with rapid expansion of embryonic cells (Gillaspy et al., 1989). Pericarp auxin levels remain low during this second peak (Slovin and Cohen, 1993). The low levels of auxin seen in immature tomato fruit are maintained by reduced production, by the conjugation of IAA to aspartate and by an increase in the IAA oxidizing capacity of the fruit (Catala et al., 1992; Slovin and Cohen, 1993). Conjugation of indole-3-acetic acid to indole-3-acetylaspartic acid (IAAsp) deactivates auxin as IAAsp as IAAsp is further metabolised to as yet unidentified conjugates (Catala et al., 1992). This capacity to form IAAsp diminishes in mature green fruit pericarp (Catala et al., 1992) and reaches very low levels in fully ripe fruit (Slovin and Cohen, 1993). At the same time there is an increase in the reversible conversion of IAA to its glucosyl conjugate, indole-3-acetyl-b-D-glucose (IAGluc) (Catala et al., 1992). In short, whatever the role of auxin in pre-ripening development, it is evident that there are extensive changes in auxin production and metabolism throughout tomato fruit development.

It has been postulated that the presence of endogenous IAA represses fruit ripening as exogenous auxin treatments can provide a delay in fruit ripening in tomato and other crop species (Vendrell, 1985; Cohen, 1996). Vendrell (1985) demonstrated that the application of an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) to tomato fruit can have a dual effect on ripening depending on the method of application. When applied externally to whole fruit, 2,4-D increases ethylene production which promotes ripening. When the auxin is vacuum-infiltrated into pericarp discs there is a delay in the onset of ripening. Vendrell (1985) concluded that the effect depends on the uniformity of the auxin distribution and upon its concentration. They also showed that the auxin led to a sustained increase in ethylene production when compared to control fruit discs, while at the same time it delayed ripening compared to control discs. This suggests that auxin is capable of inhibiting the onset of

ripening, despite an increase in ethylene production, which, given the acceleration of ageing seen with continuously high levels of ethylene, would normally be expected to ripen earlier than the control. In addition, p-chlorophenoxy isobutyric acid, an anti-auxin, has been shown to accelerate the onset of ripening in Bartlett pears, another climacteric crop specie (Frenkel and Haard, 1973). These factors make auxin an interesting candidate for the study of a role for hormones other than ethylene in tomato fruit development.

## *CHAPTER II*

# *Materials and Methods*

---



## A- Plant Material and culture conditions

During the course of our research, tomato was used as main model plant. Different tomato cultivars such as *Micro-Tom* ( $\mu Tom$ ), *Ailsa Craig* (AC) and *Kemer* are being used in our laboratory to investigate the molecular mechanisms of fruit ripening. In the present study,  $\mu Tom$  cultivar was preferred due to its small size and rapid life cycle (up to four generations per year) as well as its routine genetic transformation. Usually, results obtained in one genotype are further validated in a different genetic background.

The tomato  $\mu Tom$  seeds were sown in a MS based medium and were let for *in vitro* culture in a culture chambers for 21 days. After one day's acclimatization, plantlets were transplanted either in growth chambers or greenhouse (with standard growth condition). For those transplanted in growth chambers, plantlets were planted into small plastic pots (*Soparco*, 7x7, 6.2 cm) containing a mixture of sub-irrigation of sphagnum peat, perlite and clay granules (*Klasmann & Deilmann* RHP 15). The growth chambers maintained at a controlled photoperiod of 14 hours during the day at temperature 25 °C with 80% humidity with intense luminosity of 250  $\mu\text{Mol.M}^{-2}\text{S}^{-1}$  and 10 hours during the night at a temperature of 20°C. For *Ailsa Craig*, the seeds were sown same as  $\mu Tom$ , after one week acclimatization, plantlets were transplanted in greenhouse with standard growth condition. Plant (both WT and AS) were also grown in hydroponics culture described by Hoagland and Arnon (1950). The culture medium is described in the annex. This method of culture was adopted in order to get clean roots.

## B- Physiological analysis

### *B-1- Hormone treatment*

#### **B-1-1- Seeds germination and seedling culture**

At a sterile condition, tomato seeds were first surface-sterilized by soaking in 50 % household bleach solution for 10 minutes, The seeds were rinsed 7-9 time in sterile distilled water and dried on waterman's paper, before being sown in recipient Magenta<sup>®</sup> vessels containing 50 mL of 50% MS (Murashige and skoog, 1962) culture medium added with R<sub>3</sub> Vitamin( 0,5 mgL<sup>-1</sup> thiamine, 0,25 mgL<sup>-1</sup> nicotinic acid, 0,5 mgL<sup>-1</sup> pyridoxine), 1,5% (W/V) sucrose, and 0,8% (W/V) agar; pH 5,9. The Magenta vessels carrying the seeds were then set in standard growth chambers condition as sited before.

### B-1-2- Auxin response in hypocotyl elongation

➤ *Auxin dose response in hypocotyl segment*

5-days-old hypocotyls segments were isolated, 8,1 mm in length, immediately below the cotyledons nodes with a double-bladed cutter, and floated in sucrose/MES buffer( 1% sucrose (w/v), 5 mM Mes /KOH, pH 6,0) in 8 cm Petri dishes. After 1-2 hr pre-incubation to deplete the endogenous auxin influence, those hypocotyl segments were distributed to fresh buffer solutions with or without NAA (*α-Naphthalene acetic acid*) and incubated with gentle agitation at room temperature. The segments were photocopied in the Petri dishes 0 and 23 hr after transfer. The segment length was determined from the photocopies using a ruler. *α*-NAA solution was obtained from Sigma Chemical Co..

➤ *Kinetic analysis of auxin and NPA response elongation of hypocotyl segment*

5-days-old hypocotyls segments were isolated, 8,1 mm in length, immediately below the cotyledons nodes with a double-bladed cutter, and floated in sucrose/MES buffer( 1% sucrose (w/v), 5 mM Mes /KOH, pH 6,0) in 8 cm Petri dishes. After 1-2 hr pre-incubation to deplete the endogenous auxin influence, those hypocotyl segments were distributed to fresh buffer solutions with or without NAA and incubated with gentle agitation at room temperature. The segments were photocopied in the Petri dishes from 0 to 23 hr as demonstrated in the figure after transfer. The segment length was determined from the photocopies using a ruler. NPA [*N-(1-naphthyl)phthalamic acid*] stock solution was dissolved in DMSO at 100 mM.

### B-1-3- Auxin and cytokinin interaction in cotyledon and hypocotyl explants

11-days-old cotyledons and hypocotyls segments were cut from the seedling and placed on MS medium with R<sub>3</sub> Vitamin( 0,5 mgL<sup>-1</sup> thiamine, 0,25 mgL<sup>-1</sup> nicotinic acid, 0,5 mgL<sup>-1</sup> pyridoxine), 1,5% (W/V) sucrose, and 0,8% (W/V) agar. The medium was supplemented with 16 different hormonal ratios of auxin: cytokinin (NAA: BA) before it solidified: 0/0; 0/0,4; 0/4; 0/40; 0,4/0; 0,4/0,4; 0,4/4; 0,4/40; 4/0; 4/0,4; 4/4; 4/40; 40/0; 40/0,4; 40/4; 40/40 μM respectively. Then was cultured in standard growth chamber as

described in A-1-, after one month their callus, roots and shoots formation was compared and photographed, and their fresh weight were measured also.

#### **B-1-4- NPA, GA, Paclobutrazol treatment in seedling**

The medium prepared as described in B-1-1, before it solidified, the properly concentration of hormone were supplied into the medium before solidification. NPA stock solution was dissolved in DMSO at 100 mM. GA (gibberellin acid) was obtained from Sigma Chemical Co. Paclobutrazol was obtained from Duchefa, Netherlands; stock solution was dissolved in DMSO at 10 mM. Then the seeds were sowed and grown as described in B-1-5.

#### **B-1-5- Auxin treatment in seedling and different stage fruit for gene expression analysis**

Tomato seedling or fruits were harvested and treated in the 50% MS buffer containing 20  $\mu$ M IAA or not (as mock treatment) for the time indicated (15 min, 30 min, 3 hr), tissues were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction.

### ***B-2- Fruit analysis***

#### ***➤ Ethylene measurement***

The fruits or were placed in a sealed container at room temperature ( $25^{\circ}\text{C}$ ) for 2 to 3 hours. One-ml samples were withdrawn for ethylene measurement by gas chromatography.

#### ***➤ Firmness measurement***

Different stage fruit were harvest and proceeded with firmness measure within one hour, according to the supplier's instructions.

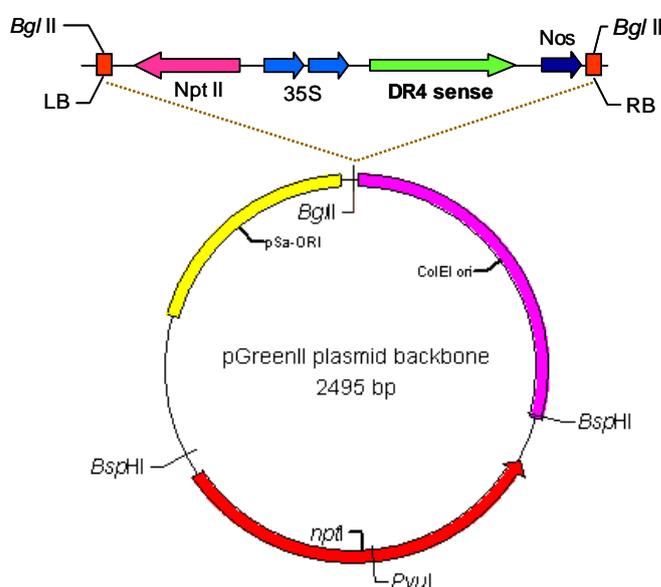
#### ***➤ Fruit juice pH and total soluble content***

Fully mature fruit (fully red) fruit were harvest and smashed up into paste, then centrifuge at 10000 rpm for 20 min, the fruit juice were collected proceeded with pH

measurement with pH meter, and total soluble content with refractometer, according to the supplier's instructions.

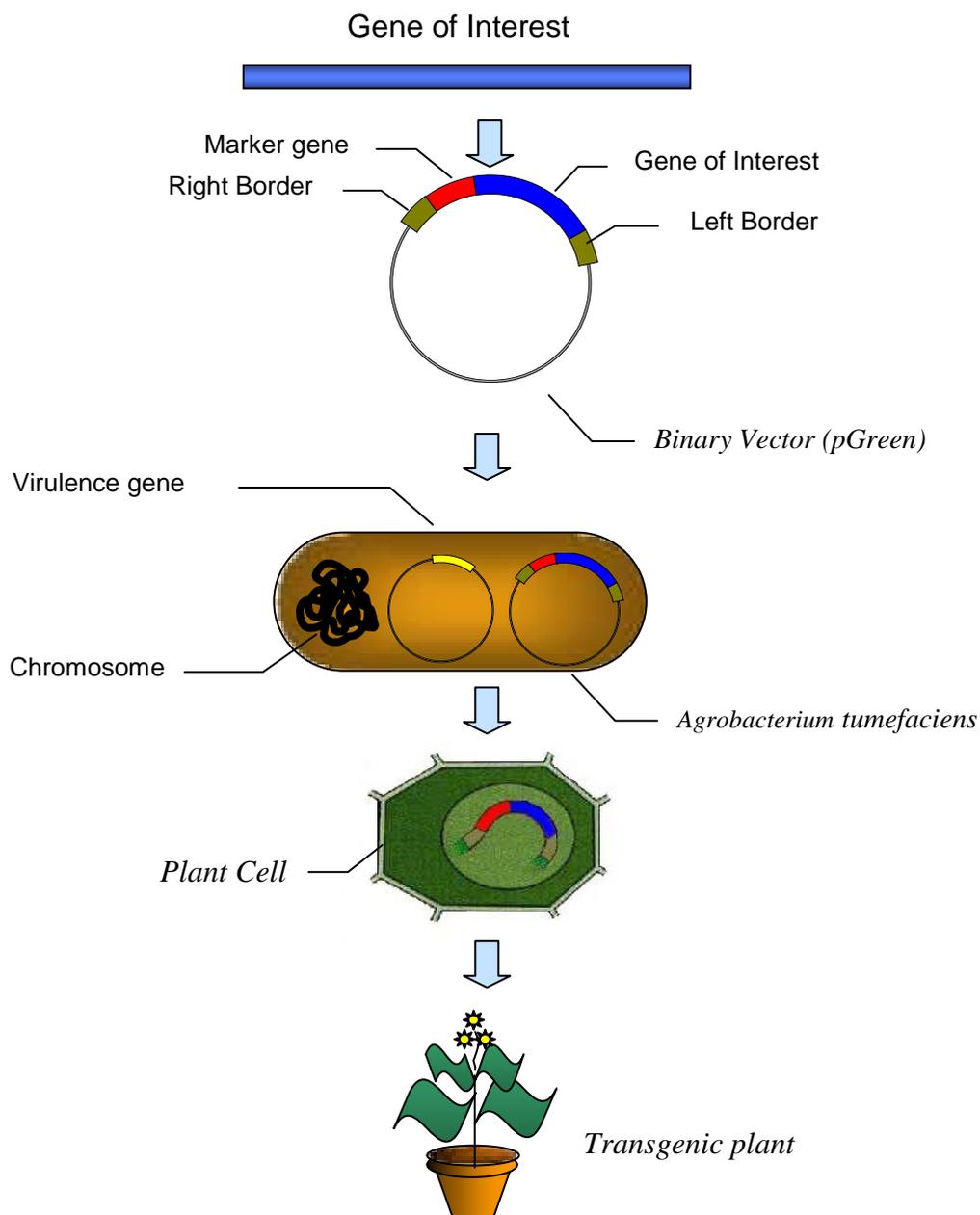
### C- Genetic transformation

The genetic transformation was carried out on tomato using the pGreen vector (Figure II-1) for the sense construct or pGA vector for the antisense construct as described by (Bird et al., 1988) for a subsequent transgenic plant with several minor changes, including the use of 8-d-old cotyledons, the introduction of a 24 hr pre-conditioning period prior to *Agrobacterium* infection, a reduction of the duration of subcultures for shoot formation to 15 days, an increase of kanamycin concentration to  $75 \text{ mgL}^{-1}$  and other minor changes in the composition of the culture medium. Homozygous transgenic lines and untransformed controls were grown under standard culture conditions. The plants were cultured in (*in vitro*) chambers maintained at a controlled photoperiod (14 hours during the day and 10 hours during the night), with 80% humidity with an intense luminosity of  $250 \mu\text{molM}^{-2}\text{S}^{-1}$ . The simplified scheme of transformation of plant is illustrated in Figure II-3.



**Figure II-1.** Map of pGreen binary vector/DR4 sense used for ectopic expression of a sense construct in transgenic plant. 35S: cauliflower mosaic virus 35S promoter; Nos: nopaline synthase (Nos) terminator; *nptII*: Kanamycin resistance gene.





**Figure II-3.** Simplified scheme of transgenic plants generation (Chaves, 2001)

### **C-2- Transformation of *Agrobacterium***

The constructed vectors with desired inserted genes were transformed into *Agrobacterium* C58C (the same as 'GV3101'). 150  $\mu$ L frozen *Agrobacterium* competent cell were added 10  $\mu$ L vectors with desired inserted gene (up to 10  $\mu$ g), thaw the tube with warmth of hand and flick to mix for 5 to 10 second, and freeze in liquid nitrogen for 5 min,

which provoked the transformation. Remove the tube and thaw, take 5-10 min in 28 °C, the add 1 mL LB in 20 mL sterile tube; incubate with shaking 150 rpm over night. After incubation, the Agrobacterium were plate on Petri dishes prepared by LB containing kanamycin (50  $\mu\text{g mL}^{-1}$ ), rifampicin (50  $\mu\text{g mL}^{-1}$ ), gentamycin (25  $\mu\text{g mL}^{-1}$ ), and tetracycline (10  $\mu\text{g mL}^{-1}$ ). Finally the Petri dishes were kept for incubation for two or three days at 28 °C in order to grow colonies.

### ***C-3- Tomato cotyledon explants transformation***

The cotyledon of explants started emerging eight days after seed sowing in the medium as mentioned in B-1-1. Cotyledons were first placed on waterman's sterilized paper and eventually were cut with the help of a sterilized scalpel and sharp blade which were then placed over pre-prepared Petri-dishes containing KCMS solid medium (Mineral elements for Murashige and Skoog (1962); 20  $\text{g L}^{-1}$  of sucrose, 200  $\text{g L}^{-1}$  of  $\text{K}_2\text{HPO}_4$ ; 0,9 of the thiamine;

0,2 of 2,4-D; 0,1 of Kinetin; 200 $\mu\text{M}$  (3,5-dimethoxy-4-hydroxy-acetophenone); Agar 8 $\text{g L}^{-1}$ , pH5,8). Finally they were placed in the dark, maintained at 25°C for 24 hours before starting genetic transformation. After agitated incubation for 24 hr in obscurity, the Agrobacterium culture was checked till have the optical density ( $\text{OD}_{600\text{nm}}$  0,2-0,8) by using a spectrophotometer (Becman DU<sup>®</sup> 640B). The culture was then centrifuged at 3000 rpm for 10 minutes at room temperature. The in-oculated cotyledons of the explants from the KCMS solid medium were then dipped in KCMS liquid medium containing [20  $\text{g L}^{-1}$  of sucrose; 200  $\text{mg mL}^{-1}$  of  $\text{KH}_2\text{PO}_4$ ; 0.9  $\text{mg mL}^{-1}$  of thiamine; and 200  $\mu\text{M}$  (3,5-dimethoxy-4-hydroxy-acetophenone) pH 5.8] for 30 minutes. Finally, the co-cultured cotyledons were dried on filter paper and placed over a new KCMS solid medium prepared as mentioned above and were incubated for 48 hr at 25°C in obscurity. After 48 hr, the cotyledons were placed on a 2Z medium containing mineral elements for [Murashige and Skoog, (1962) 4.4  $\text{g L}^{-1}$ ; 8 $\text{mL L}^{-1}$  of augmentin; 30  $\text{g L}^{-1}$  of sucrose; 2  $\text{mL L}^{-1}$  vitamin of NITSCH ; 2  $\text{mg L}^{-1}$  zeatine riboside and 100  $\text{mg L}^{-1}$  of kanamycin; pH 5.8], for 15 days of regeneration. As buds started appearing from the cotyledons, the explants were transplanted in recipient Magenta vessels on a rooting medium prepared with mineral elements [Murashige and Skoog, (1962) 2.2  $\text{g L}^{-1}$ ; 10  $\text{g L}^{-1}$  of sucrose; 2.5  $\text{g L}^{-1}$  of phytigel; 2  $\text{mL L}^{-1}$  vitamin of NITSCH; 75  $\text{mg L}^{-1}$  of kanamycin and 2.5  $\text{mL L}^{-1}$  of augmentin].

Transformed plants were selected on the basis of a PCR on DNA obtained by R<sub>0</sub> generation to test the germination in the presence of kanamycin. Finally from the R<sub>1</sub> the transformed plants were selected on the basis of homozygote.

## **D- Gene expression analysis**

### ***D-1- RNA extraction***

Total RNA were extracted according to Hamilton et al. (1990). Plant tissues were ground in liquid nitrogen. About 5 g of powdered tissues (15 mL) of were mixed with same volume (15 ml) RNA extraction buffer and phenol/chloroform (15 mL) in 50 mL Nunc tube, and tubes were vortex for 5 min until the material was homogenized. The tubes were then centrifuged at 4°C with a speed of 4000 rpm for 45 min to separate the phases. Supernatants containing RNAs were transferred to fresh Nunc tubes and add in 1/10 volume sodium acetate (3M, pH 4,5) and same volume ice cold isopropanol, vortex briefly then incubated at -20°C overnight to precipitate the nucleic acid (also mixed with carbohydrates). After an overnight precipitation, the tubes were centrifuged at 4000 rpm for 45 min at 4°C, the supernatants were discarded and the pellet was washed with 5 mL of ice cold ethanol 70%, and was further centrifuged for 5 min at 4°C. Finally the pellets were dried in a speedvac and were re-suspended thoroughly in 4 mL DEPC treated water; immediately, add equal volume of CTAB 2% extraction buffer and ½ volume CTAB 1% precipitate buffer with temperature maintaining superior to 18 °C due to CTAB itself precipitate under 18 °C. Then the tubes were centrifuged at 4000 rpm for 50 min at 25 °C. The supernatant was discarded and the pellet was re-suspended in 2 mL DEPC treated water, immediately add equal volume of CTAB 2% extraction buffer and ½ volume CTAB 1% precipitate buffer. Centrifugation was carried out at the speed of 4000 rpm for 30 min at 25°C. Finally the pellet was resuspended in 1,4 M NaCl (2 mL) and 2,5 volume ice cold ethanol absolute (5 mL) and was incubated at -20 °C at least 3 hours. After the precipitation, the tubes were centrifuged at 4000 rpm for 30 min at 4 °C, discard the supernatant and the pellet was washed with 1 mL of ice cold ethanol 70% and transfer to a fresh sterile eppendorf tube of 1,5 mL. The tubes were further centrifuged at 13600 rpm 10 min at 4°C. Discard the supernatant and the pellet was dried in a speedvac and was re-suspended in DEPC treated water. The RNA was treated with RQ1 DNase (Promega) to eliminate DNA trace according to the supplier's instructions.

## D-2- RT-PCR analysis of mRNA levels

Oligonucleotide primers were designed from the different clone in TIGR tomato gene database for use in semi-quantitative RT-PCR gene expression studies (Table II-1).

Table II -1 Sets of RT-PCR primers used

Gene	Primer sequence (5' → 3')	Gene	Primer sequence (5' → 3')
<i>DR1/IAA1</i>		<i>LeTKn1</i>	
Forward	ATGGAGTGTGTTTTGGCTCATGAG	Forward	TGGAGGAAATCAAGTTCTTGGTGGTGCTG
Reverse	AACTCCACATCCTAATCCTCTTGC	Reverse	GGGTCCTTGAAACATCTCTGTCCGGTCAA
<i>LeIAA2</i>		<i>LeT6/Kn2</i>	
Forward	AAGCGAGCTATGTTAAAGTGAGCA	Forward	GGTCAATTGTTGCGTAAGTACAGCGG
Reverse	CCGTTGTATCCATCTGTTTCTGAA	Reverse	CCAATCCCCTTGATTACAGCTAGTGC
<i>DR3/IAA3</i>		<i>LePhan</i>	
Forward	TCCTCACAATTCACAGCAATTC	Forward	ATGGGTCGAGCTAGAACTGGGATGCC
Reverse	TAGACATGCTAGACCTTTAGC	Reverse	AGCAGTTCGACCTGGTACTTCAGCTGTCT
<i>DR4/IAA4</i>		<i>DR4 sense</i>	
Forward	TGGCCACCCATTTCGATCTTTTAG	Forward	ATGTCTCCGCCGCTCCTTGGTG
Reverse	CGCAACACACATTAGTTTGCAG	Reverse	ATTTCTGCTCCGACACTTTTC
<i>DR8/IAA8</i>		<i>DR4antisense</i>	
Forward	ATGACTGAGCTAACTCTCGGCTTA	Forward	TGGCCACCCATTTCGATCTTTTAG
Reverse	ACTCGACGATCCCCAGGTGTTCT	Reverse	AGACAAACTCCAATATCAAACGG
<i>LeIAA10</i>		<i>DR4H</i>	
Forward	GACTTCTCAAAGCTTGATCGAGAG	Forward	CCATTGCCCTAGTTACTGCTACTTCC
Reverse	TGAAATCTTTCATTCTTGGACAA	Reverse	GGAAGTAGCAGTAACTAGGGCAATGG
<i>DR11/IAA11</i>		<i>DR4P</i>	
Forward	GTGCTTACCGGAAAAGAACAG	Forward	CAGCTGTTTTACAATAGGTCAATATGG
Reverse	ACTTATCTGCATCCTCCAATGC	Reverse	CCATGAGATCCATATTGACCTATTGT
<i>DR12/ARF4</i>		<i>DR4PH</i>	
Forward	CATGCTGATTTTCGTTGTACCTTAC	Forward	GTGGACCTCAGAACCTGTTCTGCATAC
Reverse	GTCTAAAAGAGCACTCCCTC	Reverse	GTATGCAGAACAGGTTCTGAGGTCCAC
<i>SAUR</i>		<i>DR4L</i>	
Forward	GGCTATCCGTATGCCTCGTA	Forward	ATTAAGGTCAATATGGATCTCATGGAG
Reverse	CCACCCATCGGATGATTAATA	Reverse	GCTCCATGAGATCCATATTGACCTTAAT
<i>LeACS4</i>		<i>Ubi3</i>	
Forward	GCGGAAAATCAGCTTTTCAGTTG	Forward	CTAACGGGGAAGACGATCACCC
Reverse	CTGGGTAATAGGGTGTGGGGAC	Reverse	TCCCAAGGGTTGTACATACATC
<i>GH3</i>		<i>Ubi3 short</i>	
Forward	AGCTCGTCATCACAACATACGC	Forward	AGAAGAAGACCTACACCAAGCC
Reverse	CAACTCGCCCTTGTCGATAAAC	Reverse	TCCCAAGGGTTGTACATACATC

The RT-PCR analysis was carried out as described previously (Lasserre et al., 1996) except that, after the reverse transcription step, the resultant cDNA products were diluted four times and then used as templates for the amplification of the clone of interest using: 50 p.mol of gene-specific primers; and 50 p.mol of tomato *ubiquitin* (*Ubi3*) specific primers as the internal control (the later added to the reaction mixture after n cycles in order to be amplified in non-saturated manner). The PCR products were separated on a 1,4% to 2,0% agarose gel, transferred to a GeneScreen Plus<sup>®</sup> nylon membrane (NEN<sup>®</sup> Life Science) for at least 15 hours, and hybridized. Probes were labeled with [<sup>32</sup>P]dCTP using random-primer kit Ready-to-go

(Amersham Pharmacia Biotech). Washes were carried out under high stringency, and exposed to Amersham Hyperfilm-MP at -80°C for 30-60 min.

### ***D-3- Northern blot analysis***

Denatured (70°C 10min, then keep in ice immediately for 5 min) total RNA (15 µg) of different samples were fractionated on a 1,2% (w/v) agarose gel containing 0,66 M formaldehyde, 1X MOPS buffer. Then transferred on GeneScreen Plus<sup>®</sup> nylon membrane (NEN<sup>®</sup> Life Science) for at least 15 hours, and was fixed by cross-link using a Stratalinker 800 (Stratagene). *DR4* probes were labeled with [<sup>32</sup>P]dCTP using random-primer kit Ready-to-go (Amersham). To check for equal loading a reverse picture of the ethidium bromide-stained gel was used. Washes were carried out under high stringency.

## **E- Transient expression of GFP-fusion protein**

The coding sequence of *DR4* was cloned in pGreen-GFP vector. This coding sequence was amplified by PCR with *Vent*<sup>®</sup> polymerase (Clontech) from a cDNA of tomato leaf. This vector allows the transient expression of DR4 protein tagged with GFP under the control of cauliflower mosaic virus 35S promoter. Protoplasts used for transfection were obtained from suspension-cultured tobacco BY-2 cells according to the method described previously (Bouzayen et al., 1989). Protoplasts were transfected by a modified polyethylene glycol method as described by (Abel and Theologis, 1994). Typically, 0,2 ml of protoplast suspension (0,5 x 10<sup>6</sup>) was transfected with 50 µg of shared salmon sperm carrier DNA and 30 µg of either 35S::*DR4*-GFP or 35S::*GFP* (control) plasmid DNA. Transfected protoplasts were incubated at 25°C for 16h and were analyzed for GFP fluorescence by confocal microscopy. All transient expression assays were repeated at least three times.

### ***E-1- DR4-GFP construction***

The coding sequence of *DR4* (ORFs) was amplified by PCR with *Vent*<sup>®</sup> polymerase (Clontech) from tomato cDNA using primers DR4-F, 5' ATG TCT CCG CCG CTC CTT GGT 3', and DR4-R, 5' ATT TCT GCT CCG ACA CTT TTC CAT 3'. The ligation was carried out in plasmid (pGreen-GFP) digested by *Sma I* (100 ng/µL) with T<sub>4</sub> DNA ligase. This

ligation was further subject *Sma I* digest to eliminate the auto-ligation of vector (false positive). Then it transformed into to *E. coli* DH5 $\alpha$ . After overnight culture, a midi-prep was preceded to harvest the desired plasmid contain inserted gene *DR4*.

### ***E-2- Preparation of protoplasts***

#### ***➤ Preparation of cells***

Twenty mL of tobacco BY-2 cell suspension were taken from one week old culture and were centrifuged at 3500 rpm for 5 min, the supernatant was discarded and the pellet was rinsed once by pouring 40 mL of Tris-MES 25 mM buffer and mannitol 0,6 M maintained at pH5,5 and were again let for centrifugation as mentioned above. The supernatant was discarded and 2 g of cell culture was placed on a Petri dish.

#### ***➤ Enzymatic digestion***

For enzymatic digestion, 20 mL enzymatic solution was prepared which contained the following: 1  $\mu\text{g mL}^{-1}$  Caylase 345 (*CAYLA*, Toulouse, France), 2  $\mu\text{g mL}^{-1}$  pectolyase Y-23, 1  $\mu\text{g mL}^{-1}$  BSA, in Tris-MES 25mM Mannitol 0,6M, pH 5,5 and was set for slight agitation at room temperature for 1 hour. The tubes were then centrifuged for 5 min at 3000 rpm to eliminate remaining insoluble precipitates. The solution was then filtered on 0,45  $\mu\text{m}$  of filter gauze and 2 g of culture cells were taken and poured into the tube already containing 20 mL of enzymatic solution. These were then set for slight agitation at a speed of 30-40 rpm and maintained at 37°C for 90 min in obscurity. Finally, the digestion was verified by microscopy.

#### ***➤ Purification of protoplast***

The cell suspension was purified using a nylon towel of about 30  $\mu\text{m}$ ; furthermore the filtered suspension was centrifuged for about 3 min at 1000 rpm. The supernatant was discarded and the pellet was washed by 20 mL of buffer (Hepes-KOH 25 mM, Sorbitol 0.6 M, pH 6.7), and placed on ice. Then the tubes were centrifuged for 3 min at 1000 rpm. The supernatant was discarded and the pellet was washed by pouring 5 mL of W5 solution which contained: 154  $\mu\text{M}$  of NaCl, 125  $\mu\text{M}$  of CaCl<sub>2</sub>, 5  $\mu\text{M}$  KCl, 5  $\mu\text{M}$  glucose and 0.1 % MES maintained at pH 5.6-6. The suspension was then centrifuged for 5 min at 1000 rpm, the

supernatant was eliminated and the pellet was retrieved by pouring 1-2 mL of W5 solution and the protoplast solution was then stabilised for 30 min on ice. The suspension was recentrifuged for 3 min at 1000 rpm, the supernatant was eliminated and the pellet was retrieved by pouring buffer Mannitol/Mg which contained: 0.55 M Mannitol, 15 mM MgCl<sub>2</sub> and 0.1 % MES maintained at pH of 5.6), the final concentration was obtained at  $0.5 \times 10^6$  protoplasts/mL. Finally, 200  $\mu$ L of protoplast suspension was distributed in tubes.

### ***E-3- Transient transformation and microscopic observation***

Heat shocks were given to tubes carrying protoplast suspension at 45°C for 5 minutes in a hot water tub and were re-cooled immediately for 5 min on ice. Transformation was carried out by using 25  $\mu$ g of carrier DNA. The DNA was poured into the protoplast suspension as mentioned above (2.5  $\mu$ L of Salmon DNA at 10 mg.mL<sup>-1</sup>, Clontech). 10-25  $\mu$ g/tube of plasmid containing the construction in GFP was poured and incubated for 5 mins at room temperature. The same volume of PEG solution (polyethylene glycol) at 10 mg.mL<sup>-1</sup> was then poured and incubated for 20 min at room temperature. Finally 2-4 mL of MS solution containing 20 g.L<sup>-1</sup> sucrose and 80 g.L<sup>-1</sup> mannitol was mixed with the protoplast and was incubated in obscurity for 16 hours. After 16 hours of incubation the transformed protoplast suspension was centrifuged at 1000 rpm for 5 min, the supernatant was discarded and 20 mL of the pellet was taken. The confocal microscope fluorescence (Leitz, objective x 40 Fluo) was used to make and to clear observation of the protoplasts fluorescence, which has an ability to take an image of 40 protoplasts one by one at the same time, quantified with the help of a software *Image Pro Plus*. Finally the subcellulaire localisation of GFP was realised by a confocal microscope,  $\lambda \sim 488$  nm (Leica).

## *CHAPTER III*

### *Results and Discussion*

---



## Introduction

This chapter presents the results and discussion of the functional characterization of the gene *DR4*, a member of the tomato *Aux/IAA* gene family. The DR4 protein subcellular localization was investigated by transient expression of *DR4-GFP* fusion via tobacco protoplast transfection assay. Transgenic plants under-expressing *DR4* were generated (referred to as DR4AS or AS throughout the text and figures), in order to explore the physiological role of the gene in tomato plants. Physiological and molecular experiments were carried out to detect the impact of down-regulating the *DR4* gene expression on plant growth and development. Anatomical analyses were performed to investigate the altered vascular system in the DR4AS plants.

Section A of this chapter describes the isolation of the *DR4* gene and structural characterization of the DR4 protein. Sequence analysis of the predicted protein, phylogeny studies and protein subcellular localization are also reported in this section. Section B addresses the biological function of the *DR4* gene using reverse genetics approach. Based on the main phenotypical features of the transgenic plants, the physiological analyses were directed to particular developmental processes of DR4AS plants, aiming at gaining further insights on the putative roles of DR4. In the light of precocious fruit set and parthenocarpy phenotypes, emasculation and genetic crosses assay were performed in order to decipher the causal mechanism underlying parthenocarpy. Hormone treatments were carried out on DR4AS seedlings to detect altered hormone responses and apical dominance. In addition, several ethylene responses were tested to investigate putative interactions between auxin and ethylene signalling that could be mediated by DR4. Spatio-temporal gene expression patterns were also studied in wild type and DR4AS plants. Section C reports on the discovery and cloning of three different *DR4* transcribed sequences.

Our study concluded that *DR4*, the new tomato *Aux/IAA* gene, plays a key role in the leaf morphogenesis and fructification, as well as in a number of auxin-mediated plant growth and developmental processes and indicated that DR4 protein acts as a negative regulator of auxin signalling.

## A- DR4: A tomato Aux/IAA-like gene

*Aux/IAA* genes were first isolated on the basis of their rapid induction by auxin, and subsequently several members were identified based on sequence homology and grouped in a large multi-gene family. In recent years, accumulating data on *Arabidopsis Aux/IAA* genes revealed that they are involved in auxin-dependent processes via the modulation of auxin-responsive gene expression. These studies also indicated that *Aux/IAA* protein turnover is essential for auxin responses. The tomato gene *DR4* isolated in our laboratory was assigned as a member of *Aux/IAA* transcription factor family based on sequence homology, and the main goal of the present study is to uncover its biological function in plants, especially to gain insight on its role in fruit development.

### A-1- Isolation

*DR4* was isolated among other tomato *Aux/IAA* clones from tomato fruit cDNA library using *Aux/IAA* gene-family-specific degenerate primers designed from conserved sequences in pea (Theologis et al., 1985), soybean (Ainley et al., 1988), mung bean (Yamamoto, 1994), and *Arabidopsis* (Conner et al., 1990; Abel et al., 1995a). Initial search in the database revealed that *DR4* corresponded to the partial GenBank clone *LeAux/IAA4*, submitted by Nevenfuehr and Lomax (1997). The orthologs of *DR4* in *Arabidopsis* are *IAA9* and *IAA8*. *DR4* was named as Developmentally-Regulated (DR) with the same numeration as used for the GenBank sequence (Jones et al., 2002).

### A-2- Sequence analysis

The longest fragment which probably corresponds to the full length ORF of *DR4* is 1,047 bp long and potentially encodes 349 amino acid (aa). The predicted protein comprises the four typical conserved amino acid sequence motifs known as domain I, II, III, IV of the *Aux/IAA* gene family. Several nuclear localization signal (NLS) sequences were found, comprising the essential bipartite structure of NLS: the invariant basic doublet KR amino acids in domain II, as well as a second SV40-type NLS located in domain IV (Figure III-1A). *DR4* predicted protein shares 97% identity with potato *StAux/IAA* (GenBank accession number AAM29182) which is the closest neighbor plant species to tomato in plant phylogeny.

It shares 92% similarity with *NtIAA9* of tobacco (CAD10639) which also belongs to the *Solanaceae* family, and 80%, 78%, 74% and 70% similarity to *VvAux/IAA* of *Vitis vinifera* (AAL92850), *CsIAA2* of cucumber (BAA85821), *ZeARP* of *Zinnia elegans* (AAM12952) and *AtIAA9* of *Arabidopsis* (AAM64650), respectively. These data indicate that the *DR4* gene has been highly conserved during the evolution. The phylogeny of *DR4* orthologs parallels that of plant evolution, that is, closer are the species in the phylogenetic tree and higher is the homology of their *DR4* sequences.

Members of the *Aux/IAA* family are characterized by four conserved domains I-IV which are separated by intervening regions of variable length and sequences (Conner et al., 1990). Phylogenetic analysis of *DR4* gene with its nearest homologs in 6 other species, 4 other tomato *Aux/IAA* genes isolated by our lab and all published sequences of *Arabidopsis Aux/IAAs* was conducted to assess the relationship of *DR4* to the other *Aux/IAA* member genes. *AtIAA15* was not included as it was assigned as a pseudogene. Alignments of the predicted protein sequences were performed with ClustalX (Jeanmougin et al., 1998) and GenDoc (Nicholas and Nicholas, 1997) and the unrooted tree was visualised with *Tree view* program (<http://taxonomy.zoology.gla.ac.uk/rod:rod.html>). The unrooted family tree shown in Figure III-1B suggests that the members of the *Aux/IAA* family can be grouped into five subfamilies. A sixth subfamily consisting of IAA24-ARF5 related proteins that only have conserved regions III and IV forms an out-group (Ulmasov et al., 1999b). For most branches our analysis confirmed the tree topology published by Nebenführ et al. (2000) which was obtained by only using amino acid sequences between domains II and IV. Interestingly, the *DR4* homolog proteins of other plants such as Grape, Cucumber, *Zinnia*, *Arabidopsis*, fall into the same subfamily IV, whilst, 4 tomato *Aux/IAA* proteins fall into three of those five main subfamilies. In those lineages, the tomato proteins are highly similar to the proteins from *Arabidopsis*. It has been proposed that the proteins of each subfamily perform similar functions, which are distinct from the function of polypeptides in other lineages.

Based this phylogenetic analysis *DR4* was assigned to subfamily IV which amino acid sequence was significantly longer (> 300 aa) than the other members (~ 200 aa) of this family. Specifically subfamily IV has a relative longer amino acid sequence (40~60 aa more) before domain I and longer sequence (>60 aa more) between domain I and II, and also contain several high conserved amino acid sequence motifs (Figure III-1A) unique to this subfamily IV, especially in the intervening region between domain I and II contain a short sequences of xPPx or (Pxx)<sub>n</sub>, indicative of a polyproline II (PPII) conformation, Left-handed polyproline II helices are contiguous elements of protein secondary structure. PPII-helices tend to occur on

the surface of the protein and, having few main-chain hydrogen bonds with the rest of the protein, tend to be the more mobile segments of the molecule. They are important in structural proteins in unfolded states and as ligands for signaling proteins. It was suggested that the specific “filler” loops between the conserved domains that enable the conserved domains to adopt the correct specific tertiary structure or they might mediate interactions with other proteins, thereby conferring functional specificity (Reed, 2001).

## A

```

Le-DR4      MSPPLLGVGE-EEGQSNVTLLASSSTSLGSI CKKGSALKE RNYMGLSDCSSVDS SNI STSSEEDNNGCGLNLK 70
St-Aux/ IAA MSPPLLGVGE-EEGQSNVTLLASSSTSLGSI CKKGSALKE RNYMGLSDCSSVDS SNI STSSEEDNNGCGLNLK 70
Nt- IAA9    MSPPLLDVRE-EEGQSNVTLLASSASLGSIC KKGSE-LKE RNYMGLSDCSSVDSCTI STSSEEDNNACGLNLK 70
Vv-Aux/ IAA MSPPLLGVGE-EEGQSNVTLLASSASME SVQI SSG-LKE RNYMGLSECSSVDSSAI STDS DGNKSS-LNLK 69
Cs- IAA2    MSPPLLDVGVVEESRSNVTL LASSNME SVSPNNLE-FEE RNYMGLSDSSSEDS CMTATKSDGNKPS-LNLK 70
Ze-ARP      MPPPLLGVGE--DGLGNVSLMTSASTMES I FQKNAE-LTE RNYLGLSDCSSSFD STAVSGISEV KRNN-LNLK 68
At- IAA9    MSP-----E-EEEQSNVSVASSPTSNCTSRNTLGGLKE RNYLGLSDCSSVGSSTLSPLAEDDKAT- I SLK 64

```

```

Le-DR4      ATELRLGLPGSQSPERGEETCPVISTKVDEKLLFPLHP SKDTAFSVSQKTVVSGNKRGFSDAM DGFSEGKFL 142
St-Aux/ IAA ATELRLGLPGSQSPERGEETCPVISTKVDEKLLFPLHP AKDTAFSVSQKTVVSGNKRGFSDIM DGFSEGKFL 142
Nt- IAA9    ATELRLGLPGSLSPERGIETCPLAS---NEKLLFPLHP AKDSALAVSQKTVVSGNKRGFSDAM DGFSEGKFM 139
Vv-Aux/ IAA ATELRLGLPGFSLSPGREPELCLLSSTKLDEKPLFPLHP SKDLTYTSSQKTVVSGNKRGFADAMNGFSEGKFL 141
Cs- IAA2    ATELRLGLPGSESPERDPDNC LRSSQLDEKPLFPLHPSSDGLYSSQKTVVSGNKRGFSDAMNEFSEEKYH 142
Ze-ARP      ATELRLGLPGSQSPERDVDVDLVNSES LDEKPLFPLHP SKDGI CSNSQKVFVSGNKRGFSDAID---EGKWM 137
At- IAA9    ATELRLGLPGSQSPARDTELNLLSPA KLDEKPLFFPLHP SKDEICSSSQKNNASGNKRGFSDIM DQFAEAKSS 136

```

## Domain I

```

Le-DR4      SNS-----GVKAGDT---KETS RVQPPKMKDANTQSTVPERPSAVN-----DASN RAGSGAPATKAQVV 198
St-Aux/ IAA SNS-----GVKSGDA---KETS RVQPSKMKDANTQSTVPERPSAVN-----DASN RAGSGAPATKAQVV 198
Nt- IAA9    PNS-----GLKAGDT---KETS RVQPPKMK EATNQNTVPERPSAVN-----GASN RVSGAPATKAQVV 195
Vv-Aux/ IAA ANSEVNVMLSPRPSPN---KENLGSQPAKMKEMASPKIVQERPRATNETPPNHTGAGNNNSGAPATKAQVV 209
Cs- IAA2    AN-----IGLKAGSL---LENLGSQPMGKVEPTTQKAVQER PQENSESRPSHNEGANNNSTPVS KAQVV 204
Ze-ARP      FGS-----SGTDSETSNMNGKISSGACPVMIKDATSKVVTQEQTHATFG---TNLNI VNTSNPPAKAQVV 200
At- IAA9    VYT-----EKNWMPFAAAATQSVTKKDVPQNI PKQS-----STTNNS SPPAKAQIV 185

```

## Domain II

```

Le-DR4      GWPPIRSF RKN TLASASKNNEEVDGKAGSPALFIKVSMDGAPYL R KVDLRTCSAYQELSSALEKMFSCFTIG 270
St-Aux/ IAA GWPPIRSF RKN TLASASKNNEEVDGKAGSPALFIKVSMDGAPYL R KVDLRTCSAYQELSSALETMFSCFTIG 270
Nt- IAA9    GWPPIRSF RKN TLASASKNNEKVDGKAGSPALFIKVSMDGAPYL R KVDLRTCSAYQELSSALEKMFSCFTIG 267
Vv-Aux/ IAA GWPPIRSF RKN TLATSKN-TEVDGKAGPCALFVKVSM D GAPYL R KVDLRTCSAYQELSSALEKMFSCFTIG 280
Cs- IAA2    GWPPIRSF RKN TLATSKNNEEVDGKAMAGALFIKVSMDGAPYL R KVDLRTCSAYQELSSALEKMFSCFTIG 274
Ze-ARP      GWPPVRSFRKN TLATNSKTND EVDGKPCGALFVKVSM D GAPYL R KVDLRSYSTYQQLSSALEKMFSCFTIG 272
At- IAA9    GWPPVRSYRKN TLATCKNSDEVDGRPGSALFVKVSM D GAPYL R KVDLRSYTN YQELSSALEKMFITFTLG 257

```

## Domain III

```

Le-DR4      QYGSHGAPGKQDMLSESKLKDLLHGSEYVLT YEDKDGWMLVGDVPWEMFIDTCKRLRIMKGS DAIGLA--PR 340
St-Aux/ IAA QYGSHGAPGKQDMLSESKLKDLLHGSEYVLT YEDKDGWMLVGDVPWEMFIDTCKRLRIMKGS DAIGLA--PR 340
Nt- IAA9    QYGSHGAPGKQDMLSESKLKDLLHGSEYVLT YEDKDGWMLVGDVPWEMFIDTCKRLRIMKGS DAIGLA--PR 337
Vv-Aux/ IAA QYGSHGAPGREMLSESKLKDLLHGSEYVLT YEDKDGWMLVGDVPWOMFIDTCKRLRIMKGS DAIGLA--PR 350
Cs- IAA2    QYGAHGALGMEKMSK LKDLLHGSEYVLT YEDKDGWMLVGDVPWEMFIDTCKRLRIMKSS DAIGLA--PR 346
Ze-ARP      CCGSQGAPGRESLSESKLRDLLHGSEYVLT YEDKDGWMLVGDVPWDMFIDTCKRLRIMKGS DAIGLA--PR 342
At- IAA9    CCGSNGAAGKQDMLSETK LKDLLNGKDYVLT YEDKDGWMLVGDVPWEMFIDVCKK LKIMKGS DAIGLAAAPR 329

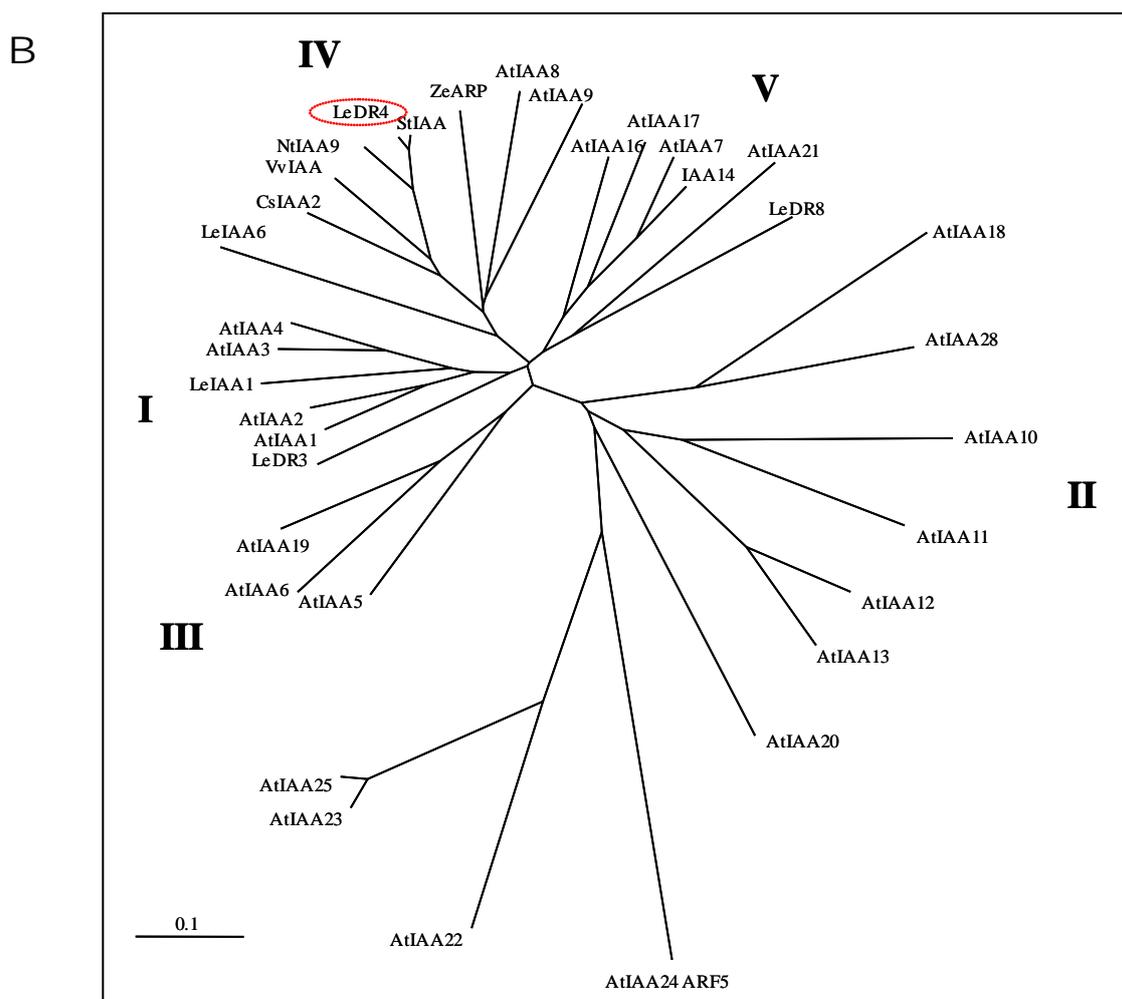
```

## Domain IV

```

Le-DR4      AMEKCRSRN 349
St-Aux/ IAA AMEKCRSRN 349
Nt- IAA9    GMEKCRSRN 346
Vv-Aux/ IAA AVEKCKNRN 359
Cs- IAA2    AVEKCRNRS 355
Ze-ARP      AMEKSKNRN 351
At- IAA9    AMEKSKMRA 338

```



**Figure III-1.** Sequence analysis of DR4.

(A) An alignment of predicted protein sequences from related *Aux/IAA* genes using ClustalX (Jeanmougin et al., 1998) and GenDoc (Nicholas and Nicholas, 1997). The four conserved domains I, II, III, and IV are indicated by underlining. Conserved basic residues that function as NLS are indicated by (▼) on the top of alignment; a putative polyproline II conformation residues are indicated by (●). Conserved residues are shaded in black, dark grey shading indicates similar residues in six out of seven of the sequences and grey shading indicates similar residues in four or five out of seven of the sequence. *Le*, *Lycopersicon esculentum*, *St*, *Solanum tuberosum* (GenBank accession number AAM29182); *Nt*, *Nicotiana tabacum* (CAD10639); *Vv*, *Vitis vinifera* (AAL92850), *Cs*, *Cucumis sativus* (BAA85821); *Ze*, *zinnia elegans* (AAM12952) and *At*, *Arabidopsis thaliana* (AAM64650).

(B) Phylogenetic analysis of the DR4 (LeDR4 in the figure) and selected *Aux/IAA* proteins (see text). Alignments of the predicted protein sequences were performed with ClustalX (Jeanmougin et al., 1998) and the unrooted tree was visualised with Tree view program (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>). Branch lengths represent the calculated distance between the sequences. Roman numerals designated individual subfamilies.

### A-3- DR4 protein

*Aux/IAAs* are primary/early auxin responsive genes encoding short-lived, nuclear-localized proteins, and function as transcriptional regulators (Abel et al., 1994; Guilfoyle,

1999; Reed, 2001; Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). Aux/IAA proteins typically have *in vitro* half-lives of several minutes (Abel and Theologis, 1996), and thus have properties needed of factors that mediate rapid and transitory responses to signals. Moreover, the Aux/IAs are thought to act as repressors of auxin-induced gene expression in general, and auxin responses require Aux/IAA proteins degradation. The highly conserved domain II was demonstrated to confer instability to the Aux/IAA protein, and almost all of the *aux/iaa* mutants obtained so far were mutated in this domain. Direct-DNA-binding capacity of the Aux/IAA proteins has not been shown, but it was well demonstrated by yeast two-hybrid assay that Aux/IAA and ARF (Auxin Response Factor) can homodimerize and heterodimerize with some selectivity, via the C-terminal domain III and IV which were found in both kind of proteins. It has therefore been hypothesized that the Aux/IAA proteins regulated transcription by modifying proteins ARF activities, which were demonstrated bind to auxin-responsive *cis*-acting promoter elements (AuxREs) (Abel et al., 1994; Guilfoyle, 1999; Reed, 2001; Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). *DR4* predicted polypeptide sequences contained perfectly the highly conserved domains I, II, III, and IV (Figure III-1A), confer theoretically the abilities be degraded quickly (domain II) and homo/hetero-dimerization (domain III, IV). The theoretical pI/Mw determinations for the full length predicted polypeptide was shown in the table III-1:

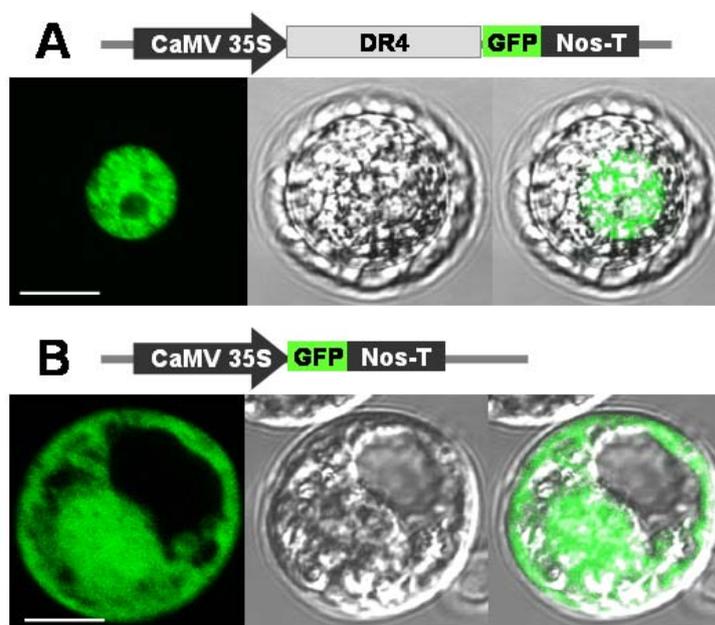
**Table III-1.** The theoretical pI/Mw determinations of DR4 predicted polypeptides

Coding Seq length (bp)	Polypeptide length (aa)	pI	Mw (KD)
1047	349	7,99	7,11

#### **A-4- Subcellular localization of DR4 protein**

Given its strong sequence homology with *Aux/IAA* transcription factor gene family and the presence of a putative nuclear localization signal in the predicted sequence (Figure III-1A) the *DR4*-encoded protein was expected to be targeted to the nucleus. To analyze the subcellular localization of the DR4 protein, we fused green fluorescent protein (GFP) to the COOH- terminus of the DR4 protein expressed under the control of the 35S promoter. Plasmids bearing either the *GFP* gene along or the *DR4-GFP* fusion were transiently transformed into tobacco protoplasts. Fluorescence microscopy analysis associated with image overlay techniques demonstrated that control cells transformed with GFP alone

displayed fluorescence distributed throughout the cytoplasm, in accordance with the expected localization of the GFP protein (Figure III-2B). In contrast, the DR4-GFP fusion protein was exclusively localized to the nucleus (Figure III-2A), indicating that DR4 protein itself was able to fully redirect the GFP from the cytosol to the nucleus. This is in agreement with a putative transcriptional regulation function.



**Figure III-2.** Nuclear localization of DR4 protein fused to GFP tag. Constructs consisting of either *35S::GFP* (B) or *35S::DR4-GFP* (A) were used to transiently transform tobacco protoplasts. The subcellular localizations of the DR4-GFP fusion protein or GFP alone were analyzed by confocal laser scanning microscopy (left panel). Light micrographs (medium panel) and fluorescence (left panel) images are merged (right panel) to illustrate the different location of the two proteins. The length of the scale bar corresponds to 10  $\mu$ m.

## B- Biological function of *DR4*

Biological function of *DR4* was studied by cellular biology and reverse genetic approaches. First the “antisense (AS)” and “sense” reverse genetic approach were used to generate transgenic plants with down-expression or over-expression of the gene *DR4*, then the *DR4* antisense transgenic plants were laboriously characterized, including the morphological analysis, physiological analysis, genes expression analysis and also anatomical analysis, to further explore the biological function of this gene.

### **B-1- Reverse genetic approach**

As for any protein, to study its biological function, the most appropriate approach is complete inhibition of their expression by targeted gene replacement. Since this technique is few applicable yet for plants, reverse genetic approaches such as construction of mutants by over-expressing sense or antisense RNA to manipulate levels of genes expression, or by insertional mutagenesis are the current methods used (Abel et al., 1995a). In our lab, reverse genetic approach antisense and sense strategy was used to study the biological function of a new tomato *Aux/IAA* gene *DR4*, especially to explore its roles during the fruit development.

#### **B-1-1- Antisense: down-expression of *DR4***

To further address the functional significance of the *DR4*-encoded protein, and to explore its physiological roles especially during the fruit development, transgenic tomato lines were generated expressing antisense constructs of the gene under the control of constitutive 35S promoter, as described in the chapter II. Of the eleven transgenic plants (selected by kanamycin resistance), six independent antisense lines in two different genotypes (*Micro-Tom*,  $\mu$ *Tom*; *Ailsa Craig*, *AC*) were found to be *DR4*-down regulated, they are five lines in  $\mu$ *Tom* (line AS58, AS70,1, AS111, AS213, and AS250) and one line in *AC* (line AS268) (Figure III-3A), the other 5 lines were found a similar level of *DR4* transcripts than the WT control, although they showed also kanamycin resistance. Plants that were regenerated as described in chapter II, and confirmed to be transgenic by PCR analysis.

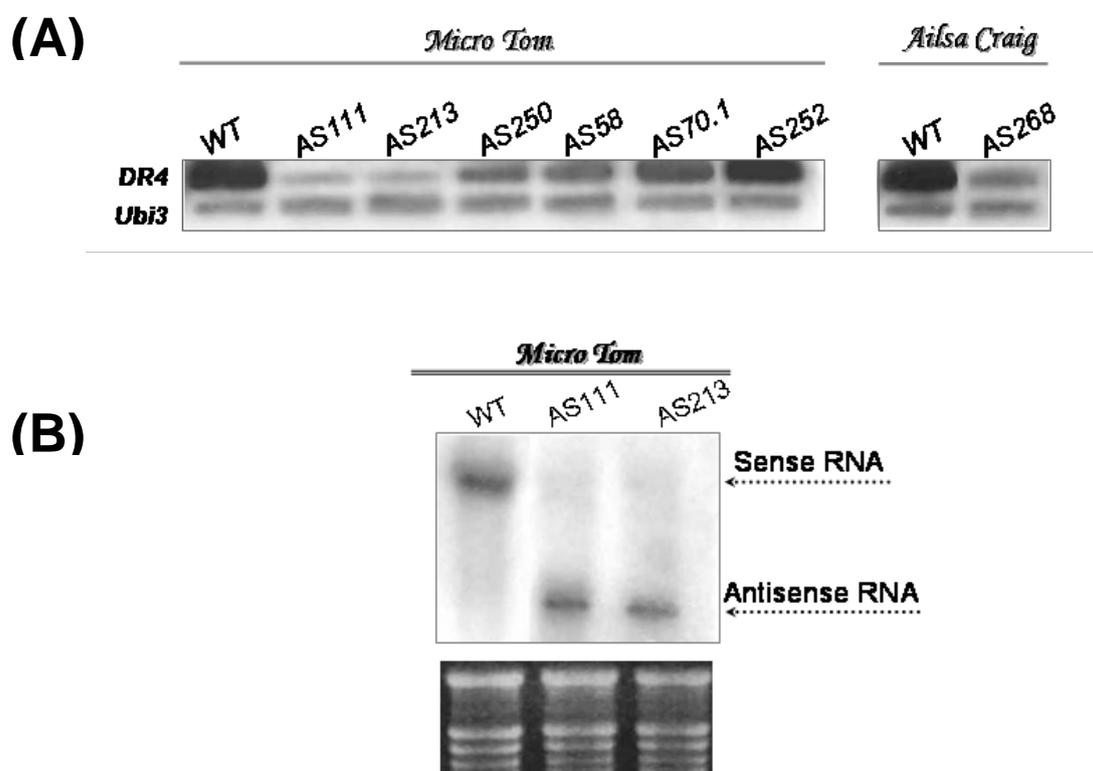
#### **B-1-2- Sense: gene over-expression of *DR4***

Generation of *DR4* sense transgenic tomato plants were carried out as antisense lines, using the pGreen binary vector and expressing the *DR4* full length ORF constructs under the control of constitutive 35S promoter. However, there is no sense line obtained so far. The table III-2 showed the original data of the transformation and generation *DR4* sense line work. The reason for this is unclear. However as *DR4* is hypothesized as auxin response repressor, over-expression *DR4*, probably decreased or blocked certain auxin responses, so that the *DR4* over-expressing transgenic plants maybe die early during the *in vitro* culture. In brief, the *DR4* sense constructs maybe is lethal to tomato plant. In spite of all of these above

suppositions, the generation work of *DR4* sense lines are still currently going on in our lab with some modification compared with the standard method, tempting to obtain the sense lines, such as application of supplemental low dose auxin in the media to promoting root generation, as we remarked that the regenerated plantlet showed difficulty in rooting. The *DR4* orthologs in Arabidopsis are *AtIAA8* and *AtIAA9*, in contrast of unceasing of *aux/iaa* mutants obtained by T-DNA insertion or the other means, there is almost no mutant obtained on this two genes to date, except for one in which have T-DNA insertion on last several amino acid sequence of *IAA8*, and no further reports for its characterization work.

**Table III-2.** Transformation and generation of *DR4* sense line

Quantity of transformation	Number of transformation times	Plants R <sub>0</sub> obtained	Rate of transformation	Number of kana resistant plant
2510 cotyledons	10	14	0,56%	0



**Figure III-3.** The expression of *DR4* in antisense transgenic plants (A) By RT-PCR analysis, five antisense lines (line AS58, AS70,1, AS111, AS213, and AS250 in  $\mu$ Tom genotype) showed down-regulated *DR4* transcripts accumulation; while the false-positive transformed line AS252 which is kanamycin resistant showed similar *DR4* transcripts level as WT; the *Ailsa Craig* genotype background line DR4AS 268 showed also down-regulated *DR4* transcripts accumulation. In each PCR reaction, the internal reference *ubi3* was co-amplified with the *DR4* gene as described in chapter II. (B) Northern blot analysis of sense and antisense *DR4* transcripts accumulation in WT and two

DR4AS lines (AS111 and AS213), it showed the important *DR4* sense transcripts in WT, but largely down-expressed (almost absent) in the two DR4AS lines; on the contrary the *DR4* antisense transcripts showed important accumulation in the two antisense lines but absent in the WT control. Total RNAs were extracted from 21-d-old seedlings.

### **B-1-3- Ectopic over-expression of tomato *DR4* in Arabidopsis**

Ectopic over-expression of the tomato *DR4* in Arabidopsis was also carried out and currently going on in our lab, in order to further address this gene function and compensate the research work in tomato, as the availability of the exist *aux/iaa* mutants in Arabidopsis.

## ***B-2- Morphological analysis***

*DR4*-inhibited lines exhibited a visible pleiotropic phenotype, including precocious fruit set and parthenocarpy, abnormal leaf morphology, multi-organs fusion, aberrant vascular tissue patterning. These phenotypes of *DR4* down-regulated plants were found in both two different background genotypes  $\mu Tom$  and *AC*. Further studies suggested that the down-regulation of *DR4* expression accounts for those phenotypes of the transgenic plants.

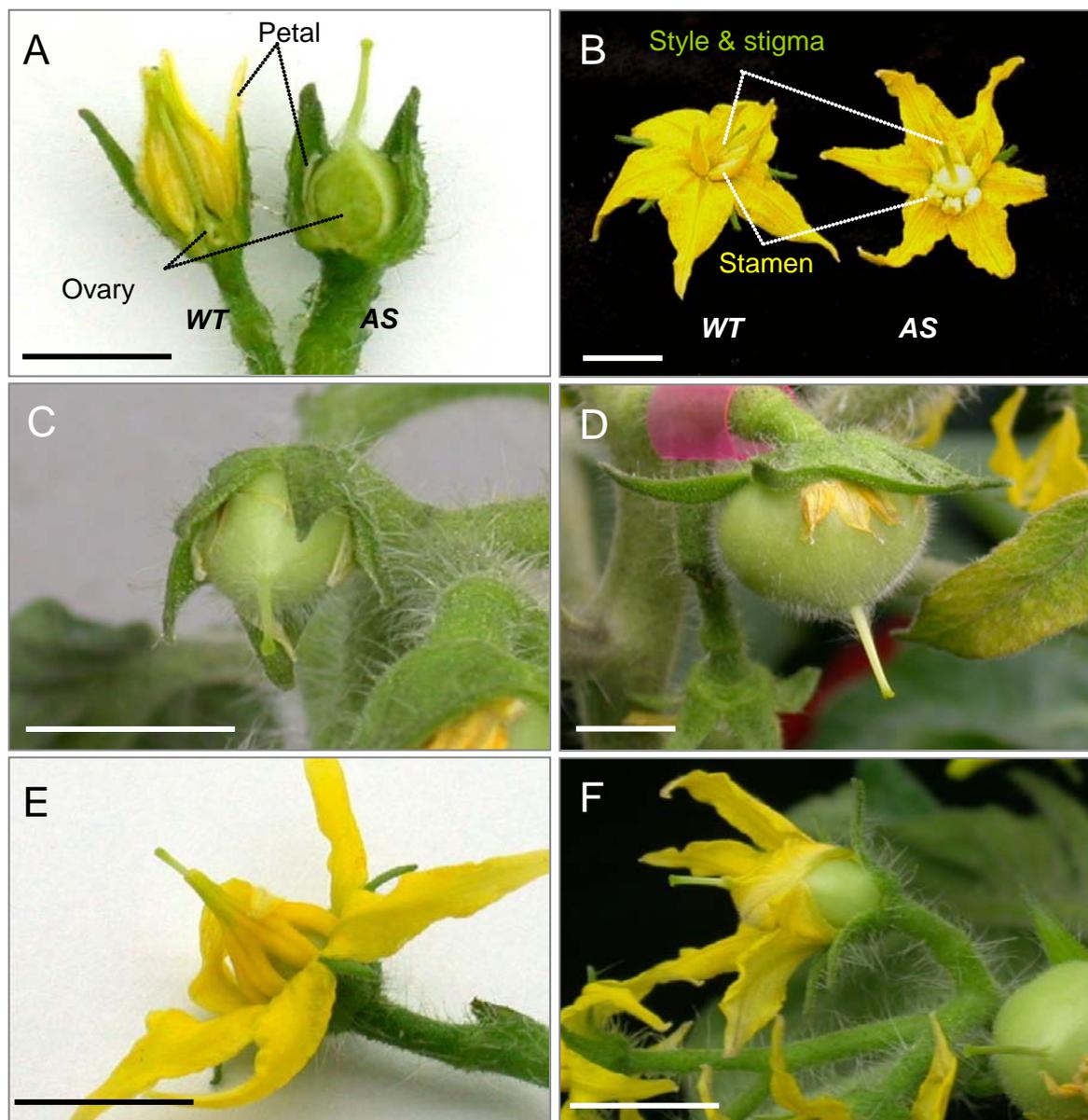
### **B-2-1- Phenotypes of *DR4* antisense transgenic tomato plants**

There are significant morphogenetic changes in those *DR4*-inhibited transgenic plants, from the vegetative organs to reproductive organs; moreover, the phenotypes are consistent with each other in two genotypes:  $\mu Tom$  and *AC*.

#### ***B-2-1-1- Precocious fruit set and parthenocarpic fruit***

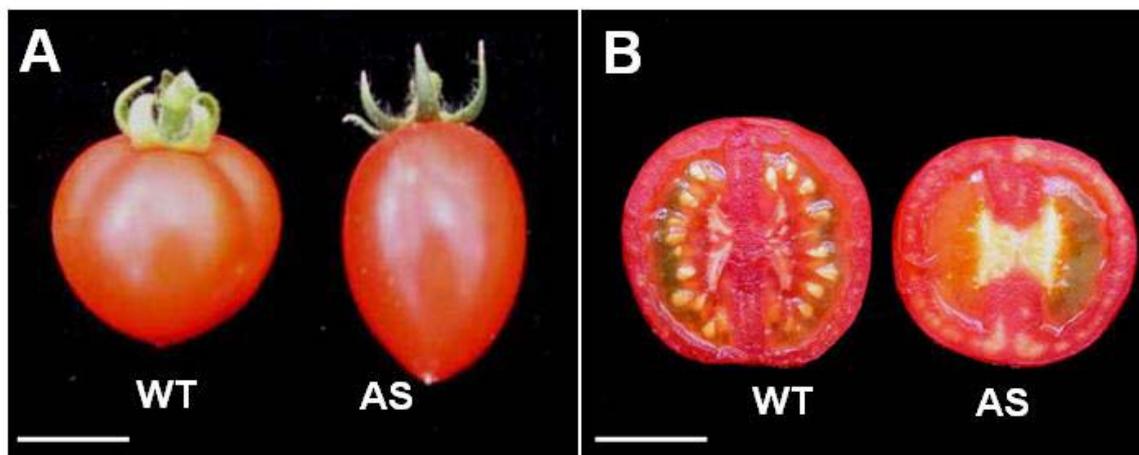
Tomato fruit development goes many stages as it initiate from a flower primordial, those normally development stages were demonstrated in chapter I, Figure I-20. When the bud first forms the petals are protected by the sepals, as the bud matures the sepals separate to expose the pale yellow-green petals. The petals later turn to deeper yellow when the flower is mature. Once the flower is mature (anthesis) it will either be fertilized to develop into a fruit, or it will be abscised. If the flower is not fertilized, it will separate from the plant at the abscission zone, this causes the flower to wither and die. If the flower is fertilized it will

undergo senescence, during senescence the flower's petals, style, and stigma are shed, and the ovary begins to enlarge and develop into the fruit (Fruit set).



**Figure III- 4.** *DR4AS* exhibit precocious fruit set. (A)-(B) Comparison of flower WT and AS; (A) the WT flower bud (left) is at 1 days before anthesis, note that the ovary is still small, about 1 mm in diameter; while the AS 'flower bud' (right) is still in early immature stage, theoretically more than one week before the anthesis, the petal is still pale yellow-green and small, note that the ovary is already started enlargement, 6,0 mm in diameter. Particularly, there is no stamen formation and development. (B) Comparison of anthesis of WT and AS; the fused cylindrical cone of stamens are torn open artificially to show the ovary, in WT the ovary is less than 1 mm and almost invisible in the end of style in the photo, while AS shows clearly the enlargement of the ovary (about 4 mm) with style and stigma still alive tagged on the top of fruit. (C)-(D) showing the development of petals in precocious setted fruit. (C) Precocious fruit set in the early flower stage, the petals are still pale and under well-developed. (D) The petals develop as well as the fruit, but finally it can not fully develop into a normal flower, although its color turn to deeper yellow, but the size stays much smaler, noted that stamen

development is absent; and the unfertilized stigma stay alive long time with the style on the top of the enlarged fruit (two weeks). **(E)-(F)** showing precocious setted fruit develop at the same time with flower, the stamen corn **(E)** and the petals with stamen corn **(F)** are torn open by the quick enlargement of fruit. The bars represent 10 mm.



**Figure III-5.** *DR4AS* parthenocarpic fruit. **(A)** Except the elongated shape, the *DR4AS* ( $\mu$ *Tom*) fruit has appearance quite similar as the normal WT tomato fruits, including the size, skin and flesh color, flesh consistency, and fresh weight. **(B)** Naturally formed parthenocarpic fruit of *DR4AS* showed quite similar appearance as that of WT (in AC background), except no seeds in locule and white placenta. The scale bars represent 10 mm **(A)** and 30 mm **(B)** respectively.

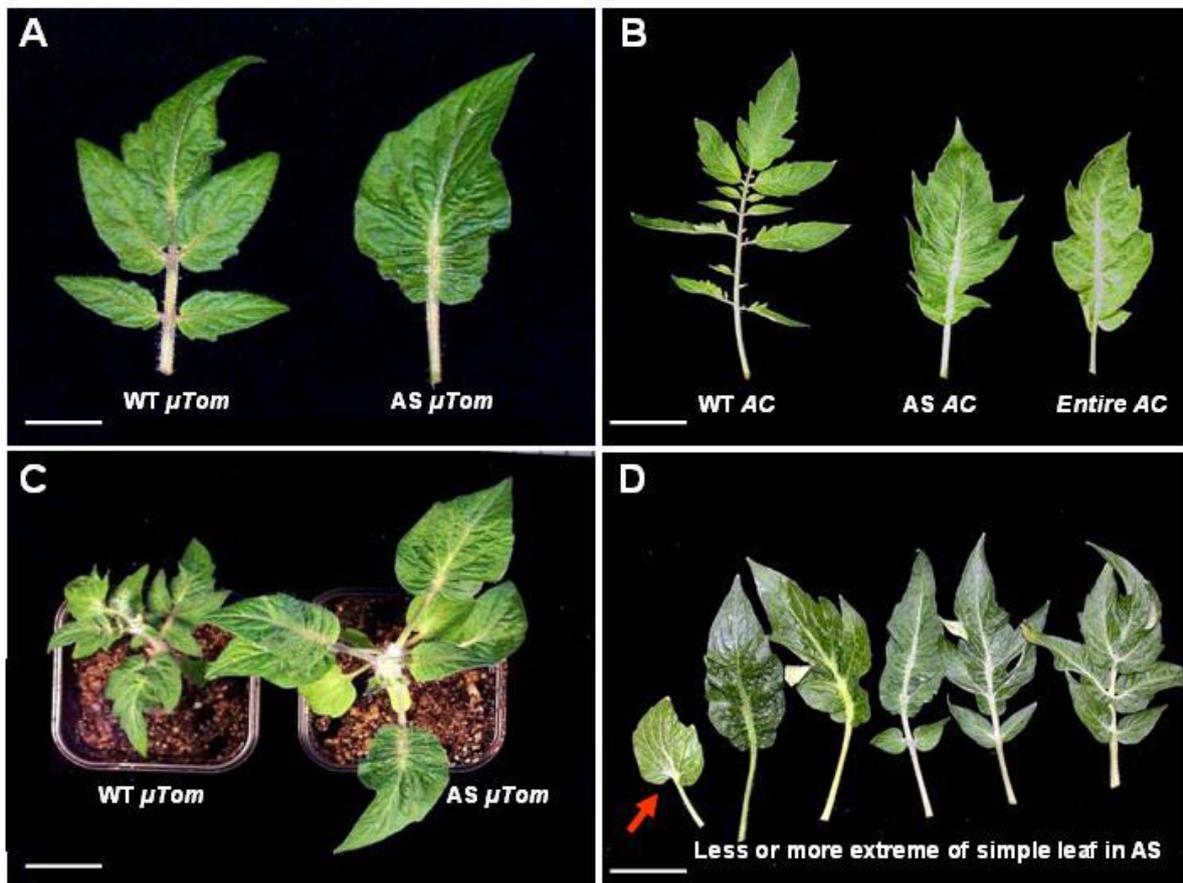
All independent lines of *DR4AS* plants exhibit precocious fruit set even in the early flower buds stage (Figure III-4A, C), the further observation showed the fruit set can take place in any point of time during the flower development, result in the phenomena that fruits develop at the same time with flowers. Due to the quick enlargement of those precocious developed fruits, the fused stamens cones of tomato are often torn open (Figure III-4E, F). The green style and stigma stayed long time viable (till two weeks after the initiation of fruit set) on the fruit top in the absence of fertilization (Figure III-4D), in contrast with the normal program in WT that the ovary won't begins to develop into the fruit till the styles and stigmas shed. The development of stamen is even absent if the fruit set and enlargement take place in the early flower bud stage (Figure III-4A, C, D). Finally most of those precocious developed fruits are parthenocarpic with the quite similar appearance as the normal fertilized WT tomato fruits, including the size, skin and flesh color, flesh consistency, and fresh weight. With regard to the fruit development program from fruit set to mature green, break, turning, red, then overripe, those parthenocarpic fruits were indistinguishable from the normal WT fruits. Only one parameter changed in the parthenocarpic fruits was the fruit shape, it was often more elongated in longitudinal order (increase in fruit height) (Figure III-5).

### ***B-2-1-2- Simple leaves***

Tomato leaves are imparipinnate-compound in nature, and exhibit a basipetal order of leaflet initiation and maturation. Upon maturation each leaf carries major and minor leaflets, most of which are lobed to various degrees, which exhibit plagiotropic growth. The adult foliage leaves of 'Ailsa Craig (AC)' tomato are triangular and imparipinnate with three pairs of large lateral leaflets. Each large leaflet has one to six lobes, some of which may be deeply incised; some of the basal leaflets are compound, making the whole leaf bipinnately compound (Figure III-6B left), typically, leaves also bear one to two pairs of less-lobed lateral leaflets, which are intermediate in size, and one to four pairs of small entire leaflets. Leaflets usually are present in opposite pairs or may occur singly or in subopposite pairs. The first leaves produced on the seedlings are small and bear two to four leaflets only. Transitional forms are characterized by increased area and additional lateral leaflets, including the intermediate and small ones. Leaves at the fifth or sixth nodes above the cotyledons reach the full size and complexity of the cultivar. The adult foliage leaves of  $\mu$ Tom are similar as AC, carried two to four pairs of large lateral leaflets and one terminal leaflet (Figure III-6A left).

Adult leaves of DR4AS are usually characterized by light lobed simple leaves, but they vary in form, from semi-compound leaves to perfect entire margin simple leaves dependent on different antisense genetic background (Figure III-6D). The strong lines carry only simple leaves, while the weak lines carry both simple leaves and semi-compound leaves. In fact, in the simple leaves, one or more pair(s) of leaflets is merged with the terminal leaflet, and the borderlines between the terminal and the laterals leaflets are often still remarkable. One or two small proximal leaflets may also be present. The lamina is often asymmetrical and is rugous or wrinkled in texture. The first-formed foliage leaves are small and usually have palm leaf venation (red arrow in Figure III-6D); the adult form is reached by the fifth or sixth node above the cotyledons. The recessive spontaneous mutant 'entire (*en*)' (background genotype *Ailsa Craig*), exhibits exactly the same simple leaf phenotype as the DR4AS (AC), including asymmetrical and wrinkled lamina, formation of less and more extreme 'simple' leaf arrangement depending on the genetic background (Figure III-6D). The mutation of *ENTIRE* was located in Chromosome IV (Dengler, 1984; Hareven et al., 1996). In addition, over-IAA-synthesis tomato transgenic plant by ectopic expressing *iaaM* under 35S promoter, also exhibit simple leaves phenotypes; spontaneous tomato mutant *lanceolate* also exhibit simple leaves phenotype, and the studies showed the impaired auxin transport in the lamina and increased auxin accumulation on the leaves. The last two suggested that the simple leaves

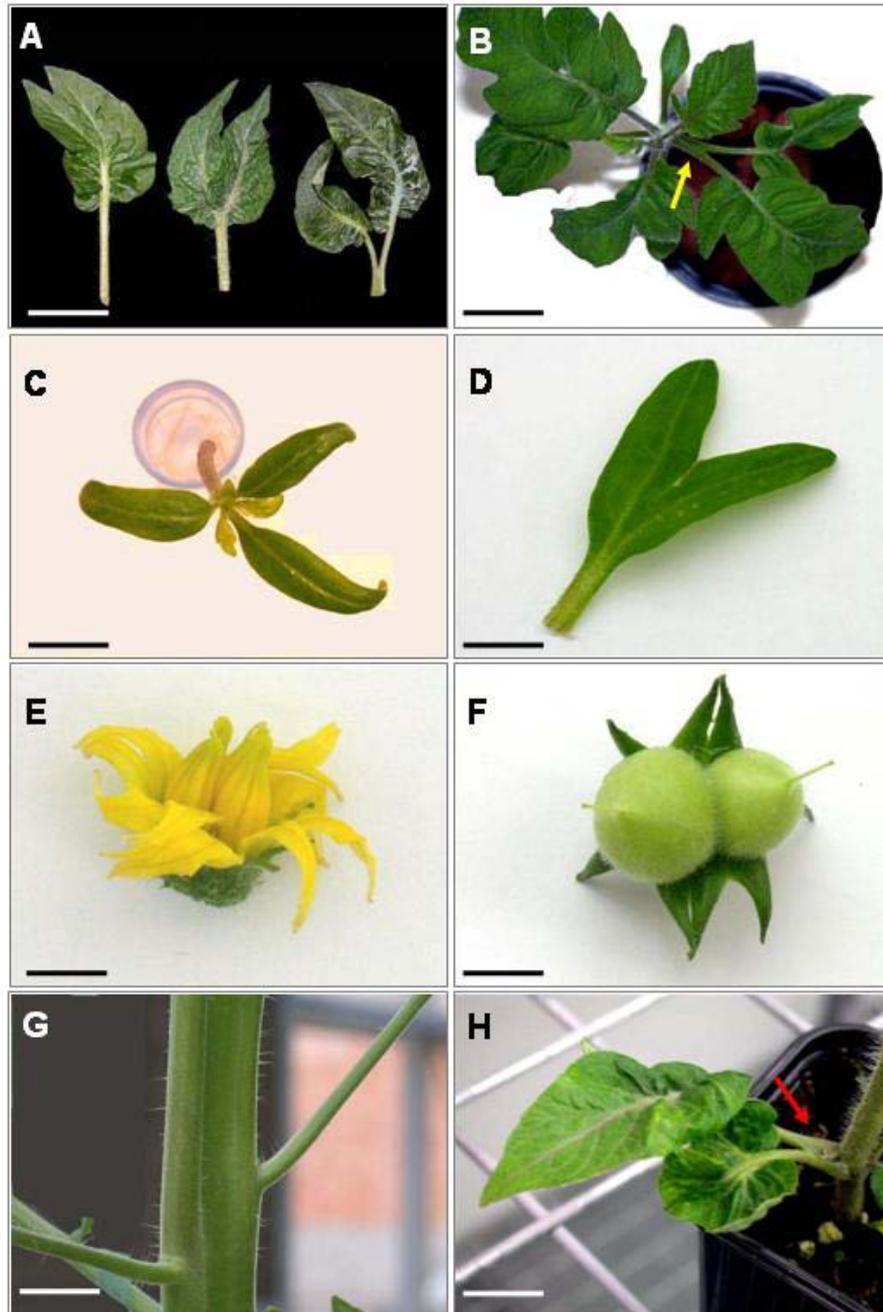
formation in *DR4AS* may be resulted from the over-auxin responses in the leaves or during the leaves ontogenesis.



**Figure III-6.** *DR4AS* exhibit simple leaf (A) Comparison of isolated leaves of WT and AS ( $\mu Tom$ ). (B) Comparison of isolated leaves of WT, AS and *entire* (*AC*). (C) Comparison of WT and AS plant ( $\mu Tom$ ), WT showing compound leaf structure, while AS showing simple leaf structure. (D) Showing less or more extreme of ‘simple’ leaves arrangement depending on their antisense genetic background in  $\mu Tom$ , the strong lines carry only simple leaves, while the weak lines carry both simple leaves and semi-simple leaves (pseudo-simple leaves), red arrow indicating that the first-formed foliage leaves are usually smaller and have palm-leaf venation. Scale bars represent 20 mm (A), 100 mm (B), 30 mm (C), 25 mm (D).

### ***B-2-1-3- Organ fusion***

Both in  $\mu Tom$  and *AC* genotype, the *DR4AS* exhibit a wide range of organ fusion including fusion of leaves, stems, flowers and fruits (Figure III-7). First, *DR4AS* plants showed frequently abnormal vein branching resulting in the appearance of fused twin leaves, which is position-dependant and the majority of these fused leaves were found only between the first to the third pair of real leaves, both in  $\mu Tom$ , *AC*, and *entire* mutant. The less or more extreme of leaf fusion showed in the Figure III-7A, including basically one leaf with two leaf



**Figure III- 7.** *DR4AS* plants exhibit multi-organ fusion (A) The less or more extreme of leaf fusion in *DR4AS μTom*, including basically one leaf with twin terminal leaf tips (left), one petiole bearing two leaf blades (medium), bifurcated petiole with two leaf blades (right). (B) A twin leaf with bifurcated petiole of *DR4AS AC* (yellow arrow head). (C) *DR4AS μTom* seedling exhibit often 3 cotyledons. (D) A twin cotyledon of *DR4AS μTom*, showing one cotyledon bearing two midrib and two tips. (E) Fused flower of *DR4AS μTom*. (F) Fused fruits of *DR4AS μTom*, noted also that the styles and stigmas stay alive on the fruit tops in the absence of fertilization. (G) A fused stem in *DR4AS AC*. (H) One twined-leaf arising from the same leaf node (red arrow head). Scale bars represent 35 mm (A), 15 mm (B), 9 mm (C), 5 mm (D), 6 mm (E), 5 mm (F), 35mm (G), 25mm (H).

apexes, or one petiole bearing two leaf blades, or bifurcated petiole with two leaf blades and one twined-leaf arising from the same leaf node (Figure III-7H). The “pin”-forming petioles

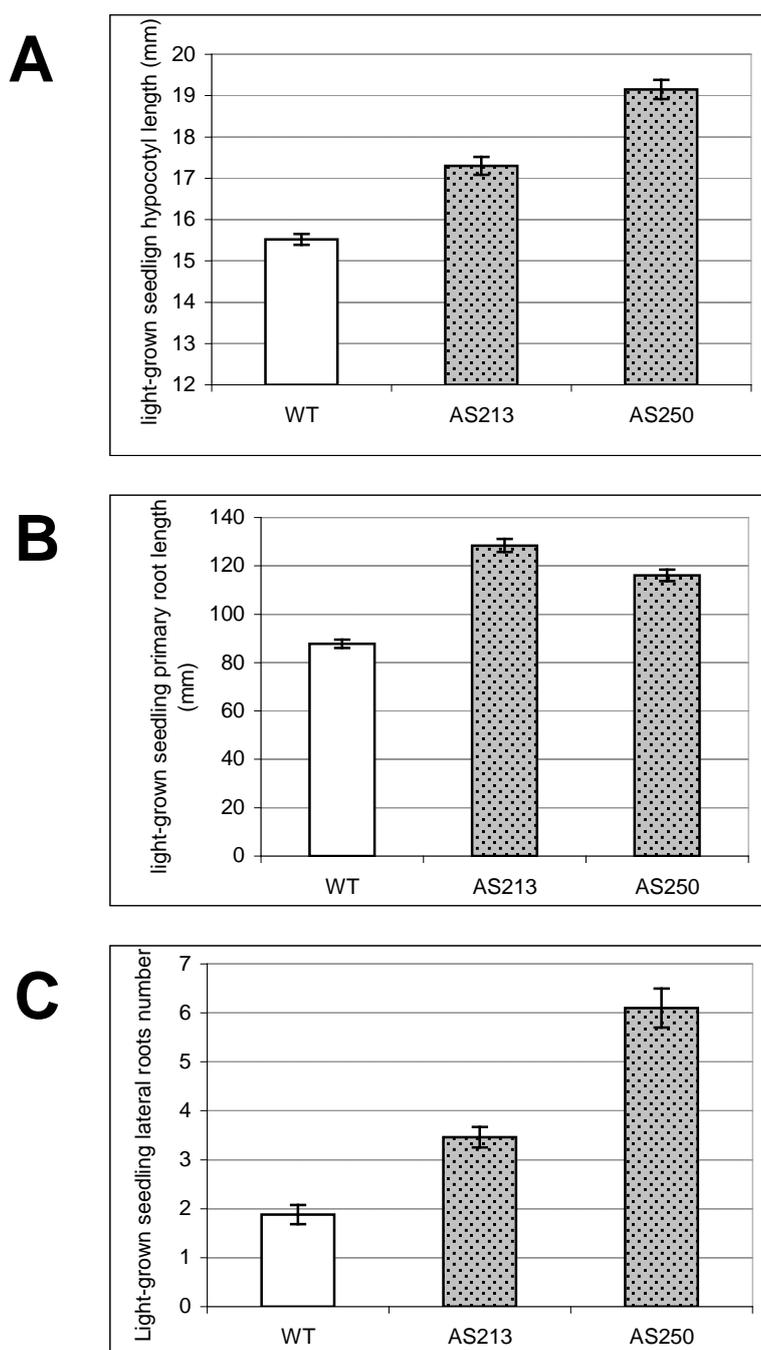
were also found with the twin leaves (red arrow). These obvious phenotypes (leaves fusion and pin-form structure) were also found in the *Arabidopsis pin1* mutant too. PIN was auxin efflux carrier protein which was detected at the basal end of auxin transport-competent cells in vascular tissue. The mutations in this gene distorted normal polar auxin transport and altered the vascular development. In addition, enhanced vascular tissue differentiation has also been observed in plants that overproduce auxin. Taken together, all of these data strongly suggest the abnormality of auxin responses in DR4AS plants. The DR4AS seedling showed frequently or occasionally twin or bifurcated cotyledon (Figure III-7D), aberrant cotyledon positioning and triple or four cotyledons (Figure III-7C), depending on the antisense line genetic background, the strongest line DR4AS 213/7 ( $\mu Tom$ ) exhibit 80% triple or four cotyledon, while the weak line DR4AS58 ( $\mu Tom$ ) and DR4AS 268 (*AC*) only exhibits the triple cotyledon occasionally. Note also that triple-cotyledons is also naturally happen in the WT ( $\mu Tom$ ) occasionally (should less than 1%) The same fusion history was happened with DR4AS stem (Figure III-7G), flowers and consequently the fruits (Figure III-7E, F), might due to the abnormal vascular tissue development or patterning. In addition, the *entire* carried the same multi-fusion phenotype.

#### ***B-2-1-4- Light-grown seedling: more lateral roots and longer hypocotyl***

White light-grown DR4AS seedlings showed increased hypocotyl elongation, more lateral roots and longer primary root length than those of WT (Figure III -8 and Figure III- 9). The *Arabidopsis* gain-of-function *aux/iaa* mutants *iaa3/shy2-2*, *iaa6/shy1-1*, *iaa7/axr2-1*, and



**Figure III-8.** Comparison of WT and DR4AS light-grown seedlings (19-d-old). DR4AS seedling (right) showed increased hypocotyl elongation, enhanced lateral roots formation and primary root elongation than their WT counterparts grown under the same conditions.



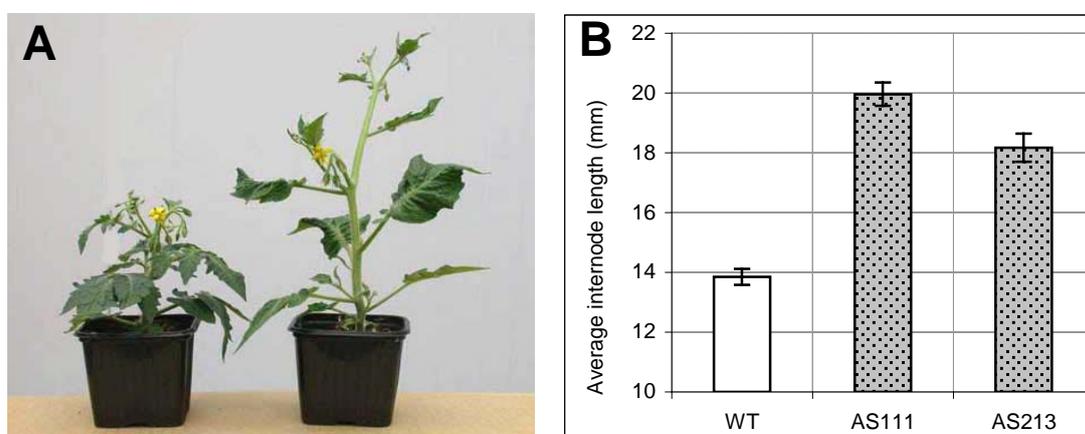
**Figure III-9.** DR4AS seedling showed longer hypocotyl and primary root, more lateral roots. DR4AS seedling showed increased hypocotyl (A) and primary root elongation (B), enhanced lateral roots formation (C). Seedling were grown as described in chapter II, Results represent the average  $\pm$  SE of at least 80 individual seedlings. Similar results were obtained in three independent experiments.

*iaa17/axr3-1* seedlings have shorter hypocotyls than wild-type, whereas the *iaa18-1* mutant has a longer hypocotyl than the WT, and all of them are generally summarized reduced auxin responses mutants. In addition, many of these *aux/iaa* mutants have altered numbers of lateral roots and/or inflorescence branches, suggesting that Aux/IAAs regulated cell division in both

lateral shoot meristems and lateral root initials in the pericycle. Consistent with the supposition that being tomato Aux/IAA protein DR4 mediates auxin responses, these DR4AS seedling phenotypes are in processes known to be influenced by auxin: promoting hypocotyl elongation and inducing the lateral roots formation. No significant difference was observed between the dark-grown seedling of WT and DR4AS (data not shown). In the light of several *Arabidopsis aux/iaa* mutants exhibiting leaf formation in the dark, this processes was also examined; however, the DR4AS etiolated seedling do not performed the leaf formation.

### ***B-2-1-5- longer internodes***

DR4AS plants are usually higher and than WT plants, due to longer internodes (increased stem elongation) (Figure III-10), and it also depends on the antisense genetic background: the stronger antisensed, the longer internodes formed. In  $\mu Tom$ , the two strong antisense lines showed much longer internodes, however the two weak lines showed similar internodes as the WT plants; the AS in AC also showed longer internodes (data not shown).

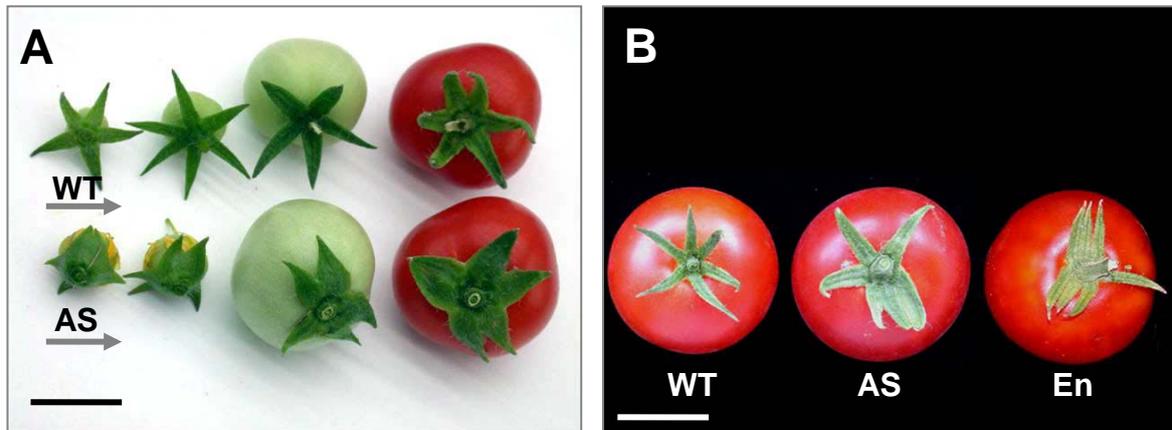


**Figure III -10.** DR4AS plants have increased stem elongation. (A) DR4AS plant is high than WT due to longer internodes. (B) Mean internodes length for WT and 2 strong lines AS ( $\mu Tom$ ) after 50 days growth. The plants were grown as described in Materials and Method, Results represent the mean  $\pm$  SE of at least 68 individual plants, similar results were obtained in three independent experiments

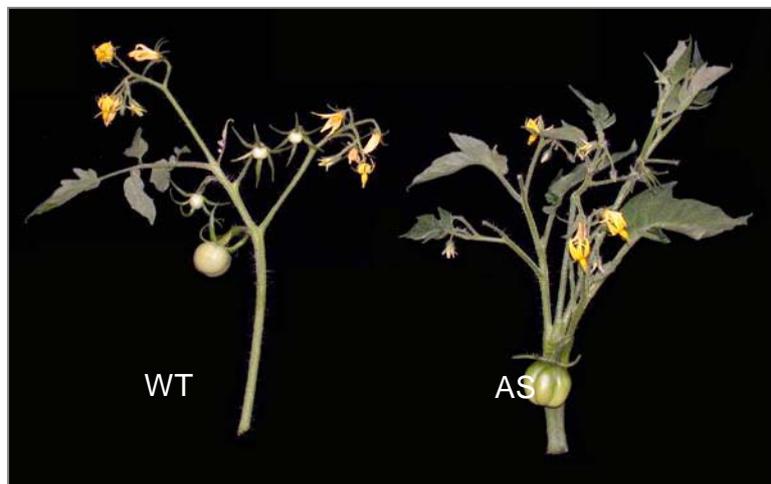
### ***B-2-1-6- Aberrant sepals arrangements***

The normal WT tomato carry five or six symmetrically arranged sepals, while the DR4AS tomato invariably had party fused, asymmetrically arranged sepals (Figure III-11), which was also observed in *entire* mutant. This phenotype was also found in tomato

spontaneous mutant “*clausa*”, which was concluded, at least partly, rendering the *LeT6/TKn2* gene (class II *Knox* gene) silence in specific tissues during the development.



**Figure III-11.** Calyx asymmetry DR4AS plant and entire plant. (A) WT ( $\mu$ Tom) fruit (up row) showing normal, symmetrically arranged sepals during the fruit development; while the DR4AS ( $\mu$ Tom) fruit (down row) exhibiting asymmetrical calyx with partly fused sepals. (B) Partial fused asymmetrical calyx in DR4AS (AS) and entire mutant fruit. The scale bars represent 15 mm (A) and 30 mm (B) respectively.



**Figure III- 12.** Comparison inflorescences in WT (left) and DR4AS (Right.) WT (left) exhibits well arranged dichotomously branched cyme inflorescence, the flowers on a given inflorescence develop in an acropetal order; while AS (right) often showed disorganized epiphyllous inflorescence. In the photo the two inflorescences are in Ailsa Craig genotype.

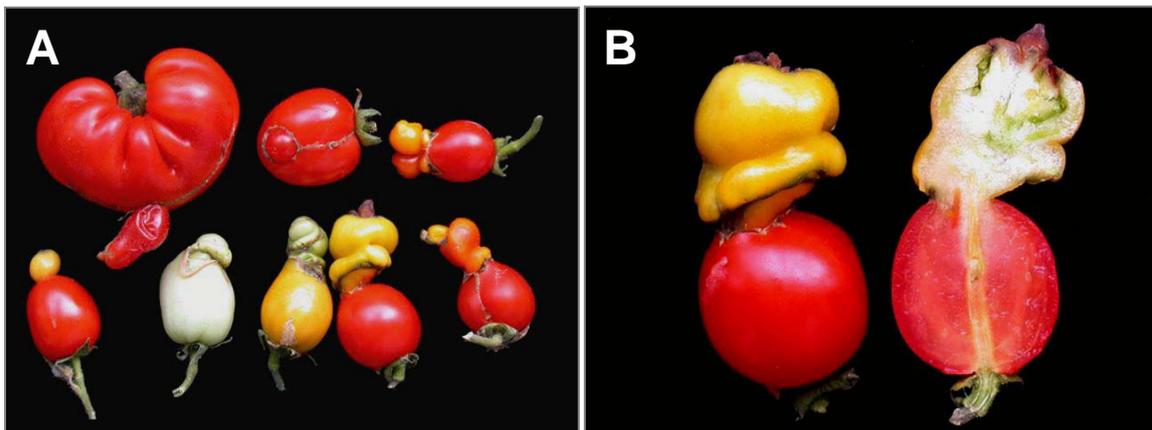
#### ***B-2-1-7- Aberrant inflorescences***

WT plants have well arranged cyme or dichotomously branched-cyme inflorescence, the flowers on a given inflorescence develop at different times; a single inflorescence may have developing buds, mature flowers and developing fruit at one time. However aberrant inflorescences were often observed in DR4AS plants (Figure III-12) (both  $\mu$ Tom and AC),

which usually can be characterized as epiphyllus, and this aberrance was preferable took as results of disorder of vascular tissue development.

### ***B-2-1-8- Navel-like fruits***

Navel-like fruits were also found in DR4AS plants, however it happen frequently only in one transgenic AS lines (AS213) (Figure III-13). In those queer-looking navel-like fruits, fruit like structures emerged from the stylar end of the fruits, and the placenta tissue occupied almost the whole volume of the locule and no seed formation. Once more, this phenotype was also found in the ‘*clausa*’ mutant, in which, scanning electron microscopy (SEM) analysis at the navel region on the mutant fruit showed ectopic development of ovule-like structures, various vegetative primordial, as well as “forests” of stalked glandular trichomes commonly found on tomato leaves, stems and sepals (Avivi et al., 2000). Recently, this similar phenotype was also found in *TM29* antisense tomato plants, and *TM29* was a tomato *SEPALLATA* homolog, *MADS* box gene (Ampomah-Dwamena et al., 2002). However this kind of the phenotypes should be treated carefully, as the transgenic plants was derived from the *agrobacterium* bacterium transfected cotyledon, whether ‘navel-like fruits’ is the DR4AS phenotype or is the effect of *agrobacterium* transfection, should be further confirmed.

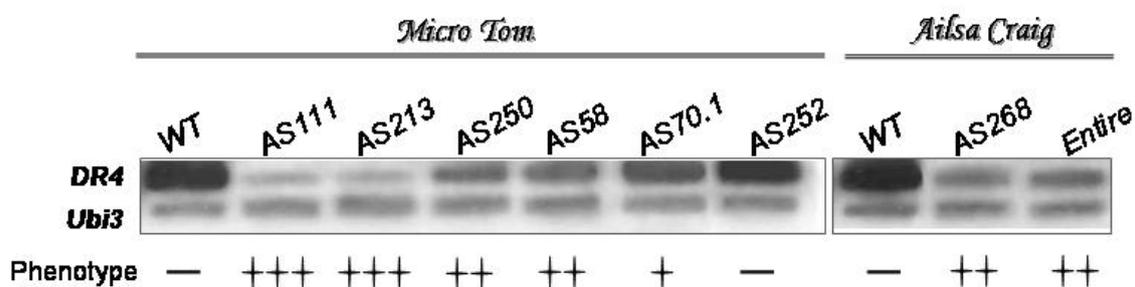


**Figure III-13.** Fruit-like structures protruding from the stylar end of the fruits of DR4AS.

(A) Showing different extreme of navel-like fruits. (B) Longitude section of one navel-like fruits, note that the placenta tissue is full up the tomato locule and the fruit-like structure is initiated from the end of vascular bundle.

### **B-2-2- Down-regulation of *DR4* expression account for the transgenic phenotypes**

To determine that the transgenic phenotypes are related to an altered *DR4* expression, we assessed the levels of *DR4* transcripts present in selected antisense transgenic lines using double strand antisense probes. The antisense probe detected a high level of *DR4* sense transcripts in the WT control plants, lower level in five AS transgenic lines (AS58, AS70,1, AS111, AS213, AS250), as well as two AS lines (AS252 and AS225) showed a similar level to the WT control (AS225 in Figure III-14). Among those five down-regulated *DR4* mRNA transcripts level lines, two lines (AS111 and AS213) showed almost virtually down-regulated of *DR4* transcripts, which later were found carried strong phenotypes such as carried almost complete simple leaves, represented high percentage (till 98%) of parthenocarpic fruits, and high frequencies of organs fusions, so they were assigned as strong lines; while those two lines (AS250 and AS58), who showed also largely down-regulated levels, exhibited moderate phenotypes such as carried both simple leaves and semi-simple leaves, moderate percentage of parthenocarpy and less frequencies of organs fusion, so they were assigned as weak lines; the last one line AS70,1, whose *DR4* mRNA transcripts also down-regulated but less than those strong and weak lines, present only occasionally semi-simple leaves and parthenocarpy. The rest two lines (AS252 and AS225), who showed a similar level as the WT control, exhibit the normal phenotype as WT control.



**Figure III-14.** *DR4* sense expression in WT, *DR4AS* and *entire*. By RT-PCR analysis, level of down-regulation of *DR4* in different transgenic  $\mu$ Tom and *Ailsa Craig* genotypes was showed the positive correlation between the severity of phenotypes in both  $\mu$ Tom and *Ailsa Craig* genotypes. In the spontaneous mutant *entire*, which showed quite similar phenotypes as *DR4AS* in *AC* background, *DR4* transcripts are also large down-regulated. *DR4* transcripts accumulation was analyzed using RNA samples extracted from leaves of WT control, *DR4AS* and *entire* mutant lines. In each PCR reaction, the internal reference *ubi3* was co-amplified with the *DR4* genes as described in the experimental procedure of chapter II. The symbols of the phenotype: -, no phenotypes; +++, strong phenotype, showing completely simple leaves, high percentage of parthenocarpy (60-100%), assigned as strong lines; ++ moderate phenotypes, showing both semi-compound leaves and simple leaves, moderate percentage of parthenocarpy (30-40%), assigned as weak lines; + weak phenotypes, only showing semi-compound leaves and occasionally parthenocarpy.

Later the DR4AS transgenic plants were generated in the AC background, base on the kanamycin resistance selection, and four transformed lines were obtained, only one line AS268 showed much lower level *DR4* sense mRNA transcripts. Once again, those phenotypes found in  $\mu Tom$  AS plants were recurred the this line AS268 AC, the rest three false-antisense lines have similar *DR4* sense mRNA transcripts level and exhibit similar phenotype as the WT control, and so further confirmed that down-regulation of *DR4* expression accounts for the transgenic phenotypes.

### ***B-3- Physiological analysis***

#### **B-3-1- The studies on the DR4AS precocious fruit set and parthenocarpic fruits**

The need of pollination to achieve fruit set has been known since the beginning of human history; however the DR4AS plants exhibit precocious fruit set before pollination even in the early immature flower bud stage (Figure III-4). In fact, the further observation found that this precocious fruit set could take place in any point during the flower development till maturation, and so resulted often that the fruits developed at the same time with the flowers, finally most of those precocious developed fruit were parthenocarpic. To further investigate this exiting phenomena, we carried out emasculatation and cross assay.

##### ***B-3-1-1- DR4AS is able to set fruit in the absence of pollination***

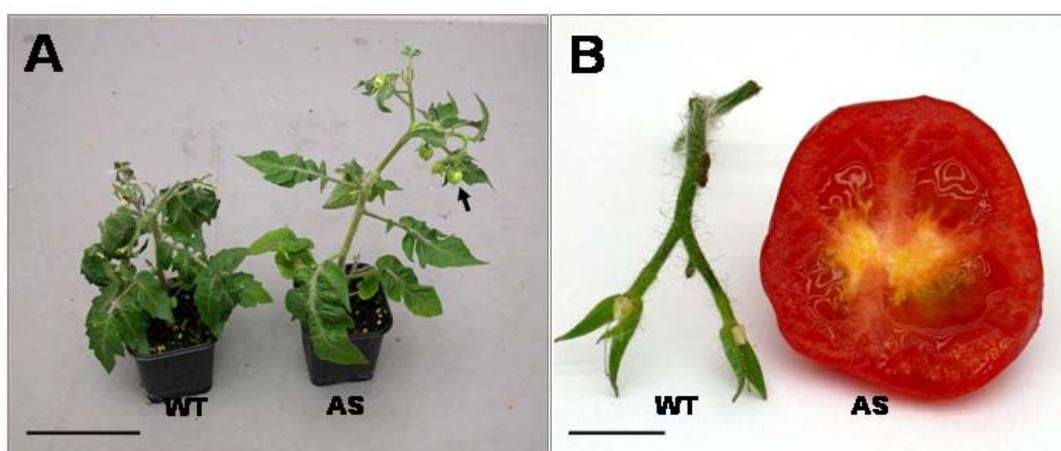
The emasculatation results demonstrated that DR4AS is able to set fruit in the absence of pollination and fertilization (Table III-3). Tomato flowers (WT and DR4AS) were emasculated before dehiscence of anthers (pre-anthesis), the result showed that, all of the tested four independent transgenic DR4AS tomato ( $\mu Tom$ ) emasculated flowers were able to set fruit and develop apparently normally; whilst the WT control plants did not set fruit at all (Table III-3 and Figure III-15). This experiment was performed in growth chamber, under the favorable condition for tomato growth and fruit development. All of the DR4S tomato plants showed percentages of fruit setting in unpollinated flowers ranging from 48% to 63% (Table III-3), this were quite similar to those in WT naturally grown tomato plant. Those unpollinated-fruits later proved were all seedless obviously, and the appearance of those fruits were quite similar as the normal tomato fruits, including the size, skin color, flesh consistency, and fresh weight. With regard to the fruit development program from fruit set to

mature green, break, turning, red, then overripe, those parthenocarpic fruits were indistinguishable from the normal naturally grown WT fruits.

**Table III-3.** Emasculation assay: DR4AS was able to set fruit in the absence of pollination

lines	Fruit set (%)	Fruit set/ emasculated flower	Seeds number/fruit
WT	0	0/100	0
AS111	63	28/44	0
AS213	68	39/56	0
AS250	49	9/17	0
AS58	48	7/14	0

The results were represent two individual trials independent



**Figure III-15.** DR4AS plant showed fruit set in emasculated flowers. (A) Emasculated DR4AS plant showed fruit set (indicated by arrow head) (right) while no fruit set in the WT counterparts (left). (B) The DR4AS fruit (right) derived from emasculated flower was seedless (parthenocarpic), with regard to parameters such as fruit size, skin color, flesh consistency, those parthenocarpic fruits were indistinguishable from the normal naturally grown WT fruits, obviously there was no fruit set in the WT counterparts (left).

### ***B-3-1-2- Both the ovule and pollen of DR4AS are fertile***

To further investigate whether the sterility of pollen or ovule is account for the parthenocarpy, cross assay was performed. The results demonstrated that both of the ovule and pollen of DR4AS are fertile (Table III-4). With WT as female recipients and DR4AS as pollens donor, 65 fruits set among 82 crossed flowers, showing 79% fruit set; all of those fruit were seeded, showing the successful pollination and fertilization, that proved the pollens of DR4AS are viable; all of those seeds were kanamycin resistant, indicating that it was the pollen of DR4AS (kanamycin resistance selection marker) caused the successful fertilization.

The same, with DR4AS as female recipients and WT as pollens donor, 20 fruit set took place among 37 crossed flower, showing 54% fruit set; among these fruits, although only 35% fruits showed seeded, it is enough to demonstrate that the ovules of DR4AS were fertile. The rest 65% fruits were seedless, that because the DR4AS ovaries were able to fruit set in the absence of fertilization; obviously their seeds F1 were 100% kanamycin resistant as the DR4AS ovule game carried with kanamycin selection marker. The control trials carried with WT female and WT pollen, only those fertilized ovaries performed fruit set and so all of the fruits were seeded, reasonably, all of their F1 seeds were kanamycin sensitive.

**Table III-4.** Cross assay demonstrated that both of the ovule and pollen of DR4AS are fertile

Female Recipient ♀	Pollen donor ♂	Fruit set/		F1 Kanamycin resistance (%)*
		Crossed flowers	Seeded fruit (%)	
WT	AS	65/82	100	100
AS	WT	20/37	35	100
WT	WT	7/7	100	0

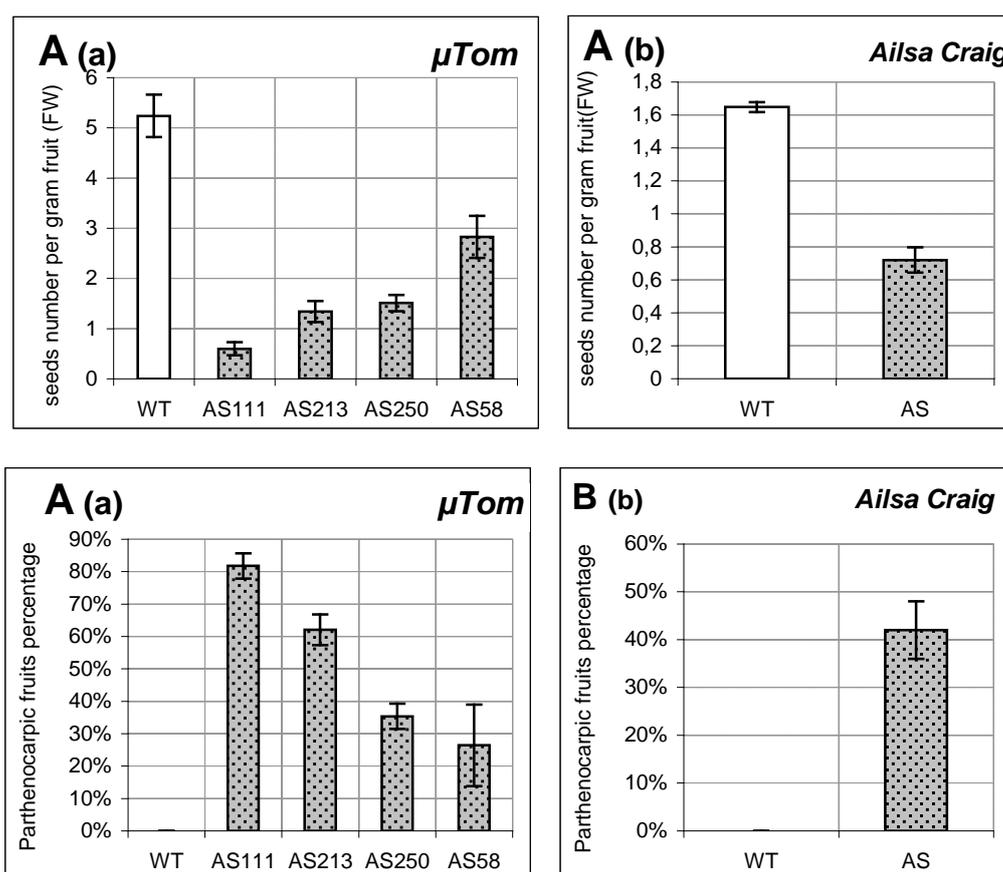
\*F1 seeds were assessed on a selective medium with kanamycin, 70 mg l<sup>-1</sup>

### ***B-3-1-3- Fully or partially parthenocarpy results in reduced seed production***

Productions of seed were dramatically reduced in DR4AS plants (Figure 16A, in two genotype  $\mu Tom$  and AC), in which the AS111 (strong line) had reduced 89% compared with it WT counterparts. Obviously the reduction of seeds production was due to the fully or partially parthenocarpy of their fruits (Figure III-16B), which varied from 82% (strong line AS111) to 28% (weak line AS58) in  $\mu Tom$ , and 42% in AC antisense background.

The onset of ovary development into fruit (fruit set) and fruit development are triggered by signals from pollination and fertilization. Auxins are produced by the pollen tube as it grows through the style, and also by the fertilized embryo and endosperm in the developing seeds, fruit set and growth depends on these sources of auxin (Levetin and McMahan, 1998); in accordance, the higher level of auxin was also found in the stage of fruit set (Gillaspy et al., 1993). Fertilization-independent fruit set can also occur either naturally in parthenocarpic fruits (genetic parthenocarpy) or by induction via exogenous application of plant growth regulators (PGRs) such as auxin or gibberellins or cytokinins to flower pistils (Rotino et al., 1997; Vivian-Smith and Koltunow, 1999; Fos et al., 2001; Balbi and Lomax,

2003). Artificial induction via auxin has long been used to study parthenocarpy in tomato (Gustafson, 1937); application of auxin transport inhibitors that block export of auxins from the ovary also stimulates the development of parthenocarpic fruits (Beyer and Quebedeaux, 1974), the higher auxin levels of auxin was reported in ovaries of parthenocarpic tomato fruits (Mapelli et al., 1978; Mapelli and Mombardi, 1982). All of these suggest that auxin and/or gibberellins responses mechanism or metabolism may be critical in fruit set and also the parthenocarpic fruit to proceed with development. Furthermore, transgenic plant *DefH::iaaM*, allowing ovule-specific expression (promoter *DefH*) a bacterial gene *iaaM* which confer over-auxin synthesis, showed over auxin synthesis and accumulation in flowers and placenta of fruits, seedless fruits were produced by emasculated flowers in the transgenic plants, providing clear evidence that high levels of auxin in the ovule can substitute for fertilization and trigger the fruit set.



**Figure III-16.** DR4AS produced fully or partially parthenocarpic fruits resulting in dramatically reduced number of seeds. (A) Seeds number of DR4AS in 2 background genotype  $\mu$ Tom (a) and AC (b). Results of the number of seeds are expressed as a number of seeds per gram fresh mass of matured fruit tissue. (B) Percentage of parthenocarpic fruit in  $\mu$ Tom (a) and AC (b), where the parthenocarpic fruits percentages of WT are 0 in both two genotypes. Error bars represent  $\pm$ SE of at least 5 independent traits. (a) n=840-3870 g, (b) n=1776-4845 g.

*DR4* showed high homology to *Aux/IAA* gene, and hypothesized as *Aux/IAA* protein intervenes of auxin responses. Most of the *Aux/IAA* proteins were demonstrated act as auxin response negative regulators (inhibitors), all of the *aux/iaa* mutants obtained so far are gain-of-function mutants, in which the *Aux/IAA* proteins are steadier, and result in reduced auxin responses. As a member of *Aux/IAA*, *DR4* was reasonably hypothesized act as an auxin responses inhibitor too. In the *DR4AS* transgenic plants, *DR4* transcripts were dramatically silenced, so genetically exempts or much diminishes this negative regulator effects on auxin responses, and the plants should have over-auxin responses in several fields (which were controlled by *DR4*) in hypothesis. In fact, as there is no problem with the ovule or the pollen, as they were proved both fertile in the cross assay, so the successful precocious fruit set and parthenocarpic fruit development well demonstrated that it was resulted from the fruit set mechanism which was usually trigger by auxins or gibberellins. Taking all of the information together, it is strongly suggested that in *DR4AS* plants there might have over-auxin responses in the ovaries and fruits, which in turn cause precocious fruit set and successful parthenocarpic fruits development.

### **B-3-2- DR4AS showed different hormones responses compared with WT in seedling**

It is well known that plant morphogenesis is governed by coordinated cell division and cell expansion. A large body of evidence suggests that plant hormones are involved in regulating these events but little is known about how they interact to bring about environmentally and developmentally regulated growth (Collett et al., 2000). The seedling with relative simple structure is a convenient system in which to study the hormones responses and as well as the hormones interactions in cell division, expansion and differentiation. For example, the *Arabidopsis* hypocotyl has a simple structure: from apex to base the cell number is relatively invariable: approximately 20 cells and during its development, although the hypocotyl may increase more than ten-fold in length, there are no significant cortical or epidermal cell division (Collett et al., 2000). Hypocotyl elongation is very plastic and is influenced strongly by factors that regulate cell division, elongation, and differentiation in the adult plant such as light, plant hormones, temperature, and touch; thereby it is efficient to monitor the effects of hormone treatments. There are mutants with altered hormone biosynthesis or signaling and many of these mutations affect hypocotyl elongation, primary root elongation, lateral roots and root hairs formation, and tropism etc. With combination of this rather simple structure, availability of hormone mutants; much

easier to carry out experiment with maximum diminish the environmental and developmental differences, as well as the accumulated endogenous hormones difference, when compared with control; relative shorter experimental periods and less experimental spaces required; the seedling is a rather satisfying experiment system to study hormones effects and their interactions effects.

Since *DR4* was hypothesized as a tomato *Aux/IAA*, the modified auxin responses were expected found in the *DR4* silenced transgenic plants (*DR4AS*), as it was well demonstrated *Aux/IAA* proteins intervene in most of auxin responses (Timppte et al., 1994; Kim et al., 1996; Leyser et al., 1996; Reed et al., 1998; Nagpal et al., 2000). Thereby auxin treatments were first performed to detect whether there was modified auxin response in *DR4AS* transgenic plants or not. The other hormones treatments were also carried out, attempting to acquire maximum possible information on this tragic genetic modified plant, that ought to reflected, if it is not demonstrated directly, the possible function of *DR4*; at the same time, those result would also contribute to elucidate the auxin roles in hormones interaction mechanism, and further to discover more about this interactions between hormones in the regulation of plant development and growth, which is still little known.

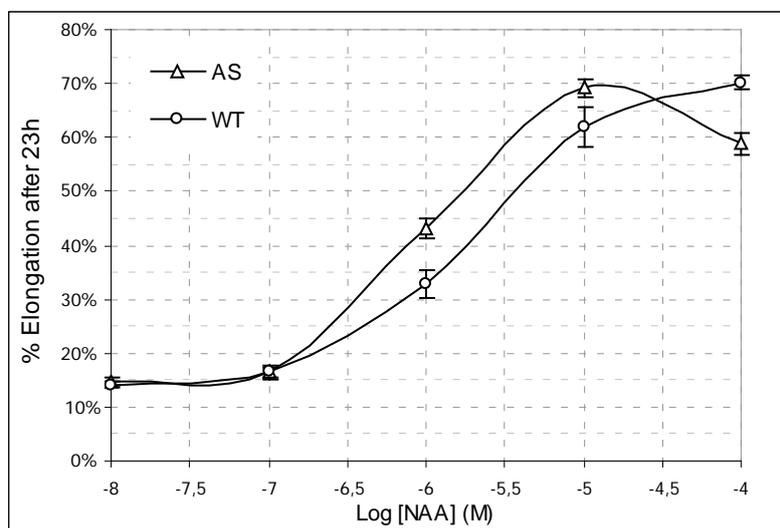
### ***B-3-2-1- Auxin response***

Auxin has long been thought of as important in cell elongation. When added to isolated stem segments and coleoptiles, auxin is able to induce elongation. This response begins within 10 min and can result in a five- to ten- fold increase in growth rate that can persist for days (Evans, 1985), and it was this elongation response led the scientist discover the first phytohormone auxin. So in the physiological characterization of *DR4AS* plants, first at all, the auxin response experiments were carried out to detect whether there was modified auxin responses. They are including the auxin dose response in hypocotyl segments and in cotyledon explants, and kinetic analysis of auxin-induced-elongation of hypocotyl segments.

#### ➤ *Auxin dose response curves in hypocotyl segments*

The dose-dependent auxin-induced elongation of isolated hypocotyl segments of *DR4AS* ( $\mu$ Tom) and WT tomato light-grown seedlings was investigated. In our condition (described in Chapter II), *DR4AS* showed more sensitive to auxin than WT, after 23 hour, elongation of the WT hypocotyl segments was stimulated by auxin concentrations from  $10^{-7}$

M to  $10^{-4}$  M (the maximum concentration of NAA could we obtain), while in the case of DR4AS was  $10^{-7}$  M to  $10^{-5}$  M, and during this field, in one certain concentration, AS showed higher percentage of elongation effects (Figure III-17).

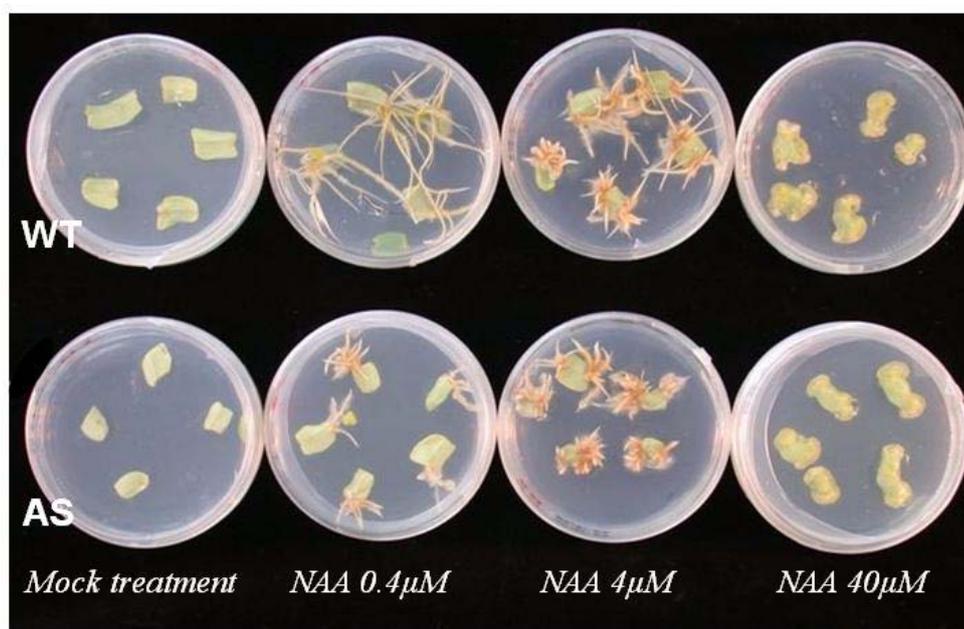


**Figure III-17.** Auxin dose response in hypocotyl segments. Auxin dose response curves in WT and DR4AS hypocotyls segments from 5-d-old light-grown seedling. Segment (7,6 mm) were prepared as described in Chapter II. Elongation is given as the percentage increase in final length over the initial length after 23 hours incubation in the solution containing the indicated NAA concentration. Error bars represent  $\pm$ SE,  $N \geq 20$ . Similar results were obtained in the dark-grown seedling.

➤ *Auxin dose response in root and callus formation in cotyledon explants*

The auxin dose response experiments was also perform on the cotyledon explants, it was demonstrated clearly that the promotion of root organ regeneration and development as well as the callus tissue formation was auxin dose-dependent (Figure III-18). There was no root formation in the medium containing any hormone (NAA 0). Appropriate concentration of NAA ( $0,4 \mu\text{M}$ ) promote root regeneration and development, roots are white and showed well typical tomato taproot system, the tap root ( same as primary root in the seedling) was the first emerged and larger in diameter and longer in the length than the lateral roots, lateral roots which originate from the pericycle, branch off from the taproot, and subsequent lateral roots can branch off sub-lateral roots; in subsuitable concentration, for example higher than optimal concentration ( $4\mu\text{M}$  in the photo), auxin still promote root regeneration, but they can not well developed, such the roots elongation dramatically reduced the roots diameter increased (short fat roots) the proper taproot system was disrupted, on the contrary, formed 'fibrous-like' roots system which was often found in the monocots plants. In brief, the higher is NAA

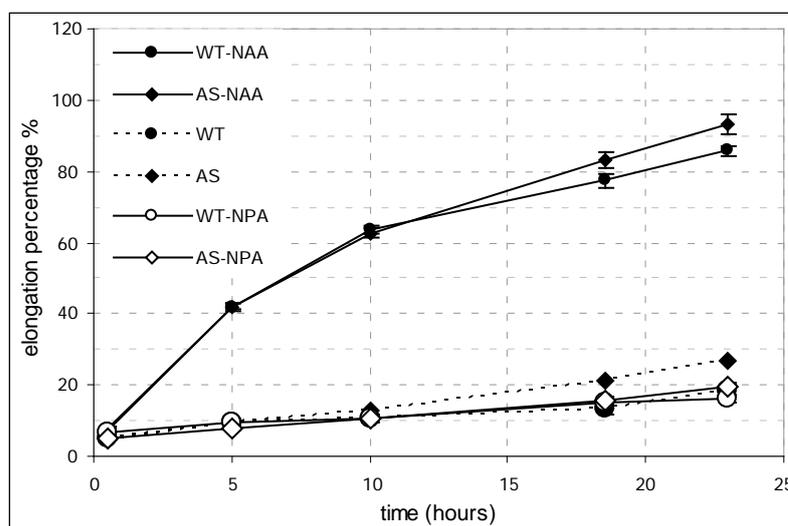
concentration, the shorter and fatter are roots. (Figure III-18, top row). When auxin concentration was too high, such as 40  $\mu\text{M}$  NAA, the roots formation were completely suppressed, but slightly formed callus tissues. While in the case of AS, there were also no roots formation in the absence of auxin (NAA 0  $\mu\text{M}$ ), while when at the concentration 0,4  $\mu\text{M}$ , which appeared quite appropriate for the WT to generate and develop taproot system, the DR4AS exhibited over-dosed effect of NAA, there were roots generation, but they could not well elongated but increased in the root diameter, and formed fibrous-like roots system, showing the more similar appearance than the WT in higher auxin concentration (4  $\mu\text{M}$ ). Obviously in the higher concentration the roots generation and formation was completely suppressed also (Figure III-18, bottom row).



**Figure III-18.** Auxin dose response in cotyledon explants. Cotyledon explants from 11-d-old and DR4AS seedling incubated on the Murashige-Skoog medium containing the indicated auxin (NAA) concentration for 10 days in standard growth chamber. It showed that auxin effect on explants organ regeneration was dose-dependent, appropriate concentration auxin can promote roots formation and development, but higher concentration will inhibit roots development, too higher concentration completely suppressed root formation. Neither organ generation nor callus formation in WT and AS explants when put on the medium in the absence of any hormone. 0,4  $\mu\text{M}$  NAA showed properly for root regeneration and development in WT, but showed over-dosed in AS which have roots generation and development as the WT treated with 4  $\mu\text{M}$ .

➤ *Kinetic analysis of auxin-induced elongation of hypocotyl segments*

The kinetic auxin-induced elongation of isolated hypocotyl segments was also investigated (Figure III-19). Both WT and DR4AS showed auxin-induced elongation, the effect was most significant at the first 5 hours; the growth rate was 9% elongation per hours, and it dropped quickly the following 5 hours, showed about 4% per hours; after the first 10 hours, it appeared that elongation rate was quite stable at about 2% per hours. The kinetic auxin-induced elongation profile between DR4AS and WT was quite similar, both showed equal quickly induced elongation at the first 10 hours and after that much slower elongation, there was only slight difference during 10 to 23 hours, DR4AS (2,3%) showed a little more quickly growth than their WT counterparts segments (1,7%). The auxin transport inhibitor had almost no effect on the auxin-induced elongation; and it was quite reasonable, as all of the hypocotyl segments were floating in the buffer.



**Figure III-19.** Kinetic analysis of auxin-induced elongation of hypocotyl segments. The kinetic auxin-induced elongation profile between DR4AS and WT was quite similar, both showed equal quickly induced elongation at the first 10 hours and slower after that. There was only slight difference during the 10 to 23 hours. Segment (8,1 mm) were prepared as described in chapter II. Elongation is given as the percentage increase in final length over the initial length after indicated hours incubation in the standard growth chamber in solution contain auxin (NAA 5  $\mu$ M), auxin transport inhibitor (NPA 10  $\mu$ M). Error bar represent  $\pm$ SE,  $N \geq 22$ .

Auxin has long been thought of as important in cell elongation. Genetic approaches have identified many genes that are needed for auxin-induced hypocotyl segment elongation (Collett et al., 2000). The phenotypes of some of these mutants are consistent with a role for auxin in hypocotyl elongation. For example, many of Arabidopsis *aux/iaa* gain-of-function mutants, exhibit shorter hypocotyl, such as *iaa3/shy2-2*, *iaa6/shy1-1*, *iaa7/axr2-1*, and *iaa17/axr3-1* (Timpte et al., 1994; Kim et al., 1996; Leyser et al., 1996; Reed et al., 1998;

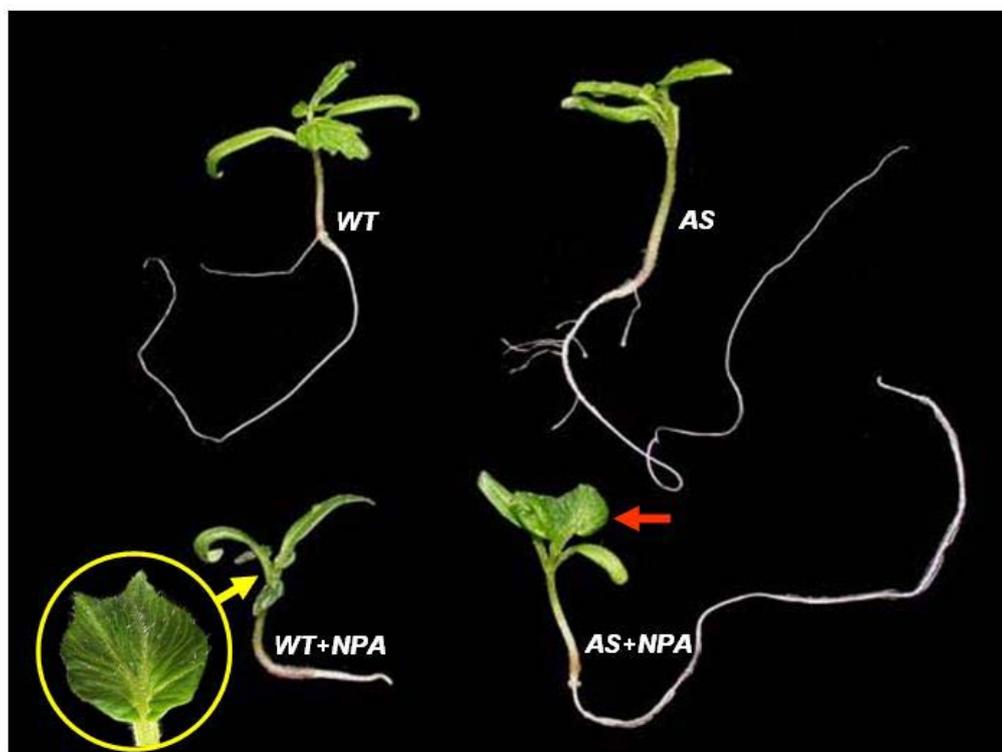
Nagpal et al., 2000). Reduced auxin response mutant *axr1* has shorter hypocotyl too. In transgenic 35S-*iaaL* plants, in which a bacterial enzyme is expressed that conjugates auxin to Lys, free auxin levels are reduced, show reduced hypocotyl elongation in the light, plant size, leaf size, apical dominance and fertility (Romano et al., 1991; Jensen et al., 1998). The single-gene, recessive *diageotropica* (*dgt*) mutant of tomato have reduced sensitivity to auxin, exogenously applied IAA does not enhance elongation growth of isolated hypocotyl segments and does not promote ethylene biosynthesis (Kelly and Bradford, 1986).

The auxin responses experiments demonstrated that DR4AS isolated hypocotyl segments were hyposensitive to exogenous auxin treatment, which is accord with the hypothesized profile of DR4 protein: acting as an auxin responses repressor (negative regulator). All of the *aux/iaa* mutants obtained so far are gain-of-function mutants, in which the Aux/IAA proteins are steadier, and can be summary as having reduced auxin responses, and many of them showed shorter hypocotyl; on the contrary, the DR4AS seedling showed increased hypocotyl elongation than WT when grown in light (Figure III-8), which, on the other way, suggesting that over-auxin responses were proceeding in DR4AS seedling, so, one more time, agree with its hypothesized auxin negative regulator profile.

### ***B-3-2-2- Auxin transport inhibitor response***

Auxin is thought to be synthesized locally in plants shoot apical tissues, and it is actively transported polarly to the base of the plant (Davis, 1995), and multiple plant developments aspects were dependent this polar transport. In roots, the situation is less clear, except for shoot apical tissues droved the mainly auxin flow, it has also been reported that a root tip can synthesize and direct auxin flow within the central cylinder is associated with an opposite flow in the root epidermis (Mattsson et al., 1999). Auxin polar transport can be blocked by several chemical compounds, such as NPA, TIBA and HFCA, similar phenotypical abnormalities were evoked by each of the three auxin transport inhibitor substances, and the most popular one used by scientists is NPA. When treated with NPA, the auxin is kept synthesizing in plant, but it will be blocked and accumulated in the site of synthesis, and obviously the other part out of the synthesis site will be lack of the auxin, the crucial hormone for normal growth and development. Experiments were performed to detect whether DR4AS showed different NPA response than WT, especially to detect the difference in the region lack of normal auxin flow caused by NPA treatment such as the root. Since DR4 was assumed act as auxin responses inhibitor, some kind of auxin responses was expected to

detect in the DR4AS transgenic plant in this region of root which normally no auxin responses undergoing because of the absence of auxin source by NPA treatment.



**Figure III-20.** Comparison of NPA responses in WT and DR4AS light-grown seedlings. WT mimicking DR4AS phenotype with application of NPA (1 $\mu$ M) including simple leaf formation (yellow arrowhead) and leaves fusion (shown in detail in the small photo beside); while with NPA application DR4AS showed more resistant to inhibition of NPA in primary root elongation and leaf blade expansion, as well as showed more frequency of leaves fusion. Where the top row are WT (left) and DR4AS (right) mocked treatment control (-NPA), and the bottom row are WT (left) and DR4AS (right) with NPA (1  $\mu$ M) treatment (+NPA). Similar results were obtained in four independent experiments, also in the antisense line in *Ailisa Craig* background genotype DR4268.

19-d-old WT seedlings grown on media without NPA had 1 pair of well expanding basical compound or deeply lobbed simple leaves, the cotyledons were fully expanded and curved backward, primary root elongation attain about 80 mm with one or two lateral roots (Figure III-20); while in DR4AS seedlings, first pair of leaves well expanding had simple leaf structures and showed palm-like venation, hypocotyls and primary roots (about 130 mm, 60% more compared with their WT counterparts) elongation increased, lateral roots number also increased, cotyledons development appear quite similar with that of WT (Figure III-20). Grown on media supplemented with 1  $\mu$ M of NPA, WT Seedlings had 1 pair of delayed emerged smaller leaves, which showed simple leaf structure with entire leaf margin; the cotyledons fully extended only in longitude order so appear very narrow and showed over-

curved backward, the primary root elongation was dramatically reduced, comprising enlarged root diameter and no lateral roots. While DR4AS seedling, showed less sensitive to NPA, seedling grown on media containing NPA, still showed active primary root elongation, rather similar leaves expanding with only more frequently leaf fusion, and the leaf margin appear more entire than that of with NPA treatment; however, always no lateral roots formation (Figure III-20). The green color of seedling grown on media containing NPA was darker than that on media without NPA in both WT and DR4AS.

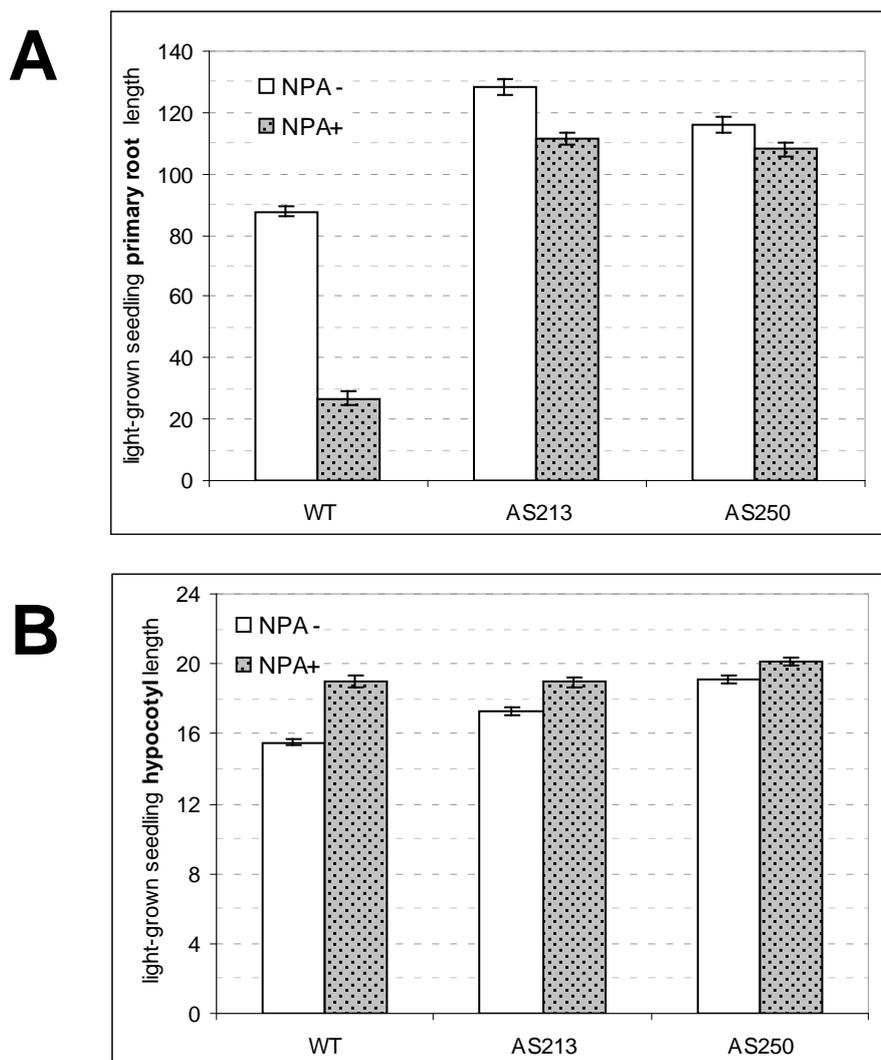
➤ *WT mimicking AS phenotype including simple leaf formation and fusing leaves*

From this experiment, it was shown that, with application of NPA, which block the export of auxin outside and accumulated in the first pair of real leaves, the WT leaves lost their natural compound leaf structure and showed simple leaves with entire margin, mimicking DR4AS simple leaves phenotype. That mean, over-accumulation of auxin can induce simple leaf formation. That strongly hints that DR4AS simple leaves were resulted from the up-auxin responses during the leaf ontogenesis, as the DR4, the assumed auxin-response inhibitor, was silenced. The frequent leaves fusion as the phenotype of DR4AS was also found in the WT when treated with higher NPA concentration (10  $\mu$ M and 20  $\mu$ M), suggesting that there were some similar proceedings undergoing between the DR4AS leaves and the WT leaves with the NPA application. And the DR4AS when treated with NPA, it showed more frequently of leaves fusion, which indicated when auxins were over-accumulated; the antisense phenotype leaves fusion was aggravated. The molecular mechanisms controlling leaf morphogenesis and vascular differentiation during plant organ development are still unknown; however, the capacity of the plant hormone auxin to induce vascular differentiation is well established. Auxin-overproducing transgenic plants have increased amounts of vascular tissues (Klee et al., 1987) and application of auxin can induce the formation of new vascular strands from parenchymatic cells of mature organs (Jacobs, 1952), as well as that auxin transport inhibition also showed to result in increased vessel differentiation, and dramatically altered the venation pattern. The same results were also obtained in the DR4AS in AC genotype in both simple leaf formation and the multi-organs fusion. On conclusion, our experiments clearly demonstrated that, the abnormal leaf morphogenesis in DR4AS was induced with large possibility by aberrant auxin signalling.

➤ *DR4AS are resistant to NPA in primary root elongation and leaf blade expansion*

From this experiment, it was also showed that DR4AS seedlings were less sensitive to NPA in primary root elongation and leaf blade expansion. In WT, NPA treatment dramatically reduced the primary root elongation, showing 70% reduction (88 mm mean length of without NPA (-NPA) against 26 mm of with NPA (+NPA)) (Figure III-21A), while in DR4AS, the same NPA treatment only reduced 2% in line AS250 (108mm of -NPA against 116 mm of +NPA) and 13% in line AS213 (112 mm of -NPA against 128 mm). That indicated that, , roots can not well elongated without the natural auxin flows (NPA application), while in the case of DR4AS, the primary roots were able to elongate even in the absence of auxin flows, which was probably due to the knock out an auxin-response inhibitor: DR4. However, the lateral root information was completely suppressed by application of NPA in both WT and DR4AS, indicating that knock-out the auxin-response inhibitor DR4 is not enough to restore the induction the lateral root formation in the absence of natural auxin.

The WT leaves emergence was retarded when treated with NPA, as well as the leaves expansion was also largely inhibited, while DR4AS showed neither retard in leaves emergence or leaves expansion, indicating the less sensitiveness to NPA than WT. The auxin biphasic dose response was well established both by physiological and molecular biological experiments (Abel et al., 1995a). Only a certain field concentration of auxin showed promoting on growth and development, too high auxin even completely inhibit growth or “kill” the plants. The inhibition of leaf expansion in WT with NPA application then was spectacled as results of too much auxin accumulation in the leaf. On the contrary, to be surprised, it was shown that NPA promoting slightly light-grown seedling hypocotyl elongation in both WT and DR4AS (Figure III-21B). With NPA application, hypocotyl of WT increased 22%, AS213 and AS250 increased 9% and 5% respectively. It has showed the less sensitiveness to NPA in DR4AS, however the reason of this slightly promotion on hypocotyl is still unknown.



**Figure III-21.** *DR4AS* showed less sensitive to NPA treatment. **(A)** Light-grown seedling of *DR4AS* was less sensitive to inhibition of primary root elongation with NPA application. Error bar represent  $\pm$ SE,  $N \geq 63$ . **(B)** Light-grown seedling hypocotyls elongation showed increased with NPA application in both WT and *DR4AS*, and *DR4AS* showed slightly less percentage of elongation effect than WT. Error bar represent  $\pm$ SE,  $N \geq 2$ . NPA treatment and seedling (19-d-old) were grown as described in chapter II. Similar results were obtained in four independent experiments, also in the antisense line in Ailsa Craig background genotype *DR4268*.

Root elongation needed the shoot apex derived auxin flows, removal of shoot apex (decapitation) inhibited the growth of primary roots of WT *Arabidopsis* seedling; application of auxin to the site from which the shoot apex had been removed restored the roots of decapitated seedlings to normal (Fu and Harberd, 2003). Thus shoot apex-derived auxin passes to the root via the polar auxin transport stream and promotes root growth. It was also documented that taproot elongation and lateral roots formation are strongly inhibited by auxin transport inhibitors (Mattsson et al., 1999). When *Arabidopsis* applied with NPA, it comprised only a very short, swollen primary root with no lateral roots; root tips were radially

expanded. Further anatomy analysis showed that in NPA treated root, the central vascular cylinder was dramatically enlarged and misshapen; the oversized vascular cylinder contained a large number of xylem strands randomly distributed in the central cylinder; individual vessel elements were only partly elongated and improperly aligned to each other and vessels were frequently interrupted (Mattsson et al., 1999). At last, it is remarkable note that it is still not unknown that the inhibition of root elongation by NPA is exactly due to lack of auxin in the elongation region of roots, or too high auxin accumulated in the root tip that inhibits cell division and/or expansion, or resulted from both of the two effects.

### ***B-3-2-3- GA and GA-inhibitor (Paclobutrazol) response***

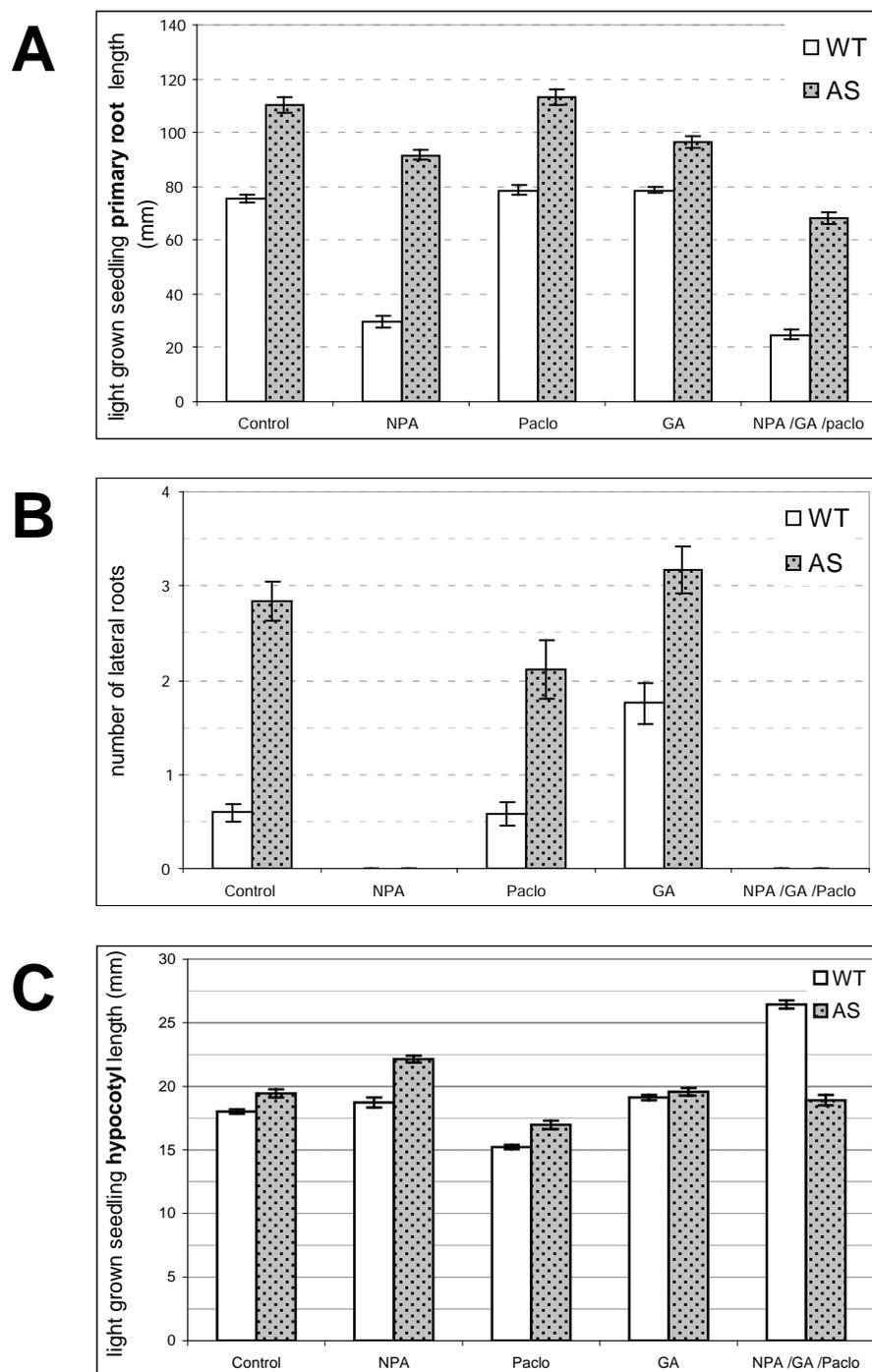
Being discovered by effect on stem elongation, gibberellins are involved in the regulation of diverse developmental processes including stimulate cell division and elongation (such as stimulate stem and hypocotyl elongation (Ross et al., 2000), root elongation (Fu and Harberd, 2003)), seeds germination, as well as fruit set and parthenocarpic fruits development (Vivian-Smith and Koltunow, 1999). Recently it was discovered also that exogenous auxin application promotes gibberellins biosynthesis in the decapitated stems of pea and tobacco. Being assumed auxin-response mutant, DR4AS showed enhanced stem elongation, hypocotyl and primary root elongation, precocious fruit set and parthenocarpic fruits development, the proceedings were also been reported regulated by gibberellins. Referring to changed vascular patterning and leaf morphology in DR4AS, gibberellins were also reported regulated the leaf vascular patterning and leaf morphogenesis by mediating *KNOTTED1*-Type homeobox function (Hay et al., 2002); moreover, it was rather recently reported that auxin promotes Arabidopsis root growth by modulating gibberellin response (Fu and Harberd, 2003). Regarding with the DR4AS's capacity of root elongation under NPA application, if compensate gibberellin treatment in the NPA media, whether it can restore the root elongation in WT; on the contrary if inhibit normal gibberellin responses in DR4AS seedling, whether the NPA-insensitive-root elongation will be inhibited. Thereby, the seedling gibberellins responses experiments were envisaged to going the further investigation.

Seedlings were treated with active gibberellin GA<sub>3</sub> and gibberellin synthesis inhibitor paclobutrazol (paclo) as described in chapter II, the seedlings under the paclobutrazol treatment were first grown in the media without paclobutrazol, than after 7 days were transferred to the correspondent paclobutrazol supplemented media, as paclobutrazol inhibits gibberellin biosynthesis, which is necessary for the seed germination. The results were

displayed in the Figure III-22. It showed several interesting results. First, in our experimental condition, the GA largely promoted lateral roots formation in WT seedling (lateral roots number increased 195%) (Figure III-22B), and slightly promoted that of DR4AS (increased 11%); on the other hand, paclo treatment showed clear inhibition on DR4AS lateral roots formation, with paclo application, the lateral number of DR4AS decreased 26%. Regarding together with the GA promotion on WT seedling lateral root formation and paclo inhibit the DR4AS lateral root formation, it imply that the increased lateral root formation in DR4AS was effected via GA response pathway, that mean, if enhance GA response in WT roots, it will promote the lateral root formation resulting in the increase of lateral roots number (like the DR4AS), conversely, if inhibit the GA response in the DR4AS seedling roots, it will inhibit the lateral root formation and the numbers will decreased. And that was exactly what was observed in our experiments.

Second, as WT primary root elongation was dramatically reduced with NPA treatment, GA supplement in NPA medium can not restore the normal primary root elongation in WT (Figure III-22A), however in DR4AS the NPA-insensitive-root elongation can be further inhibited by gibberellin synthesis inhibitor paclo (inhibited 26%). Those suggest that, GA response contribute the primary root elongation, inhibition of the GA response will reduce the root elongation; however the auxin response is necessary and indispensable for the primary root elongation and GA only is not enough to keep and promote the normal primary root growth.

Third, GA showed very slightly promotion of hypocotyl elongation on WT (increased 6% with GA) (Figure III-22C), while DR4AS hypocotyl elongation appeared same level with or without GA treatment. Similarly, the gibberellin inhibitor paclo showed also effects on hypocotyl elongation, with application of paclo the WT hypocotyl inhibited 15% and inhibited 12% in DR4AS. However GA showed already clearly the promotion on the stem elongation, the internode length between the cotyledon node and the first pair of real leaves node was largely increased with the GA treatment in both WT and DR4AS seedling (data not shown). That mean, the hormones effects were district organ and tissue specific, the same hormones have different effects in the different place. To be surprised, the combination of NPA and GA treatment largely promoted the hypocotyl elongation (increased 47%), the reason is unknown. However we should noted these results were still somewhat preliminary as this experiment was performed only once, although it already showed the tendency.



**Figure III-22.** Effects of GA, GA synthesis inhibitor (*paclo*) and NPA in seedling growth. **(A)** Effects on primary roots elongation. It showed that only GA, and *paclo* treatment separately did not have significant effects on primary roots elongation, and GA supplement can not compensate the inhibition by NPA, while GA synthesis inhibition (*paclo* treatment) further reduced DR4AS primary root elongation. **(B)** Effects on lateral root formation. GA promoted lateral root formation (much largely in WT), while *paclo* decreased the lateral roots formation in DR4AS; neither GA nor *paclo* can restore the complete suppression of lateral root formation by NPA treatment. **(C)** Effects on hypocotyl elongation. GA treatment showed light promotion on WT hypocotyl elongation while almost showed no effects on DR4AS; *paclo* showed light inhibition on hypocotyl elongation both in WT and AS. NPA also showed light promotion on hypocotyl elongation as described in Figure III-21B, however, the combination of NPA and GA treatment largely promoted the hypocotyl elongation. NPA /GA

/Paclo represent combination of NPA and GA treatment in WT and combination of NPA and paclo treatment in DR4AS respectively. Error bars represent  $\pm$ SE,  $n \geq 60$ .

#### ***B-3-2-4- Auxin and cytokinin interaction in cotyledon and hypocotyl explants***

The hormones auxin and cytokinin control plant development through a multitude of complex interactions. Classic experiments by Skoog originally demonstrated that auxin and cytokinin synergistically regulated tobacco pith cell proliferation, exposing callus cultures to a high auxin-to-cytokinin ratio results in root formation, while a low ratio of these hormones promotes shoot development (Skoog and Miller, 1957). To further investigate whether the gene *DR4* also involved cytokinin responses, we performed the experiment to characterize the effect of down-regulation of *DR4* expression on the auxin (Naphthalene acetic acid, NAA) and cytokinin (BA) responses of tissues cultured in vitro (Figure III-23, for each treatment, a representative wild-type explant was shown to the left of the corresponding DR4AS explant).

We compared the regeneration of organs and callus production by WT and DR4AS cotyledon and hypocotyl explants in culture, to investigate auxin-cytokinin interactions in variety of differentiation processes. First of all, in the absence of exogenous hormones, the DR4AS explants tissues for both cotyledons and hypocotyls showed different changes during tissue growth (Figure III-23 A and B top-left). DR4AS cotyledons lose more quickly the green color maybe due to the enhanced degradation of chlorophyll compared the WT who kept the green color after one month incubation on in vitro culture media; the WT hypocotyl explants slightly regenerate both the roots and leaf-like structure, while the DR4AS only regenerate the leaf and no root regeneration, but the hypocotyl segment much more swollen during the tissue growth. Those suggest the difference endogenous hormones balance between the WT and DR4AS. The fresh weights of them were measured and showed no significant differences (Figure III-24), the WT hypocotyl segments were observed with less swelling in comparison to DR4AS, though there was no difference in their weight.

Second, in the absence of auxin the DR4AS showed different response to cytokinin depend on the special tissues. In relative low cytokinin concentration (0,4  $\mu$ M) the DR4AS cotyledon explants lose their color quickly, but callus tissue formed in both WT and DR4AS (Figure III-23A left); the augmentation of cytokinin concentration delayed the fading to yellow in DR4AS cotyledon, and promote leaves regeneration, but too high concentration also promote fading to yellow and inhibit leaves regeneration, however in 40  $\mu$ M the leaf regeneration was completely inhibited in the WT but not in DR4AS, which indicated that

DR4AS cotyledon explants were less sensitive to exogenous cytokinin. Whilst, in hypocotyl segments DR4AS showed more sensitive to exogenous cytokinin (Figure III-23B), with the augmentation of cytokinin concentration, the leaf regeneration showed more vigorous and prolific in DR4AS, although it was suppressed in too high concentration in both WT and DR4AS. Their fresh weights were correlate with their phenotype (Figure III-24), such as in low concentration (0,4  $\mu\text{M}$ ) the WT cotyledon explants were significant heavy than that of DR4AS due to the quickly chlorophyll degradation and tissue senescence in DR4AS, with in the case of hypocotyl segment, the weight of DR4AS was significant higher than that of WT as its promotion on leaf regeneration. While too high concentration inhibits the leaf and root regeneration in both WT and AS, no significant difference was observed.

Third, in the absence of cytokinin, the DR4AS cotyledon explants showed hypersensitive to auxin (Figure III-23A), the same as described in Figure III-18; the hypocotyl segments of DR4AS also showed hypersensitive to auxin (Figure III-23B): the relative low concentration 0,4  $\mu\text{M}$ , showed positive promotion on root regeneration in DR4AS, however that is not enough to promote root regeneration in WT; and at the NAA concentration of 4  $\mu\text{M}$ , it was showed positive root regeneration both in WT and DR4AS, however, there were only WT showed positive leaf regeneration, that suggested that in DR4AS the balance of ratio of auxin and cytokinin was too high resulting the inhibition of leaf regeneration. Simultaneously the significant differences in fresh weight were observed between WT and DR4AS (Figure III-24A and B). When at NAA concentration of 40  $\mu\text{M}$ , the organ regeneration was completely suppressed in both WT and AS, which only formed callus tissue.

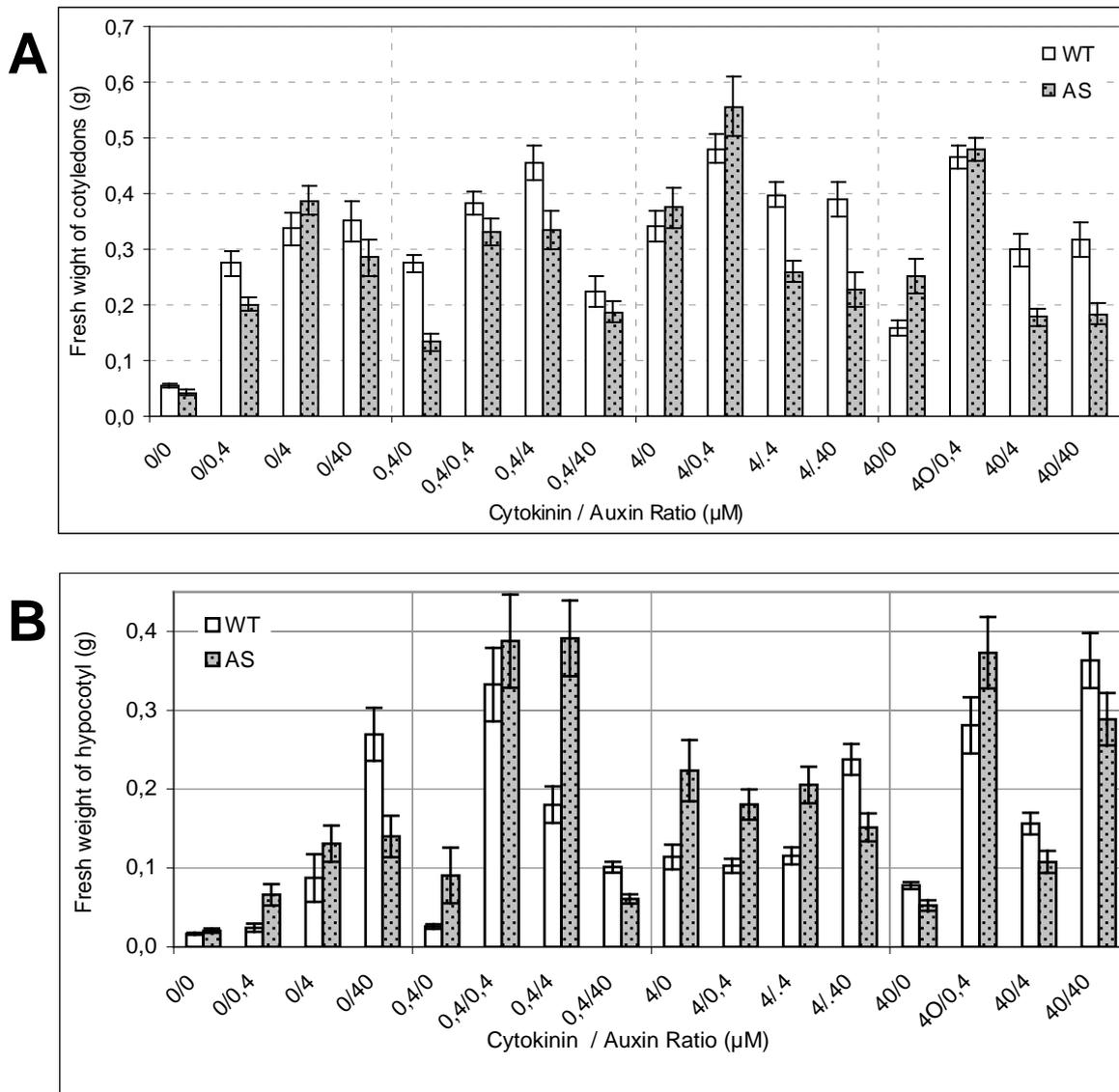
Forth, in the response to auxin and cytokinin with balanced ratio, in general, when the auxin-to-cytokinin ratio over 1, the cultured WT cotyledon explants regenerate vigorous and prolific roots although too high ratio inhibit this regeneration (such as the case of auxin/cytokinin 40/0, and 40/0,4); too high auxin concentration inhibit root generation (40  $\mu\text{M}$ ), but this inhibition can be antagonistically reduced by augmentation of cytokinin concentration, for example auxin/ cytokinin 40/4, or 40/40 there were still positive root formation in WT, while no root formation DR4AS in these cases, moreover, too high auxin concentration (40  $\mu\text{M}$ ) caused cell death in DR4AS. And when the auxin-to-cytokinin ratio is over 1 there was no shoot or leaf formation, conversely, when the ratio is less 1, it was showed preferable for the shoot (leaf) formation, at this condition, the WT and DR4AS explants cultures looked quite similar with each other. The hypocotyl segments showed the same auxin response tendency difference between WT and DR4AS as cotyledon (Figure III-23B), and the fresh weight after one month cultured explants showed the corresponding to the roots or shoots

(leaves) regeneration, the more roots (or shoots) formed, the heavier weighted. From this experiment we showed that there were significant auxin, cytokinin and their combination responses between WT and DR4AS seedling explants in vitro cultures. However to reply the question: whether *DR4* is involved cytokinin responses pathway directly or via changing the normal auxin-to-cytokinin natural ratio pathway, still needs the further experiments.



**Figure III-23.** Auxin (NAA) and cytokinin (BA) effects on callus production and root or leaf formation by WT and DR4AS seedling *cotyledon* (A) and *hypocotyl* (B) explants. Cotyledon explants and hypocotyl segments from 11-d-old, light-grown seedlings were incubated for one month

in the standard growth chamber on Murashige-Skoog media containing the indicated concentration of auxin (NAA) and cytokinin (BA). For each treatment, a representative WT explant is shown to the left of the corresponding DR4AS explant. Each treatment represents fifteen repeats in three independent experiments



**Figure III-24.** Fresh weight of WT and DR4AS cotyledon (A) and hypocotyl (B) explants culture under different combination of auxin (NAA) and cytokinin (BA) treatment. Cotyledon explants and hypocotyl segments from 11-d-old, light-grown seedlings were incubated for one month in the standard growth chamber on Murashige-Skoog media containing the indicated concentration of auxin (NAA) and cytokinin (BA). Error bars represents  $\pm$ SE from fifteen repeats in three independent experiments.

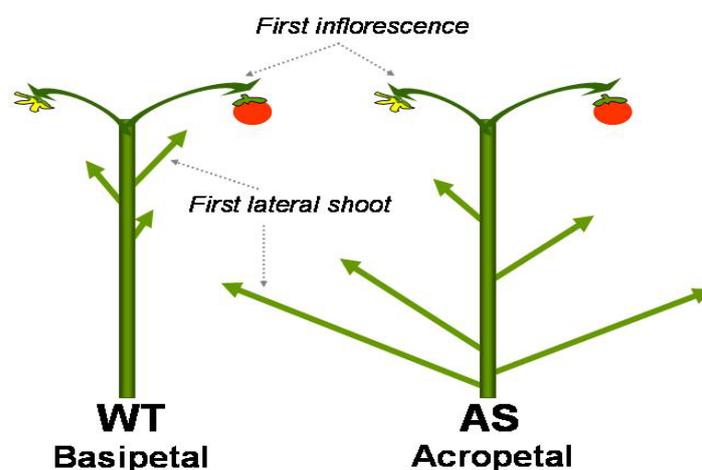
It is well established that auxin and cytokinin balance control the plant growth and development, such as the formation of roots, shoots, and callus tissue in vitro (Skoog and Miller, 1957), and outgrowth of shoot axillary buds (Wickson and Thimann, 1958; Cline,

2000). Cytokinins synergistically enhance auxin-induced ethylene production in pea stem sections, and in tobacco leaf discs, whereas they inhibit the auxin-induced elongation of sunflower and soybean hypocotyl segments. The mode of interaction between auxins and cytokinins can therefore be synergistic, antagonistic, or additive and is dependent on the type of tissue and on the plant species in which the interaction occurs. Although the molecular mechanisms underlying most of these auxin-cytokinin interactions are unknown, they are thought to include mutual control of auxin and cytokinin metabolism, interactions in the control of gene expression, and post-transcriptional interactions (Coenen and Lomax, 1997, 1998; Coenen et al., 2003). Mutants that are disrupted in their response to hormones or environmental signals can often be used to demonstrate or exclude interactions between signal transduction pathways. For example, tomato auxin insensitive mutant *diageotropica* (*dgt*) have been used and the studies showed that cross-resistance of the *dgt* mutant to cytokinin was limited to a small subset of auxin- and cytokinin-regulated growth processes affected by the *dgt* mutation, indicating that auxin and cytokinin regulate plant growth through both shared and separate signalling pathways (Coenen and Lomax, 1998). Here our experiment also demonstrated that DR4AS can be served as a new mutant tool to the further research of plants hormones signalling and interaction regulation.

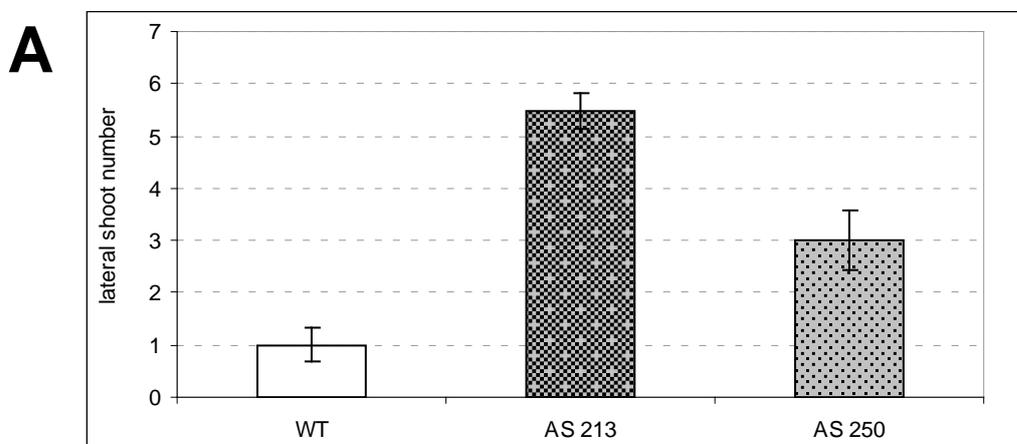
### **B-3-3- DR4AS has reduced apical dominance**

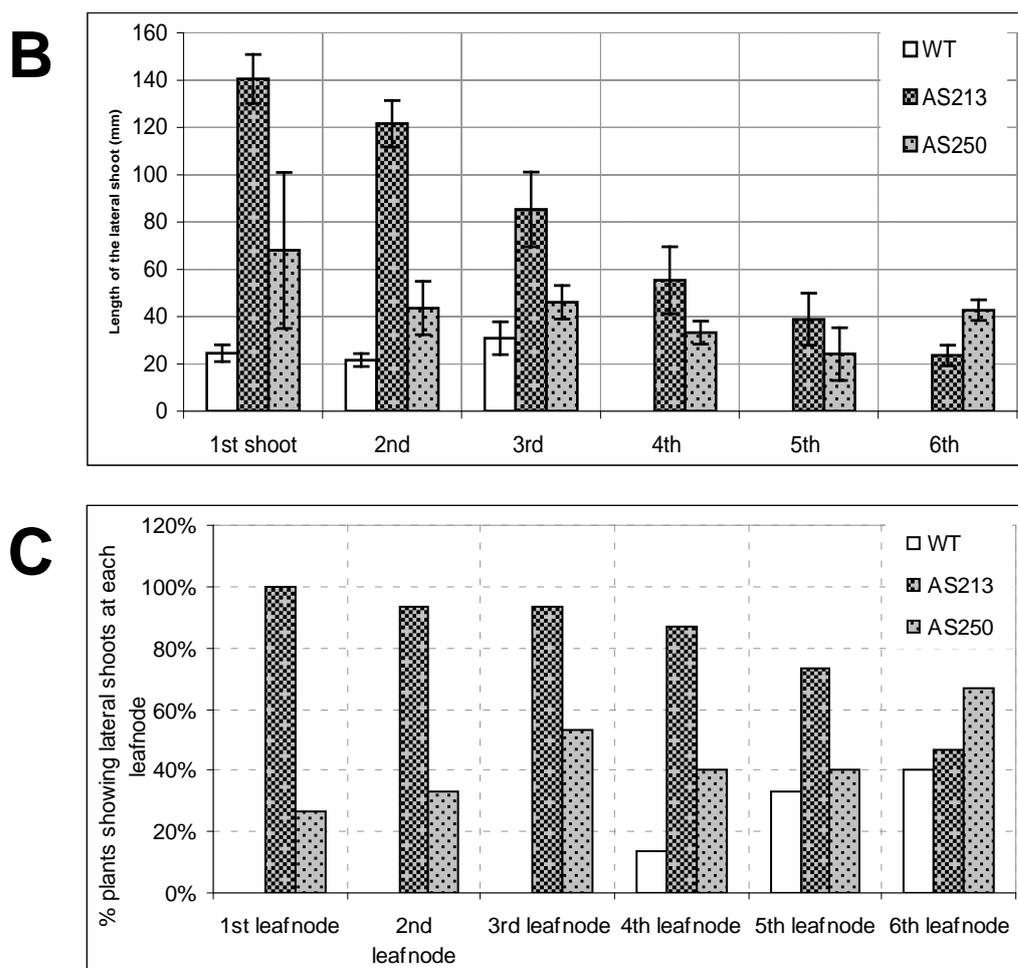
The apical dominance was largely reduced in DR4AS plants. It showed increased lateral shoot formation, and also changed the lateral shoot development pattern (Figure III-25, 26). During the vegetative growth, the normal WT young tomato plants have relative strong apical dominance and normally no lateral shoot formation; after floral transition, lateral shoots (axillary shoots) arise from the axillary buds along the primary shoot axis, and grow out to form lateral inflorescences in an apical-basal sequence (basipetally), the first lateral shoot arise from the last leafnode just below the first inflorescence (Figure III-25, Figure III-26B,C). On the contrary, in DR4AS, the lateral shoots grow out in an basal-apical sequence (acropetally), the first lateral shoot arose from the first leafnode just above the cotyledon nodes (Figure III-25, Figure III-26B,C). This decreasing of apical dominance was showed dependent the antisense transgenic background, the strong lines (lineAS213) had more serious reduction in apical dominance resulting in more lateral shoot's development (Figure III-26A, C), the weak line AS250 showed more lateral shoots than WT but less than strong line AS213 (Figure III-26A) and the lateral shoot development pattern was shown somewhat between the

WT and DR4AS strong line AS213 (Figure III-26C). Moreover, if in the favorable growth condition, these lateral shoots developed much vigorous than that of WT, results in more lateral shoots number (Figure III-26A), as well as longer length of lateral shoot (Figure III-26B), some of them even could reached the same length as the primary shoot length. In addition, although the axillary meristem formation was also after the floral transition, their development was rather quickly than that of WT.



**Figure III-25.** Diagram depicting the reduced apical dominance in DR4AS plants and the inverted order of axillary shoot development. In WT (left) the lateral shoots grow out in an apical-basal sequence (basipetally), the first lateral shoot arise from the last leafnode just below the first inflorescence; inversely, in DR4AS (right) the lateral shoots grow out in an basal-apical sequence (acropetally), the first lateral shoot arouse from the first leafnode just above the cotyledon nodes.





**Figure III-26.** DR4AS plant has decreased apical dominance. (A) DR4AS plants develop much more lateral shoots than WT. This decreasing of apical dominance was dependent the antisense transgenic background, the strong lines (lineAS213) showed more serious reduction in apical dominance resulting in more lateral shoot's development. (B) DR4AS lateral shoots developed more vigorous resulting in longer shoot length. The clones represent the means of shoot length at each leafnode in WT and two antisense lines AS213 and AS250 respectively, while in WT there was no lateral shoot occurred in the first three leafnode. (C) DR4AS showed different lateral shoot ontogenesis, the first emergence of lateral shoot took place in the first leaf axial and showed acropetal development pattern; while the WT form lateral inflorescences basipetally, the first lateral shoot arise from the last leafnode just below the first inflorescence. In general DR4AS produce more lateral shoots than WT. Error bars represents  $\pm$ SE of at least 15 plants.

Shoot apex derived auxin has long been known to inhibit lateral bud growth. Many obtained mutation or transgenes that affect auxin content, transport, or sensitivity, in Arabidopsis, showed altered in apical dominance, demonstrated clearly the normal auxin response is indispensable to keep the natural apical dominance. A role for auxin in the regulation of shoot branching was described originally in the Thimann and Skoog model, which proposes that apically derived auxin is transported basipetally directly into the axillary buds, where it inhibits their growth. Subsequent observations in many species have shown that

auxin does not enter axillary buds directly, although basipetal auxin transport in the stem is required (Stirnberg et al., 1999; Chatfield et al., 2000; Booker et al., 2003). Consistent with this finding, endogenous auxin levels in axillary buds do not correlate with the degree of bud inhibition, in some species, the level of auxin in buds released from apical dominance remains constant or even increased as they grow out (Booker et al., 2003), in contrast to the predictions of the Thimann and Skoog model. The other experiment also demonstrated that apical application of auxin can inhibit the growth of small buds, but larger buds were found to have lost competence to respond. Thereby, it has been proposed that its inhibitory effect is mediated by a second messenger. Experimental data eliminate ethylene and abscisic acid as the second messenger for the auxin-mediated bud inhibition and the rest cytokinin has been the candidate, whose biosynthesis and export from the roots is controlled by auxin and which can enter buds and stimulate their growth (Bangerth, 1994; Booker et al., 2003). However further experiments demonstrated that the cytokinin act independently to regulate the bud growth (Chatfield et al., 2000), and the kinetics of bud break are faster than those of cytokinin increase in the bud, so cytokinins may not be the second messenger for bud growth. More recently, it was demonstrated that apically derived auxin can suppress branching by acting in the xylem and interfascicular schlerenchyma to mediate apical dominance.

On the other hand, it was also documented that many auxin-response relative mutants showed altered apical dominance. Plants expressing *iaaM*, which come from *Agrobacterium tumifaciens* and confer over-auxin-biosynthesis, showed increased apical dominance (Klee et al., 1987; Romano et al., 1993; Romano et al., 1995), while plants expressing *iaaL*, which come from *Pseudomonas savastanoi* and conjugate IAA to Lys then reduce free IAA level in plants, showed reduced apical dominance. Several auxin signalling mutants also showed altered apical dominance such as Arabidopsis *Aux/IAA* gain-of-function mutant *axr3/IAA17* have enhanced apical dominance. Comparing with DR4AS, hypothesized as an *Aux/IAA* loss-of-function mutant, it showed reduced apical dominance. Attempting to explain it, we proposed one possible model, it was that over-auxin responses in the DR4AS axillary buds broke the inhibition and caused their enhanced growth and development, as *DR4* was down-regulated in DR4AS plant and who was assumed worked as auxin response inhibitor. The explaining of this hypothesis in detail was described as following. In the WT, normally the auxin is basipetally transported from the shoot apex to the roots, the auxin gradient was thereby formed with high auxin concentration in top and low auxin concentration in bottom, as well as there is no auxin transported into the axillary buds; after the floral transition, the release of inhibition of lateral shoots development was correlated with the auxin gradient, the

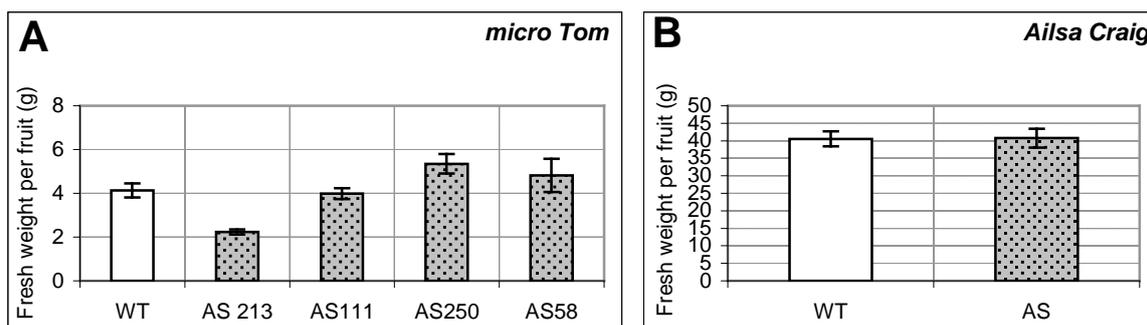
first developed take place at the last lateral buds just under the inflorescence, suggesting that lateral buds out-growth needed the presence of auxin and subsequently the auxin responses. While in the DR4AS, the lateral buds inhibition was broken earlier than WT in general, as the auxin responses inhibitor DR4 was genetically suppressed, so the lateral shoots out-growth was independent of auxin gradient but followed the same order as the leaf development, the first lateral shoot came from the first leafnode and developed rapidly and vigorously, thereby showed acropetal lateral shoots ontogenesis pattern.

#### **B-3-4- DR4AS Fruits character analysis**

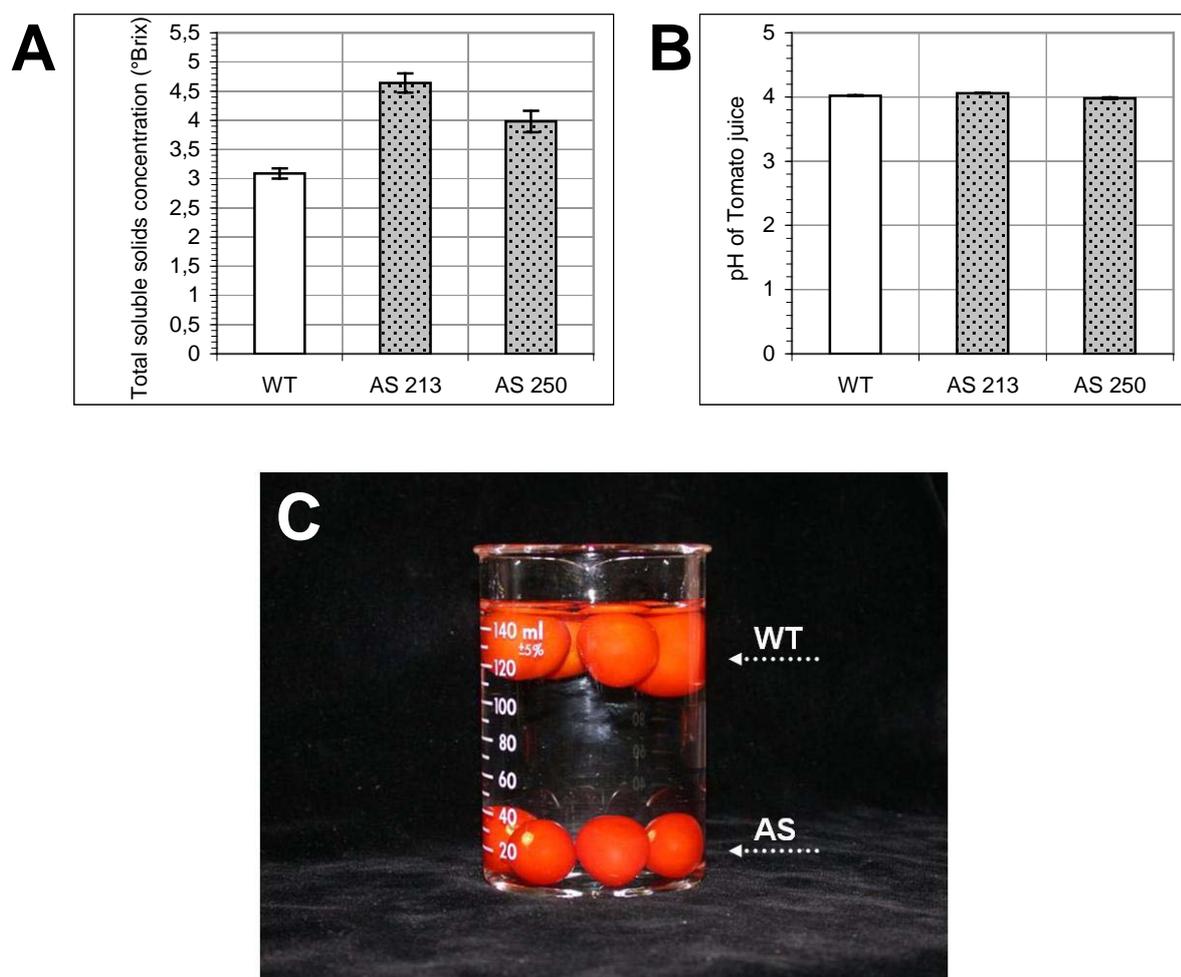
Major alteration occur throughout fruit ontogeny in the levels of several classes of phytohormones, suggesting a dynamic involvement in the fruit development (Gillaspy et al., 1993). It is well established that ripening of climacteric fruit such as the tomato is triggered by ethylene (Lelievre et al., 1997). The auxin plays a role in cell division and cell expansion during the fruit development, and also regulates fruit ripening process as exogenous applications of this hormone delay fruit ripening in many crop species (Vendrell, 1985; Cohen, 1996; Jones et al., 2002). DR4AS as a tomato *aux/iaa* loss-of-function mutant produces high percentage fully or partially parthenocarpic fruits. Several fruit character parameters were investigated. We measured the individual fruit fresh weight, total soluble solids concentration, pH, density and firmness during the fruit development, as well as the program of fruit development to ripening.

##### ➤ *Fruit fresh weight, shape and color*

The fully mature fruit fresh weight was measured in both two genotype  $\mu Tom$  and AC. It was no difference between the WT and DR4AS individual fruit fresh weight in the AC, with average weight 40,5 g in WT and 40,7 g in DR4AS (Figure III-27 B); in the genotype  $\mu Tom$ , four transgenic lines were investigated, all three lines (one strong line AS111, and two weak lines AS250 and AS58) showed the same or heavier fruits than WT (Figure III-27 A), except the one strong lines (AS213) showed less than WT. The further observation showed that the strong line AS213 produced much more fruits than WT, because it had the strong potentiality to fruit set independent on fertilization, and almost every flower was capable to develop into fruits, while the plant capacity of nutrition supply was limited, so intense concurrence of nutrition between these fruits results in less yield of individual fruit fresh weight.



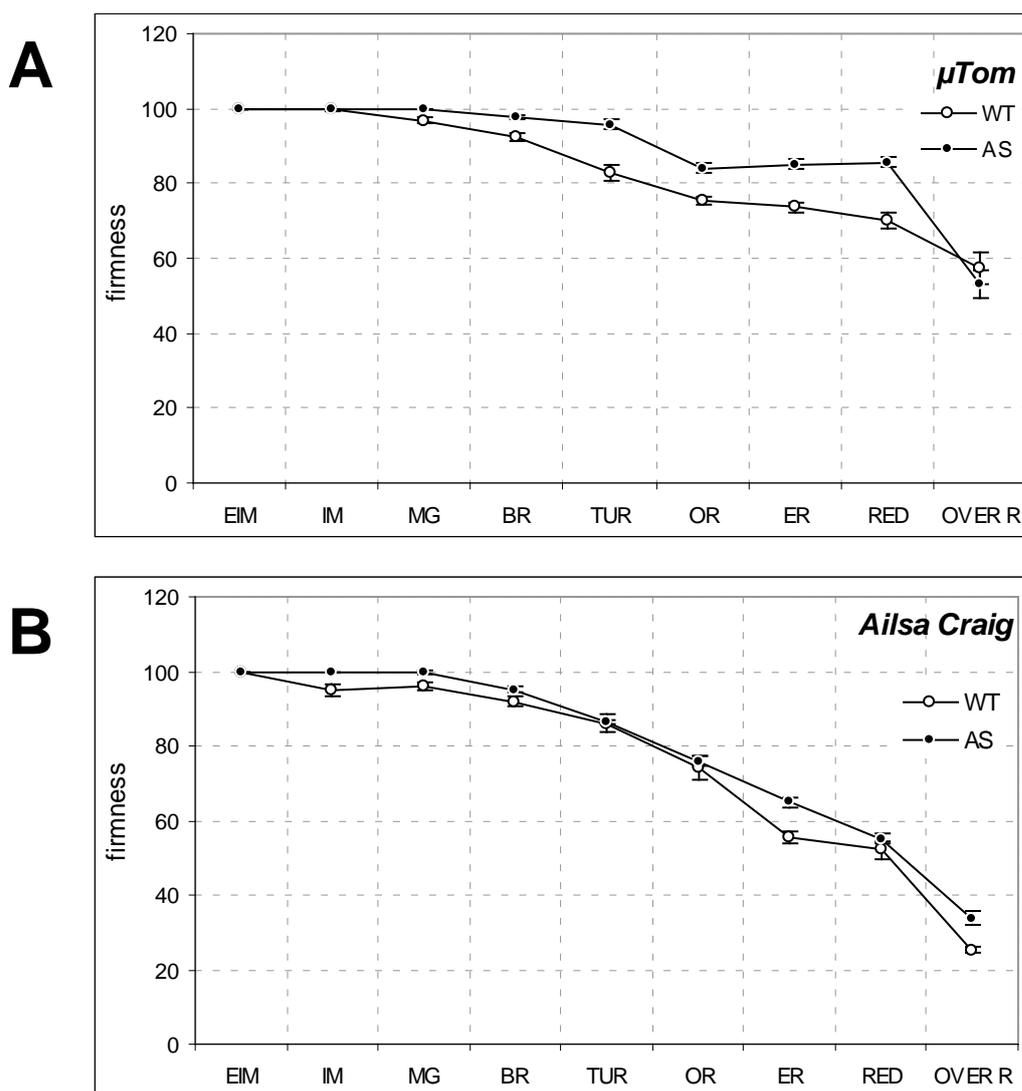
**Figure III-27.** Fresh weight per fruit in WT and DR4AS ( $\mu$ Tom and Ailsa Craig). (A) In  $\mu$ Tom genotype, three independent DR4AS lines showed similar or heavier fruits than WT, except the strong line AS213 which had smaller and light fruit because of more serious nutrition concurrence among the parthenocarpic fruits. (B) In Ailsa Craig genotype, there was not difference in individual fruit fresh weight between WT and DR4AS. Error bars represent  $\pm$ SE of at least five independent experiments.



**Figure III-28.** Total soluble solids concentration, pH and density (in  $\mu$ Tom genotype). (A) DR4AS showed significant increased total soluble solids concentration. (B) There was no difference in pH between WT and DR4AS. (C) Photo showing DR4AS have higher density than WT, consistent with the elevated total soluble solids concentration in DR4AS. Error bars represent  $\pm$ SE of at least three independent experiments.

➤ Total soluble solids concentration, pH and density

The total soluble solids concentration was significantly elevated in the DR4AS fruits, especially in those parthenocarpic fruits (Figure III-28A), consistent with this, the density of DR4AS fruits were higher than WT (Figure III-28C), while the pH was not different among them (Figure III-28B).



**Figure III-29.** Firmness evolution during the fruit development. DR4AS fruits have similar firmness evolution during the fruit development with that of WT in two genotypes  $\mu Tom$  (A) and *Ailsa Craig* (B). EIM, early immature green; IM, immature green; MG, mature green; BR, break; TR, turning; OR, orange; OR/RED, early red; RED maturation red; OVER R, over ripe. Error bars represent  $\pm$ SE of 17 fruits.

➤ Firmness evolution during the fruit development

The firmness investigation showed that DR4AS fruits have the rather similar firmness evolution during the fruit development (Figure III-29), although that many DR4AS fruits were fully or partially parthenocarpic, the locular cavities often have less gelatinous tissue, and also have relative retarded placenta maturation. But the fruit pericarp appears quite similar with that of WT (photo in Figure III-5B).

We also measured the time needed for fruit development (from fruit set to break) and ripening (from break to red), while they showed important variation themselves in both WT and DR4AS, for example, the fruit development in WT ranges from 25 days to 55 days, and there was no significant difference shown between WT and DR4AS.

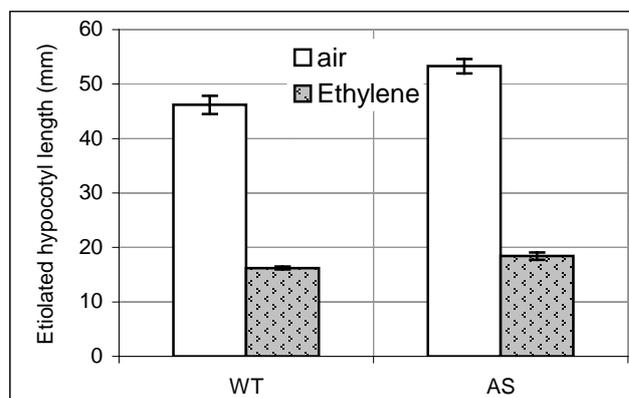
Parthenocarpy was engineered in tomato by ectopically expressing the *DefH9-iaaM* chimeric gene. The transgenic tomato can have fruit set in the absence of fertilization. The *iaaM* gene confers auxin synthesis, while the *DefH9* controlling regions drive gene expression specifically in the ovules and placentas. Seedless fruits were obtained from emasculated flowers and fruits with seeds also could be obtained from pollinated flowers. The *DefH9-iaaM* transgenic plants produced parthenocarpic fruits with similar characters with DR4AS fruits including have the similar fruits fresh weight and pH, elevated total soluble solids concentration, while their density and firmness was not reported.

### **B-3-5- Ethylene response in DR4AS**

It is well known that auxin interact with ethylene to regulate many developmental and growth processes during the plant life. Applied ethylene inhibit hypocotyl elongation growth in etiolated seedling as part of the “triple response” phenomenon, and high ethylene leads to growth inhibition and decreased in tropic responses. There is evidence that the reduction in elongation caused by ethylene occurs via an interaction between ethylene and auxin. Whereas ethylene production is stimulated by auxin, ethylene can suppress polar (basipetal) transport of auxin and can also influence asymmetric distribution of auxin (Madlung et al., 1999). Based on the context of wide range of auxin and ethylene interaction, several experiments were performed to investigate the ethylene response in DR4AS plants, including the classic ethylene response assay “triple response”, the ethylene production and during the fruit development, the ethylene production of vegetative tissues as well as the wounding ethylene production.

#### ➤ *Triple response*

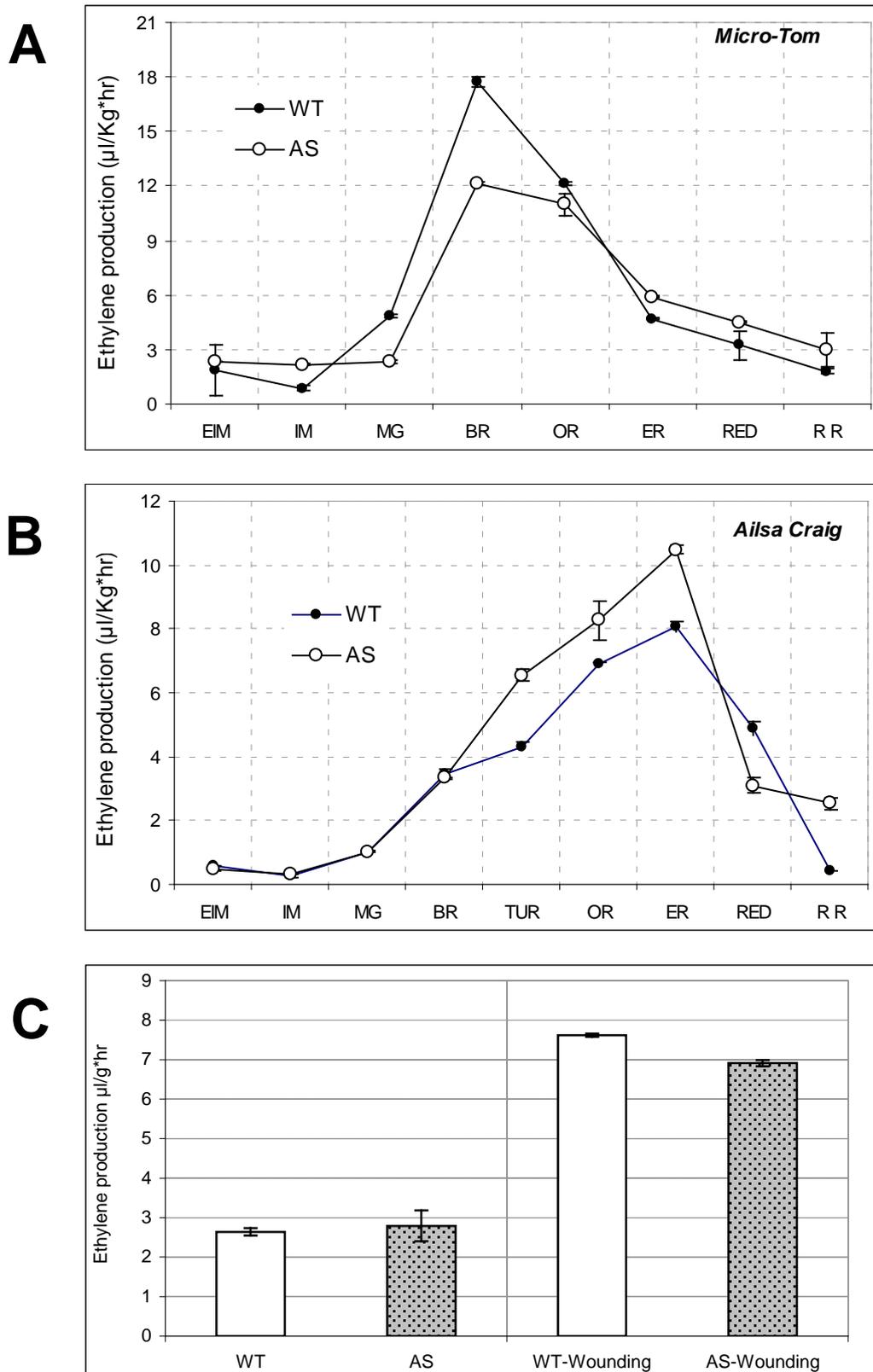
Etiolated seedling of WT and DR4AS were grown in the sealed container without or with ethylene (0,5 ppm). Both WT and DR4AS ( $\mu Tom$ ) showed typical ethylene-induced triple response: inhibition of hypocotyl elongation (Figure III-30), exaggerate hook formation, increased hypocotyl diameter. No significant difference was found in DR4AS.



**Figure III-30.** Ethylene inhibit hypocotyl elongation in both WT and DR4AS ( $\mu Tom$ ) etiolated seedlings. Error bars represent  $\pm$ SE of at least 15 seedlings.

➤ *Fruit, vegetative tissue and wounding ethylene production*

Ethylene production during fruit development was measured and compared between WT and DR4AS in both genotype  $\mu Tom$  and AC, it was shown that (Figure III-31A,B), the climacteric fruit typical ethylene production model during fruit development was remained well in DR4AS and showed the same evolution pattern as WT. While the ethylene production of DR4AS was slightly enhanced in AC genotype, it showed a slight decrease in  $\mu Tom$ . The same, vegetative tissue (shoots and leaves) and their wounding ethylene production did not show significant difference between WT and DR4AS (Figure III-31C). Many plant growth and development processes were reported being controlled by auxin and ethylene interaction, for example to control the apical hook formation (Lehman et al., 1996; Raz and Ecker, 1999), stem or hypocotyl elongation (Madlung et al., 1999), the root and root hair development (Masucci and Schiefelbein, 1994; Pitts et al., 1998; Rahman et al., 2001; Rahman et al., 2002), gravi-/phototropic response (Madlung et al., 1999; Harper et al., 2000), and leaf abscission (Beyer and Morgan, 1971). Our ethylene responses experiments showed that there were not difference between WT and DR4AS at least in the processed tested, and indicating that *DR4* may not involved in these processes.



**Figure III-31.** Fruit, vegetative tissue and wounding ethylene production. DR4AS in background genotype  $\mu$ Tom (A) and AC (B) both showed similar ethylene production during the fruit development. (C) There was no significant difference in vegetative tissue (shoots and leaves) of AC and their wounding ethylene production. EIM, early immature green; IM, immature green; MG, mature green; BR, break; TR, turning; OR, orange; OR/RED, early red; RED maturation red; OVER R, over ripe. Error bars represent  $\pm$ SE

### **B-3-6- DR4AS has normal photo-/ gravi- tropism**

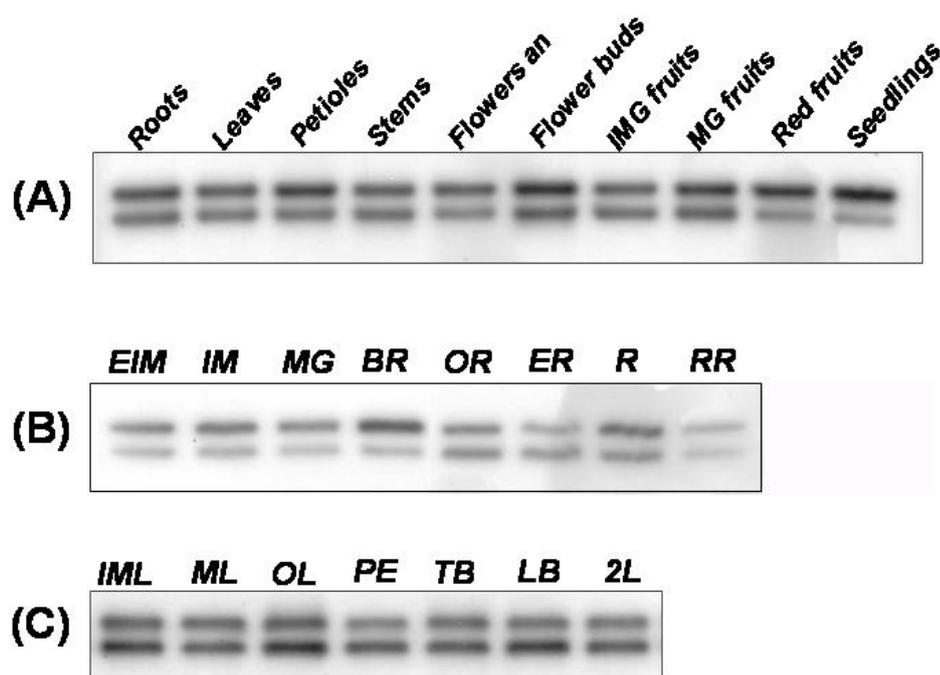
It is well established that photo-/gravi- tropism was mediated by auxin and also numerous auxin metabolism, transport and signalling mutant including many of *aux/iaa* mutants in Arabidopsis showed altered photo-/gravi- tropism phenotypes; the DR4AS showed normal photo-/gravi- tropism (data not shown).

## ***B-4- Genes expression analysis***

### **B-4-1- DR4 expression pattern**

We used RT-PCR and RNA gel blot analysis to assay the spatial and temporal expression pattern of *DR4* gene in WT plants, and the results of both methods showed that, the *DR4* mRNA is constitutively and ubiquitously accumulated in various organs such as roots, stems, leaves, flowers and fruits (Figure III-32A) at important level, as well as during the fruit development (Figure III-32B); in addition, from the TIGR tomato EST bank, the *DR4* was also detected expressed in seed and in vitro cultured callus tissues. These sites of expression are consistent with the sites of phenotypic alterations in the *DR4* down-regulated mutant DR4AS transgenic plants. In contrast, the majority of *Aux/IAA* genes are organ-preferentially expressed in Arabidopsis, for example, *AtIAA28* is only concentrated in the roots and inflorescence (Rogg et al., 2001), both *IAA3/SHY2* and *IAA7/AXR2* are expressed strongly in stems and flowers, moderately in leaves, and weakly in roots (Abel et al., 1995a). However *AtIAA9* and *AtIAA8*, who are the *DR4* orthologs in Arabidopsis, are more ubiquitous and constitutive in various organs (Abel et al., 1995a), showing the same expression profiles as the *DR4* in Tomato. In addition, compared with the other members isolated in tomato, the *DR4* showed the most important transcripts accumulation (Nebenfuhr et al., 2000), once more *AtIAA9* and *AtIAA8* were found the most abundant natural basal transcripts accumulation when compared with all other member of *Aux/IAA* gene family (Abel et al., 1995a). The *DR4* distinct expression pattern with the other members of *Aux/IAA* gene family reinforces the specific, maybe unique roles of *DR4* in plant morphogenesis and development, suggested by the wide range of DR4AS transgenic plant phenotypes. In addition, as DR4AS have changed morphology, showing simple leaf architecture instead of natural compound leaf, the *DR4* temporal and spatial expression pattern was also examined in the leaf (Figure III-32C), and

the same constitutive and ubiquitous expression pattern was found; it indicating that the proper *DR4* expression is indispensable for the normal leaf ontogenesis. Moreover, recently several genes were isolated who showing changes during the programmed cell death in a model system of suspension-cultured tomato cells, one clone *CTD1* was shown dramatically down-regulated during the programmed cell death, after the careful amino acid sequence alignment, and comparison, it has shown 100% identity between *CTD1* and *DR4*, so *DR4* was demonstrated regulated by programmed cell death.

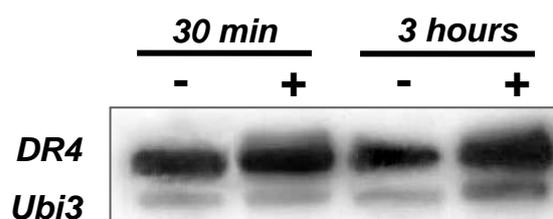


**Figure III-32.** RT-PCR analysis of *DR4* spatial and temporal expression pattern. (A) *DR4* spatial expression in various major organs, showing constitutive and ubiquitous expression pattern. The various organs were marked in the top of the figure, where: Flowers an, flowers in anthesis; IMG Fruits, immature green fruits; MG Fruits, mature green fruits. (B) *DR4* temporal expression pattern during the fruit development, at stage: EIM, Early immature green; IM, immature green; MG, mature green; BR, break; OR, orange; ER, early red; R, red; RR, over ripe. (C) *DR4* temporal and spatial expression pattern in the compound leaves, in: IML, expanding immature leaves; ML, expanded mature leaves; OL, old turning yellow leaves; PE, petioles; TB, terminal-leaflet blades; LB, lateral-leaflet blades; 2L, the second leaf counted from the cotyledon node, in which the leaves fusion is the most frequently happened. The RNA gel blot also showed the similar results.

#### B-4-2- *DR4* mRNA transcripts levels are slightly and slowly induced by auxin

*Aux/IAA* genes were originally identified because of their strong and rapid transcriptional induction in response to auxin (Theologis et al., 1985). Subsequent analysis *Aux/IAA* genes including several of them identified based on sequences homology, has shown

that *Aux/IAA* genes can be grouped into several subgroup based on their auxin induction kinetics. Some of these genes respond to auxin with large increases in transcript levels with several minutes, whereas others respond more weakly and slowly, only two of them (*IAA7* and *IAA8*) showed only minor or no induction (Abel et al., 1995a); in addition, more recently it was also found that one *Aux/IAA* gene *IAA28* in Arabidopsis showed slightly decrease of transcripts accumulation in response to auxin treatment (Rogg et al., 2001). To determine whether *DR4* expression is similar as the other *Aux/IAA* genes in the responses to auxin treatment, we assayed the levels of *DR4* transcripts accumulation after auxin treatment in WT. In contrast of the majority of members of *Aux/IAA* gene family, which show rapid and strong induction by auxin, we found that *DR4* mRNA level only showed minor and slowly induction in response to auxin. This slightly and slowly induction by auxin treatment is distinct and unique among the *Aux/IAA* genes (Abel et al., 1995a).

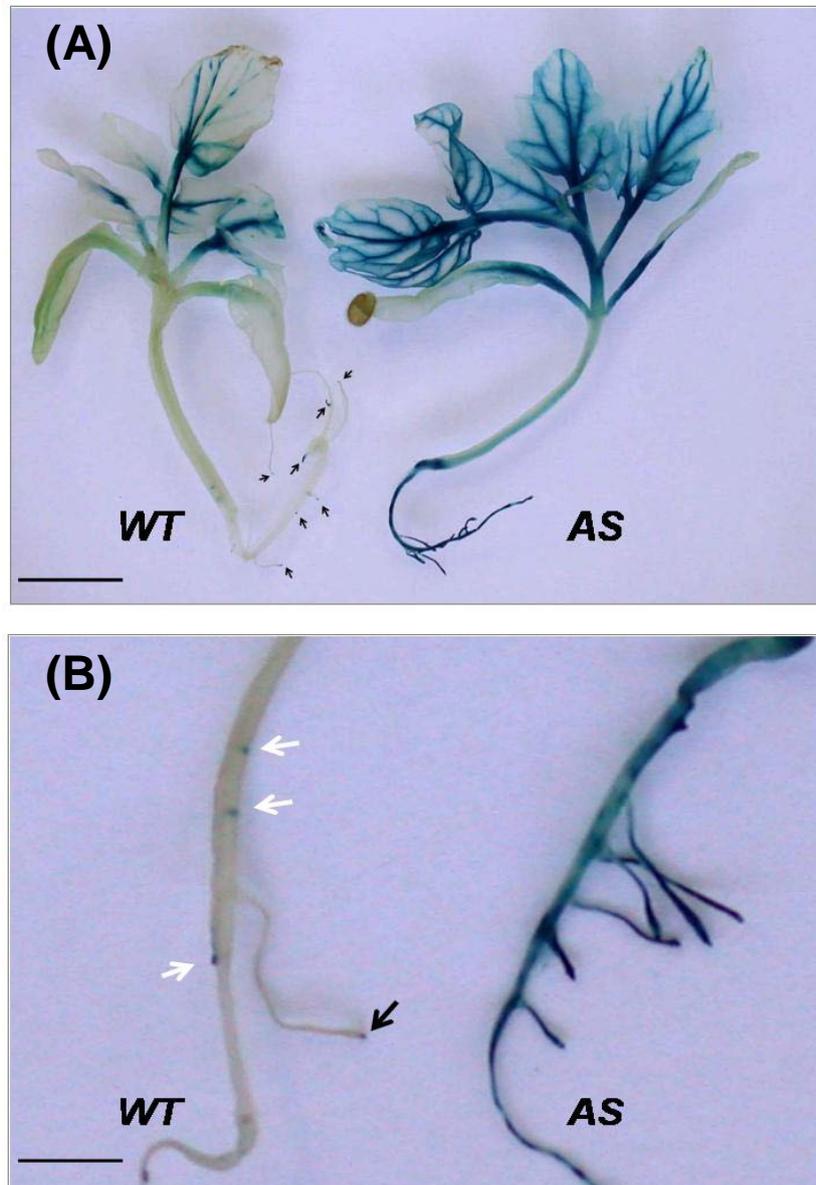


**Figure III-33.** *DR4* transcripts accumulation show slight and slow induction to auxin. RT-PCR analysis showed slight and slow increase of *DR4* mRNA transcripts accumulation to auxin incubation (IAA 20  $\mu$ M for 30 min or 3 hours) in WT background. In each PCR the *DR4* was amplified only 25 cycles, showing *DR4* have important basal transcripts level. The internal reference *ubi3* was co-amplified with the *DR4* gene as described in chapter II. Total RNAs were extracted from light-grown seedling.

#### B-4-3- Comparison of genes expression in different tissue of WT and AS

*Aux/IAA* genes are thought to encode transcriptional modulators that regulate (especially repress) the auxin-inducible gene expression, Arabidopsis *Aux/IAA* proteins repressed transcription on auxin-responsive reporter genes in protoplast transfection assays. The Arabidopsis *aux/iaa* gain-of-function mutants, in which the corresponded *Aux/IAA* proteins are steadier, show reduction in the expression levels or ectopic expression activity of some auxin-regulated genes, and also show reduced auxin responses and altered morphology in general. As a member of *Aux/IAA* transcription factors gene family, *DR4* was also thought to regulate some of auxin regulated genes expression then mediate the auxin responses and

exhibit auxin-related phenotypes. So some of the auxin-regulated genes' expressions were investigated in *DR4AS* transgenic plants by both RT-PCR analysis, and GUS activity analysis which is under the control of auxin-responsive gene's promoter.



**Figure III-34.** Auxin-inducible gene *DR3* is up-regulated in the *DR4AS* seedling. (A) Promoter *DR3*::GUS in WT background Seedling (left) and in *DR4AS* background (right), the GUS staining is much higher all over in the *DR4AS* plants, indicating that the auxin primary response gene *DR3* expression is up-regulated in the *DR4* down-regulated plant. The black arrowhead indicated GUS staining in the WT roots. (B) GUS staining is limited in the root tip (indicated by black arrowhead) and absent in all of other root system in WT (left), the emerging point of lateral roots are showing GUS staining also and indicated by white arrowhead; whilst, the GUS staining is all over the root system in *DR4AS*, indicating that *DR3* is up-regulated in all over the root system. The scale bars represent 10 mm (A) and 3 mm (B).

***B-4-3-1- Auxin-inducible gene DR3 is up-regulated in DR4AS seedling***

We examined the expression of a reporter gene driven by a promoter of auxin inducible gene *DR3/IAA3*, which is a tomato *Aux/IAA* gene and showed rapidly induction by auxin application (Nebenfuhr et al., 2000; Jones et al., 2002) (Figure III-36). In addition, the promoter DR3::GUS construct is rapidly and strongly induced by auxin in the tobacco protoplast transfection assay (Wang et al., 2002). After crossing this promoter DR3::GUS (P-DR3::GUS) construct into the DR4AS plant, we compared the *GUS* expression in WT background P-DR3::GUS plants and that of in DR4AS background. It was well showed the highly increased staining of DR4AS plants when compared with the WT seedling (Figure III-34); particularly, the *DR3* is only expressed in the root tips, and absent in all of the other region in WT background, however, it is very strongly induced in all over the root system in DR4AS. That strongly suggests the over-auxin responses in the roots of DR4AS. In accordance, it might be able to explain the DR4AS seedling phenotypes such as enhanced lateral roots formation and increased primary root elongation.

***B-4-3-2- Down regulation of DR4 does not alter examined genes expression levels***

The RT-PCR analysis first examined the auxin-inducible gene expression including the three major early/primary auxin response gene families and the ACC synthase which was showed up-regulated by auxin. Second, three genes involved vascular tissue patterning and development were also examined, as the mutants of these genes either showed several similarity in the phenotypes as DR4AS, or showed the capacity to govern the vascular tissue patterning and development.

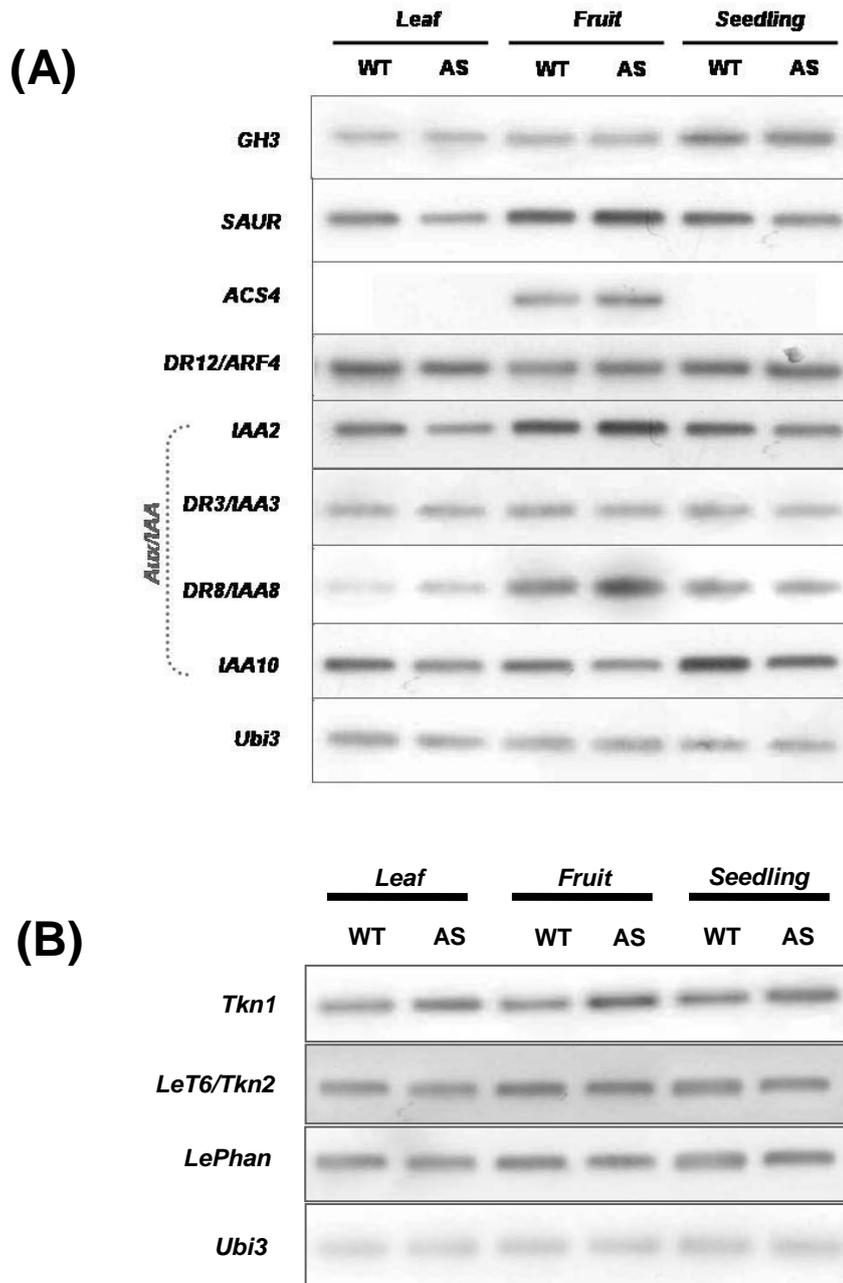
➤ *Auxin-inducible genes expression analysis*

It is well established that auxin activates early/primary gene families at the transcription level, three major classes of them are *GH3* family, the *SAUR* family, and the *Aux/IAA* family. In addition, the ACC synthase (*ACS4*) was also showed up-regulated by auxin and thereby confer the enhanced ethylene production. In order to explore, at the molecular level, the potential role of *DR4* in mediating the auxin response, these auxin inducible genes (at least one representative of the three major classes) as well as an tomato *ARF* (auxin response factor) *DR12* gene were examined in the DR4AS plants. As many of

these auxin-early-response, or auxin-mediated genes show distinct organ preferential and temporal expression pattern, those auxin inducible genes expression were thereby examined in the different plant tissues: the expanding leaves were chosen to represent the vegetative tissue, and the early immature green fruits were chosen to represent the reproductive tissue, as it was unique period (initiation of cell expansion) that have the high level of endogenous auxin accumulation in the pericarp, and auxin level fall rapidly after that and maintain at low level during the fruit development and ripening. To be surprised that all of those tested auxin regulated genes expression did not shown significant different expression levels in the tested tissue of WT and DR4AS (Figure III-35A), including *DR3* which has been demonstrated showed up-regulated in the roots of DR4AS seedling. These data suggest that although *DR4* expression show ubiquitous and constitutive pattern, but its transcription regulation role is highly organ specific and temporal, for example the *DR3* is largely up-regulated in the root of DR4AS seedling, but showed slightly induction in the shoot apex and the first leaves and almost the same level in the hypocotyls; while the almost no up-regulation of *DR3* in the tissue of expanding leaves and early immature green fruits of mature plants.

➤ *Leaf morphogenesis involved genes expression analysis*

The leaf is a coordinated mosaic of developmental domain, the acquisition of symmetry axes in the emerging leaf on the flanks of the apical meristem is a process coordinated by hormones (such as auxin, gibberellins, and cytokinins), and the expression of classes of genes. So far the most characterized genes involved in leaf initiation, vascular tissue patterning/development and leaf morphogenesis are *Knox* gene family and the *ARP: as1* (*Asymmetric leaves1* in Arabidopsis), *rs2* (*Rough sheath2* in Maize), *phan* (*Phantastica* in *Antirrhinum*) genes family (Pozzi et al., 2001). The *Knox* genes orthologs in Tomato *Tkn1* and *LeT6/Tkn2* (Hareven et al., 1996; Janssen et al., 1998; Avivi et al., 2000), and the *ARP* gene in Tomato *LePhantastica* (koltai and Bird, 2000) were examined in DR4AS (Figure III-35B). However, the results showed that there is no significant difference expression levels of those genes between the DR4AS and WT in the three tissues examined. This data indicated either the *DR4* does not via regulation of these three genes transcription to control the leaf morphogenesis, or *DR4* disturbs the cell-type-expression pattern (Avivi et al., 2000) of those genes, without having the significant change in transcripts accumulation in the whole leaves, or seedling, during the leaf ontogenesis. And the further *in situ* hybridization assay can confirm the later hypothesis.



**Figure III-35.** RT-PCR analysis comparing the genes expression levels in *WT* and *DR4AS*. **(A)** Expression analysis of auxin-responses related genes in *DR4*-inhibited plants (*AS*) and no significant difference was found when compared that of wild-type (*WT*), the special tissues chosen are marked at the top of the figure. The genes specific primers were designed after *GH3* (Liu et al., 1994), *SAUR* (Gil et al., 1994), *ACC* synthase (*ACS4*) (Yip et al., 1992), *IAA2* and *IAA10* (Balbi and Lomax, 2003), *DR12*, *DR3* and *DR8* (Jones et al., 2002). **(B)** Expression analysis of leaf morphogenesis involved genes, the genes specific primers were designed after *Tkn1* (Hareven et al., 1996), *LeT6/Tkn2* (Janssen et al., 1998; Avivi et al., 2000), *LePhantastica* (koltai and Bird, 2000).

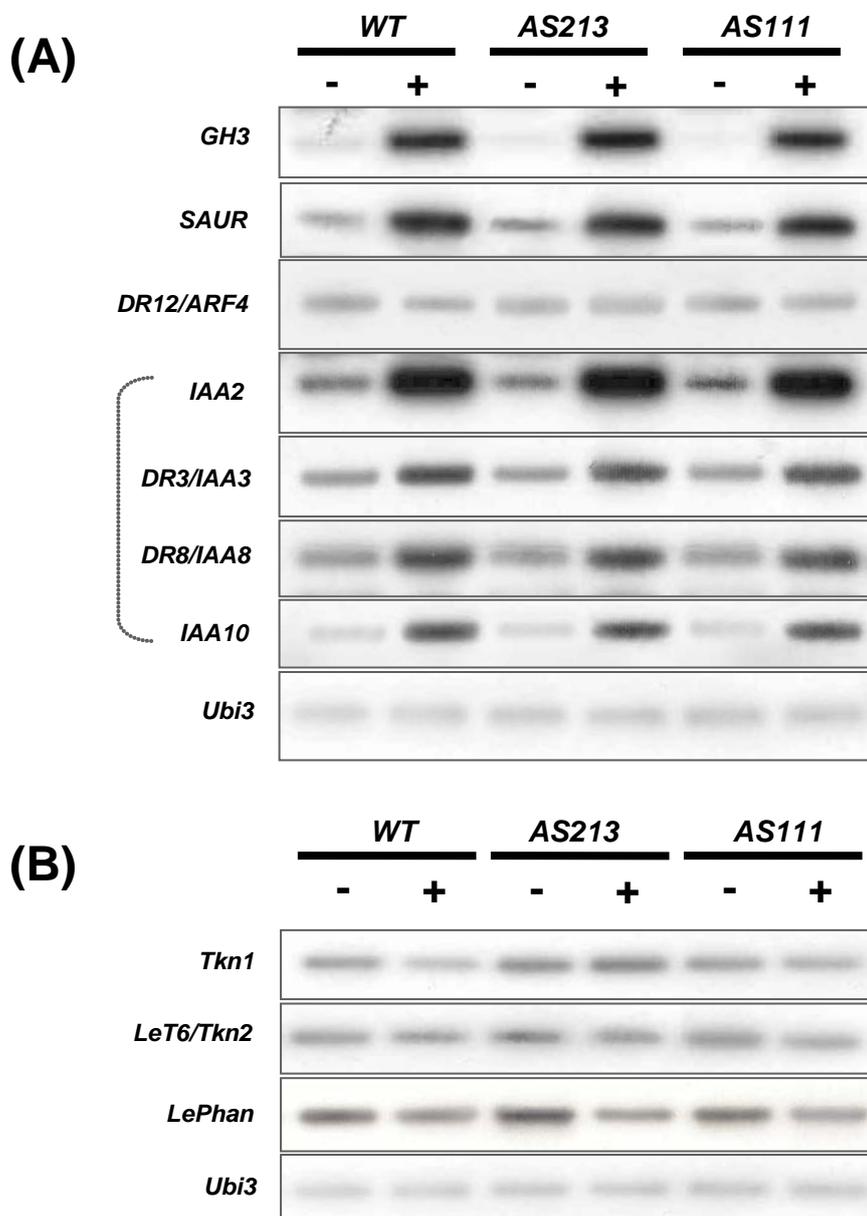
The *Knox* genes are expressed in the SAM (Shoot Apical Meristem) but are down-regulated in founder cells at the time of leaf initiation (Jackson et al., 1994). They are

implicated in maintaining division or preventing differentiation of cells in the SAM. Loss-of-function mutations in the Arabidopsis *Knox* gene *SHOOT MERISTEMLESS (STM)* result in embryos that lack a SAM (Barton, 2001). Recessive mutations in the *Kn1* gene of maize are also defective in meristem maintenance. In contrast, gain-of-function mutants that result in ectopic expression of *Knox* genes in maize disrupt normal leaf development causing distal displacement of the sheath and auricle tissue into the blade (Reiser et al., 2000). In dicotyledonous plants, such as Arabidopsis and tobacco, misexpression of homeobox transgenes results in lobed leaves, distally displaced stipules, and occasional ectopic meristems on the adaxial leaf surface; when the over expressing the maize *Kn1* gene in tomato results in “super compound” with each leaf having up to 2000 leaflets (Hareven et al., 1996). *PHANTASTICA (PHAN)* in Antirrhinum is transcribed in organ founder cells and is required to prevent expression of an *STM*-like *KNOX* gene in the lateral organs, *phan* mutant showed variable defects in leaf patterning, affecting both dorsoventral and proximodistal axes. *PHAN* has a myb domain found in several transcription factors and its homologue in maize is *ROUGH SHZATH2 (rs2)*, the mutant *rs2* showed ectopic expression of homeodomain proteins in developing leaves and revealing the role of *RS2* in repressing *KNOX* gene expression. *ASYMMETRIC LEAVES1 (AS1)* is the *PHAN* ortholog in Arabidopsis, the *as1* lose-of-function mutant showed deeply lobbed leaves, in which the leaf lamina is subdivided into prominent outgrowths. In brief, these two gene families play very important roles in leaf morphogenesis, the *Knox* genes gain-of-function and *ARP* loss-of-function mutant cause either “super-compound” or further lobbed leaves, which is the inverse proceeding compared with the *DR4* loss-of-function mutant (*DR4AS*) which have less-compound leaves. However whether there is molecular relationship between them should be a very interesting subject of further experiments.

#### **B-4-4- Compare genes expression in WT and AS in response to auxin**

The genes expression with application of auxin was also examined in order to investigate the potential role of *DR4* in mediating the auxin responses at the molecular level. The same, we examined two groups of genes as: auxin inducible genes and leaf morphogenesis involved genes. The seedling was chosen as the plant material undergoing IAA (20  $\mu$ M) treatment or mock treatment for 3 hours. The results were showed in the Figure III-36, all of the examined genes transcripts accumulation of auxin treatment was quite similar, in WT and *DR4*-inhibited seedling. That mean, *DR4* does not regulate these genes

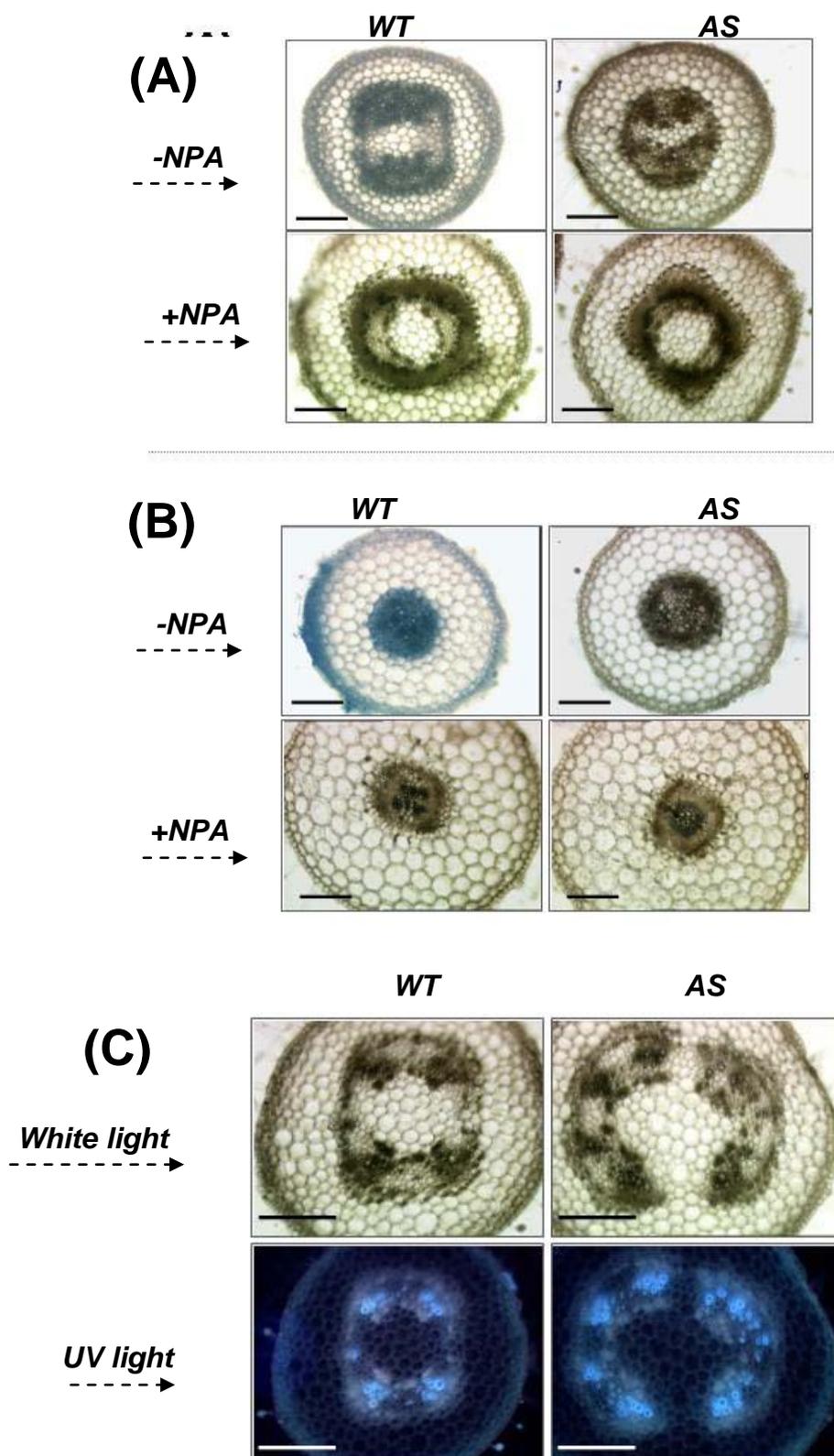
expression. Of course, the auxin regulated genes are not limited to these genes we examined in our experiments, and the micro-array is currently going on in our lab in order to gain the DR4 regulated genes.



**Figure III-36.** RT-PCR analysis comparing the genes expression levels with auxin application between WT and DR4AS. **(A)** Expression analysis of auxin-responses related genes in DR4-inhibited plants (AS) with auxin application (20  $\mu$ M, 3 hours) and no significant difference was found when compared that of wild-type (WT), the symbols marked at the top of the figurer represent mock treatment (-) and auxin treatment (+) respectively. **(B)** Expression analysis of leaf morphogenesis involved genes with auxin application (20  $\mu$ M, 3 hours).

### ***B-5- Anatomical analysis***

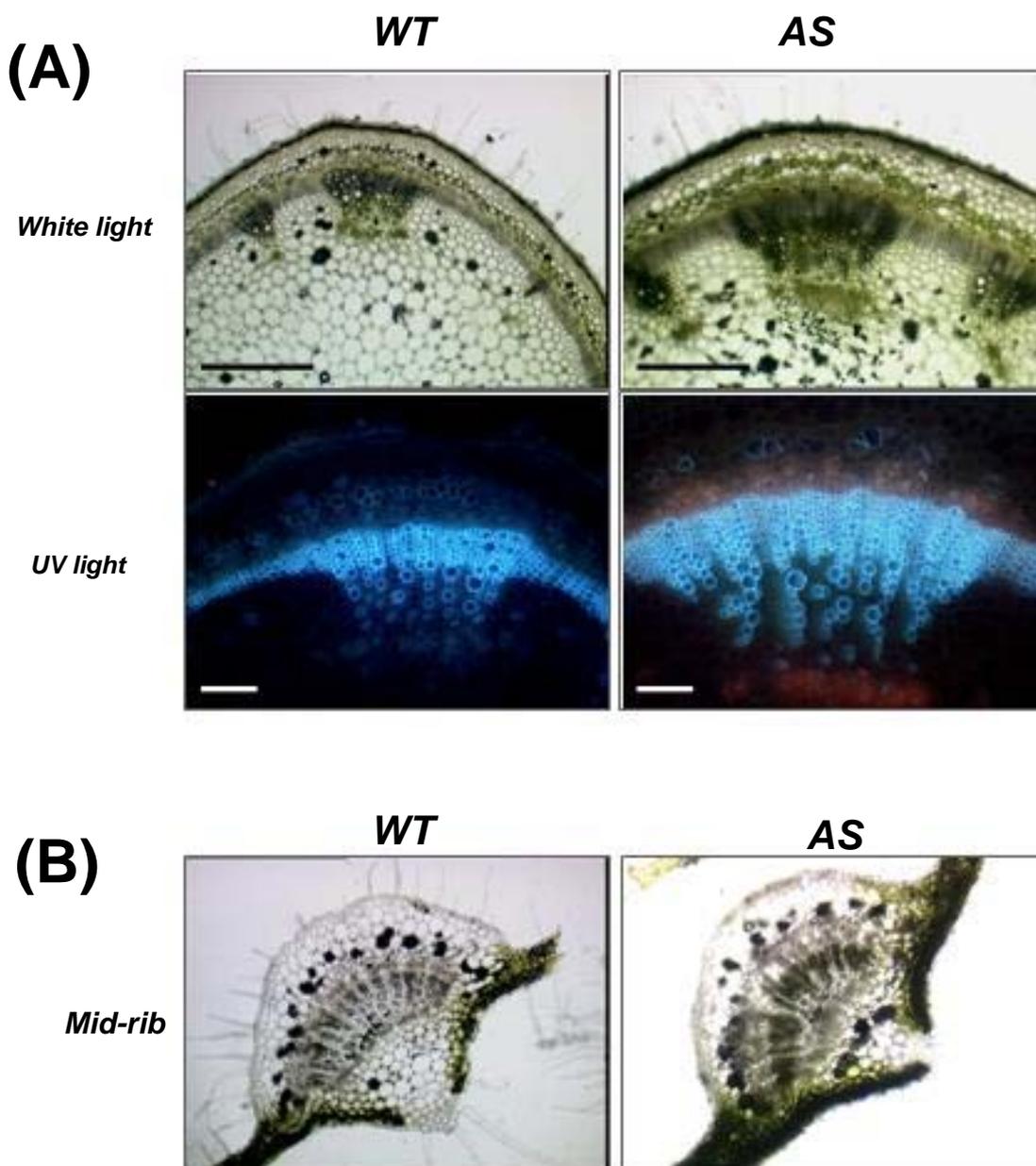
DR4AS plants show altered leaf-venation patterning, multi-organs fusions due to the abnormal vein branching such as the fusion of leaves, flowers and fruits, disrupted the normal sepal arrangement, as well as the frequent exhibition of triple or four cotyledons. The results of anatomical analysis further demonstrated the abnormality in the vascular patterning and development. In accordance with the frequency of aberrant cotyledon positioning or three or four cotyledon, it was found that the vascular bundles were dis-organized in the DR4AS hypocotyl (Figure III-37). In WT, two basal vascular bundles were formed towards the two cotyledons, while the vascular bundles were scattered around the “stele” (Figure III-A, C). The auxin transport inhibitor NPA induced enhanced xylem proliferation in both WT and DR4AS, although the WT mimics several of the DR4AS phenotypes such as the simple leaf formation and leaf-fusion with the NPA treatment. It was demonstrated in Arabidopsis that the NPA-caused massive radial xylem proliferation in the vascular bundles was serious adjacent to the leaves, because the normal auxin drainage from the leaves was blocked by NPA and accumulated locally, and cause the extensive xylogenesis in the vascular bundles (Galweiler et al., 1998). In accordance, the enhanced vascular tissue differentiation has also been observed in the plants that overproduce auxin, supporting the role of auxin gradients in radial vascular pattern formation (Klee et al., 1987). The vascular patterning in the stems and leaves were also examined, and the results showed that in the DR4AS often (but not always) showed the heavier xylem proliferation than its WT counterparts (Figure III-38A). While the profiles of petioles and mid-rib are rather similar or the DR4AS showed a slightly enhanced xylem proliferation (Figure III-38B). On conclusion, the anatomical analysis supported the enhanced auxin responses in the DR4AS plants.



**Figure III-37.** Analysis of vascular patterning in DR4AS seedlings.

(A) & (B) The 4-d-old light-grown seedling of WT (left colonna) and DR4AS (AS, right colonna) were grown without (-NPA) or with (+NPA) the presence of auxin transport inhibitor NPA (1  $\mu$ M). (A) Cross sections were cut in the high region of hypocotyl, just below the cotyledon node in which the vascular patterning is toward the stem structure. In WT, it forms the two basal vascular bundles

towards the two cotyledons (1), while the AS has more scattered vascular bundles (2). **(B)** Cross sections were cut in the base region of the hypocotyl, just above the adjacency of root, in which the vascular patterning is toward the root structure. The WT and DR4AS showed rather similar profiles. The NPA caused massive radial xylem proliferation was observed in both WT and DR4AS. **(C)** The cross section (just below the cotyledon node) of 7-d-old light-grown seedling further confirm the abnormal scatter of vascular bundles in white-light microscopy (the top-row) and in the UV-light microscopy (the bottle-row), the xylem shows the specific bright blue-fluorescence under the UV light. Scale bars represent 1mm.



**Figure III-38.** Analysis of vascular patterning in DR4AS stems and leaves

**(A)** Hand-cut cross section of stem. The up-row represents white-light microscopy, and the bottle-row is under the UV-light condition, in which the xylem shows the specific bright blue-fluorescence. The enhanced xylem proliferation was observed in the DR4AS stems (right) compared with its WT counterparts (left). Cross sections of stem were cut at the same level between the WT and AS. **(B)**

Cross section of mid-rib. The *DR4AS* exhibit quite similar profiles or slightly increased xylem proliferation. The similar results were also observed in the cross section of petiole (data not shown). Scale bars represent 1mm.

## C- Three *DR4* sequences

Oligonucleotide primers *DR4C-F* and *DR4C-R* were designed in order to amplify the C-terminal sequence of *DR4* gene. Unexpected, the PCR products using tomato cDNA as template showed always two bands when migrated on agarose gel 2% and ethidium bromide-stained, in which one was correspond to the expected size (720 bp) the other one is one hundred base pairs more (830 bp). Therefore both two bands were cloned in *pGEM-T easy* vector (Promega), the plasmids containing each of those bands were collected by mini-prep, and then performed DNA sequencing. The second time unexpected, three different sequences were obtained, they were named as *DR4H*, *DR4B*, and *DR4L* respectively (Figure III-39A). Where, *DR4B* correspond the 'classic' one we obtained before, corresponding to the partial GenBank clones *LeAux/IAA4*. While the *DR4H*, the longest one, and the *DR4L*, the shortest one, were obtained for the first time. Based on sequence analysis, the longest one *DR4H* was tentatively concluded as pre-message RNA for *DR4B*, as the 117 base pairs sequence (more than *DR4B*) in *DR4H* segment shows a typical intron structure: 5'GT .....AG 3'. However, it arise new questions: first, are they issued different genes or issued the same gene but undergone different splicing? Second, the pre-message RNAs were unstable and rare to detect, if it was true that *DR4H* was pre-mRNA, why it presented abundantly? Third, does this pre-mRNA transcripts accumulation have organ or tissue preference? Fours, as in the *DR4AS* plant pre-mRNA transcripts *DR4H* was present (Figure III-Figure III-39B), instead of degraded by the antisense gene silence mechanism, so why it was not silenced? At first, we proposed one tentative model, that *DR4H* transcripts work as the stock of *DR4B* transcripts, the *DR4B* protein are normally thought would be degraded quickly in responses the special signal such as auxin, at this condition, the *DR4H* would be quickly processed to its mature mRNA: *DR4B*, hence, formed the sensitive and efficient feedback mechanism. However, the further study of expression pattern showed that *DR4H* seemed not respond to the auxin treatment. Thereby, *DR4H*-stock hypothesis does not work. The further study may explain the "three *DR4* sequences story"

## A

## Alignment of 3 Different DR4 fragments

```

1
DR4H ACAGGTTGTT GGTGGCCAC CCATTCGATC TTTTAGAAAG AACACTCTAG CCTCTGCCTC GAAGAATAAC
DR4B ACAGGTTGTT GGTGGCCAC CCATTCGATC TTTTAGAAAG AACACTCTAG CCTCTGCCTC GAAGAATAAC
DR4L ACAGGTTGTT GGTGGCCAC CCATTCGATC TTTTAGAAAG AACACTCTAG CCTCTGCCTC GAAGAATAAC

71
DR4H GAAGAGGTTG ACGGAAAAGC TGGCTCACCA GCTCTTTTTA TTAAGGTCAG CATGGATGGT GCTCCCTATT
DR4B GAAGAGGTTG ACGGAAAAGC TGGCTCACCA GCTCTTTTTA TTAAGGTCAG CATGGATGGT GCTCCCTATT
DR4L GAAGAGGTTG ACGGAAAAGC TGGCTCACCA GCTCTTTTTA TTAAGG---- ----

141
DR4H TGAGGAAAGT GGACCTCAGA ACCTGTCTG CATACTAGGA GCTATCTTCT GCTCTTGAAA AAATGTTTCA
DR4B TGAGGAAAGT GGACCTCAGA ACCTGTCTG CATACTAGGA GCTATCTTCT GCTCTTGAAA AAATGTTTCA
DR4L -----

211
DR4H CTGTTTTTACA ATAGGTTAGC TATTTTGGAG CCTTTAGTAG CTCATAATTT CATTGATTGG ATTTAAATAG
DR4B CTGTTTTTACA ATAG----- ----
DR4L -----

281
DR4H TAAGATACAT GTTCATGATA TTTCCATTGC CCTAGTFACT GCTACTTCCT TGTTATGACA GGTCAAATATG
DR4B ----- -GTCAATATG
DR4L ----- --TCAATATG

351
DR4H GATCTCATGG AGCTCCTGGG AAGGATATGT TAAGTGAGAG CAAATTGAAG GATTGCTTC ATGGATCTGA
DR4B GATCTCATGG AGCTCCTGGG AAGGATATGT TAAGTGAGAG CAAATTGAAG GATTGCTTC ATGGATCTGA
DR4L GATCTCATGG AGCTCCTGGG AAGGATATGT TAAGTGAGAG CAAATTGAAG GATTGCTTC ATGGATCTGA

421
DR4H GTATGTTTCT ACTTACGAAG ATAAGGATGG GGAAGGATGG CTTGTCCGTTG ATGTCCCCTG GGAGATGTTT
DR4B GTATGTTTCT ACTTACGAAG ATAAGGATGG GGAAGGATGG CTTGTCCGTTG ATGTCCCCTG GGAGATGTTT
DR4L GTATGTTTCT ACTTACGAAG ATAAGGATGG GGAAGGATGG CTTGTCCGTTG ATGTCCCCTG GGAGATGTTT

491
DR4H ATCGATACTT GCAAAAGGTT GAGGATCATG AAAGGTTTCA ATGCCATTGG CCTGGCCCCA AGGGCTATGG
DR4B ATCGATACTT GCAAAAGGTT GAGGATCATG AAAGGTTTCA ATGCCATTGG CCTGGCCCCA AGGGCTATGG
DR4L ATCGATACTT GCAAAAGGTT GAGGATCATG AAAGGTTTCA ATGCCATTGG CCTGGCCCCA AGGGCTATGG

561
DR4H AAAAGTGTCT GAGCAGAAAT TAGCCTATCT ACACATGATT AAAGCTATCC ATCCAGCATT TCTAGTAGGC
DR4B AAAAGTGTCT GAGCAGAAAT TAGCCTATCT ACACATGATT AAAGCTATCC ATCCAGCATT TCTAGTAGGC
DR4L AAAAGTGTCT GAGCAGAAAT TAGCCTATCT ACACATGATT AAAGCTATCC ATCCAGCATT TCTAGTAGGC

631
DR4H GAGGAAGACA CAAAAACGG GTGAAGAGCT CATAAAGTTG AATCCTTTTT AAAGGCATGA ACAGTAGCTG
DR4B GAGGAAGACA CAAAAACGG GTGAAGAGCT CATAAAGTTG AATCCTTTTT AAAGGCATGA ACAGTAGCTG
DR4L GAGGAAGACA CAAAAACGG GTGAAGAGCT CATAAAGTTG AATCCTTTTT AAAGGCATGA ACAGTAGCTG

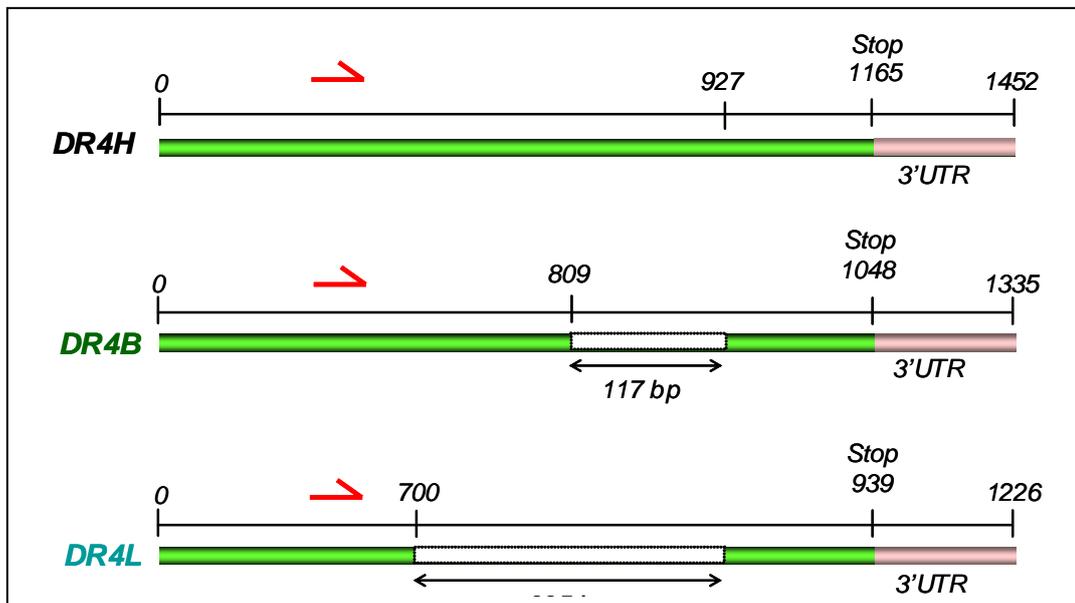
701
DR4H GTTGTTGGTC TTGGATAGCT CAGCATGGGT AGATGCTTTA CCTGATTACG ACATGGTGTT CTTTATATT
DR4B GTTGTTGGTC TTGGATAGCT CAGCATGGGT AGATGCTTTA CCTGATTACG ACATGGTGTT CTTTATATT
DR4L GTTGTTGGTC TTGGATAGCT CAGCATGGGT AGATGCTTTA CCTGATTACG ACATGGTGTT CTTTATATT

771
DR4H TGCCTAATAT ATGTCCTCTT TTAAGTTTTT TTTTATCATG TCTATTTAAA AAACCGTTTG ATATTGGAGT
DR4B TGCCTAATAT ATGTCCTCTT TTAAGTTTTT TTTTATCATG TCTATTTAAA AAACCGTTTG ATATTGGAGT
DR4L TGCCTAATAT ATGTCCTCTT TTAAGTTTTT TTTTATCATG TCTATTTAAA AAACCGTTTG ATATTGGAGT

841
DR4H TTGTCTGCAA ACTAATGTGT GTTGCAGAA
DR4B TTGTCTGCAA ACTAATGTGT GTTGCAGAA
DR4L TTGTCTGCAA ACTAATGTGT GTTGCAGAA
868

```

## B



**Figure III-39.** 3 DR4 sequences. (A) An alignment of three DR4 nucleotide sequences using Multalin version 5.4.1 (<http://prodes.toulouse.inra.fr/multalin>). The typical intron structure is shared in grey: 5'GT.....AG 3'. The identical sequences are in red, two out of three identical sequences are in blue, the unique sequence is in black (fragment in DR4H). (B) Scheme of 3 different DR4 nucleotide sequences. 3'UTR: 3 terminal untranslated region. Stop codon is marked by stop with the position. Red fletch present the relative position of prime used for sequencing. Start codon is marked by 0. The discontinuous lines represent the gap in DR4B (117 bp) and in DR4L (225 bp) respectively, their position is indicated also.



## *CHAPTER IV*

### *General Discussion and Perspectives*

---



## A- General Discussion

### A-1- The first phenotype associated with *aux/iaa* loss-of-function mutant

The *DR4/IAA4* is the new discovered member of the tomato *Aux/IAA* gene family. Screens for Arabidopsis mutants with altered auxin responses or morphological phenotypes have identified mutations in nine different *Aux/IAA* genes; all of these mutations are believed to cause a gain-of-function of the corresponding genes. Moreover, each of these mutations changes a single amino acid in domain II of the corresponding protein and confers more stability to these short-lived nuclear proteins. Loss-of-function *aux/iaa* mutations have been reported by screening for intragenic suppressors of gain-of-function alleles (Timpote et al., 1994; Rouse et al., 1998; Tian and Reed, 1999), or for insertions among T-DNA-insertional mutant populations (Nagpal et al., 2000). Several of the intragenic suppressors create premature stop codons (*iaa3/shy2-24*) or shift in the reading frame; others affect single amino acids in one or the other conserved domains. The T-DNA insertion mutation that has been described in *AXR2/IAA7* lacks the corresponding transcript and is probably a null allele (Nagpal et al., 2000). However, in contrast to their gain-of-function counterparts, all of the examined loss-of-function *aux/iaa* mutations have relatively minor effects on growth and development (Reed, 2001; Liscum and Reed, 2002). The subtlety of these phenotypes suggests that other *Aux/IAA* proteins may compensate for absence of one of them in these mutants. This could occur if the genes are functionally redundant, as it seems likely considering the phylogeny (Figure III-1B). A feedback regulatory mechanism might also operate to buffer the effects of the loss of a single protein function. Efforts are currently continuing in several laboratories to find insertion mutations in additional *Aux/IAA* genes (Liscum and Reed, 2002) or to cross mutants affected in different members of the *Aux/IAA* gene family to create double and multiple mutations in these target genes.

*DR4* was initially isolated in our laboratory from a tomato cDNA library using degenerate primers targeted to conserved domains of *Aux/IAA* gene-family (Jones et al., 2002). The isolated clone was found to correspond to the partial GenBank clone *LeAux/IAA4* (Nebenfuhr et al., 2000), a tomato *Aux/IAA* gene. In the present work, antisense strategy was carried out to efficiently down-regulate *DR4* transcript accumulation in tomato transgenic plants DR4AS lines, which mimics situation encountered in loss-of-function *aux/iaa* mutants.

Interestingly, in contrast to all loss-of-function mutants described to date in Arabidopsis, the DR4AS lines exhibit distinct pleiotropic phenotypes which reveal overlapping and distinct roles of *Aux/IAA* genes in development. The characterisation of these *DR4* knock-down lines provides a new insight on the function of *Aux/IAA* genes that could not be acquired from the gain-of-function mutants. *Aux/IAA* genes are present as multigene families in Arabidopsis (Abel et al., 1995a), tobacco (Dargeviciute et al., 1998), tomato (Nebenfuhr et al., 2000), soybean (Ainley et al., 1988), pea and mung bean (Yamamoto, 1994), Arabidopsis contains 29 *Aux/IAA* genes (Reed, 2001). The Arabidopsis *IAA9* and *IAA8* are most likely the orthologs of tomato *DR4*. These closely related genes form a subclade quite distinct from the other members (Figure III-1B) (Abel et al., 1995a; Nebenfuhr et al., 2000; Rogg et al., 2001; Liscum and Reed, 2002). Tomato *DR4* and related genes from other species differ from other *Aux/IAA* members in several aspects such as the high level of their basal expression and its ubiquitous and constitutive spatial and temporal pattern, as well as the slow and slight auxin induction kinetics. In recent years, accumulating data on several *Aux/IAA* genes were produced on the model plant Arabidopsis, yet there is no report on mutations affecting either *IAA9* or *IAA8* with the exception one T-DNA insertion on the last several amino acid sequence of *IAA8* but with no further description of this mutant. To our knowledge, the phenotypes associated with *DR4* down-regulation presented here provide the first phenotypic characterisation of what could be considered as a loss-of-function mutation. These data reveal novel and distinct roles for *Aux/IAA* genes such as the control of fruit set and development, leaf morphogenesis and vein branching.

#### **A-2- *DR4*: a key regulatory gene in fruit set and development**

It is well known for a long time that auxin is involved in fruit set and development, but the molecular mechanism on how auxin regulates these processes still mostly unknown. Recent studies suggested that early stages of fruit development in tomato are regulated, at least in part, by auxin-mediated gene expression (Balbi and Lomax, 2003). Our data clearly show that auxin is involved in tomato fruit set and development and bring direct evidence that this hormone action is mediated by *DR4*, a member of the *Aux/IAA* family, who acts as a negative regulator of auxin response.

It has been already reported that exogenous application of auxin or auxin transport inhibitors induce fructification in the absence of pollination and fertilisation, then stimulates formation parthenocarpic fruit. Higher auxin levels were also found in ovaries of

parthenocarpic fruits (Mapelli et al., 1978; Mapelli and Mombardi, 1982). The fruit set and development are usually triggered immediately following flower fertilisation. It was then hypothesized that pollination activates auxin signalling where auxins were produced by the pollen tube as it grows through the style and also by the embryo and endosperm in the developing seeds after the fertilisation. It is largely accepted that fruit set and growth depend on these sources of auxin. Two peaks of auxin level occur distinctly during the time course of fruit development which is indicative of their important role in this process (Gillaspy et al., 1993). The first peak occurs 10 days after anthesis, coinciding with the beginning of cell expansion; the second peak appears in the final embryo development stage, but is absent in the pericarp. In non-parthenocarpic tomato varieties, a positive correlation exists between the number of seeds and sustained fruit growth (Hobson and Davies, 1976). Lopsided or malformed fruit form correspond to fruit where seeds do not develop in one part of the fruit while retarded fruit development corresponding to the absence of seeds (Nitsch et al., 1960). Embryo is believed to be the source of the second auxin peak (Balbi and Lomax, 2003) and accordingly, in parthenocarpic fruits, this second peak is not detected and fruits are correspondingly smaller (Mapelli et al., 1978). The above arguments indicate that the presence of auxin is positively correlated with successful fruit set and subsequent fruit development. However, the necessary link between auxin signalling and fructification remains questionable. In the tomato *dgt* mutant, fruit set is dramatically reduced and fruit weight is decreased along with a reduction in the number of locules and seeds. In *dgt* mutants, auxin metabolism and transports are not affected but auxin responsiveness is partially disrupted indicating that disruption of auxin signalling may affect normal fruit set and development. Aux/IAAs are thought to act as transcriptional repressor of auxin-regulated gene expression (Guilfoyle, 1999; Liscum and Reed, 2002) and for the DR4AS lines described in this study, lowering the level of DR4 proteins may largely release auxin-dependent mechanisms that are responsible for inducing fruit set in the absence of fertilization and successful fruit. Several *Arabidopsis aux/iaa* mutants also exhibit reproductive alterations as part of their phenotypes. The *iaa28-1* mutant has a lower seed yield, smaller siliques and shorter inflorescence internodes (Rogg et al., 2001). Similarly, the *axr3/iaa17* have reduced seed set, and exhibits unbranched inflorescence associated with enhanced apical dominance (Leyser et al., 1996).

The fertilization-independent fruit set can also occur either naturally in genetic parthenocarpic fruits or as a result of plant growth regulator (PGRs) applications such as gibberellins (mainly) and cytokinins. Genetic parthenocarpy has been widely studied in

tomato, such as *pat*, *pat-2*, and *pat-3/pat-4*. In these three genetic systems, the mechanism necessary for fruit set and development seems to be switched on before pollination and fertilization (Fos et al., 2003). Recent studies revealed higher accumulation of GAs during parthenocarpic fruit set and growth in *pat-2* and *pat-3/pat-4* mutants (Fos et al., 2000; Fos et al., 2001; Fos et al., 2003). Consistent with this, parthenocarpic tomato fruits induced by exogenous application of auxin contain high levels of GAs (Koshioka et al., 1994) and it was also shown that in pea shoots and tobacco stems, auxin promotes gibberellin biosynthesis (Ross et al., 2000). Very recently, it was reported that auxin promotes Arabidopsis root growth by modulating gibberellin signalling (Fu and Harberd, 2003). In addition, the fruit set can be inhibited by paclobutrazol (a gibberellins synthesis inhibitor) and can be further reversed by GA<sub>3</sub> application (Fos et al., 2000). Therefore, whether auxin induces parthenocarpy via a gibberellin signalling pathway or whether the two hormones induce development of parthenocarpic fruit by parallel pathways is still to be elucidated.

Parthenocarpy is advantageous for the consumer, the producer and the food industry. The main advantage is that fruit set is not inhibited by the unfavorable environmental conditions; for example, tomato pollination is impaired when either night temperature is lower than 13°C or when day temperature is higher than 38°C for five hours (Pandolfini et al., 2002). Thus, successful development of fruit in the absence of pollination ensures yield stability. Moreover, parthenocarpic fruits are seedless, which improves the quality of the fruits in many species because seeds are usually hard to taste and to digest, and often their presence is associated with bitter substances (such as eggplant). In addition, parthenocarpy can also be used to have an earlier harvest or a late fruit ripening (Varoquaux et al., 2000; Pandolfini et al., 2002). Parthenocarpic cultivars exist in many species; however, they have not been widely used in horticulture because high yield (the fruits are much smaller) and good fruit quality have been seldom combined with parthenocarpy. Interestingly, DR4AS parthenocarpic fruits have the same appearance than their wild type counterparts including the size, the skin and flesh colour, flesh consistency and fresh weight. Even better, the parthenocarpic fruits accumulate higher percentage of total soluble solids which is expected to have a positive impact on their sensorial and technological characteristics. The feature described above for DR4AS parthenocarpic fruits represent a real advantages that contribute to improve both yield and flavour and further reduce the processing costs for the industrial tomatoes.

In the literature, auxin and ethylene have been often reported to have antagonist effects on fruit ripening. However, preliminary characterisation of DR4AS transgenic fruit indicated

that down-regulation of the *DR4* gene did not significantly affect the ripening process. With the exception of the increased sugar accumulation mentioned above, DR4AS fruit did not exhibit any visible alteration of ripening-associated processes such as the climacteric burst of ethylene production, pigment biosynthesis and flesh softening. Furthermore, there is no significant delay or precocious ripening. However, these data do not definitely rule out the possibility that auxin could play an active role in the triggering and spreading out of the ripening process via a signalling pathway independent from the *DR4* gene. On the other hand, the absence of altered ripening phenotypes may only result from a functional redundancy within the *Aux/IAA* multigene family. Genetic crosses between lines affected in different *Aux/IAA* genes aimed at creating double and multiple mutants may be required to escape this phenomenon of functional redundancy and to efficiently unmask putative ripening-related phenotypes.

### **A-3- *DR4*: a crucial gene in leaf morphogenesis**

One of the most obvious phenotype exhibited by the DR4AS plant is the simple leaf architecture instead of the normal tomato unipinnate compound leaves. Our data clearly indicate that down-regulation of *DR4* accounts for this distinct phenotype and reveal that *DR4* is a key switch gene in the control of leaf morphogenesis, as well as in the vascular vein system patterning. Interestingly, our study reveals for the first time that the expression level of *DR4* is also largely down-regulated in the natural tomato mutant *entire* who exhibits a simple leaf phenotype.

A number of observations have implicated auxin in the formation of vascular tissues in plant organs. These include vascular strand formation in response to local auxin application (Sachs, 2000), the effects of impaired auxin transport on vascular patterns (Mattsson et al., 1999) and suggestive phenotypes of Arabidopsis auxin-response mutants such as *pin1*, *mp*, *axr6*, and *bdl* (Table I-1) (Przemeck et al., 1996; Galweiler et al., 1998; Hobbie et al., 2000; Gaedeke et al., 2001; Hamann et al., 2002). The recent studies showed also that IAA distribution coincides with vascular differentiation pattern during Arabidopsis leaf ontogeny, by using both immunocytochemistry and the IAA-driven GUS activity of *Aux/IAA* promoter::GUS constructs to detect IAA (Avsian-Kretchmer et al., 2002). In young Arabidopsis leaf primordia, molecular auxin response patterns presage sites of procambial differentiation, and auxin signals are instrumental in patterning Arabidopsis leaf vasculature (Mattsson et al., 2003). All of the above arguments indicated that the abnormality of auxin

signalling will result in the defects in normal leaf ontogeny and/or leaf morphogenesis. In accordance, the tomato auxin over-synthesis mutant, generated by over-expressing *iaaM* gene (the auxin-biosynthesis gene) under the 35S promoter, also exhibits the simple leaf phenotype (personal communication). Moreover, our data show that treatment with auxin transport inhibitor, which leads to over-accumulation of auxin in the young leaves (the site of synthesis), also results in tomato leaves exhibiting the simple leaf architecture (Figure III-20). It further indicates that in a tomato genetic background corresponding to compound-leaves, an auxin over-response results in the induction of simple leaf architecture. Taking together, these data strongly suggest that the simple leaf of DR4AS is the consequence of the over-auxin responses during leaf ontogeny and/or in several loci in the leaves.

Although it was demonstrated that *DR4* is a key player in the switch from simple to compound leaves, it might not be the only factor impacting this process. Therefore, we examined the *DR4* expression in other tomato leaf-morphology mutants like *lanceolate* who shows simple-leaf and *petroselinum* who shows super-compound leaves. The results did not reveal any difference in the *DR4* expression level between wild type and these latter leaf mutants. Moreover, it was reported that over-expression of the maize homeobox gene *Knotted-1 (Kn1)* in the tomato resulted in “super compound” phenotype with each leaf having up to 2000 leaflets. However, our study shows that the expression level of *TKn1*, the tomato ortholog of *Kn1*, was not affected in the leaves and other organs of DR4AS lines. These data are suggestive of a number of independent and possibly redundant pathways that control leaf ontogeny and morphogenesis.

#### **A-4- Down-regulation of *DR4* reveals overlapping and distinct roles for *Aux/IAA* genes**

The *aux/iaa* gain-of-function mutations have been shown to affect various tissues and developmental responses, including root or shoot gravitropism, lateral root formation, shoot apical dominance, stem elongation, leaf expansion and leaf formation in the dark (Table I-3). In general, the phenotype can be explained in terms of reduced auxin response, and consistently, some auxin-induced genes are expressed at lower levels in *axr2-1* and *shy2-2* mutants (Reed, 2001). Most of the gain-of-function mutants like *iaa3/shy2-2*, *iaa6/shy1-1*, *iaa7/axr2-1* and *iaa17/axr3-1*, have shorter hypocotyls than wild type whereas DR4AS seedlings present longer hypocotyl than wild type suggesting that Aux/IAA proteins, including DR4, repress hypocotyl elongation. The majority of gain-of-function mutants such as *iaa3/shy2-2*, *iaa6/shy1-1*, *iaa7/axr2-1*, *iaa12/bdl*, *iaa18-1* and *iaa17/axr3-1*, have up-

curled leaves, abnormalities in vascular venation system formation or leaves in dark whereas DR4AS exhibits asymmetrical simple leaf with the rogues lamina. This indicates that Aux/IAA proteins guide the normal vascular venation system formation, as well as the leaf ontogeny and morphogenesis. Similarly, DR4AS and *iaa18-1* mutant seedling often have fused cotyledons or leaves (Liscum and Reed, 2002), suggesting that Aux/IAA proteins can also regulate phyllotaxic pattern.

The *iaa12/bdl-1* mutant embryos lack roots due to abnormality in the vascular tissue pattern (Hamann et al., 1999). While DR4AS showed increased primary root elongation and displayed more lateral roots formation (Figure III-7 and 8) several mutants have been described that have altered root system formation. These *aux/iaa* mutants reveal either similar or different and sometimes opposite phenotypes suggesting some of the complexity permitted by the large number of Aux/IAA genes. The *iaa28-1* mutant did not initiate normal number of lateral roots even when treated with exogenous auxin, but have the normal gravity responses (Rogg et al., 2001). Mutants *iaa17/ax3-1* have short primary roots and develop adventitious roots (Leyser et al., 1996); *iaa3/shy2* have short primary roots with more root hairs (Tian and Reed, 1999) and *iaa7/axr2-1* have roots of normal length with fewer root hairs (Wilson et al., 1990). It will be interesting to investigate whether these differences reflect expression in different cells or tissues, or whether they reflect functional differences among the corresponding Aux/IAA proteins. However, some Aux/IAA mutations result in distinct phenotypes such as *iaa12/bdl-1* who lacks embryonic root (Hamann et al., 1999), *iaa14/slr* which has no lateral roots (Fukaki et al., 2002), and *iaa19/mgs2* that shows aphototropic hypocotyl (Watahiki and Yamamoto, 1997). The particular feature of DR4AS resides in its ability to set and develop fruit in the absence of pollination and fertilization (Figure III-4, 14; Table III-2).

Overall, these overlapping and distinct roles of Aux/IAA genes suggest that they do mediate distinct cellular processes at virtually all stages of plant growth and development. However, as guides to the normal functions of these genes, the phenotypes should be also regarded with some caution because they might arise from abnormal levels of Aux/IAA protein activities that are higher or lower than any reached in the normal course of the plant development. Ideally, one should compare these results with phenotypes of both gain-of-function and loss-of-function mutations in the same genes. Given the number of Aux/IAA proteins, their possible promiscuity through dimerization and the putative feedback regulation, it will take considerable effort to sort out the precise functions of each one (Reed, 2001).

### A-5- DR4, a negative regulator of auxin response

An increasing number of evidences identified Aux/IAA proteins as key factors mediating auxin responses and revealed their role as transcriptional regulators of auxin-induced gene expression. According to the most widely admitted scheme, at basal auxin levels in plant tissues, Aux/IAAs are relatively stable and can dimerize with ARF proteins preventing their binding to canonical AuxREs (Auxin-response elements) that reside in the promoters of target genes thus blocking their transcription. When auxin levels increase in the tissues, the auxin transduction pathway generates a signal that leads to the degradation of Aux/IAAs proteins by 26S proteasome via SCF complex which results in the release of activated ARF proteins. These active ARF proteins become free to direct transcription of auxin-responsive genes that in turn carry out the required auxin responses (Figure I-15). Apparently, such a model hardly reconciles the auxin inducibility of Aux/IAA with their role as negative regulators of ARFs proteins. A better understanding of the system emerged from recent studies (Tatematsu et al., 2004), that postulate that Aux/IAA and ARFs may form a negative feedback loop to regulate auxin-dependent differential growth responses. Therefore, negative feedback has been proposed to allow tight regulation between auxin abundance and target gene expression depending on cell and tissue type and/or on the particular Aux/IAA proteins themselves (Reed, 2001) which might both activate or inhibit auxin responses during the development.

Abnormality in DR4AS mutants are due to the down-regulation of this tomato *Aux/IAA* gene, which affects the auxin signalling in a way that fits to the model described above and results in altered auxin-dependent growth development. However, our data showed that not all auxin responses are disrupted in DR4AS lines since hypocotyl segments still exhibit auxin-induced elongation. Though auxin metabolism and transport have not yet been investigated in the DR4AS plants, several phenotypes suggest that they are not affected, such as the photo-/gravitropism which are well known to require proper auxin metabolism and transport. In the same line of evidence, DR4AS lines showed even more vigorous growth than wild type plants suggesting that auxin metabolism and transport have not been seriously affected. Taking together, our data suggest that down-regulation of *DR4* only disrupts a specific auxin-regulated process. A tentative scheme depicting the putative mechanism of DR4 action, drawn on the light of the informations gained from the characterisation of DR4AS lines is given in Figure IV-1. That is, down-regulation of *DR4* releases ARF proteins

that can be active in transcribing auxin-regulated genes which results in auxin hypersensitivity and in some cases partial constitutive auxin response. In wild type plants degradation of DR4 protein occurs in the presence of auxin, which decrease the amount of DR4, mimics the situation encountered in DR4AS plants in the absence of auxin. While in the *aux/iaa* gain-of-function mutants, Aux/IAA proteins are more stable and less sensitive to auxin-induced degradation. This enhances the abundance of Aux/IAA proteins and promotes their dimerization with ARFs lowering at the same time the amount of free ARFs that could be available for carrying out the transcription of auxin-regulated genes and therefore results in reduced auxin responses.

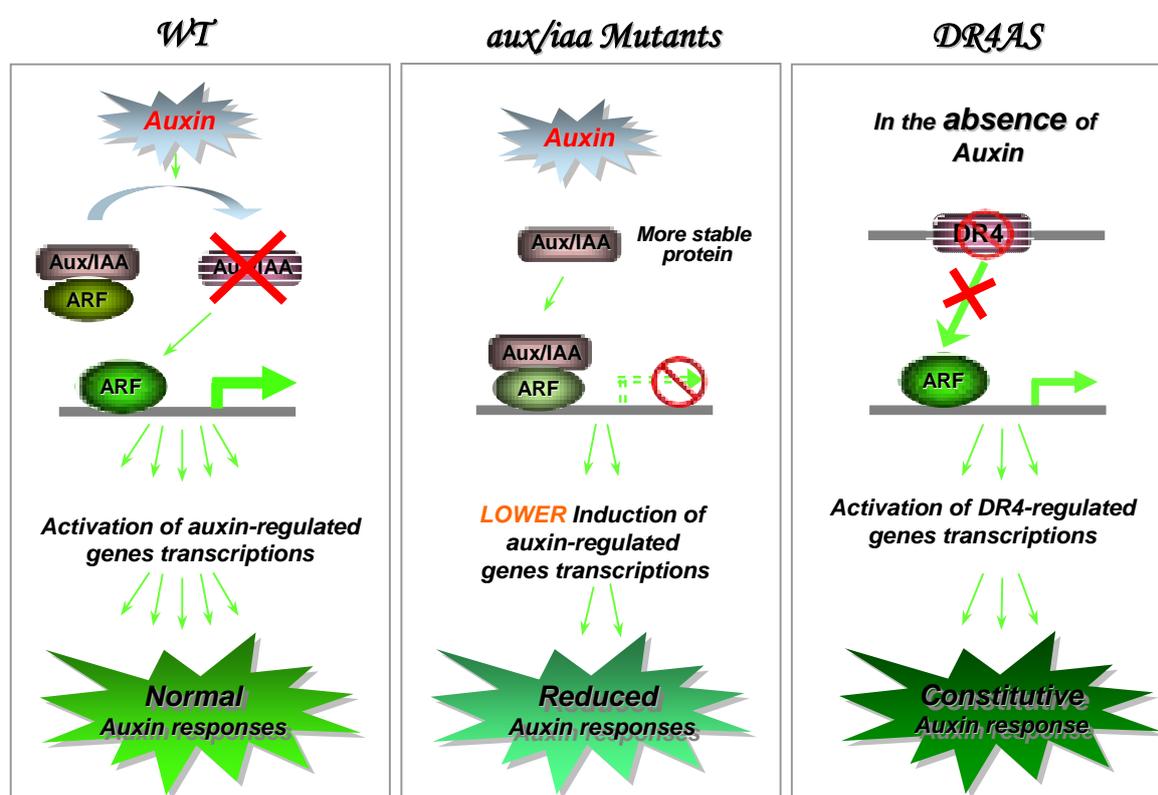


Figure IV-1. Working models for Aux/IAA and DR4 in auxin signalling

In WT, at basal levels of auxin, Aux/IAAs are relative stable and dimerize with ARF proteins, reducing transcriptions by preventing the recruitments of activating ARFs on AuxREs of auxin induced genes; increasing auxin levels cause an increasing proportion of Aux/IAAs to be degraded, allowing the formation of a greater number of free ARF or ARF-ARF dimmers on AuxREs, and hence higher levels of transcription of auxin-responsive genes, which, in turn, carry out the required auxin responses. While in the *aux/iaa* gain-of-function mutants, each mutation confer the corresponding Aux/IAA protein more stable even with higher auxin level, and occupying with the corresponding ARFs then block their transcription activation, in turn, the mutant plants exhibit reduced auxin responses. On the contrary, in DR4AS plant, due to the antisense technique, the *DR4* transcripts are largely down-regulated, therefore few DR4 protein yield and then liberate its ARF partners, who keep

the transcriptions activating even in the absence of auxin signal, and the transcription products of carry out required responses, hence the DR4AS plants exhibit partial constitutive auxin responses.

## **B- Outcome of the study and perspectives**

While our study clearly demonstrated the role of *DR4*, a tomato *Aux/IAA* gene, in specific developmental processes; the target genes regulated by DR4 and hence the molecular mechanisms by which it impacts auxin-dependent plant growth and development are yet to be elucidated. Identifying both DR4-target executor and regulatory genes as well as interacting partners is mandatory to decipher the complex process of auxin signalling that remains poorly understood. In particular, this will represent an important step towards uncovering the mechanism of differential growth and developmental responses to auxin in different plant tissues and organs. The unique genetic and biological resources generated in this study offer new opportunities to address the role of auxin in plant growth and organ differentiation.

The use of high throughput techniques to explore gene expression is a dedicated approach to unravel the transcriptional changes associated with *DR4* down-regulation in DR4AS lines. Tomato DNA microarrays containing more than 4000 unigenes expressed in the fruit are being produced by our group and should be available for use in the near future. These global transcriptomics studies are expected to reveal the regulatory targets of DR4 proteins, and identify the executor genes that account for fruit initiation, thereby providing clues on how auxin-regulated developmental processes unfold. Moreover, to gain further insight on the developmental functions of *DR4*, over-expressing plants (*DR4* Sense) have been generated and their phenotypical and molecular analyses are currently underway. Comparing the molecular and physiological data that will emerge from the characterisation of down-regulated and over-expressing lines will enlarge the picture on the role of *DR4* in various developmental processes.

As part of the general project aiming at the elucidation of the role of auxin in plant development, the knock-down of other *Aux/IAA* (*DR3*, *DR8*) or *ARF* (*DR12*) genes in the tomato undertaken in our laboratory and their subsequent characterisation will help identifying specific roles for each of these transcriptional regulators in mediating various auxin responses. However, a functional redundancy within the *Aux/IAA* multigene family is likely expected and may therefore mask any effect in these transgenic lines, so double and

multiple mutants will be generated by genetic crosses between DR3AS, DR8AS, DR12AS and the DR4AS line. Physiological and molecular analysis of these multiple mutants could reveal some yet unidentified aspects of the Aux/IAA functions. On the other hand, a number of tomato *Aux/IAA* (*DR1*, *DR3*, *DR4* and *DR8*) have been over-expressed in Arabidopsis aiming at mimicking the gain-of-function mutations described in this model plant. This ectopic expression is expected to unmask the specific role, if any, of each of these Aux/IAs.

Since it is postulated here that *DR4* might modulate auxin-responsive gene transcription through interaction with ARF proteins, yeast two-hybrid system will be used to carry out protein/protein interaction assays in order to identify specific ARFs that interact with DR4 protein. The promoter region of two tomato *Aux/IAA* that show different pattern of expression have been isolated and fused to *GUS* or *GFP* reporter genes in order to carry out histological analyses of their activities and to study their responsiveness to different hormones and non-hormones signals.



## *Bibliographic References*

---



---

## Reference

- Abel, S., and Theologis, A.** (1994). Transient transformation of Arabidopsis leaf protoplasts: a versatile experimental system to study gene expression. *Plant J* **5**, 421-427.
- Abel, S., and Theologis, A.** (1996). Early genes and auxin action. *Plant Physiol* **111**, 9-17.
- Abel, S., Oeller, P.W., and Theologis, A.** (1994). Early auxin-induced genes encode short-lived nuclear proteins. *Proc Natl Acad Sci U S A* **91**, 326-330.
- Abel, S., Nguyen, M.D., and Theologis, A.** (1995a). The PS-IAA4/5-like family of early auxin-inducible mRNAs in Arabidopsis thaliana. *J Mol Biol* **251**, 533-549.
- Abel, S., Nguyen, M.D., Chow, W., and Theologis, A.** (1995b). ACS4, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in Arabidopsis thaliana. Structural characterization, expression in Escherichia coli, and expression characteristics in response to auxin [corrected]. *J Biol Chem* **270**, 19093-19099.
- Ainley, W.M., Walker, J.C., Nagao, R.T., and Key, J.L.** (1988). Sequence and characterization of two auxin-regulated genes from soybean. *J Biol Chem* **263**, 10658-10666.
- Ampomah-Dwamena, C., Morris, B.A., Sutherland, P., Veit, B., and Yao, J.L.** (2002). Down-regulation of TM29, a tomato SEPALLATA homolog, causes parthenocarpic fruit development and floral reversion. *Plant Physiol* **130**, 605-617.
- Anai, T., Kono, N., Kosemura, S., Yamamura, S., and Hasegawa, K.** (1998). Isolation and characterization of an auxin-inducible SAUR gene from radish seedlings. *DNA Seq* **9**, 329-333.
- Asahira, T., and Hosoki, T.** (1977). In vitro studies of controlling tomato puffiness by growth regulators. *Sci. Hort.* **7**, 319-328.
- Avivi, Y., Lev-Yadun, S., Morozova, N., Libs, L., Williams, L., Zhao, J., Varghese, G., and Grafi, G.** (2000). Clausa, a tomato mutant with a wide range of phenotypic perturbations, displays a cell type-dependent expression of the homeobox gene LeT6/TKn2. *Plant Physiol* **124**, 541-552.
- Avsian-Kretchmer, O., Cheng, J.C., Chen, L., Moctezuma, E., and Sung, Z.R.** (2002). Indole acetic acid distribution coincides with vascular differentiation pattern during Arabidopsis leaf ontogeny. *Plant Physiol* **130**, 199-209.
- Balbi, V., and Lomax, T.L.** (2003). Regulation of early tomato fruit development by the diageotropica gene. *Plant Physiol* **131**, 186-197.
- Baldi, B.G., Maher, B.R., Slovak, J.P., and Cohen, D.J.** (1991). Stable isotope labelling, in vitro, of D- and L-tryptophan pools in lemna gibba and the low incorporation of label into IAA. *Plant Physiol* **95**, 1203-1208.
- Bandurski, R.S., Schulze, A., Jensen, P., Desrosiers, M., Epel, B., and Kowalczyk, S.** (1992). The mechanism by which an asymmetric distribution of plant growth hormone is attained. *Adv Space Res* **12**, 203-210.
- Bangerth, F.** (1994). Response of cytokinin concentration in the xylem exudate of bean (*Phaseolus vulgaris* L.) plants to decapitation and auxin treatment, and relationship to apical dominance. *planta* **194**.
- Barton, M.** (2001). Leaving the meristem behind: regulation of KNOX genes. *Genome Biol* **2**, REVIEWS1002.

- Bauly, J.M., Sealy, I.M., Macdonald, H., Brearley, J., Droge, S., Hillmer, S., Robinson, D.G., Venis, M.A., Blatt, M.R., Lazarus, C.M., and Napier, R.M.** (2000). Overexpression of auxin-binding protein enhances the sensitivity of guard cells to auxin. *Plant Physiol* **124**, 1229-1238.
- Becker, D., and Hedrich, R.** (2002). Channelling auxin action: modulation of ion transport by indole-3-acetic acid. *Plant Mol Biol* **49**, 349-356.
- Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A.** (1996). Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. *Science* **273**, 948-950.
- Bennett, S.R.M., Alvarez, J., Bossinger, G., and Symth, D.R.** (1995). Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. *Plant J* **8**, 505-520.
- Berleth, T., and Mattsson, J.** (2000). Vascular development: tracing signals along veins. *Curr Opin Plant Biol* **3**, 406-411.
- Berleth, T., Mattsson, J., and Hardtke, C.S.** (2000). Vascular continuity and auxin signals. *Trends Plant Sci* **5**, 387-393.
- Beveridge, C.A., Ross, J.J., and Murfet, I.C.** (1994). Branching Mutant rms-2 in *Pisum sativum* (Grafting Studies and Endogenous Indole-3-Acetic Acid Levels). *Plant Physiol* **104**, 953-959.
- Beyer, E.M., and Morgan, P.W.** (1971). Abscission: the role of ethylene modification of auxin transport. *Plant Physiol* **48**, 208-212.
- Beyer, E.M., and Quebedeaux, B.** (1974). Parthenocarpy in cucumber: mechanism of action of auxin transport inhibitors. *J Am Soc Hortic Sci* **99**, 385-390.
- Bird, C.R., Smith, C.J., Ray, J.A., Moureau, P., Bevan, M.W., Bird, A.S., Hughes, S., Morris, P.C., Grierson, D., and Schuch, W.** (1988). The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. *Plant Mol Biol* **11**, 651-662.
- Blatt, M.R., and Thiel, G.** (1994). K<sup>+</sup> channels of stomatal guard cells: bimodal control of the K<sup>+</sup> inward-rectifier evoked by auxin. *Plant J* **5**, 55-68.
- Boerjan, W., Cervera, M.T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M., Van Onckelen, H., Van Montagu, M., and Inze, D.** (1995). Superroot, a recessive mutation in Arabidopsis, confers auxin overproduction. *Plant Cell* **7**, 1405-1419.
- Bohnert, H.J., and Bangerth, F.** (1988). Cell number, cell size and hormone levels in semi-isogenic mutants of *Lycopersicon pimpinellifolium* differing in fruit size. *Physiol Plant* **72**, 316-320.
- Booker, J., Chatfield, S., and Leyser, O.** (2003). Auxin acts in xylem-associated or medullary cells to mediate apical dominance. *Plant Cell* **15**, 495-507.
- Boonsirichai, K., Guan, C., Chen, R., and Masson, P.H.** (2002). Root gravitropism: an experimental tool to investigate basic cellular and molecular processes underlying mechanosensing and signal transmission in plants. *Annu Rev Plant Biol* **53**, 421-447.
- Bouzayan, M., Latche, A., Pech, J.C., and Marigo, G.** (1989). Carrier mediated uptake of 1-(malonylamino) cyclopropane-1-carboxylic acid in vacuoles isolated from *Catharantus roseus* cells. *Plant Physiol* **91**, 1317-1322.
- Briggs, W.R., and Huala, E.** (1999). Blue-light photoreceptors in higher plants. *Annu Rev Cell Biol* **15**, 33-62.
- Burg, S.P., and Burg, E.A.** (1966). The interaction between auxin and ethylene and its role in plant growth. *Proc Natl Acad Sci U S A* **55**, 262-269.

- Casimiro, I., Marchant, A., Bhalerao, R.P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inze, D., Sandberg, G., Casero, P.J., and Bennett, M.** (2001). Auxin transport promotes Arabidopsis lateral root initiation. *Plant Cell* **13**, 843-852.
- Catala, C., Ostin, A., Chamarro, J., Sandberg, G., and Crozier, A.** (1992). Metabolism of indole-3-acetic acid by pericarp discs from immature and mature tomato (*Lycopersicon esculentum Mill*). *Plant Physiol* **100**, 1457-1463.
- Celenza, J.L., Jr., Grisafi, P.L., and Fink, G.R.** (1995). A pathway for lateral root formation in Arabidopsis thaliana. *Genes Dev* **9**, 2131-2142.
- Chatfield, S.P., Stirnberg, P., Forde, B.G., and Leyser, O.** (2000). The hormonal regulation of axillary bud growth in Arabidopsis. *Plant J* **24**, 159-169.
- Chen, R., Rosen, E., and Masson, P.H.** (1999). Gravitropism in higher plants. *Plant Physiol* **120**, 343-350.
- Chen, R., Guan, C., Boonsirichai, K., and Masson, P.H.** (2002). Complex physiological and molecular processes underlying root gravitropism. *Plant Mol Biol* **49**, 305-317.
- Christensen, S.K., Dagenais, N., Chory, J., and Weigel, D.** (2000). Regulation of auxin response by the protein kinase PINOID. *Cell* **100**, 469-478.
- Cleland, R.E., Buckley, G., Nowbar, S., Lew, N.M., Stinemetz, C., Evans, M.L., and Rayle, D.L.** (1991). The pH profile for acid-induced elongation of coleoptile and epicotyl sections is consistent with the acid-growth theory. *Planta* **186**, 70-74.
- Cline, M.G.** (2000). Execution of the auxin replacement apical dominance experiment in temperate woody species. *Am J Bot* **87**, 182-190.
- Coenen, C., and Lomax, T.L.** (1997). Auxin-cytokinin interactions in higher plants: old problems and new tools. *Trends Plant Sci* **2**, 351-356.
- Coenen, C., and Lomax, T.L.** (1998). The diageotropica gene differentially affects auxin and cytokinin responses throughout development in tomato. *Plant Physiol* **117**, 63-72.
- Coenen, C., Christian, M., Luthen, H., and Lomax, T.L.** (2003). Cytokinin inhibits a subset of diageotropica-dependent primary auxin responses in tomato. *Plant Physiol* **131**, 1692-1704.
- Cohen, J.D.** (1996). In vitro tomato fruit cultures demonstrate a role for indole-3-acetic acid in regulating fruit ripening. *J Amer Soc Hort Sci* **121**, 520-524.
- Collett, C.E., Harberd, N.P., and Leyser, O.** (2000). Hormonal interactions in the control of Arabidopsis hypocotyl elongation. *Plant Physiol* **124**, 553-562.
- Colon-Carmona, A., Chen, D.L., Yeh, K.C., and Abel, S.** (2000). Aux/IAA proteins are phosphorylated by phytochrome in vitro. *Plant Physiol* **124**, 1728-1738.
- Conner, T.W., Goekjian, V.H., LaFayette, P.R., and Key, J.L.** (1990). Structure and expression of two auxin-inducible genes from Arabidopsis. *Plant Mol Biol* **15**, 623-632.
- Dargeviciute, A., Roux, C., Decreux, A., Sitbon, F., and Perrot-Rechenmann, C.** (1998). Molecular cloning and expression of the early auxin-responsive Aux/IAA gene family in Nicotiana tabacum. *Plant Cell Physiol* **39**, 993-1002.
- Darwin, C.** (1880). *The power of Movements in Plants*. John Murray, London.
- David, K., Carnero-Diaz, E., Leblanc, N., Monestiez, M., Grosclaude, J., and Perrot-Rechenmann, C.** (2001). Conformational dynamics underlie the activity of the auxin-binding protein, Nt-abp1. *J Biol Chem* **276**, 34517-34523.

- Davis, P.J.** (1995). Plant hormones: Physiology, Biochemistry and Molecular Biology. Martinus Nijhoff, Dordrecht, Netherlands.
- del Pozo, J.C., and Estelle, M.** (1999a). The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. *Proc Natl Acad Sci U S A* **96**, 15342-15347.
- del Pozo, J.C., and Estelle, M.** (1999b). Function of the ubiquitin-proteasome pathway in auxin response. *Trends Plant Sci* **4**, 107-112.
- del Pozo, J.C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W.M., and Estelle, M.** (2002). AXR1-ECR1-dependent conjugation of RUB1 to the Arabidopsis Cullin AtCUL1 is required for auxin response. *Plant Cell* **14**, 421-433.
- DeLong, A., Mockaitis, K., and Christensen, S.** (2002). Protein phosphorylation in the delivery of and response to auxin signals. *Plant Mol Biol* **49**, 285-303.
- Dengler, N.** (1984). Comparison of leaf development in normal (+/+), *entire* (e/e), and *Lanceolate* (La/+) plant of tomato, *Lycopersicon esculentum* 'Ailsa Craig'. *Bot Gaz* **145**, 66-77.
- Deshaies, R.J.** (1999). SCF and cullin/RING-H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* **15**, 435-467.
- Eckert, M., and Kaldenhoff, R.** (2000). Light-induced stomatal movement of selected Arabidopsis thaliana mutants. *J Exp Bot* **51**, 1435-1442.
- Eklof, S., Astot, C., Sitbon, F., Moritz, T., Olsson, O., and Sandberg, G.** (2000). Transgenic tobacco plants co-expressing Agrobacterium *iaa* and *ipt* genes have wild-type hormone levels but display both auxin- and cytokinin-overproducing phenotypes. *Plant J* **23**, 279-284.
- Emmanuel, E., and Levy, A.** (2002). Tomato mutants as tools for functional genomics. *Current Opinion in Plant Biology* **5**, 112-117.
- Ephritikhine, G., Fellner, M., Vannini, C., Lapous, D., and Barbier-Brygoo, H.** (1999). The *sax1* dwarf mutant of Arabidopsis thaliana shows altered sensitivity of growth responses to abscisic acid, auxin, gibberellins and ethylene and is partially rescued by exogenous brassinosteroid. *Plant J* **18**, 303-314.
- Evans, M.L.** (1985). The action of auxin on plant cell elongation. *CRC Crit Rev Plant Sci* **2**, 317-365.
- Ficcadenti, N., Sestili, S., Pandolfini, T., Cirillo, C., Rotino, G., and Spena, A.** (1999). Genetic engineering of parthenocarpic fruit development in tomato. *Molecular Breeding* **5**, 463-470.
- Firmin, J., and Palme, K.** (2002). Polar auxin transport - old questions and new concepts. *Plant Mol Biol* **49**, 273-284.
- Fischer, C., Speth, V., Fleig-Eberenz, S., and Neuhaus, G.** (1997). Induction of Zygotic Polyembryos in Wheat: Influence of Auxin Polar Transport. *Plant Cell* **9**, 1767-1780.
- Fischer-Iglesias, C., Sundberg, B., Neuhaus, G., and Jones, A.M.** (2001). Auxin distribution and transport during embryonic pattern formation in wheat. *Plant J* **26**, 115-129.
- Fos, M., Nuez, F., and Garcia-Martinez, J.L.** (2000). The gene *pat-2*, which induces natural parthenocarpy, alters the gibberellin content in unpollinated tomato ovaries. *Plant Physiol* **122**, 471-480.
- Fos, M., Proano, K., Nuez, F., and Garcia-Martinez, J.L.** (2001). Role of gibberellins in parthenocarpic fruit development induced by the genetic system *pat-3/pat-4* in tomato. *Physiol Plant* **111**, 545-550.
- Fos, M., Proano, K., Alabadi, D., Nuez, F., Carbonell, J., and Garcia-Martinez, J.L.** (2003). Polyamine Metabolism Is Altered in Unpollinated Parthenocarpic *pat-2* Tomato Ovaries. *Plant Physiol* **131**, 359-366.

- Frenkel, C., and Haard, N.F.** (1973). Initiation of ripening in Bartlett pears with an antiauxin (p-chlorophenoxy) isobutyric acid. *Plant Physiol* **52**.
- Friml, J.** (2003). Auxin transport - shaping the plant. *Curr Opin Plant Biol* **6**, 7-12.
- Friml, J., and Palme, K.** (2002). Polar auxin transport--old questions and new concepts? *Plant Mol Biol* **49**, 273-284.
- Friml, J., Wisniewska, J., Benkova, E., Mendgen, K., and Palme, K.** (2002a). Lateral relocation of auxin efflux regulator PIN3 mediates tropism in Arabidopsis. *Nature* **415**, 806-809.
- Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jurgens, G., and Palme, K.** (2002b). AtPIN4 mediates sink-driven auxin gradients and root patterning in Arabidopsis. *Cell* **108**, 661-673.
- Fu, X., and Harberd, N.P.** (2003). Auxin promotes Arabidopsis root growth by modulating gibberellin response. *Nature* **421**, 740-743.
- Fujii, N., Kamada, M., Yamasaki, S., and Takahashi, H.** (2000). Differential accumulation of Aux/IAA mRNA during seedling development and gravity response in cucumber (*Cucumis sativus* L.). *Plant Mol Biol* **42**, 731-740.
- Fukaki, H., Tameda, S., Masuda, H., and Tasaka, M.** (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. *Plant J* **29**, 153-168.
- Fukuda, H.** (1997). Tracheary element differentiation. *Plant Cell* **9**, 1147-1156.
- Gaedeke, N., Klein, M., Kolukisaoglu, U., Forestier, C., Muller, A., Ansorge, M., Becker, D., Mammun, Y., Kuchler, K., Schulz, B., Mueller-Roeber, B., and Martinoia, E.** (2001). The Arabidopsis thaliana ABC transporter AtMRP5 controls root development and stomata movement. *Embo J* **20**, 1875-1887.
- Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K.** (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science* **282**, 2226-2230.
- Garbers, C., DeLong, A., Deruere, J., Bernasconi, P., and Soll, D.** (1996). A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in Arabidopsis. *Embo J* **15**, 2115-2124.
- Gee, M.A., Hagen, G., and Guilfoyle, T.J.** (1991). Tissue-specific and organ-specific expression of soybean auxin-responsive transcripts GH3 and SAURs. *Plant Cell* **3**, 419-430.
- Gil, P., Liu, Y., Orbovic, V., Verkamp, E., Poff, K.L., and Green, P.J.** (1994). Characterization of the auxin-inducible SAUR-AC1 gene for use as a molecular genetic tool in Arabidopsis. *Plant Physiol* **104**, 777-784.
- Gillaspy, G., Ben-David, H., and Gruissem, W.** (1989). Fruit: a developmental perspective. *Plant Cell* **5**, 1439-1451.
- Gillaspy, G., Ben-David, H., and Gruissem, W.** (1993). Fruit: a developmental perspective. *Plant Cell* **5**, 1439-1451.
- Gray, J., Picton, S., Shabbeer, J., Schuch, W., and Grierson, D.** (1992). Molecular biology of fruit ripening and its manipulation with antisense genes. *Plant Mol Biol* **19**, 69-87.
- Gray, W.M., and Estelle, I.** (2000). Function of the ubiquitin-proteasome pathway in auxin response. *Trends Biochem Sci* **25**, 133-138.
- Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M.** (2001). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature* **414**, 271-276.

- Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M.** (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes Dev* **13**, 1678-1691.
- Guilfoyle, T.** (1999). Auxin-regulated genes and promoters. In *Biochemistry and Molecular Biology of Plant Hormones*, P.J.J. Hooykaas, M. Hall, and K.L. Libbenga, eds, 423-459.
- Guilfoyle, T., Hagen, G., Ulmasov, T., and Murfett, J.** (1998). How does auxin turn on genes? *Plant Physiol* **118**, 341-347.
- Guilfoyle, T.J., Hagen, G., Li, Y., Gee, M.A., Ulmasov, T.N., and Martin, G.** (1992). Expression of auxin-responsive genes in soybean and transgenic tobacco. *Biochem Soc Trans* **20**, 97-101.
- Gustafson, F.G.** (1937). Parthenocarpy induced by pollen extracts. *Am J Bot* **24**, 102-107.
- Hadfi, K., Speth, V., and Neuhaus, G.** (1998). Auxin-induced developmental patterns in *Brassica juncea* embryos. *Development* **125**, 879-887.
- Hagen, G., and Guilfoyle, T.J.** (1985). Rapid induction of selective transcription by auxins. *Mol Cell Biol* **5**, 1197-1203.
- Hagen, G., and Guilfoyle, T.** (2002). Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Mol Biol* **49**, 373-385.
- Hagen, G., Martin, G., Li, Y., and Guilfoyle, T.J.** (1991). Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. *Plant Mol Biol* **17**, 567-579.
- Hamann, T., Mayer, U., and Jurgens, G.** (1999). The auxin-insensitive bodenlos mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo. *Development* **126**, 1387-1395.
- Hamann, T., Benkova, E., Baurle, I., Kientz, M., and Jurgens, G.** (2002). The *Arabidopsis* BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes Dev* **16**, 1610-1615.
- Hansen, H., and Grossmann, K.** (2000). Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. *Plant Physiol* **124**, 1437-1448.
- Hardtke, C.S., and Berleth, T.** (1998). The *Arabidopsis* gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. *Embo J* **17**, 1405-1411.
- Hareven, D., Gutfinger, T., Parnis, A., Eshed, Y., and Lifschitz, E.** (1996). The making of a compound leaf: genetic manipulation of leaf architecture in tomato. *Cell* **84**, 735-744.
- Harper, R.M., Stowe-Evans, E.L., Luesse, D.R., Muto, H., Tatematsu, K., Watahiki, M.K., Yamamoto, K., and Liscum, E.** (2000). The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial *Arabidopsis* tissue. *Plant Cell* **12**, 757-770.
- Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S., and Tsiantis, M.** (2002). The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. *Curr Biol* **12**, 1557-1565.
- Hobbie, L., and Estelle, M.** (1994). Genetic approaches to auxin action. *Plant Cell Environ* **17**, 525-540.
- Hobbie, L., and Estelle, M.** (1995). The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. *Plant J* **7**, 211-220.
- Hobbie, L., McGovern, M., Hurwitz, L.R., Pierro, A., Liu, N.Y., Bandyopadhyay, A., and Estelle, M.** (2000). The *axr6* mutants of *Arabidopsis thaliana* define a gene involved in auxin response and early development. *Development* **127**, 23-32.

- Hobson, G.E., and Davies, J.N.** (1976). Protein and enzyme changes in tomato fruit in relation to blotchy ripening and potassium nutrition. *J Sci Food Agric* **27**, 15-22.
- Hsieh, H.L., Okamoto, H., Wang, M., Ang, L.H., Matsui, M., Goodman, H., and Deng, X.W.** (2000). FIN219, an auxin-regulated gene, defines a link between phytochrome A and the downstream regulator COP1 in light control of *Arabidopsis* development. *Genes Dev* **14**, 1958-1970.
- Hull, A.K., Vij, R., and Celenza, J.L.** (2000). *Arabidopsis* cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. *Proc Natl Acad Sci U S A* **97**, 2379-2384.
- Jackson, D., Veit, B., and Hake, S.** (1994). Expression of maize KNOOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. *Development* **120**, 405-413.
- Jacobs, W.D.** (1952). The role of auxin in differentiation of zylem around a wound. *Am. J. Bot.* **39**, 301-309.
- Janssen, B.J., Lund, L., and Sinha, N.** (1998). Overexpression of a homeobox gene, LeT6, reveals indeterminate features in the tomato compound leaf. *Plant Physiol* **117**, 771-786.
- Jensen, P.J., and Bandurski, R.S.** (1994). Metabolism and Synthesis of Indole-3-Acetic Acid (IAA) in *Zea mays* (Levels of IAA during Kernel Development and the Use of in Vitro Endosperm Systems for Studying IAA Biosynthesis). *Plant Physiol* **106**, 343-351.
- Jensen, P.J., Hangarter, R.P., and Estelle, M.** (1998). Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown *Arabidopsis*. *Plant Physiol* **116**, 455-462.
- John, P.C., Sek, F.J., Carmichael, J.P., and McCurdy, D.W.** (1990). p34cdc2 homologue level, cell division, phytohormone responsiveness and cell differentiation in wheat leaves. *J Cell Sci* **97 ( Pt 4)**, 627-630.
- Jones, A.M., Im, K.H., Savka, M.A., Wu, M.J., DeWitt, N.G., Shillito, R., and Binns, A.N.** (1998). Auxin-dependent cell expansion mediated by overexpressed auxin-binding protein 1. *Science* **282**, 1114-1117.
- Jones, B., Frasse, P., Olmos, E., Zegzouti, H., Li, Z.G., Latche, A., Pech, J.C., and Bouzayen, M.** (2002). Down-regulation of DR12, an auxin-response-factor homolog, in the tomato results in a pleiotropic phenotype including dark green and blotchy ripening fruit. *Plant J* **32**, 603-613.
- Kelly, M.O., and Bradford, K.J.** (1986). Insensitivity of the *diageotropica* tomato mutant to auxin. *Plant Physiol* **82**, 713-717.
- Kepinski, S., and Leyser, O.** (2002). Ubiquitination and auxin signaling: a degrading story. *Plant Cell* **14 Suppl**, S81-95.
- Kieber, J.J.** (1997). The ethylene response pathway in *Arabidopsis*. *Annu Rev Plant Physiol Plant Mol Biol* **48**, 277-296.
- Kim, B.C., Soh, M.S., Kang, B.G., Furuya, M., and Nam, H.G.** (1996). Two dominant photomorphogenic mutations of *Arabidopsis thaliana* identified as suppressor mutations of *hy2*. *plant J* **15**, 441-456.
- Kim, J., Harter, K., and Theologis, A.** (1997). Protein-protein interactions among the Aux/IAA proteins. *Proc Natl Acad Sci U S A* **94**, 11786-11791.
- Kim, S.K., Chang, S.C., Lee, E.J., Chung, W.S., Kim, Y.S., Hwang, S., and Lee, J.S.** (2000). Involvement of brassinosteroids in the gravitropic response of primary root of maize. *Plant Physiol* **123**, 997-1004.
- King, J.J., Stimart, D.P., Fisher, R.H., and Bleecker, A.B.** (1995). A Mutation Altering Auxin Homeostasis and Plant Morphology in *Arabidopsis*. *Plant Cell* **7**, 2023-2037.

- Klee, H.J., Horsch, R.B., Hinchee, M.A., Hein, M.B., and Hoffmann, N.L.** (1987). The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. *Genes Dev* **1**, 86-96.
- koltai, H., and Bird, D.M.** (2000). Epistatic repression of PHANTASTICA and class 1 KNOTTED genes is uncoupled in tomato. *Plant J* **22**, 455-459.
- Koshioka, M., Nishijima, T., Yamzaki, H., Nonaka, M., and Mander, L.N.** (1994). Analysis of gibberellins in growing fruits of *Lycopersicon esculentum* after pollination or treatment with 4-chlorophenoxyacetic acid. *J Hort Sci* **69**, 171-179.
- Laskowski, M.J., Williams, M.E., Nusbaum, H.C., and Sussex, I.M.** (1995). Formation of lateral root meristems is a two-stage process. *Development* **121**, 3303-3310.
- Lasserre, E., Bouquin, T., Hernandez, J.A., Bull, J., Pech, J.C., and Balague, C.** (1996). Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). *Mol Gen Genet* **251**, 81-90.
- Leblanc, N., Perrot-Rechenmann, C., and Barbier-Brygoo, H.** (1999). The auxin-binding protein Nt-ERabp1 alone activates an auxin-like transduction pathway. *FEBS Lett* **449**, 57-60.
- Lehman, A., Black, R., and Ecker, J.R.** (1996). HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. *Cell* **85**, 183-194.
- Lelievre, J.M., Latche, A., Jones, B., Bouzayen, M., and Pech, J.C.** (1997). Ethylene and fruit ripening. *Plant Physiol* **101**, 727-739.
- Levetin, E., and McMahon, K.** (1998). *Plants and Society*, (WCB McGraw-Hill, Madison, Wisconsin). , 88-89.
- Leyser, H.M., Pickett, F.B., Dharmasiri, S., and Estelle, M.** (1996). Mutations in the AXR3 gene of Arabidopsis result in altered auxin response including ectopic expression from the SAUR-AC1 promoter. *Plant J* **10**, 403-413.
- Leyser, H.M., Lincoln, C.A., Timpte, C., Lammer, D., Turner, J., and Estelle, M.** (1993). Arabidopsis auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. *Nature* **364**, 161-164.
- Leyser, O.** (2001). Auxin signalling: the beginning, the middle and the end. *Curr Opin Plant Biol* **4**, 382-386.
- Lincoln, C., Britton, J.H., and Estelle, M.** (1990). Growth and development of the axr1 mutants of Arabidopsis. *Plant Cell* **2**, 1071-1080.
- Liscum, E., and Briggs, W.R.** (1995). Mutation in the *NPH1* locus of *Arabidopsis* disrupt the perception of phototropic stimuli. *Plant Cell* **7**, 473-485.
- Liscum, E., and Reed, J.W.** (2002). Genetics of Aux/IAA and ARF action in plant growth and development. *Plant Mol Biol* **49**, 387-400.
- Liu, C., Xu, Z., and Chua, N.H.** (1993). Auxin Polar Transport Is Essential for the Establishment of Bilateral Symmetry during Early Plant Embryogenesis. *Plant Cell* **5**, 621-630.
- Liu, Z.B., Ulmasov, T., Shi, X., Hagen, G., and Guilfoyle, T.J.** (1994). Soybean GH3 promoter contains multiple auxin-inducible elements. *Plant Cell* **6**, 645-657.
- Ljung, K., Bhalerao, R.P., and Sandberg, G.** (2001). Sites and homeostatic control of auxin biosynthesis in Arabidopsis during vegetative growth. *Plant J* **28**, 465-474.

- Ljung, K., Hull, A.K., Kowalczyk, M., Marchant, A., Celenza, J.L., Cohen, D.J., and Sandberg, G.** (2002). Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. *Plant Mol Biol* **49**, 249-272.
- Luschnig, C., Gaxiola, R.A., Grisafi, P., and Fink, G.R.** (1998). EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes Dev* **12**, 2175-2187.
- Madlung, A., Behringer, F.J., and Lomax, T.L.** (1999). Ethylene plays multiple nonprimary roles in modulating the gravitropic response in tomato. *Plant Physiol* **120**, 897-906.
- Mapelli, S.C., and Mombardi, L.** (1982). A comparative auxin and cytokinin study in normal and to-2 mutant tomato plants. *Plant Cell Physiol* **23**, 751-757.
- Mapelli, S.C., Frova, G., Torti, G., and Soressi, G.** (1978). Relationship between set, development and activities of growth regulators in tomato fruits. *Plant Cell Physiol* **19**, 1281-1288.
- Martin, D.N., Proebsting, W.M., and Hedden, P.** (1997). Mendel's dwarfing gene: cDNAs from the *le* alleles and function of the expressed proteins. *Proc Natl Acad Sci U S A* **94**, 8907-8911.
- Masucci, J.D., and Schiefelbein, J.W.** (1994). The *rh6* Mutation of *Arabidopsis thaliana* Alters Root-Hair Initiation through an Auxin- and Ethylene-Associated Process. *Plant Physiol* **106**, 1335-1346.
- Masucci, J.D., and Schiefelbein, J.W.** (1996). Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the *Arabidopsis* root. *Plant Cell* **8**, 1505-1517.
- Mattsson, J., Sung, Z.R., and Berleth, T.** (1999). Responses of plant vascular systems to auxin transport inhibition. *Development* **126**, 2979-2991.
- Mattsson, J., Ckurshumova, W., and Berleth, T.** (2003). Auxin signaling in *Arabidopsis* leaf vascular development. *Plant Physiol* **131**, 1327-1339.
- McClure, B.A., Hagen, G., Brown, C.S., Gee, M.A., and Guilfoyle, T.J.** (1989). Transcription, organization, and sequence of an auxin-regulated gene cluster in soybean. *Plant Cell* **1**, 229-239.
- Meissner, R., Jacobson, Y., Melamed, S., Levyatuv, S., Shalev, G., Ashri, A., Elkind, Y., and Levy, A.** (1997). A new model system for tomato genetics. *Plant J* **12**, 1465-1472.
- Merritt, F., Kemper, A., and Tallman, G.** (2001). Inhibitors of ethylene synthesis inhibit auxin-induced stomatal opening in epidermis detached from leaves of *Vicia faba* L. *Plant Cell Physiol* **42**, 223-230.
- Mockaitis, K., and Howell, S.H.** (2000). Auxin induces mitogenic activated protein kinase (MAPK) activation in roots of *Arabidopsis* seedlings. *Plant J* **24**, 785-796.
- Morgan, K.E., Zarembinski, T.I., Theologis, A., and Abel, S.** (1999). Biochemical characterization of recombinant polypeptides corresponding to the predicted betaalphaalpha fold in Aux/IAA proteins. *FEBS Lett* **454**, 283-287.
- Motchoulski, A.V., and Liscum, E.** (1999). *Arabidopsis* NPH3: A NPH1 photoreceptor-interaction protein essential for phototropism. *Science* **286**, 961-964.
- Muday, G.K.** (2001). Auxins and tropisms. *J Plant Growth Regul* **20**, 226-243.
- Muday, G.K., and DeLong, A.** (2001). Polar auxin transport: controlling where and how much. *Trends Plant Sci* **6**, 535-542.
- Muller, A., Guan, C., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E., and Palme, K.** (1998). AtPIN2 defines a locus of *Arabidopsis* for root gravitropism control. *Embo J* **17**, 6903-6911.

- Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M., and Reed, J.W.** (2000). AXR2 encodes a member of the Aux/IAA protein family. *Plant Physiol* **123**, 563-574.
- Napier, R.M., David, K.M., and Perrot-Rechenmann, C.** (2002). A short history of auxin-binding proteins. *Plant Mol Biol* **49**, 339-348.
- Nebenfuhr, A., White, T.J., and Lomax, T.L.** (2000). The diageotropica mutation alters auxin induction of a subset of the Aux/IAA gene family in tomato. *Plant Mol Biol* **44**, 73-84.
- Nitsch, J., Pratt, C., Nitsch, C., and Shaullis, N.** (1960). Natural growth substances in concord and concord seedless grapes in relation to berry development. *Am. J. Bot.* **47**, 566-576.
- Nogue, F., Grandjean, O., Craig, S., Dennis, S., and Chaudhury, M.** (2000). Higher levels of cell proliferation rate and cyclin CycD3 expression in the *Arabidopsis amp1* mutant. *Plant Growth Regul* **32**, 275-283.
- Normanly, J., and Bartel, B.** (1999). Redundancy as a way of life - IAA metabolism. *Curr Opin Plant Biol* **2**, 207-213.
- Normanly, J., Grisafi, P., Fink, G.R., and Bartel, B.** (1997). Arabidopsis mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the NIT1 gene. *Plant Cell* **9**, 1781-1790.
- Oeller, P.W., and Theologis, A.** (1995). Induction kinetics of the nuclear proteins encoded by the early indoleacetic acid-inducible genes, PS-IAA4/5 and PS-IAA6, in pea (*Pisum sativum* L.). *Plant J* **7**, 37-48.
- Oeller, P.W., Keller, J.A., Parks, J.E., Silbert, J.E., and Theologis, A.** (1993). Structural characterization of the early indoleacetic acid-inducible genes, PS-IAA4/5 and PS-IAA6, of pea (*Pisum sativum* L.). *J Mol Biol* **233**, 789-798.
- Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y.** (1991). Requirement of the Auxin Polar Transport System in Early Stages of Arabidopsis Floral Bud Formation. *Plant Cell* **3**, 677-684.
- Ostin, A., Ilic, N., and Cohen, J.D.** (1999). An in vitro system from maize seedlings for tryptophan-independent indole-3-acetic acid biosynthesis. *Plant Physiol* **119**, 173-178.
- Ostin, A., Kowalyczuk, M., Bhalerao, R.P., and Sandberg, G.** (1998). Metabolism of indole-3-acetic acid in Arabidopsis. *Plant Physiol* **118**, 285-296.
- Ottenschlager, I., Wolff, P., Wolverson, C., Bhalerao, R.P., Sandberg, G., Ishikawa, H., Evans, M., and Palme, K.** (2003). Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proc Natl Acad Sci U S A*.
- Ouellet, F., Overvoorde, P.J., and Theologis, A.** (2001). IAA17/AXR3: biochemical insight into an auxin mutant phenotype. *Plant Cell* **13**, 829-841.
- Palme, K., and Galweiler, L.** (1999). PIN-pointing the molecular basis of auxin transport. *Curr Opin Plant Biol* **2**, 375-381.
- Pandolfini, T., Rotino, G.L., Camerini, S., Defez, R., and Spena, A.** (2002). Optimisation of transgene action at the post-transcriptional level: high quality parthenocarpic fruits in industrial tomatoes. *BMC Biotechnol* **2**, 1.
- Petrasek, J., Elckner, M., Morris, D.A., and Zazimalova, E.** (2002). Auxin efflux carrier activity and auxin accumulation regulate cell division and polarity in tobacco cells. *Planta* **216**, 302-308.
- Pitts, R.J., Cernac, A., and Estelle, M.** (1998). Auxin and ethylene promote root hair elongation in Arabidopsis. *Plant J* **16**, 553-560.

- Pozzi, C., Rossini, L., and Agosti, F.** (2001). Patterns and symmetries in leaf development. *Semin Cell Dev Biol* **12**, 363-372.
- Przemeck, G.K., Mattsson, J., Hardtke, C.S., Sung, Z.R., and Berleth, T.** (1996). Studies on the role of the Arabidopsis gene MONOPTEROS in vascular development and plant cell axialization. *Planta* **200**, 229-237.
- Rahman, A., Amakawa, T., Goto, N., and Tsurumi, S.** (2001). Auxin is a positive regulator for ethylene-mediated response in the growth of Arabidopsis roots. *Plant Cell Physiol* **42**, 301-307.
- Rahman, A., Hosokawa, S., Oono, Y., Amakawa, T., Goto, N., and Tsurumi, S.** (2002). Auxin and Ethylene Response Interactions during Arabidopsis Root Hair Development Dissected by Auxin Influx Modulators. *Plant Physiol* **130**, 1908-1917.
- Ramos, J.A., Zenser, N., Leyser, O., and Callis, J.** (2001). Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. *Plant Cell* **13**, 2349-2360.
- Raven, J.A.** (1975). Transport of indole acetic acid in plant cells in relation to pH and electrical potential gradients, and its significance for IAA transport. *New Phytol* **74**, 163-172.
- Rayle, D.L., and Cleland, R.E.** (1992). The Acid Growth Theory of auxin-induced cell elongation is alive and well. *Plant Physiol* **99**, 1271-1274.
- Raz, V., and Ecker, J.R.** (1999). Regulation of differential growth in the apical hook of *Arabidopsis*. *Development* **126**, 3661-3668.
- Reed, J.W.** (2001). Roles and activities of Aux/IAA proteins in Arabidopsis. *Trends Plant Sci* **6**, 420-425.
- Reed, R.C., Brady, S.R., and Muday, G.K.** (1998). Inhibition of auxin movement from the shoot into the root inhibits lateral root development in Arabidopsis. *Plant Physiol* **118**, 1369-1378.
- Reiser, L., Sanchez-Baracaldo, P., and Hake, S.** (2000). Knots in the family tree: evolutionary relationships and functions of knox homeobox genes. *Plant Mol Biol* **42**, 151-166.
- Rick, C.M., and Yoder, J.I.** (1988). Classical and molecular genetics of tomato: highlights and perspectives. *Annu Rev Genet* **22**, 281-300.
- Rogg, L.E., and Bartel, B.** (2001). Auxin signaling: derepression through regulated proteolysis. *Dev Cell* **1**, 595-604.
- Rogg, L.E., Lasswell, J., and Bartel, B.** (2001). A gain-of-function mutation in IAA28 suppresses lateral root development. *Plant Cell* **13**, 465-480.
- Romano, C.P., Hein, M.B., and Klee, H.J.** (1991). Inactivation of auxin in tobacco transformed with the indoleacetic acid-lysine synthetase gene of *Pseudomonas savastanoi*. *Genes Dev* **5**, 438-446.
- Romano, C.P., Cooper, M.L., and Klee, H.J.** (1993). Uncoupling Auxin and Ethylene Effects in Transgenic Tobacco and Arabidopsis Plants. *Plant Cell* **5**, 181-189.
- Romano, C.P., Robson, P.R., Smith, H., Estelle, M., and Klee, H.** (1995). Transgene-mediated auxin overproduction in Arabidopsis: hypocotyl elongation phenotype and interactions with the hy6-1 hypocotyl elongation and axr1 auxin-resistant mutants. *Plant Mol Biol* **27**, 1071-1083.
- Ross, J.J., O'Neill, D.P., Smith, J.J., Kerckhoffs, L.H., and Elliott, R.C.** (2000). Evidence that auxin promotes gibberellin A1 biosynthesis in pea. *Plant J* **21**, 547-552.
- Ross, J.J., O'Neill, D.P., Wolbang, C.M., Symons, G.M., and Reid, J.B.** (2001). Auxin-Gibberellin Interactions and Their Role in Plant Growth. *J Plant Growth Regul* **20**, 336-353.

- Rotino, G.L., Perri, E., Zottini, M., Sommer, H., and Spena, A.** (1997). Genetic engineering of parthenocarpic plants. *Nat Biotechnol* **15**, 1398-1401.
- Rouse, D., Mackay, P., Stirnberg, P., Estelle, M., and Leyser, O.** (1998). Changes in auxin response from mutations in an AUX/IAA gene. *Science* **279**, 1371-1373.
- Rubery, P.H., and Sheldrake, A.R.** (1974). Carrier-mediated auxin transport. *Planta* **118**, 101-121.
- Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Turner, J., and Estelle, M.** (1998). The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast grl1p. *Genes Dev* **12**, 198-207.
- Sachs, T.** (1986). Cellular interactions in tissue and organ development. *Symp Soc Exp Biol* **40**, 181-210.
- Sachs, T.** (2000). Integrating cellular and organismic aspects of vascular differentiation. *Plant Cell Physiol* **41**, 649-656.
- Schiavone, F.M., and Cooke, T.J.** (1987). Unusual patterns of somatic embryogenesis in the domesticated carrot: developmental effects of exogenous auxins and auxin transport inhibitors. *Cell Differ* **21**, 53-62.
- Schopfer, P.** (1993). Determination of Auxin-Dependent pH Changes in Coleoptile Cell Walls by a Null-Point Method. *Plant Physiol* **103**, 351-357.
- Sessions, A., Nemhauser, J.L., McColl, A., Roe, J.L., Feldmann, K.A., and Zambryski, P.C.** (1997). ETTIN patterns the Arabidopsis floral meristem and reproductive organs. *Development* **124**, 4481-4491.
- Shen-Miller, J., Cooper, P., and Gordon, S.A.** (1967). Hormonal relations in the phototropic response. V. Photoinhibition of auxin transport and its bearing on phototropism. ANL-7409. ANL Rep, 93-95.
- Skoog, F., and Miller, C.O.** (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp Soc Exp Biol* **11**, 118-131.
- Slovin, J.P., and Cohen, D.J.** (1993). Auxin metabolism in relation to fruit ripening. *Acta Hort.* **329**, 84-89.
- Stirnberg, P., Chatfield, S.P., and Leyser, H.M.** (1999). AXR1 acts after lateral bud formation to inhibit lateral bud growth in Arabidopsis. *Plant Physiol* **121**, 839-847.
- Stowe-Evans, E.L., Luesse, D.R., and Liscum, E.** (2001). The enhancement of phototropin-induced phototropic curvature in Arabidopsis occurs via a photoreversible phytochrome A-dependent modulation of auxin responsiveness. *Plant Physiol* **126**, 826-834.
- Swarup, R., Parry, G., Graham, N., Allen, T., and Bennett, M.** (2002a). Auxin cross-talk: integration of signalling pathways to control plant development. *Plant Mol Biol* **49**, 411-426.
- Swarup, R., Parry, G., Graham, N., Allen, T., and Bennett, M.** (2002b). Auxin cross-talk: integration of signalling pathways to control plant development. *Plant Mol Biol* **49**, 411-426.
- Tang, Z., Sadka, A., Morishige, D.T., and Mullet, J.E.** (2001). Homeodomain leucine zipper proteins bind to the phosphate response domain of the soybean vspb tripartite promoter. *Plant Physiol* **125**, 797-809.
- Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M.K., Harper, R.M., Liscum, E., and Yamamoto, K.T.** (2004). MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. *Plant Cell* **16**, 379-393.
- Tena, G., and Renaudin, J.P.** (1998). Cytosolic acidification but not auxin at physiological concentration is an activator of MAP kinases in tobacco cells. *Plant J* **16**, 173-182.

- Theologis, A., Huynh, T.V., and Davis, R.W.** (1985). Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. *J Mol Biol* **183**, 53-68.
- Thimann, K.V.** (1963). Plant growth substances; past present and future. *Annu Rev Plant Physiol* **14**, 1-18.
- Tian, Q., and Reed, J.W.** (1999). Control of auxin-regulated root development by the *Arabidopsis thaliana* SHY2/IAA3 gene. *Development* **126**, 711-721.
- Tian, Q., Uhlir, N.J., and Reed, J.W.** (2002). *Arabidopsis* SHY2/IAA3 inhibits auxin-regulated gene expression. *Plant Cell* **14**, 301-319.
- Timpte, C., Wilson, A.K., and Estelle, M.** (1994). The *axr2-1* mutation of *Arabidopsis thaliana* is a gain-of-function mutation that disrupts an early step in auxin response. *Genetics* **138**, 1239-1249.
- Timpte, C., Lincoln, C., Pickett, F.B., Turner, J., and Estelle, M.** (1995). The AXR1 and AUX1 genes of *Arabidopsis* function in separate auxin-response pathways. *Plant J* **8**, 561-569.
- Tiwari, S.B., Hagen, G., and Guilfoyle, T.** (2003). The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* **15**, 533-543.
- Tiwari, S.B., Hagen, G., and Guilfoyle, T.J.** (2004). Aux/IAA proteins contain a potent transcriptional repression domain. *Plant Cell* **16**, 533-543.
- Tiwari, S.B., Wang, X.J., Hagen, G., and Guilfoyle, T.J.** (2001). AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *Plant Cell* **13**, 2809-2822.
- Ulmasov, T., Hagen, G., and Guilfoyle, T.J.** (1997a). ARF1, a transcription factor that binds to auxin response elements. *Science* **276**, 1865-1868.
- Ulmasov, T., Hagen, G., and Guilfoyle, T.J.** (1999a). Dimerization and DNA binding of auxin response factors. *Plant J* **19**, 309-319.
- Ulmasov, T., Hagen, G., and Guilfoyle, T.J.** (1999b). Activation and repression of transcription by auxin-response factors. *Proc Natl Acad Sci U S A* **96**, 5844-5849.
- Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J.** (1997b). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9**, 1963-1971.
- Varoquaux, F., Blanvillain, R., Delseny, M., and Gallois, P.** (2000). Less is better: new approaches for seedless fruit production. *Trends Biotech* **18**, 233-242.
- Vendrell, M.** (1985). Dual effect of 2,4-D on ethylene production and ripening of tomato fruit tissue. *Physiol Plant* **64**, 559-563.
- Vivian-Smith, A., and Koltunow, A.M.** (1999). Genetic analysis of growth-regulator-induced parthenocarpy in *Arabidopsis*. *Plant Physiol* **121**, 437-451.
- Vivian-Smith, A., Luo, M., Chaudhury, A., and Koltunow, A.** (2001). Fruit development is actively restricted in the absence of fertilization in *Arabidopsis*. *Development* **128**, 2321-2331.
- Walker, L., and Estelle, M.** (1998). Molecular mechanisms of auxin action. *Curr Opin Plant Biol* **1**, 434-439.
- Wang, H., Li, Z.G., Audran, C., Frasse, P., Jones, B., and Bouzayen, M.** (2002). Characterisation of genes encoding auxin transcription factor homologues expressed in tomato fruit. *Biotechnology of the Plant Hormone Ethylene 2002 S6-P9*, 140.

- Watahiki, M.K., and Yamamoto, K.T.** (1997). The massugul mutation of *Arabidopsis* identified with failure of auxin-induced growth curvature of hypocotyl confers auxin insensitivity to hypocotyl and leaf. *Plant Physiol* **115**, 419-426.
- Watahiki, M.K., Tatematsu, K., Fujihira, K., Yamamoto, M., and Yamamoto, K.T.** (1999). The MSG1 and AXR1 genes of *Arabidopsis* are likely to act independently in growth-curvature responses of hypocotyls. *Planta* **207**, 362-369.
- Wickson, M., and Thimann, K.V.** (1958). The antagonism of auxin and kinetin in apical dominance. *Physiol Plant* **11**, 62-74.
- Wilson, A.K., Pickett, F.B., Turner, J.C., and Estelle, M.** (1990). A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. *Mol Gen Genet* **222**, 377-383.
- Wolbang, C.M., and Ross, J.J.** (2001). Auxin promotes gibberellin biosynthesis in decapitated tobacco plants. *Planta* **214**, 153-157.
- Worley, C.K., Zenser, N., Ramos, J., Rouse, D., Leyser, O., Theologis, A., and Callis, J.** (2000). Degradation of Aux/IAA proteins is essential for normal auxin signalling. *Plant J* **21**, 553-562.
- Wyatt, R.E., Ainley, W.M., Nagao, R.T., Conner, T.W., and Key, J.L.** (1993). Expression of the *Arabidopsis* AtAux2-11 auxin-responsive gene in transgenic plants. *Plant Mol Biol* **22**, 731-749.
- Xiao, W.Y., and Jang, J.C.** (2000). F-box protein in *Arabidopsis*. *Trends Plant Sci* **5**, 454-457.
- Yamamoto, K.T.** (1994). Further characterization of auxin-regulated mRNAs in hypocotyl sections of mung bean [*Vigna radiata* (L.) Wilczek]: sequence homology to genes for fatty-acid desaturases and atypical late-embryogenesis-abundant protein, and the mode of expression of the mRNAs. *Planta* **192**, 359-364.
- Yamamoto, K.T., and Tatematsu, K.** (2002). [Plant response to gravity: towards understanding of gravitropic growth habit]. *Tanpakushitsu Kakusan Koso* **47**, 1695-1699.
- Yang, T., and Poovaiah, B.W.** (2000). Molecular and biochemical evidence for the involvement of calcium/calmodulin in auxin action. *J Biol Chem* **275**, 3137-3143.
- Yi, H.C., Joo, S., Nam, K.H., Lee, J.S., Kang, B.G., and Kim, W.T.** (1999). Auxin and brassinosteroid differentially regulate the expression of three members of the 1-aminocyclopropane-1-carboxylate synthase gene family in mung bean (*Vigna radiata* L.). *Plant Mol Biol* **41**, 443-454.
- Yip, W.K., Moore, T., and Yang, S.F.** (1992). Differential accumulation of transcripts for four tomato 1-aminocyclopropane-1-carboxylate synthase homologs under various conditions. *Proc Natl Acad Sci U S A* **89**, 2475-2479.
- Yu, S.J., Kim, S., Lee, J.S., and Lee, D.H.** (1998). Differential accumulation of transcripts for ACC synthase and ACC oxidase homologs in etiolated mung bean hypocotyls in response to various stimuli. *Mol Cells* **8**, 350-358.
- Zenser, N., Ellsmore, A., Leasure, C., and Callis, J.** (2001). Auxin modulates the degradation rate of Aux/IAA proteins. *Proc Natl Acad Sci U S A* **98**, 11795-11800.
- Zhang, R., Zhang, X., Wang, J., Letham, D.S., McKinney, S.A., and Higgins, T.J.V.** (1995). The effect of auxin on cytokinin levels and metabolism in transgenic tobacco tissue expressing an *ipt* gene. *Planta* **196**, 84-94.
- Zhao, H., Hertel, R., Ishikawa, H., and Evans, M.L.** (2002). Species differences in ligand specificity of auxin-controlled elongation and auxin transport: comparing *Zea* and *Vigna*. *Planta* **216**, 293-301.

