THÈSE

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Titre : *Caractérisation fonctionnelle des gènes Sl-IAA3 et Sl-hls chez la tomate. Rôle dans le dialogue entre l'auxine et l'éthylène*

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RESUME

Le développement des plantes est le résultat d'une coordination complexe entre de multiples signaux endogènes essentiellement hormonaux et exogènes issus de l'environnement. En particulier, l'intégration des différentes voies de signalisation hormonales est modulée de manière organe-dépendante. Mon projet de thèse s'inscrit dans ce cadre et vise à identifier les acteurs moléculaires du dialogue hormonal entre l'éthylène et l'auxine. L'interaction entre l'éthylène et l'auxine est l'exemple choisi dans cette étude en raison du rôle prépondérant joué par ces deux hormones dans les processus de maturation et de développement des fruits. En premier lieu, la caractérisation fonctionnelle du gène Sl-*IAA3* codant pour un régulateur transcriptionnel apparenté à la famille des Aux/IAA de tomate a montré que la sous-expression de ce gène engendre des phénotypes associés à la fois à l'auxine (altération de la dominance apicale) et à l'éthylène (exagération du crochet apical et réduction de l'epinastie foliaire). Ces résultats révèlent pour la première fois que le gène Sl-*IAA3* se trouve au centre du dialogue entre les voies de signalisation de l'auxine et de l'éthylène. La formation du crochet apical constitue un système bien adapté à l'étude du dialogue hormonal en raison du rôle déjà démontré de l'auxine et l'éthylène dans ce processus. L'étude réalisée ici montre qu'en plus de l'altération du crochet apical, le mutant *hls1* présente des phénotypes nouveaux associés à la lumière, au glucose et à l'ABA. Deux orthologues (Sl-*HLS1* et Sl-*HLS2*) du gène At-*HLS1* d'Arabidopsis ont été isolés chez la tomate et leur validation fonctionnelle a été réalisée par complémentation du mutant *hls1* d'Arabidopsis. L'étude de l'expression tissulaire montre que Sl-*HLS2* s'exprime au niveau de la face convexe du crochet apical à l'opposé de Sl-*IAA3* dont l'expression est associée à la face concave. Ce résultat suggère que Sl-*IAA3* et Sl-*HLS2* pourraient avoir des fonctions antagonistes sur l'élongation cellulaire aux niveaux interne et externe du crochet permettant ainsi la formation de la boucle. Au total, la caractérisation de deux gènes intervenant à la croisée des voies de signalisation de l'auxine et l'éthylène réalisée ici sur des tissus végétatifs, fournit de nouveaux outils pour aborder à l'avenir le rôle du dialogue hormonal dans le développement et la maturation des fruits.

ABSTRACT

Plant development and survival depend on the ability of these organisms to integrate many signalling which enables them to produce an appropriate response. Ethylene and auxin are phytohormones known to regulate agonistly or antagonistly many processes of plant development but yet the key integrating molecular players remain largely undiscovered. My Ph.D project deals with the identification and characterization of molecular actors that take part in this dialogue. We report that Sl-*IAA3,* a member of the tomato auxin/indole-3-acetic acid (*Aux/IAA*) gene family, is an intersection point between auxin and ethylene signal transduction pathways. *Aux/IAA* genes encode short-lived transcriptional regulators that mediate auxin responses. Sl-*IAA3* expression is controlled by both auxin and ethylene and is regulated on a tight tissue-specific basis. Down-regulation of Sl-*IAA3* via an antisense strategy results in auxin and ethylene-related phenotypes including altered apical dominance, lower auxin sensitivity, exaggerated apical hook curvature in the dark and reduced petiole epinasty in the light. These ethylene-related phenotypes in the antisense tomato lines (*AS-IAA3*) position Sl-*IAA3* firmly at the crossroads between auxin and ethylene signalling in tomato. The induction of apical hook offers an excellent system to study auxin-ethylene interplay. In Arabidopsis, ethylene acts through HOOKLESS (HLS1) to control hook formation through modulating differential cell elongation in opposite sides of the hook. Loss of function mutation in the *HLS1* gene results in the absence of hook even in the presence of exogenous ethylene. In the present study, we extended the phenotypes of the Arabidopsis *hls1* mutant to alteration of light sensitivity, glucose and ABA tolerance and gravitropic growth thus uncovering the importance of *HLS* gene in the integration of multiple signalling pathways. Two functional tomato hookless genes (Sl-*HLS1* and Sl-*HLS2*) were isolated in this study and shown to positively complement the Arabidopsis *hls1* mutant. Expression of Sl-*HLS2* in the hook is restricted to the outer face, opposite to Sl-*IAA3* whose expression is localized in the inner face of the hook curvature. The data suggest that Sl-HLS2 and Sl-IAA3 exert antagonist control of cell elongation in the inner and outer part of the apical hook. Overall, the two genes characterized in this study open new prospects towards addressing the role of ethylene and auxin cross-talk during fruit development and ripening.

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ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic Acid
ABP	Auxin Binding Protein
AC	Ailsa Craig
ACC	1-Aminocyclopropane-1-acide carboxilique
ACO	ACC oxydase
ACS	ACC synthase
AFB	Auxin signalling F-Box proteins
ARF	Auxin Response Factor
AS	Antisense
AuxRE	Auxin Response Element
BR	Brassinosteroids
CDK	Cyclin-Dependent Kinase
cDNA	Complementary desoxyribonucleic acid
Cnr	Colorless nonripening
COI1	Coronatine Insensitive 1
CTR1	Constitutive triple réponse 1
D	*DWARF*
DBD	DNA Binding Domain
dgt	Diageotropica
DPA	Days Post Anthesis
DR	Developmently Regulated
GA	Gibberellic Acid

Gr Green-ripe

GUS β-Glucoronidase

HLS Hookless

IAA Indole-3-Acetic Acid

IBA Indole-3-Butyric Acid

IPA Indole-3-Pyruvic Acid

JA Jasmonate

KUP Potassium transporter-like

MAPK Mitogen-Activated Protein Kinase

MDRs Multi-Drug Resistant-like transporters

MCP 1-Methylciclopropène

NAA α-Naphtalene Acetic Acid

NPA N-(napthyl) Phthalamic Acid

Nr Never-ripe

rin Ripening-inhibitor

RT Reverse transcription

SAM S-adenosine-méthionine

SAUR Small auxin up-regulated RNA

SP Self Pruning

Sur Super-root

TIR1 Transport Inhibitor Response 1

WT Wild Type

X-Gal 5-bromo-4-cloro-3-indolil- β-D-galactoside

OBJECTIFS ET PRINCIPAUX RESULTATS
DES TRAVAUX DE THESE

Les hormones jouent un rôle essentiel dans la coordination des processus de développement et d'adaptation des plantes à leur environnement. Au regard de la diversité de réponses développementales que les plantes sont capables de produire, l'hypothèse de la nécessité d'un mécanisme combinatoire basé sur l'intégration de différents signaux hormonaux a été depuis longtemps favorisée. Au cours des dernières décennies, les exemples de convergence entre voies de signalisation hormonales se sont multipliés parmi lesquels l'interaction entre l'éthylène et l'auxine est la plus étudiée chez les plantes. Ainsi les effets soit synergiques de l'éthylène et de l'auxine dans la régulation de la croissance racinaire, soit antagonistes dans le contrôle de l'abscission des fleurs et des fruits ont été largement décrits. Cependant, les médiateurs moléculaires de ce dialogue hormonal restent à ce jour mal connus. Grâce au déploiement des approches modernes de génétique, des avancées considérables ont été réalisées dans l'élucidation des modes d'action de chacune de ces deux hormones et les éléments clés de leurs voies de biosynthèse, de perception et de transduction ont été relativement bien caractérisés. Ceci a ouvert de nouvelles possibilités pour l'exploration, au niveau moléculaire, des intermédiaires de l'interaction hormonale entre auxine et éthylène. Le projet de ma thèse s'inscrit dans ce cadre et vise à mieux comprendre les modalités d'interaction entre l'auxine et l'éthylène à travers la caractérisation chez la tomate du gène Sl-*IAA3* codant pour un médiateur de la réponse à l'auxine apparenté à la famille des Aux/IAA et du gène Sl-*HLS* (hookless) impliqué dans la réponse des plantules étiolées à l'éthylène.

Le développement et la maturation des fruits: un exemple d'interaction entre l'auxine et l'éthylène

Dans le cas des fruits climactériques comme la tomate, l'éthylène contrôle le déclanchement et le déroulement de la maturation. Cependant, l'acquisition de la compétence à murir ne semble pas dépendre de l'éthylène mais plutôt de

l'intervention conjuguée de plusieurs facteurs hormonaux que les physiologistes avaient coutume d'appeler "la balance hormonale". La recherche des signaux impliqués dans le développement du fruit et dans l'avènement du processus de maturation a été entreprise dans notre laboratoire par un criblage différentiel qui a permis d'identifier plusieurs gènes qui s'expriment de manière différentielle au cours du développement du fruit et en réponse à l'éthylène chez la tomate (Zegzouti et al., 1998, Jones et al., 2002). Ce criblage a conduit entre autres à l'isolement de plusieurs gènes codant pour des régulateurs transcriptionnels impliqués dans la réponse à l'auxine. En particulier, les gènes qui appartiennent soit la famille des Aux/IAA ou à celle des ARF (Auxin Response Factor) présentant une double régulation par l'auxine et par l'éthylène. Ces études ont révèlé pour la première fois l'existence d'un dialogue entre la voie de signalisation de l'auxine et de l'éthylène au cours de la maturation du fruit et fourni des gènes cibles codant pour des effecteurs potentiels de cette action croisée des hormones.

Sl-*IAA3*, un membre de la famille des *Aux/IAA* intervenant dans la régulation de la réponse à l'auxine et à l'éthylène

Sl-IAA3 est apparenté aux régulateurs transcriptionnels de type Aux/IAA médiateurs de la réponse à l'auxine chez les plantes et codés par une large famille multigénique. L'intérêt de gène Sl-*IAA3* pour la présente étude vient de son profil d'expression endogène ainsi que de l'analyse des lignées transgéniques de tomates exprimant une construction fusionnant son promoteur au gène *GUS* (*proIAA3:GUS*) qui montrent que le gène Sl-*IAA3* répond à une double régulation par l'auxine et par l'éthylène. Le travail réalisé au cours de la présente thèse montre que la sous-expression du gène Sl-*IAA3* dans des lignées antisens (*AS-IAA3*) entraîne des phénotypes associés à l'auxine (dominance apicale et élongation de l'hypocotyle) et à l'éthylène (exagération du crochet apical et réduction de l'épinastie foliaire) suggérant qu'il s'agit d'un point de convergence des deux voies de signalisation hormonale. Au niveau moléculaire, nous montrons que certains médiateurs de la réponse à l'auxine (*ARF, Auxin Response Factor*) et de l'éthylène (*ERF, Ethylene Response Factor*) sont différentiellement régulés dans les lignées *AS-IAA3* et constituent de ce fait des

cibles potentielles régulées par Sl-IAA3. Ce gène s'exprime uniquement dans la face concave du crochet apical des plantules étiolées ce qui suggère que l'éthylène pourrait moduler la distribution et/ou la réponse asymétrique à l'auxine au niveau du crochet.

L'ensemble de ces résultats apporte des connaissances nouvelles sur la fonction de la famille des Aux/IAA mais surtout révèle pour la première fois que le gène Sl-*IAA3* se trouve au centre du dialogue auxine/éthylène nécessaire à certains processus de développement chez les plantes. Il faut toutefois souligner qu'alors que le gène Sl-*IAA3* a été isolé sur la base de son expression différentielle dans le fruit, aucun phénotype associé au fruit n'a pu être observé chez les plantes antisens. L'absence de phénotype visible au niveau du fruit pourrait être le résultat de la redondance fonctionnelle au sein de la famille des Aux/IAA. Cependant, ces résultats n'excluent pas définitivement l'intervention de Sl-IAA3 dans certains aspects discrets de la maturation qui n'ont pas été testés au cours de cette étude.

At-*HLS1*, un gène clé au carrefour de plusieurs voies de signalisations

Dans plusieurs espèces incluant Arabidopsis et la tomate, l'éthylène induit chez les plantules étiolées une réponse morphologique caractéristique appelée "triple réponse". La triple réponse comprend: (i) une courbure exagérée du crochet apical, (ii) un raccourcissement et un grossissement de l'hypocotyle, et (iii) une inhibition de l'élongation racinaire. La formation et le maintien de ce crochet apical est le résultat de l'intégration de plusieurs signaux hormonaux et non hormonaux et représente ainsi un système d'étude privilégié de l'interaction auxine-éthylène. Selon un modèle récent, basé sur la caractérisation du mutant *hookless* (*hls1*) chez Arabidopsis, HLS1 serait la protéine clé où convergeraient différents signaux hormonaux ainsi que le signal lumière. Une mutation au niveau de ce gène inhibe la formation du crochet même en présence de traitement exogène d'éthylène. L'étude réalisée au cours de ce travail de thèse montre qu'en plus de l'altération du crochet apical, le mutant *hls1* présente des phénotypes nouveaux associés à la lumière, au glucose et à l'ABA. A la suite de la description de l'ensemble de ces phénotypes, je propose un schéma qui

présente un modèle permettant d'expliquer le rôle du gène *HLS* dans l'intégration de ces différents signaux hormonaux.

Analyse fonctionnelle des gènes hookless de tomate

La caractérisation de Sl-*IAA3* présentée dans cette étude montre que ce gène est impliqué dans la formation du crochet apical et qu'il présente un profil d'expression en réponse à l'éthylène finement régulé au niveau de la face interne du crochet. Il ressort donc que Sl-*IAA3* comme Sl-*HLS* jouent un rôle actif dans la formation du crochet apical et que les deux gènes se trouvent à l'intersection de la signalisation auxinique et éthylénique. Afin de mieux connaître les modalités d'interaction entre ces deux gènes clés, une partie de ma thèse a été dédiée à l'isolement de l'orthologue du gène At-*HLS* chez la tomate. Deux gènes nommés Sl-*HLS1* et Sl-*HLS2* ont été ainsi isolés dont la validation fonctionnelle a été réalisée par complémentation du mutant *hls1* d'Arabidopsis. L'étude de l'expression de Sl-*HLS1* et Sl-*HLS2* montre que l'accumulation de leurs transcrits n'est pas affectée dans la lignée *AS-IAA*3 favorisant l'hypothèse que Sl-IAA3 agit soit en parallèle soit en aval de HLS dans la formation du crochet apical. Les études d'expression montrent que seul Sl-*HLS2* répond à l'éthylène et à l'auxine au niveau du crochet. L'analyse de lignées *proHLS2:GUS* montre que l'expression de ce gène est exclusive à la face convexe du crochet apical, exactement à l'opposé de celle du gène Sl-*IAA3.* Ces résultats suggèrent que la formation du crochet fait intervenir un jeu d'expression entre Sl-*HLS2* et Sl-*IAA3* qui régule l'élongation cellulaire dans les cotés convexe et concave du crochet. J'ai enfin entrepris de déréguler l'expression des gènes Sl-*HLS1* et Sl-*HLS2* dans la tomate par sur- et sous-expression de ces gènes. Ces lignées qui sont en cours d'obtention permettront de mieux disséquer les modalités d'interaction entre Sl-*IAA3* et les gènes hookless de tomate. Des croisements entre les plantes AS-*IAA3* et les plantes transformées par *proHLS2:GUS* sont également envisagés.

En conclusion, cette thèse a permis l'isolement et la caractérisation de deux gènes intervenant à la croisée des voies de signalisation de l'auxine et l'éthylène chez la tomate. Tout en étant centrée sur des processus de développement

touchant essentiellement les tissues végétatifs, cette étude ouvre de nouvelles perspectives pour aborder le rôle du dialogue hormonal dans le développement et la maturation des fruits.

Organisation du manuscrit de thèse

Après une introduction bibliographique (**chapitre I**) concernant la signalisation de l'auxine, son mode d'action et les modalités d'interactions avec d'autres hormones en particulier avec l'éthylène, le **chapitre II** de ce manuscrit est consacré à la caractérisation fonctionnelle du gène Sl-*IAA3* faisant partie de la famille des Aux/IAA codant pour des régulateurs transcriptionnels de l'auxine. Le **chapitre III** concerne la caractérisation du gène *HOOKLESS* (*HLS1*) et décrit de nouveaux phénotypes du mutant *hls1* d'Arabidopsis. Le **chapitre IV** est dédié à l'isolement et à la caractérisation fonctionnelle des gènes Sl-*HLS1* et Sl-*HLS2* de tomate. Il est montré en particulier que la formation du crochet apical est associée à une répartition fine des territoires d'expression des gènes Sl-*IAA3* et Sl-*HLS2*.

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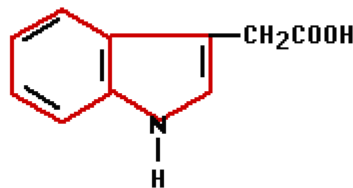
CHAPITRE I

Bibliographic review

I.Introduction

Auxin

Auxin biology is among the oldest fields of experimental plant research. Charles Darwin performed early auxin experiments, observing the effects of a hypothetical substance modulating plant shoot elongation to allow tropic growth toward light (Darwin, 1880). Darwin's experiments expanded upon Theophil Ciesielski's research examining roots bending toward gravity (Ciesielski, 1872). The term auxin was coined by scientists examining plant growth modulating substances in human urine named auxins A and B (Kögl and Haagen Smit., 1931). A structurally distinct compound with auxin activity isolated from fungi was called heteroauxin; auxins A and B were gradually abandoned for the reproducibly bioactive heteroauxin, which was later determined to be indole-3-acetic acid (IAA) (Thimann, 1977).



Indole-3-acetic acid (IAA)

In the plant life cycle, few developmental processes occur without the involvement of the phytohormone auxin from embryonic patterning to fruit dehiscence including the process of wounding.

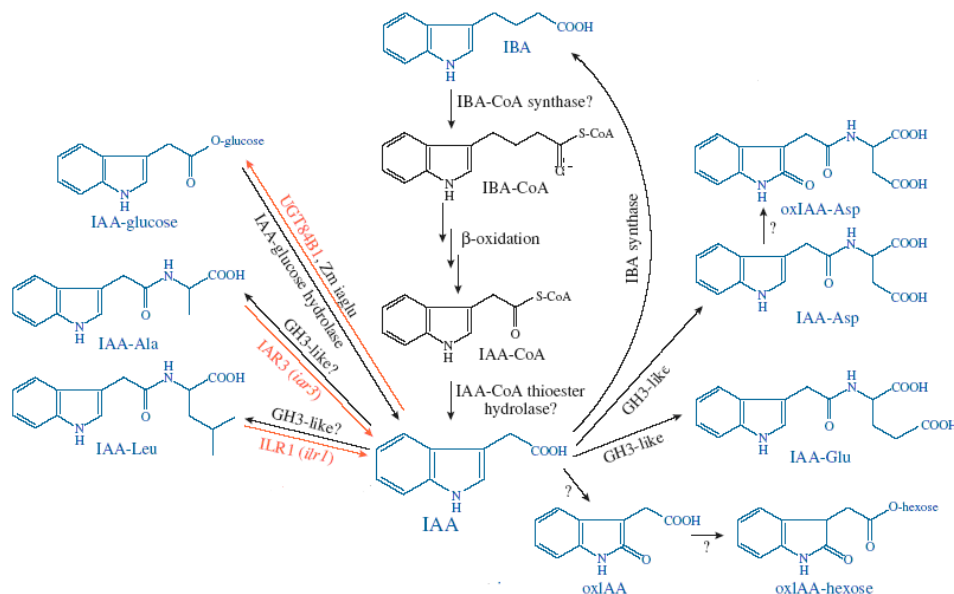
II. Synthesis of IAA

The naturally predominant auxin in all plants is IAA. In seeds and seedlings, it is synthesized primarily from storage conjugates in the endosperm, but just a few days after germination de *novo* synthesis starts and from then on it seems that young leaves close to the shoot apex are the primary, but not the sole source of IAA (Ljung et al., 2002). Some plant species synthesize additional active auxin molecules such as 4-chloroindole-3-acetic acid (CAA), phenylacetic acid (PA),

and indole-3-butyric acid (IBA), but it is likely that IBA is converted to IAA by oxidation in peroxisomes (Bartel et al., 2001).

II. 1 Auxin conjugates

In germinating seeds, IAA is produced from the breakdown of stored forms of the hormone, conjugates of amino acids, proteins, and sugars (Figure 1). The most abundant storage products in dicotyledonous plants are amino acid conjugates. Hydrolysis of these conjugates during germination precedes or



(Woodward and Bartel., 2005)

Figure 1. Potential pathways of IAA metabolism. *Compounds quantified in Arabidopsis are in blue, enzymes for which the arabidopsis genes are cloned are in red, and Arabidopsis mutants are in lower-case italics. A family of amidohydrolases can release IAA from IAA conjugates. ILR1 has specificity for IAA–Leu (Bartel and Fink, 1995), whereas IAR3 prefers IAA–Ala (Davies et al., 1999). Arabidopsis UGT84B1 esterify IAA to glucose (Jackson et al., 2001). IBA is likely to be converted to IAA–CoA in a peroxisomal process that parallels fatty acid b-oxidation to acetyl-CoA (Bartel et al., 2001). IAA can be inactivated by oxidation (oxIAA) or by formation of nonhydrolysable conjugates (IAA–Asp and IAA–Glu). IAA–amino acid conjugates can be formed by members of the GH3/JAR1 family (Staswick et al., 2002, 2005). OxIAA can be conjugated to hexose, and IAA–Asp can be further oxidized (Östin et al., 1998). IBA and hydrolysable IAA conjugates are presumably derived from IAA; biosynthesis of these compounds may contribute to IAA inactivation. Formation and hydrolysis of IBA conjugates may also contribute to IAA homeostasis.*

coincides with the start of root extension. The storage endosperm of seeds is not the only site of IAA conjugate synthesis. Conjugate synthesis is developmentally

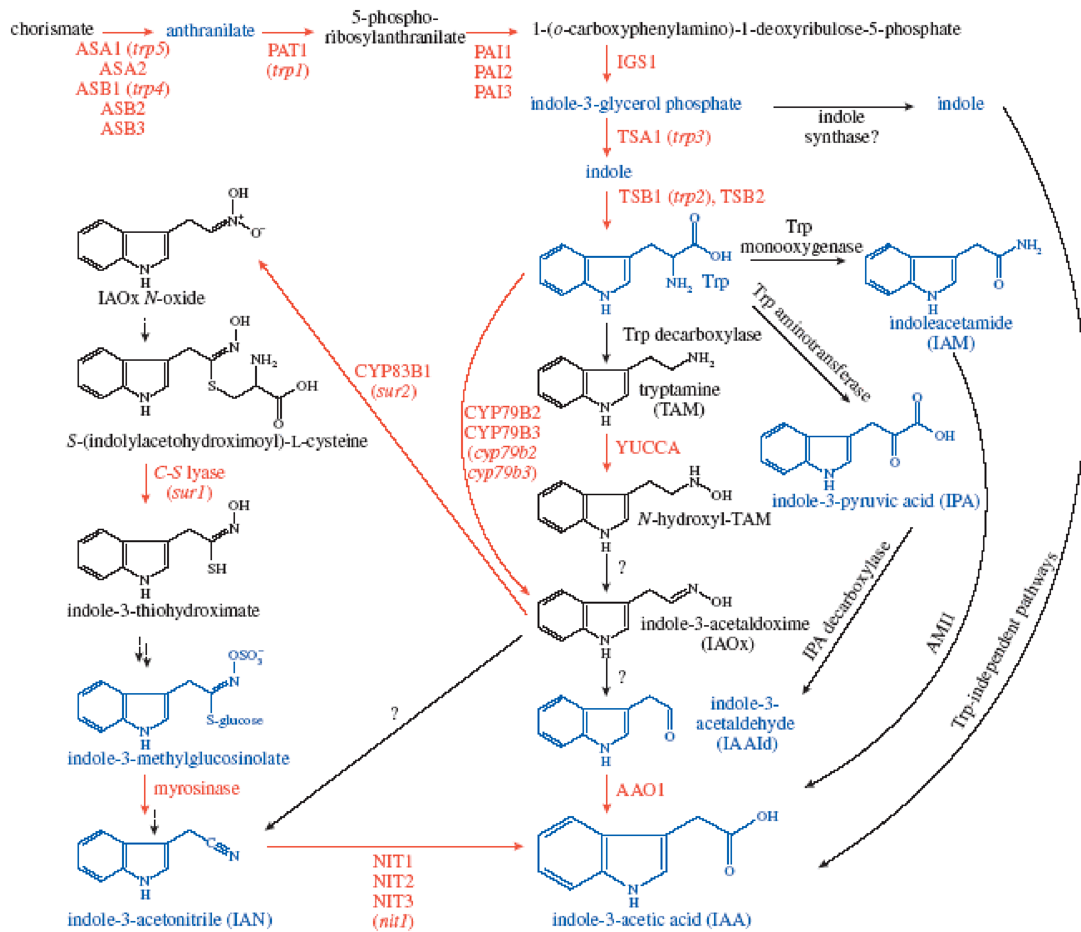
regulated and can be switched on at any time by exogenous application of auxins. Conjugates are likely to be moved into storage compartments such as the plant vacuole and, possibly, the endoplasmic reticulum.

II. 2 De novo synthesis

There are parallel biosynthetic pathways referred to as the tryptophan-dependent and tryptophan-independent pathways. Several Trp-dependent pathways, which are generally named after an intermediate, have been proposed: the indole-3-pyruvic acid (IPA) pathway, the indole-3- acetamide (IAM) pathway and the tryptamine pathway (Figure2).

An Arabidopsis mutant named *yucca* accumulates more IAA than the wild type and exhibits characteristic auxin-enriched phenotypes such as elongated hypocotyls, epinastic leaves, and increased apical dominance (Zhao et al., 2001). The YUCCA protein is a cytosolic flavin monooxygenase that has tryptamine as substrate (Figure2). There is also evidence for a pathway through indole-3-pyruvate (Bartel et al., 2001; Ljung et al., 2002). Moreover, in *Arabidopsis*, an interesting pathway using a set of cytochrome P450s and a C-S lyase (Mikkelsen et al., 2004) has been identified through the analysis of a set of auxin-enriched mutants such as the superroot lines (*sur1* and *sur2)* implicated in the synthesis of glucosinolates.

All parts of young, growing plants appear to participate in IAA synthesis, although this synthesis is tightly controlled in order to maintain homeostasis (Ljung et al., 2001). Very young leaves produce large amounts of IAA. These early high concentrations and high synthetic capacity help drive leaf cell division. As the leaf expands towards its final size, synthesis and concentrations fall. In fully grown *Arabidopsis* plants, the highest concentrations of IAA are found in expanding fruiting bodies, the siliques (Müller et al., 2002). It is likely that most of this is synthesized by seeds during embryo development. The inflorescence stalk contains more IAA than most other parts of the plant, but it is not clear if this is synthesized in situ or if it is in transit (Figure3).



(Woodward and Bartel.,2005)

Figure 2. Potential pathways of IAA biosynthesis in Arabidopsis*. De novo IAA biosynthetic pathways initiate from Trp. Compounds quantified in Arabidopsis are in blue, enzymes for which the Arabidopsis genes are identified are in red, and Arabidopsis mutants are in lower-case italics. Suggested conversions for which genes are not identified are indicated with question marks.*

II. 3 Desactivation

Control of auxin action can be mediated by removing IAA as well as by synthesis. Auxins are removed by conjugation into inactive storage compounds and by oxidation. The primary oxidation auxins product of IAA in *Arabidopsis* and other plants is likely to be 2-oxo-indoleacetic acid (OxIAA), although quantities of oxidized conjugates, OxIAAaspartate, and O-glucoside are also found (Östin et al., 1998). Free IAA is metabolized by decarboxylation by a variety of plant peroxidases in vitro, but the quantities of such products from intact tissues are generally very low suggesting that these are less important catabolic pathways in vivo.

III. Auxin distribution and transport: the road network

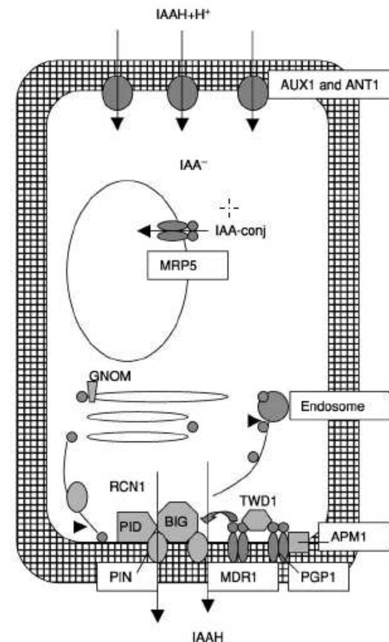
All plant hormones are moved around the plant in the vasculature where, once loaded, they move passively as passenger molecules. Auxin is so far unique among plant hormones in being actively moved around the plant by a series of transmembrane pumps or pump components (Blakeslee et al., 2005). The chemiosmotic hypothesis is a long-standing and widely accepted model for the basic operation of this system (Goldsmith et al., 1981). Auxin is a weak acid, and at the extra-cellular (apoplastic) pH a significant fraction is protonated and hence apolar. As such auxin can freely diffuse into the cell, where the pH is higher, resulting in ionisation. The auxin ions are then trapped in the cell and can only leave through active transport, energised by the electrochemical gradient across the plasma membrane. The auxin efflux activity can be localised to a specific part of the cell surface. Thus, in a file of cells that are all polarised in the same direction, auxin movement will be unidirectional. Polar auxin transport contributes to many of the important auxin-dependant response as cell division, cambial development, apical dominance and gravitropism but is not correct to suppose that all auxin movement is through the polar transport system. Considerable quantities of free IAA and auxin conjugates are carried in the vasculature, particularly the phloem. (Baker, 2000; Cambridge and Morris, 1996; Else et al., 2004).

Many of the auxin-driven responses fail if auxin is absent or in excess, or if polar auxin transport is defective. Certainly, hormone delivery is as important as the hormone itself. As a result, auxin physiology has benefited from the use of drugs that act specifically to inhibit polar auxin transport. Of these, naphthylphthalamic acid (NPA) is the most response-specific and has been used widely.

III.1 Update carriers

All Candidate proteins described for both uptake and efflux have been identified from screening mutant populations and the application of molecular genetics. The influx carrier gene, known as AUX1, was isolated from seedlings

insensitive to the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (Bennett et al., 1996). The AUX1 is an integral membrane protein, a member of the amino acid-proton cotransporter superfamily. Arabidopsis and other plant species tend to have small gene families of AUX1-like proteins (Schnabel and Frugoli, 2004). In addition, a member of the aromatic and neutral amino acid transporter family (AtANT1) has been found to carry IAA in heterologous expression studies using yeast (Chen et al., 2001) and seems likely to contribute to overall auxin influx.



(Perrot-Rechenmann and Napier., 2005)

***Figure 3. The dynamics of auxin transport**. Auxin is imported into the cell (at the top) through influx carriers, notably AUX1 and the less specific ANT1. Influx is cotransport with protons. Inside the cell, IAA dissociates to the IAA- anion and is subject to conjugation as well as export through an efflux complex. Conjugates (and possibly free IAA) are compartmentalized through carrier proteins like MRP5. The most important component of the efflux complex is one or more PIN proteins and it seems likely that the kinase PID and phosphatase RCN1 and BIG are all involved in the regulation of PIN activity. The PIN proteins are rapidly recycled away from the plasma membrane to the plant endosomal system. New PIN proteins are secreted through a GNOM-regulated ER–Golgi– vesicle pathway. Additionally, MDR1 may regulate the PIN complex and transport IAA directly. The twisted protein (TWD1) interacts with a cytosolic domain of MDR1 and also with PGP1, which in turn interacts with APM1.*

III.2 The efflux complex and the importance of vesicle cycling

There are tree classes of transmenbrane protein that have been involved in polar auxin transport. The PINs (a plant-specific transporter family), the PGP/MDRs (Multi-Drug Resistant-like transporters) and the KUPs (Potassium

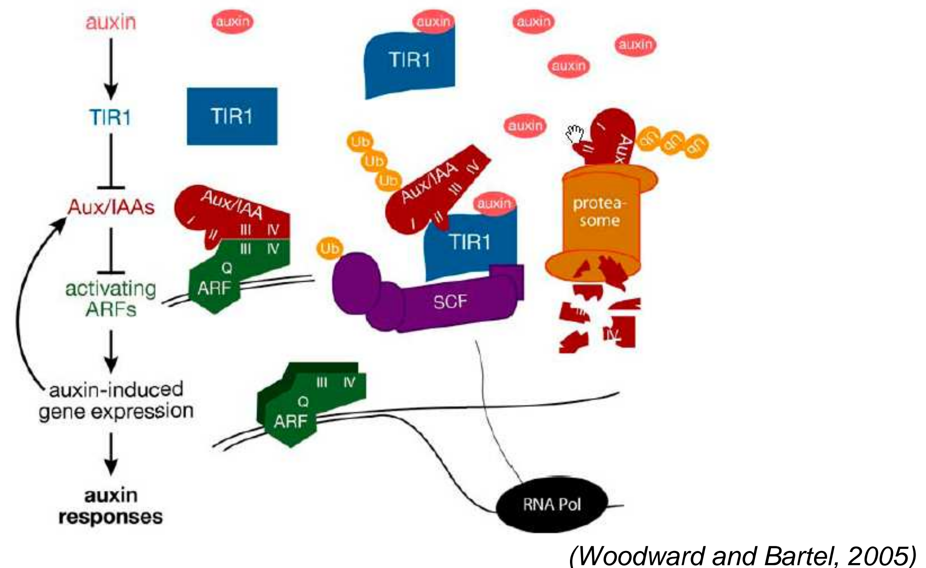
transporter-like) (Vicente-Agullo et al., 2004). The relationship between these classes is unclear; however, mutants in many of the genes encoding these proteins result in auxin transport defects. Both the PINs and the MDRs appear to be able to transport auxin directly and at least partially independently of each other (Geisler et al., 2005; Petraek, et al., 2006).

The PINs are the best characterised in planta and show an excellent correlation between PIN localisation to a particular cell face, and the direction of auxin transport (Winiewska et al., 2006). PIN targeting appears to be a highly dynamic process with continuous cycling of the PINs between the cell surface and an intracellular compartment, a process dependent on ARF-GEF proteins such as GNOM (Figure3).

IV. Auxin perception, receptor and signalling

IV.1 .Perception and receptors: TIR1 the heart of auxin-signalling

Receptor proteins are key components of signalling systems and their discovery is fundamental to both the understanding and the exploitation of hormonal regulation. In 2005, a receptor of auxin was identified as the F-box protein TIR1 (Transport Inhibitor Response 1). The TIR1 gene was first identified in a genetic screen for Arabidopsis plants tolerant to auxin transport inhibitors (NPA). However, soon after it was shown that TIR1 was involved in auxin action. TIR1 is a component of a cellular protein complex known as SCF^{TIR1} (Skp1/Cullin/F-box) (Dharmasiri et al., 2005; Kepinski et al., 2005) involved in ubiquitin-mediated protein degradation (Ruegger et al., 1998). The substrates for TIR1, Aux/IAA repressors, are recruited to the receptor in an auxin-dependent manner and, after binding to TIR1, are degraded. Identification of the TIR1 receptor suggested that auxin perception and the signalling pathway to auxin-regulated gene expression was direct and simple, but it left various questions.



(Woodward and Bartel, 2005)

Figure 4. A Model for auxin response through the TIR1 auxin receptor pathway. *Transcriptionally activating ARFs (green) are bound to auxin-responsive promoter elements but are counteracted (blunt arrows) by heterodimerization with Aux/IAA transcriptional repressors (dark red) via two domains (III and IV) conserved between ARF and Aux/IAA proteins. Auxin (pink) binds to TIR1 (blue) or a TIR1-Aux/IAA complex to promote or stabilize TIR1-Aux/ IAA domain II interaction. TIR1 tethers the Aux/IAA protein to an SCF complex (purple) that is thought to catalyze attachment of multiple ubiquitin (Ub) moieties to the Aux/IAA target protein. The ubiquitinated Aux/IAA protein is then degraded by the 26S proteasome (orange). Increased Aux/IAA degradation in response to auxin frees the activating ARF proteins from repression, allowing auxin-responsive transcription. Among the auxin-induced transcripts are those encoding the Aux/IAA repressors themselves, creating a negative feedback regulatory system. See text for references.*

Tan et al. *(*2007) expressed TIR1 complexed with ASK1 (a SCF ^{TIR1} adaptor) and demonstrated that auxin enhanced the binding of an Aux/IAA protein to the complex, they obtained crystal structures for the complex alone and for complexes bound to IAA and the two synthetic auxins (1-NAA and 2,4-D) along with an Aux/IAA peptide.

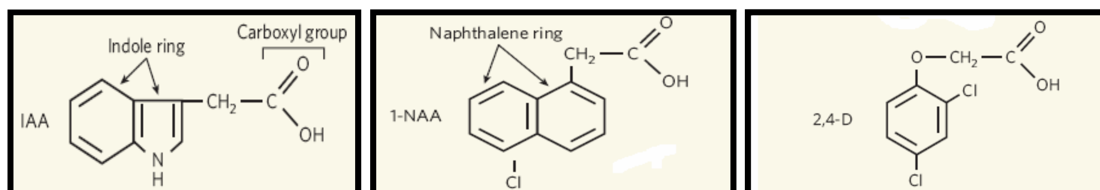


Figure 5. The main molecular players in the work of Tan et al (2007). *The three auxin ligands that the authors crystallizedin association with the TIR1 auxin receptor. IAA itself, and the synthetic auxins 1-NAA and 2,4-D, bind to a 'promiscuous' cavity in the receptor with different affinities, but all of them stabilize the interaction between the Aux/IAA repressor and the receptor.*

The crystal structures showed that the TIR1–ASK1 complex had a mushroom shape, with the leucine-rich-repeat domain of TIR1 forming the cap, and the F-

box of TIR1 along with ASK1 forming the stem. A pocket on the top of the TIR1 leucine-rich-repeat domain functions in both auxin binding and substrate recruitment. It turns out that auxin binds to the bottom of the pocket that tolerates moderately different planar ring structures (that is, natural and synthetic auxins).

(Tan et al., 2007)

Figure 6. Schematic diagram of auxin functioning as a 'molecular glue' to enhance TIR1–substrate interactions. *In contrast to an allosteric mechanism, auxin binds to the same TIR1 pocket that docks the Aux/IAA substrate. Without inducing significant conformational changes in its receptor, auxin increases the affinity of two proteins by simultaneously interacting with both in a cavity at the protein interface.*

The Aux/IAA peptide binds in close proximity to the auxin-binding site in the upper part of the pocket (Figure6). The GWPPV motif of Aux/IAA proteins (Figure7) is packed directly against auxin and covers the auxin binding site. This is thought to trap auxin in the binding pocket until the Aux/IAA peptide is released and moved along the degradation pathway.

(Tan et al., 2007)

Figure 7. Depiction of the four conserved domains of Aux/IAA repressors. *The synthetic peptide from domain II is sufficient for targeting Aux/IAA to the TIR1 auxin receptor, the core sequence being GWPPV (G, glycine; W, tryptophan; P, proline; V, valine).*

The authors compared TIR1 structures that had IAA or the two synthetic auxins in the ligand binding site. IAA binds to TIR1 with the greatest affinity of the three auxins (Figure 5). The synthetic auxins bind to TIR1 in a manner similar to IAA, but with affinities determined by how effectively their ring structures fit into and interact with the promiscuous cavity of the receptor.

Interestingly, the binding of auxin does not induce significant conformational changes in TIR1. Instead, auxin enhances the binding of Aux/IAA substrate to TIR1 by occupying a cavity between substrate and receptor, thus forming a

continuous hydrophobic core among ligand, substrate and receptor. The authors characterize this as a '*molecular glue*' that effectively strengthens the binding of the substrate to the receptor (Figure 6).

Two others F-box proteins had been identified earlier [now named Auxin signalling F-Box proteins (AFB) 1 and 2] but not studied in detail (Ruegger et al., 1998). One other is named as AFB3 (Dharmasiri et al., 2005). Each AFB protein has been shown to bind Aux/IAAs in an auxin-dependent manner (Dharmasiri et al., 2005). Seedlings of each single TIR1 and AFB mutant line resemble wild-type seedlings in light or dark, but some do display auxin-tolerant phenotypes such as a partial tolerance to auxin-induced inhibition of root elongation (Dharmasiri et al., 2005). Far greater additive effects are found in double, triple and quadruple mutants. Triple and quadruple mutant lines show high levels of arrest after germination, although some seedlings do develop and a range of phenotypes develops in the quadruple mutants (Figure8). Classified into three groups, the most severe group fails to develop a root and develops only a single cotyledon. The least severe group has seedlings with roots that respond poorly to gravity and lack hairs. In more mature plants, the triple and quadruple mutants develop rosettes with reduced highly curled leaves and the inflorescences are dwarfed and highly branched (Dharmasiri et al., 2005).

(Badescu and Napier., 2006)

Figure 8. Seedlings of the quadruple mutant tir1 afb1 afb2 afb3 show three classes of phenotype. *Seven-day-old seedlings were grown on vertical plates in the light. The most extreme phenotype (right) is rootless, shows no hypocotyl extension and often has only a single cotyledon, resembling monopteros plants at the same age. The least extreme (left) shows impaired gravitropism and few root hairs.*

All these observations are consistent with the assertion that the TIR1 family acts as a set of auxin receptors with overlapping redundant functionality between members.

IV. 2 Signalling

IV.2.a .Auxin induced transcripts

Auxin rapidly and transiently induced accumulation of at least three families of transcripts: SMALL AUXIN-UP RNAs (SAURs), GRETCHENHAGEN (GH3)-related transcripts and Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) family members.

SAUR transcripts accumulate rapidly after auxin exposure in soybean (Walker and Key., 1982) and many other species, including Arabidopsis (Gil et al., 1994). Maize ZmSAUR2 is a small nuclear protein that, like the encoding transcript, is rapidly degraded (Knauss et al., 2003). The short half-lives of SAUR mRNAs appear to be conferred by downstream elements (DSTs) in the 3' untranslated region of the message (Sullivan and Green., 1996). Arabidopsis mutants that stabilize DST-containing RNAs, and thus stabilize SAUR transcripts, have no reported morphological phenotype (Johnson et al., 2000), and the function of these small RNAs remains unknown.

GH3 transcript accumulation is also induced by auxin (Hagen et al., 1984). At least some IAA-induced GH3 genes encode IAA–amino acid conjugating enzymes (Staswick et al., 2005) (Figure 1), whereas several GH3-related proteins that are not auxin regulated function to adenylate or conjugate amino acids to molecules other than IAA, including jasmonic acid (Staswick et al., 2002; Staswick and Tiryaki., 2004). Thus, the auxin induction of GH3 genes likely serves to dampen the auxin signal by inactivating IAA via conjugation.

Aux/IAA family includes 29 proteins in Arabidopsis. Induction of some Aux/IAA genes occurs within minutes of auxin application and does not require new protein synthesis (Abel et al., 1994; Abel and Theologis., 1996). Aux/IAA genes encode proteins that generally have nuclear localization and four conserved domains (I–IV). Domain I is a transcriptional repressor (Tiwari et al., 2004). Domain II is critical for Aux/IAA instability; several mutations in this domain

have been isolated as gain-of-function alleles that stabilize the proteins and confer auxin-resistant phenotypes. Domains III and IV are involved in homodimerization and heterodimerization with other Aux/IAA proteins and with Auxin Reponse Factors (ARFs) (Kim et al., 1997; Ulmasov et al., 1999; Hardtke et al., 2004) (Figure 7).

Gain-of-function mutations in several Aux/IAA genes, including *axr5/iaa1* (Yang et al.,2004), *shy2/iaa3* (Tian and Reed., 1999), *shy1/iaa6* (Kim et al., 1996; Reed., 2001), *axr2/iaa7* (Timpte et al., 1994; Nagpal et al., 2000), *bdl/iaa12* (Hamann et al., 2002), solitary root *slr/iaa14* (Fukaki et al., 2002), *axr3/iaa17* (Rouse et al., 1998), *iaa18* (Reed., 2001), *msg2/iaa19* (Tatematsu et al., 2004), and *iaa28* (Rogg et al., 2001), have pleiotropic effects on plant growth. All of these primary mutations in Aux/IAA genes were found in highly conserved domain II, which is responsible for protein degradation. The mutations stabilize the proteins resulting in gain of function phenotype.

IV.2.b. Auxin-responsive- elemen Aux/RE and isolation of Auxin Response Factor (ARF)

Many genes with auxin-induced expression, including most SAUR, GH3 and Aux/IAA genes, share a common sequence in their upstream regulatory regions, TGTCTC or variants, first identified from the promoter region of the pea Ps-IAA4/5 gene (Ballas et al., 1993). Regions including this sequence, known as the Auxin-Responsive Element, or AuxRE, confer auxin-induced gene expression in synthetic constructs (Ulmasov et al., 1995, 1997b). More recently, genome-wide profiling experiments have revealed a wealth of auxin-induced genes (Sawa et al., 2002; Pufky et al., 2003; Cluis et al., 2004; Himanen et al., 2004), many of which contain AuxREs in putative regulatory regions (Pufky et al., 2003; Nemhauser et al., 2004). The identification of the AuxRE sequence led to the isolation of the Arabidopsis Auxin Response Factor1 (ARF1) gene (Ulmasov., 1997a) and subsequent genetic, genomic, and molecular studies have identified 23 ARF genes in Arabidopsis (Liscum and Reed., 2002). In addition to a conserved amino-terminal (N-terminal) domain that mediates AuxRE binding (Tiwari et al., 2003), most ARF transcription factors also contain carboxyl-terminal

(C-terminal) dimerization elements (domains III and IV). In between is a variable middle region (MR) that generally is either glutamine (Q)-rich or serine (S)-rich. This difference is apparently a major determinant of ARF function, with S-rich ARFs acting as transcriptional repressors and Q-rich ARFs as transcriptional activators in protoplast transfection assays (Tiwari et al., 2003).

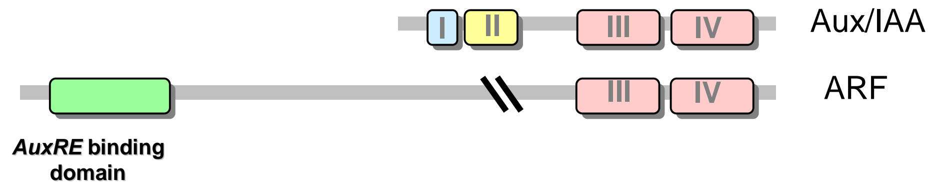


Figure 9. Diagram of Aux/IAA and ARF protein with conserved domains. *Aux/IAA genes share four conserved domains including the C-terminal domains III and IV that are found in most ARF proteins. These domains mediate Aux/IAA–ARF heterodimerization.*

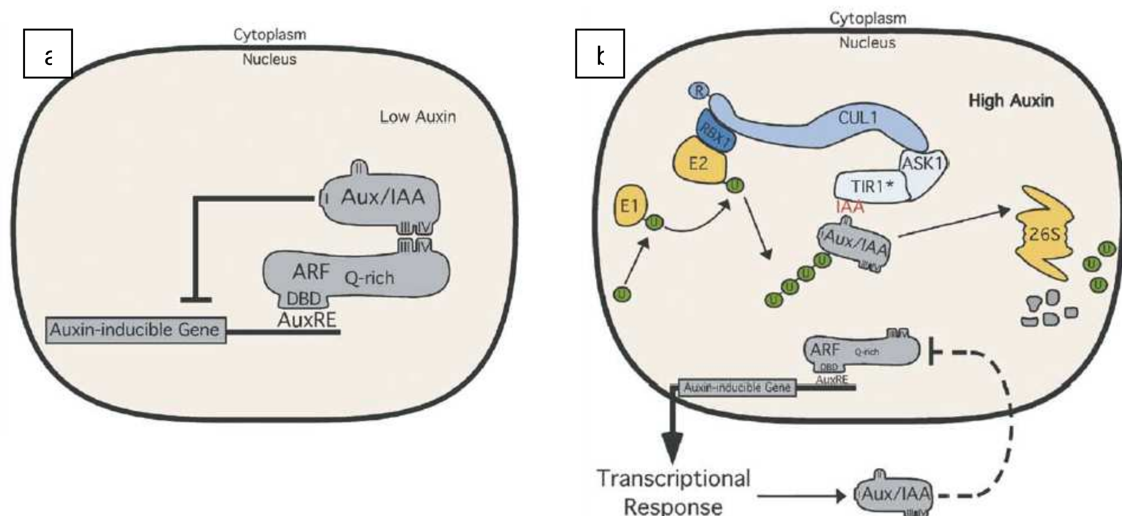
Glutamine-rich ARFs such as ARF5 and ARF7, when mutated, they often give remarkable phenotypes; for example, those seen in *monopteros* (ARF5) (rootless) and those seen in *non phototropic hypocotyl4* (ARF7) (unable to bend towards light) (Hardtke et al., 2004). There is emerging evidence that ARFs that lack a glutamine-rich middle region function act as transcriptional repressors (Tiwari et al., 2003).

IV.2.c.Transcriptional control

The effects of auxin are thought to depend on its concentration, with high and low doses eliciting different responses. At basal auxin levels, Aux/IAAs are relatively stable, homodimerize and heterodimerize with ARFs that can bind to Aux/RE in the promoters of auxin-responsive genes. The ARF-bound Aux/IAA proteins block transcription from auxin-responsive promoters by controlling the amount of free ARF transcription factors to the promoters (Figure 10a). An increase in auxin levels causes the proteasome mediated degradation of Aux/IAAs, which in turn allows for a gradually increasing number of functionally active ARF proteins and the transcriptional activation of auxin regulated genes. The Aux/IAA genes themselves are auxin-inducible. This might represent a negative feedback loop that ensures a transient response, with the nascent

Aux/IAA proteins attenuating the signalling pathway as auxin levels fall by restoring repression of the ARF transcription factors (Figure 10b).

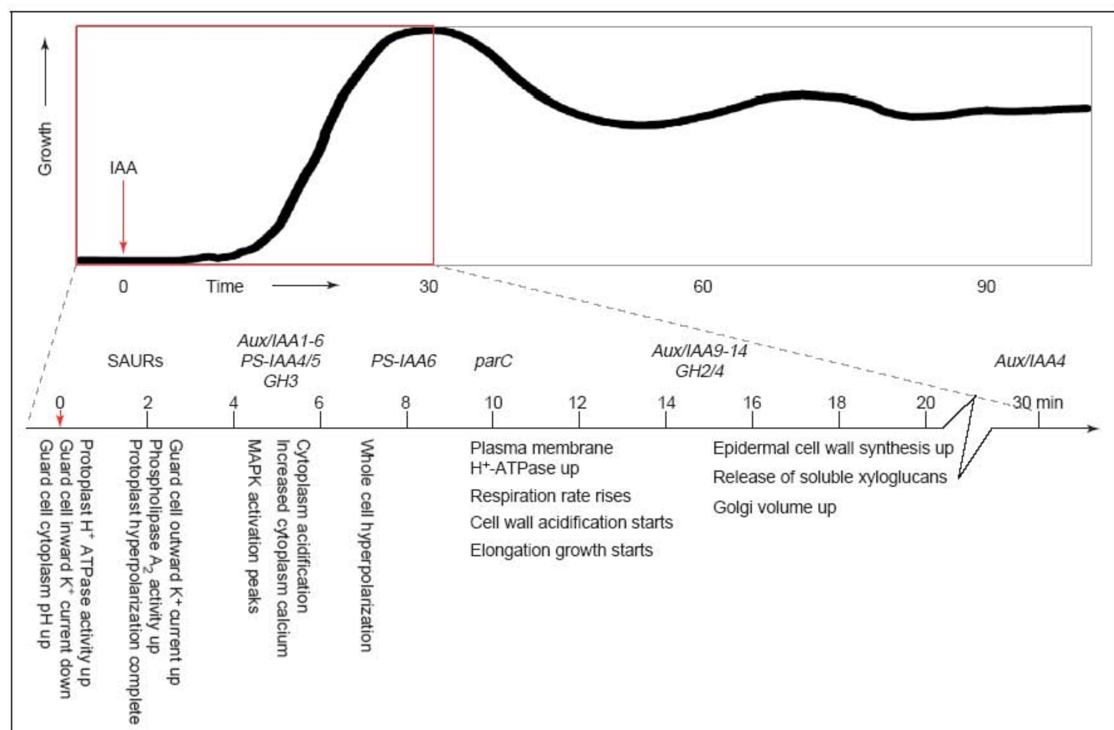
In conclusion, The SCF$^{TIR1/AFB}$-mediated proteolysis of Aux/IAA proteins is clearly responsible for many of the effects of auxin, but the resulting changes in gene expression occur too slowly to account for the most rapid auxin responses, including ion fluxes across the plasma membrane (Hager et al., 2003) and mitogen-activated protein (MAP) kinase activation (Mockaitis et al., 2000) (Figure 11). There are two further sets of responses induced by auxin for whish TIR1 is unlikely to be the receptor: those for whish the receptor is shown to be extracellular and those for which other receptor can be specified.



(Quint and Gray, 2006)

***Figure 10: Auxin regulation of gene expression**. (a) Under basal auxin concentrations the Aux/IAA proteins heterodimerize with the ARF transcription factors, thereby repressing auxin-inducible gene expression. (b) Auxin binding to the TIR1/AFB receptors promotes the recruitment of Aux/IAA proteins to the SCF complex. Subsequent Aux/IAA ubiquitinylation and proteasome-mediated degradation results in a decline in Aux/IAA protein levels, thus de-repressing auxin-inducible gene expression. DBD, DNA-binding domain; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; U,ubiquitin; R, RUB,AFB1, AFB2, or AFB3.*

This receptor might very likely be ABP1 (Auxin Binding Protein1) (Steffens et al., 2001) but the mechanism of ABP1 action and the identities of other components of this pathway await discovery. Furthermore, several factors, including the repressing ARFs, SAUR proteins, MAP kinase pathway components, have been implicated in auxin signalling but are presently without a home in current models. Clearly, much remains to be learned about the mechanics of auxin signalling.



(Badescu and Napier., 2006)

Figure 11. Early events after an auxin stimulus. *The upper panel shows a trace of the growth against time for a coleoptile after addition of exogenous auxin at time 0. There is a characteristic lag of 10–15 min after which extension rate increases rapidly before falling off after 30 min. In the continuing presence of auxin, a second peak follows after 60 min and an elevated growth rate is sustained. The first 30 min of the timeline is expanded below; against this timeline times are indicated at which specific responses occur. Some of the transcripts that increase in abundance in response to auxin are indicated in the center of the figure; changes in protein activity or cellular function are shown at the base. Many responses precede changes in transcript abundance.*

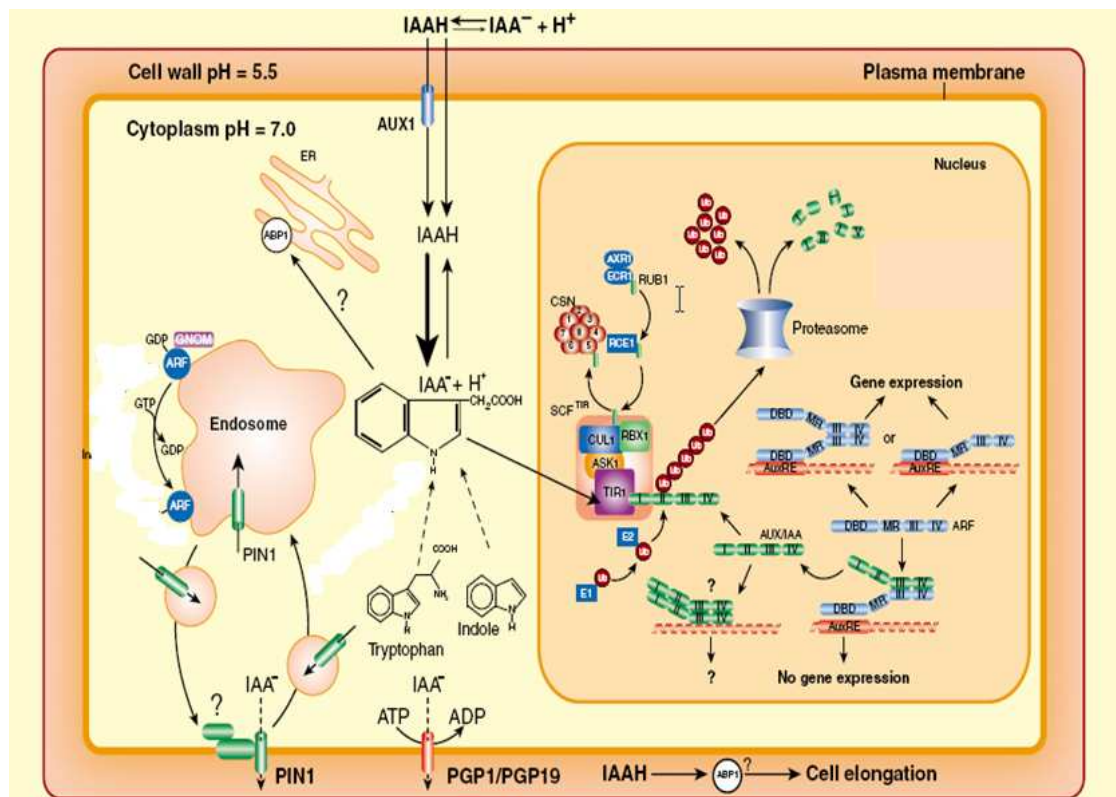
V. HOMEOSTASIS

The balance of synthesis, breakdown, conjugation, and transport is regulated rigorously to give auxin homeostasis and thus orchestrate the plant development. Clearly, changes in auxin concentration are important as plants respond to

stimuli, but homeostasis is critical for both optimal development and to keep the system primed for stimulatory responses. At present, the feedback mechanisms for homeostasis are not well characterized. However, it is clear that auxin transport plays a crucial role in both establishing and perturbing homeostasis (Figure 12).

VI. Role of auxin

Auxin is an absolute requirement for plant cell division, as well as to many other developmental and environmental responses that are mediated through auxin movements. Of these, a good number are commercially relevant, such as branching, rooting, and fruiting, but the biggest market for synthetic auxins is as selective herbicides.



(Paciorek and Friml, 2006)

Figure 12. Auxin homestasis.

VI.1 Cell division and tissue culture

One of the most profound actions of auxin on plants is the control of cell division. A primary event in the stimulation of cell division is the cytokinin-mediated initiation of cyclin D transcription (Cockcroft et al., 2000). The newly synthesized cyclin D associates with a cyclin-dependent kinase (CDK) to create an active complex at the G1 to S transition. The CDK-a/cyclin D complex leads to phosphorylation of the retinoblastoma tumor suppressor protein (Rb). Inactivation of Rb in late G1 provokes release of the transcription factor E2F, and genes controlled by E2F factors are therefore activated, driving cells into DNA replication and committing them to the cell cycle. Cells can be arrested either in G1 or in G2 phase after auxin deprivation (Planchais et al., 1997).

Generally, higher auxin concentrations stimulate cell division, and low auxin concentrations drive cell elongation, cell enlargement, and cell differentiation (Winicur et al., 1998; Zazimalova et al., 1995).

Among the most striking examples of this critical control over division is in cell and tissue culture where auxin must be included in almost all media. Furthermore, auxin and cytokinin concentrations can be manipulated to promote proliferative callus growth, regeneration of vegetative tissue, or root induction. Elevated auxin/cytokinin in ratio is favorable for rooting, whereas a reduced ratio facilitates buds regeneration. Horticultural practices, such as cutting and clonal micropropagation, are directly based on these characteristics.

VI.2 Organ patterning

VI.2.a Phyllotaxis

Phyllotaxis is the regular arrangement of leaves on a stem. Polar auxin transport inhibitors and Arabidopsis mutants defective in polar transport like *pin1* have helped us to illustrate that accumulation of IAA in cells at the side of the shoot apical meristem initiates organogenesis and determines the position of the next leaf primordium. Moreover, microapplications of transport inhibitors or IAA to

tomato shoot apex have shown that for leaves and flowers, auxin application determines primordium development in the radial dimension (Reinhardt et al., 2000). The cellular and subcellular distributions of the efflux proteins PIN and AUX1 in the surface layers of cells towards the tip of the shoot apical meristem were shown to correspond to the subsequent differentiation of leaf primordial (Reinhardt et al., 2003).

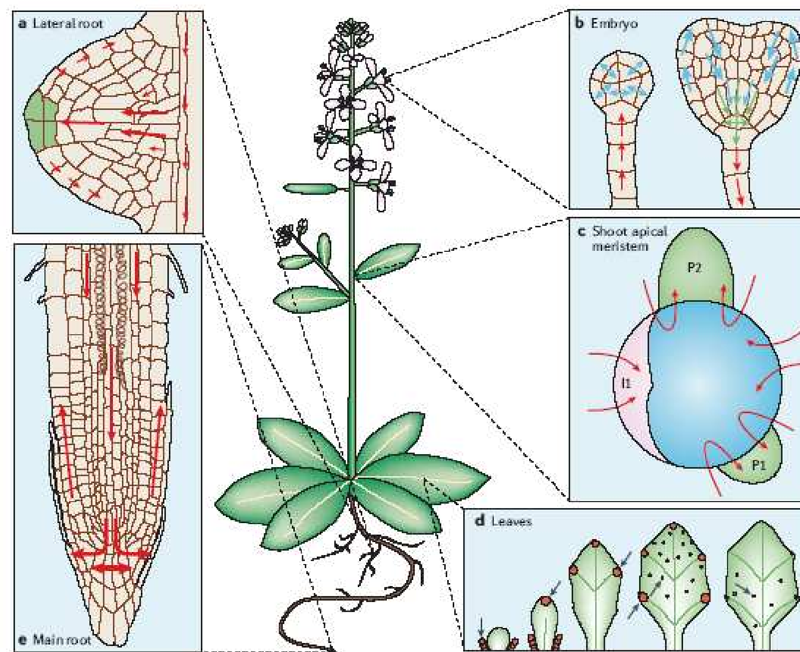
VI.2.b Root initiation and gravitropism

Auxin is transported into distinct tissues of the root drives lateral primordium development (Blilou et al., 2005; Casimiro et al., 2001). Specific PIN proteins then contribute to root patterning, particularly AtPIN4 (Friml et al., 2002), although the AUX1 protein is again an essential contributor. The center of auxin concentration in roots is at the root tip, just behind the cap (Bhalerao et al., 2002). From here both AUX1 and PIN proteins work to produce defined gradients along the root epidermal layers stretching back into the elongation zone (Ottenschlager et al., 2003). These control not just lateral rooting but also gravitropism (Friml et al., 2002; Müller et al., 1998).

VI.3. Apical dominance and branching

In addition to controlling the initiation of leaf primordia at shoot apices it has been recognized for many years that auxin transported back from the shoot apex controls the outgrowth of side branches from axillary buds (Figure 13). Gardeners and horticulturalists make use of this apical dominance to control plant structure by pruning. By removal of the stem apex, the source of auxin is removed and its inhibitory action on axillary buds is released, leading to side branch outgrowth and bushier plants. Consistent with these observations, classic experiments by Thimann and Skoog showed that if auxin is applied to the pruned apical stump, bud outgrowth is inhibited (Davies, 1995).

Apical dominance is active in roots as well as stems. Root tip-derived auxin certainly inhibits lateral root initiation close to the apex (Casimiro et al., 2001).



(*Teale et al.,* 2006)

Figure 13. The developmental processes that are controlled by auxin flux.
a *Lateral root. PINs conduct auxin from the centre of the root (stele) to the new root tip (auxin is indicated in green and auxin transport is indicated by red arrows), and then away again through the epidermis.*
b *Embryo. Auxin is taken to the very young embryo by PIN7 (left). At a later stage (right), the auxin flux is reversed as PIN1, PIN4 and PIN7 conduct auxin out of the embryo. Transport by PIN1, PIN4 and PIN7 is indicated by blue, green and red arrows, in corresponding order.*
c *Shoot apical meristem. Auxin is redirected towards the site of new leaf formation (primordial P1 and P2 and the incipient primordium) in the epidermal layer. The shoot apex is indicated in blue.*
d *Leaves. Auxin mediates vascular tissue development and patterning in the developing leaf through non-polar PIN1. The arrows indicate sites of auxin production and the redcircles indicate auxin accumulation.*
e*. Main root. PINs determine the flux of auxin towards the root tip in the centre of the root, and back again in the epidermis. This movement forms the basis of the root's ability to respond quickly to gravity.*

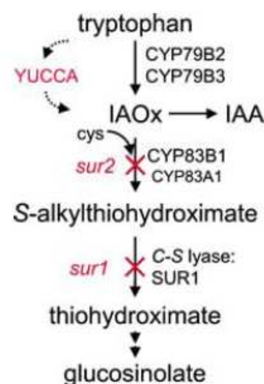
VI.4.Tropisms

Plants respond to different signals as light and gravity by differential growth. Auxin gradients, generated by polar auxin transport, have been implicated. This hypothesis is supported by the experiments of Cholodny and Went (Davis., 1995). In stems, greater auxin concentrations enhance elongation growth. In roots auxin inhibits growth. Auxin measurements have shown that the side of stems expanding more rapidly was found to contain a little more IAA (Harrison and Pickard., 1989). Recent data on the stimulus-induced redistribution of specific members of the PIN family in roots (e.g., PIN3), data from auxin measurements,

from auxin response mutants, all support this hypothesis. Other data, such as kinetic measurements of gravistimulated roots and experiments in which seedling shoots are incubated in excess IAA that are still found to be able to respond by differential growth (Edelmann., 2001), argue against the first hypothesis. Either way, it is clear that auxin is a requirement for differential and tropic growth, and many auxin sensitive mutants like *aux1* have agravitropic root phenotypes (Bennett et al., 1996).

VI.5. Adventitious rooting

Addition of auxin to cut or damaged stems often induces a strong adventitious rooting response, and horticultural industries relying on clonal propagation make good use of this response for ornamentals, trees, flowers, and general garden plants. The initiation of root primordia in stem tissues requires a redifferentiation response, and, although auxin promotes this, the molecular or genetic mechanisms are unclear. There are mutants of Arabidopsis that show precocious rooting along the seedling hypocotyl, superroot (*sur*) 1 and 2, for example (Boerjanet al., 1995). In both, auxin levels are elevated due to accumulation of endogenous aldoxime (IAOx) that is channeled into auxin (Figure 14).



(Mikkelsen et al., 2004)

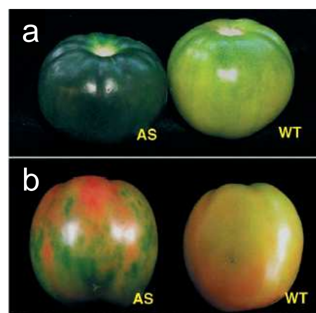
Figure 14. The role of SUR genes in auxin homeostasis. *Hight auxin mutant are schown in red.*

VI.6. Fruit growth

Auxin plays a vital role in all stages of reproductive growth. Some of the highest auxin concentrations have been found in developing fruit (Müller et al.,

2002). It has been postulated that auxin is first produced by elongating pollen tubes and then by the embryo and endosperm in the developing seeds. Subsequent development of the fruit appears to depend on these sources of auxin. In the early 1950s, Nitsch showed that achene (seed) removal from strawberry receptacles inhibited receptacle enlargement (Pennazio., 2002). Replacing the achene with a supply of auxin maintained fruit growth. Cessation of auxin supply also led to ripening in this nonclimacteric fruit. Supporting this hypothesis, the auxin-resistant tomato mutant, diageotropica, which encodes a cyclophilin has reduced fruit set, fruit weight and seed production (Balbi and Lomax., 2003; Oh et al., 2006) and the application of either auxin or auxin transport inhibitors that cause an increase in auxin in the ovary stimulate fruit set and the development of parthenocarpic fruit (Gustafson., 1937; Beyer and Quebedeaux., 1974).

Parthenocarpy has also been induced in tomato and other fruit by ovary-targeted ectopic expression of Agrobacterium *iaaM* under the control of carpel-specific promoter, which confers higher auxin production and induces seed free fruit (Ficcadenti et al., 1999). Some apple and cherry crops are sprayed with auxin (mixed with other hormones) at flowering stage to induce fruit set. Treatments of orchards to delay fruit abscission is becoming common to facilitate automated harvesting, Thus maximizing the yield of ripe fruit collected in the minimum number of passes by harvesters. In 2002, Jones demonstrated for the first time that down-regulation of DR12, an auxin-response-factor homolog, in tomato, results in a pleiotropic fruit phenotype including dark green, blotchy ripening fruit and enhanced fruit firmness (Figure 15).

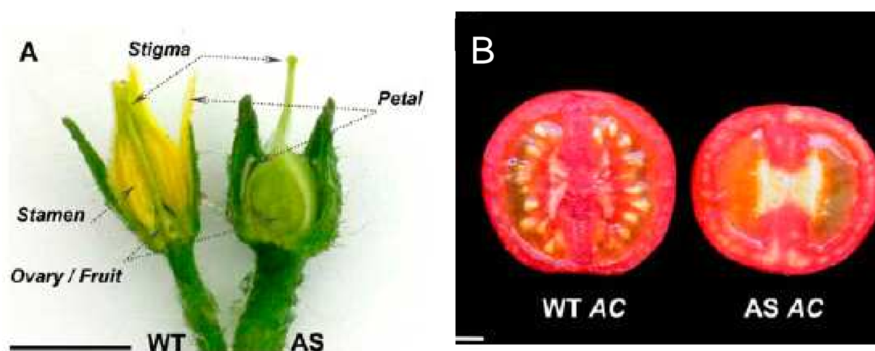


(Jones et al., 2002)

Figure 15. Altered phenotypes of DR12-inhibited plants. *(a) Dark-green phenotype of DR12 antisense fruit (AS) at anthesis +30 days compared to wild-type fruit (WT) at the same stage. (b)*

Blotchy ripening phenotype of DR12 antisense fruit (AS) at breaker +3 days compared to wild-type fruit (WT) at the same stage.

In 2005, Wang et al revealed that the antisense of Aux/IAA like gene (*IAA9*) in tomato shows precocious fruit set and marked parthenocarpy. It appears that in wild-type plants, the presence of the IAA9 protein prevents ovary development prior to pollination, potentially by acting as a negative regulator of auxin response pathways. The downregulation of this gene in the antisense lines may release the expression of target auxin-responsive genes, thus mimicking a burst of auxin produced during pollination leading to fruit set and development independent of pollination and fertilization.



(Wang et al., 2005)

Figure 16. Fruit set and parthenocarpy in AS-IAA9 lines. *(A) Flower buds at 1 d before anthesis in wild-type and AS-IAA9 lines (AS), showing dramatically enlarged ovary and underdeveloped stamen in AS-IAA9 lines. (B) Wild-type Ailsa Craig seeded fruit (WT AC) and AS-IAA9 parthenocarpic fruit (AS AC).*

VI.7. Herbicides

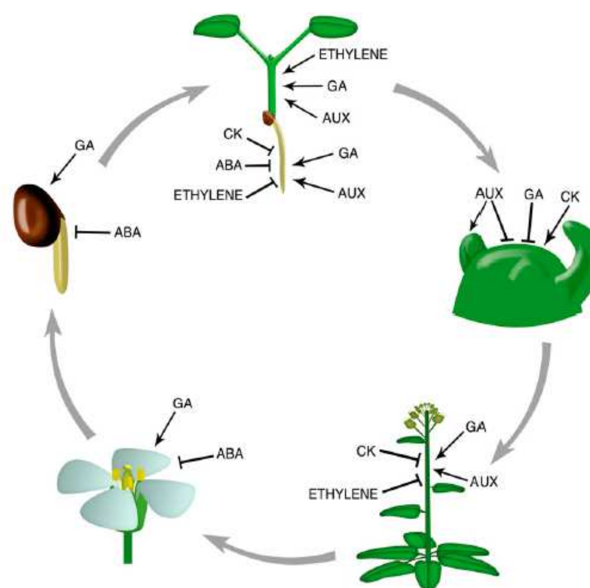
The largest commercial exploitation of plant hormone has been the use of synthetic auxins as selective herbicides. Around the world, a number of populations of auxinic herbicide-tolerant weeds have arisen, an indication of the long time over which applications have been made (Hall et al., 1996). Synthetic auxins such as 2,4-D acid were developed in the 1940s and use has been extensive, particularly because of their selectivity. The mechanisms of herbicidal action and the basis of selectivity are unclear. Induction of a massive synthesis of ethylene has been suggested to be the mechanism of activity. However, recent

work shows that inhibition of either ethylene synthesis or ethylene receptors does not prevent herbicidal activity. Therefore, although induction of the ethylene-synthesizing enzymes is a recognized effect of auxin application (Hansen and Grossmann, 2000; Kim et al., 1992), ethylene remains a symptom of herbicidal auxin and is not the sole mediator. Auxinic herbicides also induce ABA synthesis and this might be a consequence of elevated ethylene synthesis.

Saturating auxin clearly gives rise to a number of damaging symptoms, but genetic data from resistant biotypes suggest that there is a single target site for these herbicides and this seems likely to be the auxin receptor. As more becomes known about the receptor, the understanding of plant development and auxins as agrochemicals will also grow.

VII. Interaction of auxin with other hormones

Interactions with other hormones play major roles in auxin action, which necessitates the existence of efficient and sensitive cross talk mechanisms among the corresponding signalling pathways (Figure 17). Recently, several studies have focused on the molecular machinery behind the interactions between auxins and other hormones, uncovering a complex network.



(Weiss and Ori., 2007)

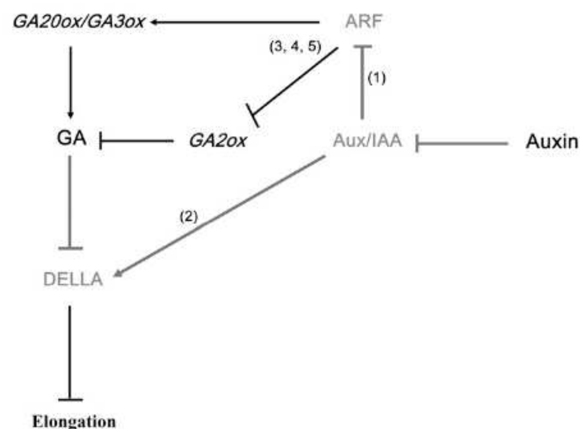
***Figure 17. Auxin interacts positively and negatively with other plant hormones throughout the plant's life cycle**. Some of the effects are shown. Aux, Auxin; CK, Cytokinin; ABA, abscissic acid; GA, Gibberllin.*

VII.1 Auxin and gibberellin (GA)

The activities of gibberellin and auxin overlap with respect to the regulation of cell expansion and tissue differentiation. Auxin affects GA signalling as well as GA biosynthesis (Figure 18). In Arabidopsis, GA stimulation of root elongation has been shown to require auxin. GA-induced root elongation was inhibited by the removal of the shoot apex that is a major auxin source and this effect was reversed by auxin application. Moreover, application of the auxin-transport inhibitor NPA or mutation in the auxin efflux regulator AtPIN1 suppressed the effect of GA on root elongation.

In addition to its requirement for GA signaling in the root, auxin also affects GA production in the stem by positively regulating the expression of GA biosynthetic genes (Nemhauser et al., 2006). Decapitation of pea (*Pisum sativum*) and tobacco (*Nicotiana tabacum*) shoot apices reduced the level of active GAs in the stems and this effect was reversed by auxin application (Ross et al., 2000; Wolbang and Ross., 2001).

As the interactions between auxin and GA involve components from the GA biosynthetic and response pathways, we briefly introduce a few relevant players in these pathways: The first few steps of the GA synthesis, from trans-geranylgeranyl diphosphate to GA12-aldehyde, are common to all species. The final steps to produce active GAs are species specific but in most cases require activity of the GA 20-oxidase (GA20ox) and GA3ox enzymes. In contrast, the enzyme GA2ox antagonizes GA activity by deactivating GAs. The level of endogenous active GA is governed by feedback regulation, where active GAs suppress the expression of the GA20ox and GA3ox genes and promote the expression of the GA2ox gene (Figure18) (Lange and Lange., 2006; Razem et al., 2006). Auxin was shown to induce the expression of the GA biosynthetic gene GA20ox in tobacco and Arabidopsis (O'Neill and Ross, 2002; Frigerio et al., 2006). This effect of auxin was shown to transducer via the degradation of auxin signaling suppressors Aux/IAA proteins (Teale et al., 2006) and the resulting activation of the transcription factor AUXIN RESPONSE FACTOR7 (ARF7).



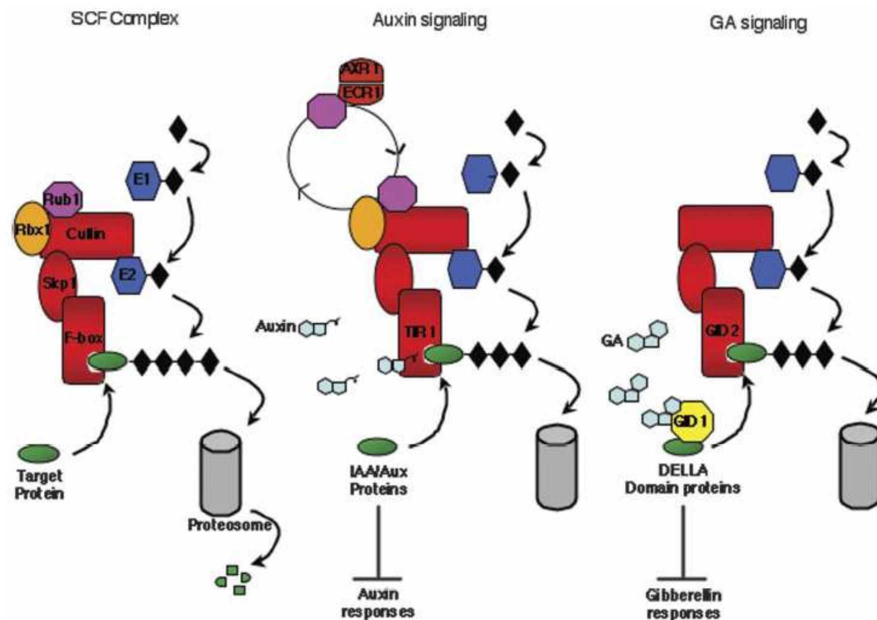
(Weiss and Ori., 2007)

Figure 18. Network of interactions between auxin and GA. *Auxin promotes GA responses by destabilizing DELLA and by promoting the expression of GA biosynthetic genes. Interactions mediated by changes in protein activity or stability are in gray and those mediated by gene expression are in black. Numbers in parentheses indicate the respective reference as follows: 1, Frigerio et al., 2006; 2, Fu and Harberd., 2003; 3, O'Neill and Ross., 2002; 4, Ross et al., 2000; 5, Wolbang and Ross., 2001.*

Moreover, loss of the auxin receptor TIR1, suppressed auxin regulation of GA biosynthetic gene expression (Frigerio et al., 2006). Therefore, auxin positively interacts with GA either at the biosynthesis level or by promoting DELLA degradation.

DELLA proteins are the most characterized GA signalling components. DELLA proteins belong to the GRAS family of transcriptional regulators and act as suppressors of GA signalling. The interaction between DELLA domain of the DELLA proteins with GA receptor (GA INSENSITIVE DWARF1 (GID1)) stimulates binding of the DELLA proteins to an SCF E3 ubiquitin ligase via specific F-box proteins (GID2), leading to polyubiquitination and degradation of the DELLA protein by the 26S proteosome (Sasaki et al., 2003; Dill et al., 2004; Griffiths et al., 2007).

In conclusion, Auxin and GA perception use a unique SCF-based proteolysis mechanism that takes advantage of F-box selectivity or target proteins. With



(Chow and McCourt., 2007)

***Figure 19. SCF-dependent hormone-regulated ubiquitination of proteins**. The left panel represents a yeast-type SCF proteolysis model in which an ubiquitin is added to the target protein via a set of reactions. Ubiquitin is activated by an E1 enzyme and conjugated to the target via the E2 and F-box proteins associated with the SCF complex. The F-box protein determines specificity of targets. The ubiquitin tail grows and causes the degradation of the target via the 20S proteosome. The middle panel depicts auxin-mediated degradation of Aux/IAA repressor targets. The TIR1F-box protein binds auxin, and this stimulates its association with the Aux/IAA protein. Targeting of Aux/IAA to the proteosome releases auxin response genes from repression. The right panel depicts GA-mediated degradation of the DELLA domain repressor targets. The GID1 protein binds the DELLA protein in a GA-dependent manner and targets it to the SCF ^{GID2} complex for ubiquitination. Degradation of the DELLA domain proteins releases GA-dependent genes from repression.*

respect to plant hormone signalling, the auxin and GA receptors are unique in that the number of components between the hormone ligand and the transcriptional factors they control is very small. The use of SCF based proteolysis as a mechanism of signalling creates potential nodes of interactions between signalling molecules.

VII.2 Auxin and jasmonic acid (JA)

Jasmonates (JAs), derived from linolenic acid, function in normal developmental pathways, but also play a crucial role in allowing plants to mount a defence to biotic challenges. JA affects processes such as pollen development and fruit ripening, and also promotes resistance to insects and pathogens (reviewed in Creelman and Mullet., 1997). The majority of papers that have

researched the interactions between jasmonic acid (JA) and auxin have concluded that the relationship between the two hormones is antagonistic. Ueda et al., 1994 and Irving et al., 1999 have shown that JA inhibits auxin regulated elongation in etiolated oat coleoptiles. Tang et al*.,* 2001 have reported that two vacuolar glycoprotein acid phosphatases (VspA and VspB) from soybean are differentially regulated by JA and auxin during early stages of seedling growth. In contrast to these findings, Wang et al (1999) have shown that upon germination, auxin and JA act synergistically to up-regulate two soybean vacuolar lipoxygenases (*LOX4* and *LOX5*).

Our understanding of the integration of auxin and JA signalling pathways has been greatly facilitated after the isolation of the JA response gene COI1 (CORONATINE INSENSITIVE 1) (Xie et al., 1998). COI1 encodes an F-box protein which interact with ASK1, ASK2, RBX1 and CUL1 in planta, suggesting that COI1 assembles into an SCF-type E3 ubiquitin ligase (Devoto et al., 2002; Xu et al., 2002). Despite the demonstrated importance of COI1 and Ubiquitin/ Proteasome system-related proteins in JA signalling, no substrates of the SCF-COI1 E3 ligase have been conclusively identified to date, but specific mutations that conferred auxin insensitivity and mapped to a Cullin subunit also altered jasmonate sensitivity by attenuating the assembly of the COI1 protein into the SCF complex (Ren et al., 2005). So, further research is needed to determine whether any integration of auxin and JA-signaling pathways is achieved though degradation of common target regulatory proteins.

VII.3 Auxin and cytokinin

Auxins and cytokinins interact in the control of many central developmental processes in plants, particularly in apical dominance and root and shoot development. The classic experiments of Skoog and Miller in 1957 demonstrated that the balance between auxin and cytokinin is a key regulator of *in vitro* organogenesis. Exposing callus cultures to a high auxin-to cytokinin ration results in root formation, whereas a low ration of these hormones promotes shoot development. Apical dominance is also one of the classical developmental events believed to be controlled by the ratio of auxin to cytokinin. This is supported by phenotypic observations in many Arabidopsis mutants impaired in different

aspects of auxin and/or cytokinin biology (Hobbie & Estelle., 1994; Catterou et al., 2002) as well as transgenic studies of plants with altered auxin or cytokinin levels (Binns et al., 1987; Eklöf., 1997). Moreover, many experiments have demonstrated the existence of synergistic, antagonistic and additive interactions between these two plant hormones, suggesting a complex web of signal interactions (Coenen and Lomax., 1997). It is clearly documented that auxin regulates cytokinin levels and vice versa. It has, for example, been observed that cytokinin overproducing tobacco had lower levels of indole-3-acetic acid (IAA), and that overproduction of IAA in tobacco leads to a reduced pool size of cytokinins (Eklöf et al., 2000).

It has been argued that cytokinins can both up- and downregulate auxin levels. The increase in auxin, for example, has been demonstrated after application of exogenous cytokinin. Although the basis for these changes is not fully understood, cytokinin-induced inhibition of enzymes that conjugate free IAA into inactive IAA aspartate has been suggested as a putative mechanism. In contrast, (Eklöf et al., 2000) studies demonstrated a decrease in auxin content of transgenic plants overproducing cytokinins. These contradictory results show the complexity of the interactions between these two hormones.

VII.4 Auxin and abscisic acid (ABA)

The abscisic acid (ABA) is a prominent regulator of seed germination that also enables plants to respond to abiotic stresses such as drought. ABA can directly affect ion transport in guard cells to alter stomatal aperture rapidly in response to changing water availability (reviewed in Roelfsema et al., 2004). ABA and auxin have been observed to interact antagonisticaly to regulate stomatal aperture (Eckert and Kaldenhoff., 2000). Auxin serves to open the stomatal pore, whereas, ABA helps to closes the stomatal pore and reduces water loss via transpiration. 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 effect the turgor of the guard cells (Grabov and Blatt., 1998). Aside from interactions of auxin and ABA at the level of guard cell aperture, genetic evidence from Arabidopsis indicates that these two hormones 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 (Wilson et al., 1990; Timpte et al., 1994; Nagpal et al., 2000) and both *axr1* and *axr2* have weak ABA-insensitive phenotype as measured by seed germination. Furthermore, *abi3* mutants that were originally identified as highly insensitive to ABA are insensitive to NPA, at the level of lateral root growth.

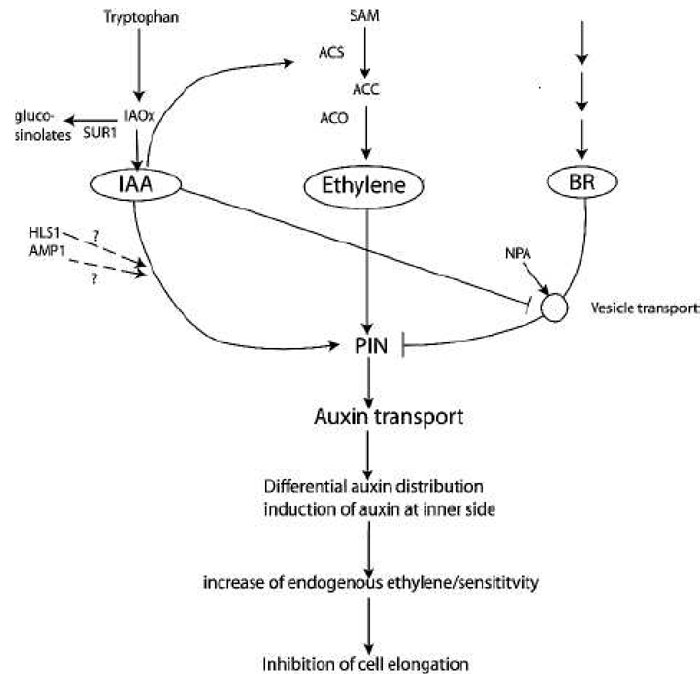
As observed for other hormones, the ubiquitin/proteasome system is implicated in regulation of ABA-responsive transcription. Many evidence of the possible involvement of an SCF-complex in ABA-signalling was uncovered through characterisation of many proteins: ABI5 (abscisic acid insensitive5), ABI3, ABF2 (ABRE Binding Factor2), ATL (Arabidopsis toxico para Levadura), TLP9 (TUBBY-Like Proteins)and finally XERICO (Smalle et al., 2003; Zhang et al.,2005; Kim et al.,2004; Serrano et al.,2006; Lai et al.,2004; Ko et al; 2006), but further research is needed to determine whether any integration of auxin and ABA signalling pathways is achieved though the ubiquitin/proteasome system.

VII.5 Auxin and brassinosteroids (BR)

Auxin response is also connected to brassinosteroids (BRs), which also interact with auxin to promote root gravitropic curvature in maize (Kim et al., 2000). Yi et al., 1999 shown that auxin and BR synergistically and antagonistically regulate expression of the ACS family of ethylene biosynthesis genes in mung bean (*Vigna radiata*) hypocotyl tissues. Other molecular link has been described between the auxin-response pathway and BR biosynthesis upon characterisation of the *sax1* mutant (Ephritikhine et al., 1999). The *sax1* mutant plant is partially restored by treatment with exogenous brassinosteroid. *sax1* root growth is hypersensitive to both ABA and auxin.

De Grauwe et al., 2005 described the involvement of (BRs) in auxin- and ethylene-controlled processes in the hypocotyls of both light- and dark-grown seedlings. They showed that BR biosynthesis is necessary for the formation of an exaggerated apical hook and that either application of BRs or disruption of BR synthesis alters auxin response, presumably by affecting auxin transport, eventually resulting in the disappearance of the apical hook (Figure 20).

Moreover, Nemhauser et al. suggested that the activity of some of the auxin signalling transcription factors, ARFs, is modulated by the formation of specific transcriptional complexes, involving input from both auxin and brassinosteroid signalling pathways. Furthermore, Nakamura et al. provided evidence that the activity of certain Aux/IAA proteins could be jointly regulated by auxin and brassinosteroids (Nakamura et al., 2006).



(*De Grauwe et al., 2005)*

Figure 20. Model of the interaction between ethylene, auxin and brassinosteroids in the hook of etiolated seedlings.

VII.6 Auxin and ethylene

Auxin and ethylene have a long history of reported interactions both at the physiological and molecular level. The antagonistic effects of these hormones in the control of abscission of fruits and flowers (Brown, 1997) as opposed to their synergistic effects in the regulation of root elongation, root hair formation, and growth in Arabidopsis thaliana (Pitts et al., 1998; Rahman et al., 2002; Swarup et al., 2002) illustrate some of the complexity of the auxin–ethylene crosstalk. Mutant analysis has uncovered additional levels of complexity in the relationship between these two hormones.

Ethylene is synthesized from the amino acid Met by the consecutive action of three enzymatic activities: S-adenosyl- L-methionine (SAM) synthase, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, and ACC oxidase (Figure 21). ACC synthase catalyzes the main regulatory step in this biosynthetic pathway, the conversion of SAM to ACC (Wang et al., 2002). Auxin is known to stimulate ethylene production by activating this particular biosynthetic step (Abel et al., 1995). In fact, transcription of eight out of the nine Arabidopsis ACS genes is upregulated by auxin, and many auxin response elements have been found in the promoters of several of these biosynthetic genes (Tsuchisaka and Theologis., 2004).

Once produced, ethylene is sensed by a family of receptors that show similarity to the bacterial two-component His kinases. Ethylene binding to the

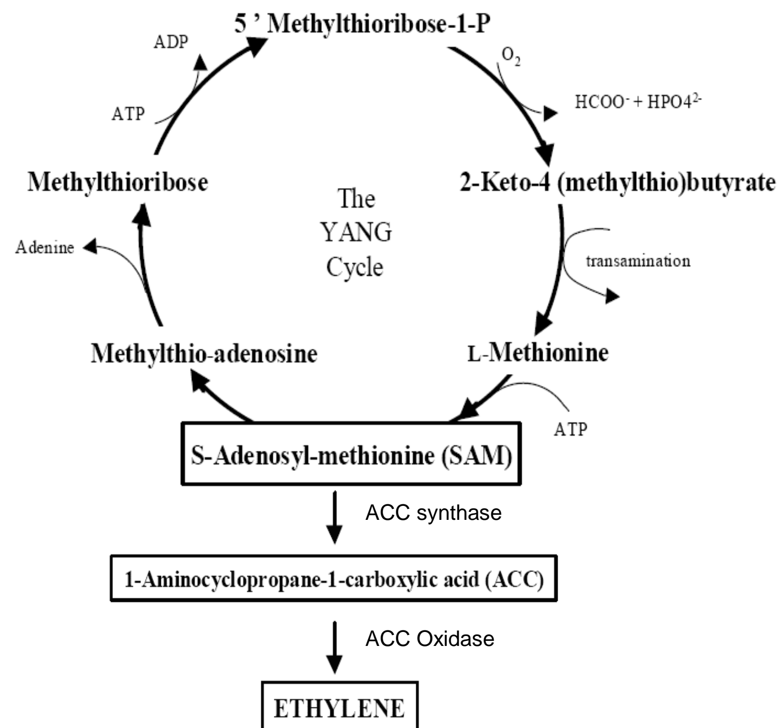
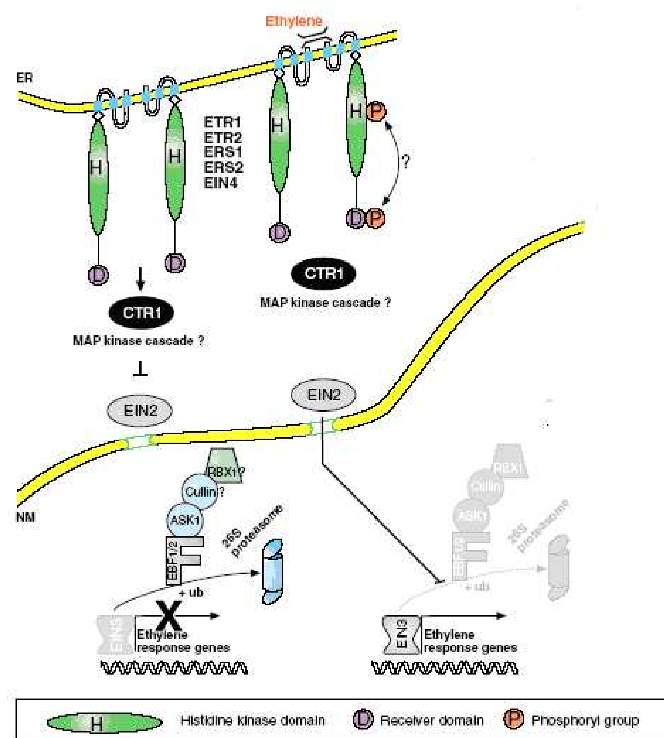


Figure 21. Ethylene biosynthesis. *The amino acid Methionine is conversed by the consecutive action of three enzymatic activities: S-adenosyl- L-methionine (SAM) synthase, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, and ACC oxidase.*

receptors causes inactivation of a Raf-like kinase CTR1 and the consequent derepression of EIN2, a protein of unknown biochemical function that is essential for the ethylene response. Downstream of EIN2, a family of transcription factors composed of EIN3 and EIN3-like proteins triggers a transcriptional cascade that results in the activation/repression of hundreds of target genes (Alonso and Stepanova., 2004; Guo and Ecker., 2004) (Figure 22). The first step in the cascade initiated by EIN3/EILs involves other transcription factors, such as the AP2/EREBP family members ERF1 (Solano et al., 1998) and EDF1-4 (Alonso et al., 2003). These different EIN3 targets probably represent branching points in the ethylene response that can be turned on or off not only by ethylene but also by other factors and therefore could be used to provide specificity in response to ethylene. In the case of ethylene and auxin, several common target genes have



(Bishop et al., 2006)

Figure 22. Ethylene signalling: *At low concentrations of the ligand, ethylene receptors (ETR1, ERS1, ETR2, EIN4, and ERS2) are active and can therefore stimulate the negative regulator CTR1, which in turn shuts down ethylene signalling by allowing EIN3 degradation. Ethylene binding inactivates the receptors and therefore they are unable to stimulate CTR1-mediated inhibition. As a result, EIN2 is active and prevents EIN3 degradation, which leads to EIN3 accumulation and activation of ethylene responsive gene transcription.*

also been identified (Zhong and Burns., 2003). However it's still unclear if whether or not they participate in common biological processes and related regulation.

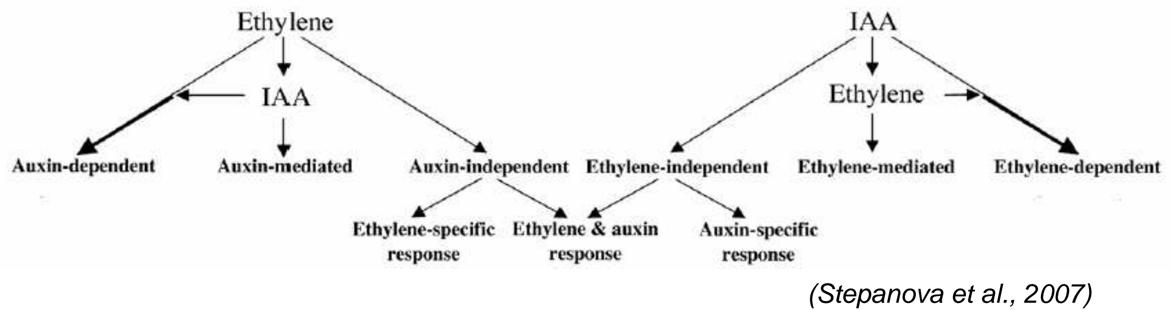
VII.6.a Multilevel interaction between auxin and ethylene in the roots

Despite the accumulated knowledge on each individual hormone signaling and response pathway, very little is known about the mechanisms that govern the interactions between ethylene and auxin. The ethylene-mediated regulation of auxin biosynthesis through the activation of WEI2/ASA1 and WEI7/ASB1, a anthranilase synthase subunits that catalyse the fist step in tryptophane biosynthesis (Figure 2) (Stepanova et al., 2005) as well as the reciprocal effect of auxin on ethylene biosynthesis through the activation of several ACC synthases, represent two elegant examples of the molecular mechanisms controlling the ethylene–auxin crosstalk.

Recently, for better understand the molecular mechanisms behind the ethylene–auxin interactions in the roots, a comprehensive study relying on physiological, cellular, genetic and genomic approaches was performed by Stepanova et al., 2007. Quantification of the morphological effects of ethylene and auxin in a variety of mutant backgrounds indicates that auxin biosynthesis, transport, signalling and response are required for the ethylene-induced growth inhibition in roots. This analysis suggests a simple mechanistic model for the interaction between these two hormones in roots, according to which ethylene and auxin can reciprocally regulate each other's biosynthesis, influence each other's response pathways and/or act independently on the same target genes. This model not only implies existence of several levels of interaction but also provides a likely explanation for the strong ethylene response defects observed in auxin mutants (Figure 23)

VII.6.b Interaction between auxin and ethylene in the hook

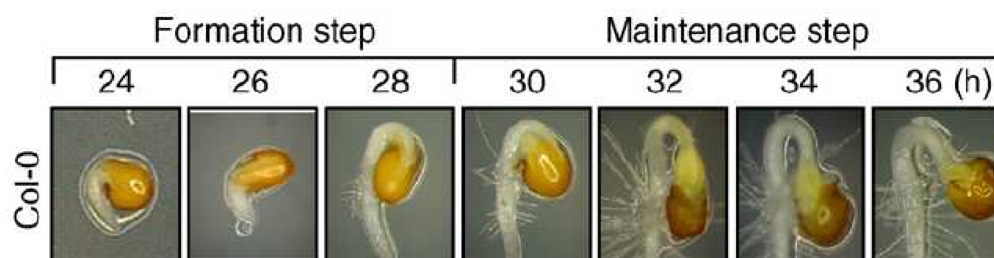
The induction of apical hook formation in *Arabidopsis* represents one of the best described examples of auxin-ethylene cross-talk in plants (Lehman et al.,



(Stepanova et al., 2007)

Figure 23. Schematic representation of the mechanistic model of ethylene–auxin crosstalk in roots of etiolated Arabidopsis seedlings. *The model assumes existence of at least three different types of molecular interactions between ethylene and auxin. A subset of ethylene responses (left side of the panel) is dependent on auxin levels. In this case, the role of auxin is restricted to promoting (or attenuating) the ethylene effect. By contrast, the auxin-mediated responses correspond to those changes in gene expression that are directly triggered by auxin, but in this case, by an ethylene-induced auxin activity. Finally, those ethylene effects that are not affected by the levels of auxin are classified as auxin independent, with some of these changes being independently stimulated by auxin. Equivalent interactions can be defined among auxin responses (right side of the panel).*

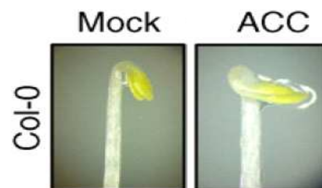
1996; Raz and Ecker., 1999). Bending (180°) of the embryonic stem (hypocotyl) at its apex just below two seed leaves (cotyledons), results in the formation of an apical hook. The structure performs an important biological function, as it places the cotyledons below the hook region so that the meristematic primordial can be protected from damage during penetration of soil (Harpham et al., 1991). The apical hook is formed by differential cell elongation on opposite sides of the hypocotyl, in which the rates of cell elongation on outside of the hook are modulated differently than of cells inside the hook (Silk and Erickson., 1978). In the hook region, the growth rate of the outer (convex) side of the hypocotyl exceeds that of the inner (concave) side, resulting in hypocotyl bending (Figure 24).



(Park et al., 2006)

Figure 24. Apical hook development during early stages of seedling germination. *Wild-type seeds were germinated in air and photographs were taken after 24, 26, 28, 30, 32, 34 and 36 hours.*

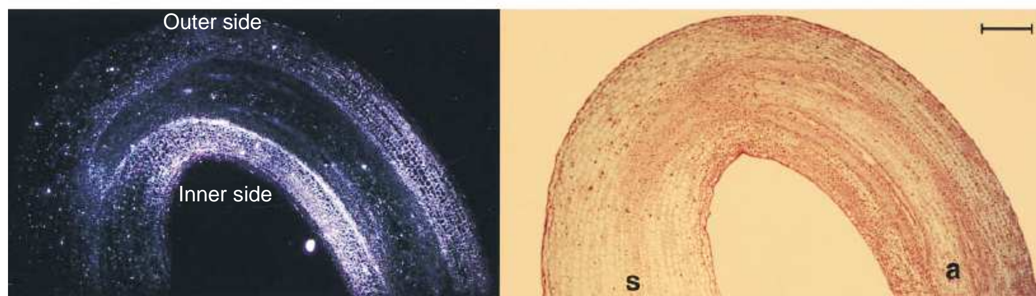
Ethylene and auxin play a major role in this differential cell elongation process (Schwark and Bopp., 1993; Schwark and Schierle., 1992). In dark-grown seedlings, ethylene exposure enhances apical hook curvature, causing cotyledons to form a 270° bend relative to the hypo cotyls (Ecker., 1995) (Figure 25). Moreover, mutants that are defective in ethylene perception, e.g., *etr1-1, ein2, ein3*, etc., do not form exaggerated hooks in response to ethylene treatment



(Park et al.,2006)

Figure 25. Ethylene enhances exagerated hook in etiolated seedlings. *Seeds were germinated in darkness in air (left panel) or ethylene (lower panel); photographs were taken of 64-, 66-, 68- and 70-hour-old seedlings. During photography, the ethylene-grown seedlings were transferred to AT plates containing 10 mM ACC.*

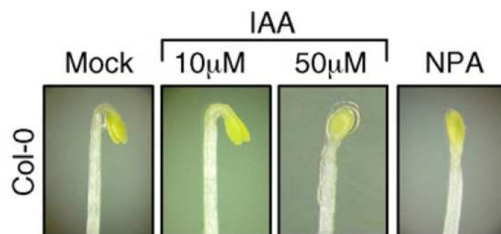
(Roman et al., 1995), whereas the constitutive ethylene responsive mutant *ctr1* develops exaggerated hooks even in the absence of ethylene (Guzman and Ecker 1990; Kieber et al., 1993). The precursor to ethylene, ACC, is asymmetrically localized in cells of the apical hook in bean (Schwark and Bopp., 1993). Expression of the gene encoding *ACO*, the terminal enzyme in ethylene biosynthesis and its enzyme activity have also been found to be higher on the concave side of the apical hook than on the convex side of pea hook (Figure26) (Peck et al., 1998). Another *ACO* gene, *AtACO2*, is predominantly expressed on the convex side of the hook (Raz and Ecker., 1999).



(Peck et al., 1998)

Figure 26. Localization of Ps-ACO1 mRNA in the apical hook of etiolated pea seedlings. *Apical hooks were isolated from air-grown etiolated 5- to 6-day-old seedlings. The apex (a) is towards the right side and the stem (s) is on the left. At left are dark-field images, and at right are bright-field images.Hybridization was performed with a 35S-labeled antisense strand of Ps-ACO1.*

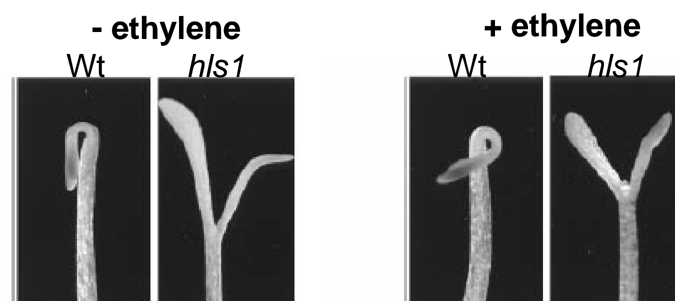
On the other hand, auxin, which stimulates cell expansion to promote hypocotyl elongation, is also unequally distributed in the apical hook (Schwark and Schierle., 1992). Inhibition of auxin transport disrupts formation of the apical hook, suggesting that auxin asymmetry or an asymmetry in the perception or response of cells to auxin may exist in the hypocotyls (Figure 27). Consistent with these observations, auxin mutants such as *axr1* (Lincoln et al., 1990), *hls3/rooty* (King et al., 1995) and *yucca* (Zhao et al., 2001) also lack normal apical hooks.



(Park et al., 2006)

Figure 26. Effects of IAA and NPA on apical hook development.

In 1996, Echer's group identified an Arabidpsis mutant that showed no differential growth in the apical region of the hypocothy (*hookless1).* This gene has been proposed to be a key regulator that integrates ethylene and auxin signalling pathways during apical hook formation of Arabidopsis seedlings (Lehman et al., 1996). Plants that lack *HOOKLESS (HLS1)* are unable to maintain an apical hook despite normal responses to ethylene in other tissues, whereas transgenic plants that overexpress *HLS1* develop an exaggerated differential growth (hook curvature) in the absence of ethylene (Lehman et al., 1996) (Figure 27).

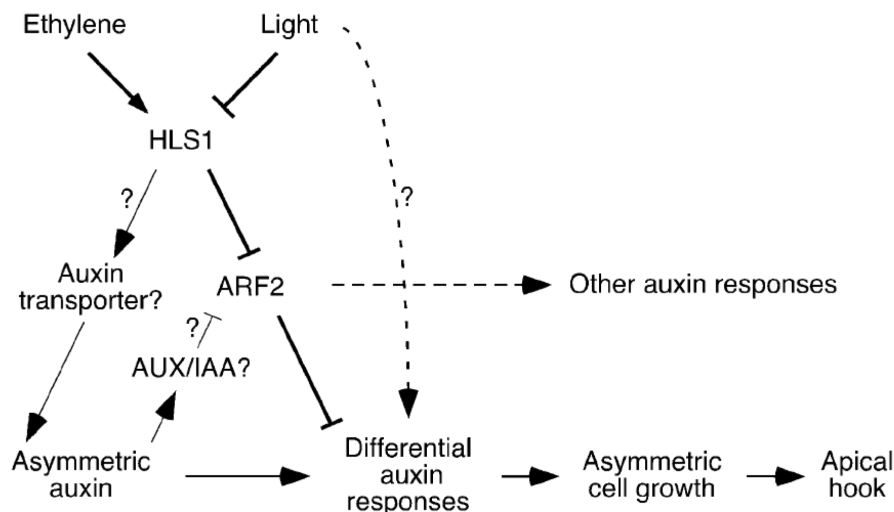


(Lehman et al., 1996)

Figure 27. Morphology of wild-type and hookless1 dark-grown Arabidopsis seedlings. *Seeds were grown either in air or 10 ml ethylene per liter of air in the dark.*

Thus, *HLS1* is required for hook formation and is sufficient to induce enhanced hook curvature in the absence of exogenous ethylene. In addition the absence of HLS1 leads to abnormal auxin-regulated gene expression in the cotyledons and apical region of the hypocotyl.

In 2004, Li et al showed that the supressors of *hls1* were identified as mutations in Auxin Reponse Factor 2 (ARF2). Exposure to light decreased HLS1 protein level and evoked a concomitant increase in ARF2 accumulation. These studies demonstrate that both ethylene and light signals affect differential cell growth by acting through HLS1 to modulate the auxin response factor, pinpointing HLS1 as a key integrator of the signalling pathways that control hypocotyls binding (Figure 28).



(Li et al., 2004)

Figure 28: Model for integration of ethylene, auxin, and light signalling in differential growth of the seedling hypocotyl. *An asymmetric auxin distribution in hook tissues is proposed to cause differential auxin responses in the region, resulting in asymmetric cell elongation of the hypocotyl and formation of the apical hook structure. Ethylene enhances apical hook bending through activation of HLS1 transcription. One of the roles for HLS1 is to inhibit the function of the auxin response factor ARF2, a negative regulator of the differential auxin response in apical hook, leading to enhanced differential growth and exaggerated hook curvature. In contrast to ethylene, light disrupts the differential auxin responses in hook tissues by decreasing HLS1 abundance. Subsequently, Fine Mapping of hss1 (hookless supressors) Mutations ARF2 protein levels increase and the hook opens.*

These findings provide another molecular link that connects ethylene and light signalling to auxin-mediated differential cell elongation process.

VII.7 Other gene bridge auxin response with other stimulus

VII.7.a Auxin and light-dependant growth

Darwin originally proposed that light and a transmissible signal (later discovered to be auxin) interact to cause phototropic curvature in plants (Darwin, 1880). The recent adoption of a genetic approach in Arabidopsis has significantly advanced our understanding of how phototropic and auxin-signalling pathways interact. Phototropic defects have been described for mutations disrupting several auxin transport and signal transduction components. For example, reverse genetic studies on the auxin efflux carrier gene *At-PIN3* have uncovered a phototropic defect (Friml and Palme, 2002). Moreover the *nph4* mutant was originally identified by its reduced phototropic response (Liscum and Briggs., 1996). The hypocotyl growth of *NPH4* mutant is resistant to the auxins IAA, 2,4-D and 1-NAA (Ruegger et al., 1997; Watahiki and Yamamoto., 1997; Harper et al., 2000), suggesting that the NPH4 protein plays a central role in auxin-mediated differential growth. The *NPH4* gene has been cloned and found to encode the auxin response factor, ARF7 (Harper et al., 2000). Recently (Salisbury et al., 2007) show that phytochrome regulate emergence of lateral roots at least partially by manipulating auxin distribution within the seedling. Thus, shoot-localised phytochrome is able to act over long distances, through manipulation of auxin to regulate root development. These results represent a new link between phytochrome and auxin.

VII.7.b Auxin and photomorphogenesis

Many genetic and biochemical experiments indicate that auxin is closely associated with photomorphogenesis (Jones et al., 1991; Behringer and Davies., 1992; Boerjan et al., 1995; Kraepiel et al., 1995; Gil et al., 2001). Several evidences suggest that members of the *GH3* family are involved in phytochrome signalling. *FIN219*, a member of the *GH3* family, is involved in *phyA* signalling (Hseih et al., 2000) and *WES1,* an Arabidopsis *GH3* Gene, encoding an auxin-

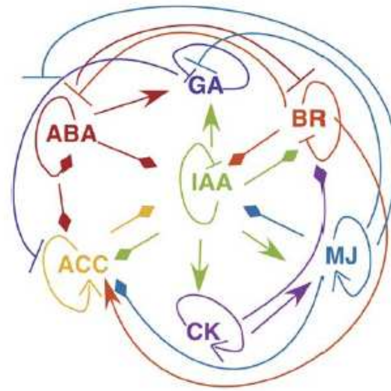
conjugating enzyme, mediates phytochrome B-regulated light signals in hypocotyl growth (Park et al., 2007b).

The Aux/IAA proteins provide an example of the proposed link between auxin signalling and light. Mutations in Arabidopsis *Aux/IAA* genes such as *AXR2/IAA7*, *AXR3/IAA17* and *SHY2/IAA3* induce photomorphogenic characteristics in dark-grown seedlings (Kim et al.,1996; Reed et al., 1998; Nagpal et al., 2000), suggesting that light may normally regulate these genes or proteins to induce morphological responses. Furthermore, Aux/IAA proteins from Arabidopsis and pea are phosphorylated by phyA in vitro. Together, these results suggest that phytochrome-dependent phosphorylation of Aux/IAA proteins may provide a molecular mechanism for integrating light and auxin signalling in plant development (Colon-Carmona et al., 2000; Tian and Reed, 2001).

Additional evidence for a link between light and IAA signalling comes from the characterisation of the constitutive photomorphogenic Arabidopsis mutant long hypocotyls 5 (*hy5*). This gene, which encodes a bZIP transcription factor, acts as a positive regulator of photomorphogenesis (Osterlund et al., 2000; Oyama et al., 1997). Loss-of-function *hy5* mutants exhibit an auxin-related phenotype and overexpressing *AXR2/IAA7* in this plants can partially rescued these phenotypes (Cluis et al., 2004).

Conclusion

Auxin is a critical phytohormone. Complex and redundant regulation of IAA abundance, transport and response allow an intricate system of auxin utilization that achieves a variety of purposes in plant development. As a result, the study of auxin biology is making an impact on our understanding of a variety of processes, from regulated protein degradation to signal transduction cascades, from organelle biogenesis to plant morphogenesis. Despite prodigious historical and ongoing auxin research, many of the most fundamental original questions remain incompletely answered. The discovery of TIR1/AFB F-box proteins that function as a auxin receptor is a surprising development that fills in a crutial piece of the auxin puzzle and might well serve our understanding of the interaction of this key hormones with other signalling pathway (Figure 29).



(Nemhauser et al., 2006)

Figure 29. Key role of auxin in hormone network. *Lines with arrowheads represent upregulation of hormone biosynthetic genes or downregulation of genes involved in hormone inactivation. Blocked arrows represent downregulation of genes involved in hormone biosynthesis or upregulation of genes involved in inactivation of a hormone.*

VIII. Tomato as model plant

VIII.1 Fruit as an important component in the human fruit diet

Fruit are an important component of the human and animal diet and are developmental systems that are unique to plant. As a result, considerable scientific study has focused on questions of fruit organogenesis, development and maturation. Ripening has an impact on fibre content and composition, lipid metabolism, and the levels of vitamins and various antioxidants (Ronen et al., 1999). Breeding or biotechnologies are the most suitable means to understand the regulatory points involved in ripening. Manipulation of the ripening associated attributes related to biosynthesis of carotenoids, flavonoids, vitamins, and flavour volatiles, will allow the improvement of manipulation of nutrition and quality characteristics. Possibly, the most convincing argument for the promotion of plant genetic engineering will be the development of modified plants or plant-derived products with direct consumer appeal such as increased quality and nutrition.

Ripening is influenced by internal and external signals, including developmental gene regulation, hormones, light and temperature. Two major classifications of ripening fruit, climacteric and non-climacteric, have been used to

distinguish fruit on the basis of respiration and ethylene biosynthesis rates. Climacteric fruit (e.g. tomato, avocado, apple, banana) are distinguished from non-climacteric fruits (e.g. strawberry, grape, citrus) by their increased respiration and ethylene biosynthesis rates during ripening (Lelievre et al., 1997). While non-climacteric fruits do not require ethylene for ripening of their fruits, ethylene has been shown to be necessary for the co-ordination and completion of ripening in climacteric fruit (Yen et al., 1995; Klee et al., 1991; Oeller et al., 1991; Lanahan et al., 1994; Wilkinson et al., 1995).

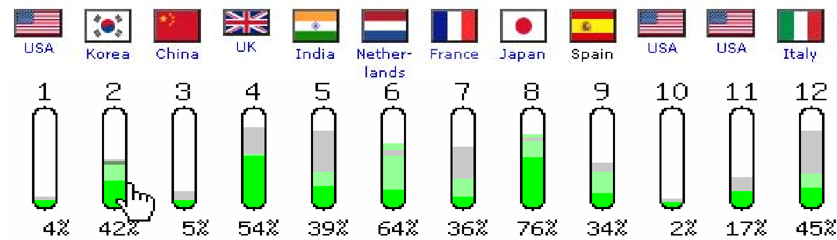
VIII.2 Tomato as a model system for fruit ripening

Tomato (*Solanum lycopersicum*) the centerpiece of the Solanaceae family, has emerged as a model of fleshy fruit development, primarily because of its importance as a food crop species and this is the species for which the genetic and molecular toolkits are most advanced. Extensive germplasm collections, well-characterized mutant stocks, high-density genetic maps, immortalized mapping populations, efficient transient and stable transformation, deep expressed sequence tag (EST) resources, microarrays and an ongoing genome sequencing efforts all contribute to the utility of this experimental system (www.sgn.cornell.edu and www.tigr.org for links to these resources). Well-characterized ripening mutations, short generation time, a long history of physiological, biochemical and molecular investigations related to fruit development and maturation and interest in the species as an important commodity crop, have fuelled considerable effort on understanding ripening in tomato.

VIII.3 The sequencing of the tomato genome

The tomato genome sequencing project is an international effort involving 12 different countries. Currently, the 12 tomato chromosomes are split up between the countries as follows: Korea (chromosome 2), China (chromosome 3), United Kingdom (chromosome 4), India (chromosome 5), the Netherlands (chromosome

6), France (chromosome 7), Japan (chromosome 8), Spain (chromosome 9), Italy (chromosome 12) and the United States (chromosomes 1, 10 and 11).



(From Solanaceae Genomic Network (SGN), June 2008)

Figure 30: The International tomato genome sequencing project. *This project aims to sequence the gene-rich euchromatic portions of the twelve tomato chromosomes. An international consortium of sequencing centers is performing most of the genomic sequencing. Each chromosome is assigned to a sequencing center. The genome is split into manageable chunks known as BACs (Bacterial Artificial Chromosomes), which are sequenced separately, then assembled together.*

The total size of the tomato genome is estimated to be approximately 950 Mb of DNA, more than 75% of which is heterochromatic, rich in repetitive sequences and largely devoid of genes. The French effort devoted to sequencing of the gene-rich portion of tomato chromosome 7 is led by our laboratory of Genomics and Fruit Biotechnology and involves Genome Express (Meylan, France) as a main sequencing partner (Delaland et al., 2007).

The sequencing of the tomato genome opens exciting new perspectives for the understanding of the genetic basis of plant morphological and physiological diversity. It is also expected that the comparative sequence information will possibly uncover the underlying mechanisms driving plant evolution.

VIII.4 Microtom: characteristics of miniature tomato

The tomato cultivar Micro-Tom was produced for ornamental purposes by crossing 3 cultivars and displays a very dwarf phenotype with small and red ripened fruits (Scott and Harbaugh., 1989) (Figure 33A). Its small size, rapid growth and easy transformation have led to its proposal as a convenient model system for research on the regulation of berry fruit development (Meissner et al., 1997; Eyal and Levy., 2002). Micro-Tom plants have a bushy appearance and their leaves are small, with deformed leaflets, and a deep green colour compared with diverse wild-type cultivars. Those phenotypic characteristics are similar to

those described for BR-deficient mutants (Altmann, 1998). It was shown that Micro-Tom has mutations in the *SELF PRUNING (SP)* and *DWARF (D)* genes. SP belongs to the CETS family of regulatory genes encoding modulator proteins that determine the potential for continuous growth of the shoot apical meristem (Pnueli et al., 2001). The *DWARF* (D) gene encodes a P450 protein involved in brassinosteroid (BR) biosynthesis (Bishopet al., 1999). The dwarf phenotype of Micro-tom is thus a consequence of these mutations in addition to the lower reponse to GA, possibly due to the reduced content of BR in this cultivar (Marti et al., 2006).

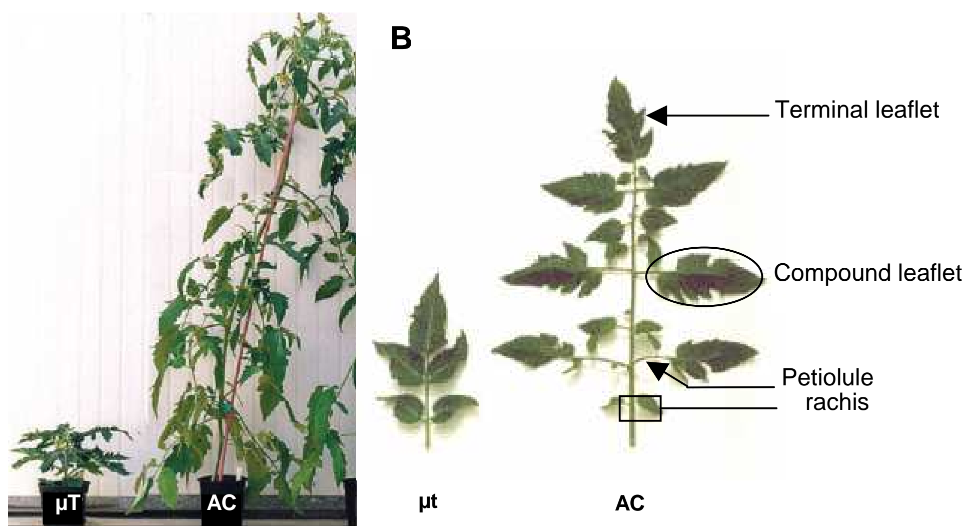


Figure 33. Plant of micro-Tom (µT), Ailsa Craig (AC). *(A) Plants at the time of flowering. (B) Compound leaf from tomato.*

VIII.5 Tomato plant characteristics

The tomato plant has compound leaves. A compound leaf 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 (Figure 33B). Most leaves are protected by a thin outer cuticle. Just inside the cuticle lies the epidermis. Note that the epidermis surrounds the leaf and is therefore visible on the abaxial (lower) and adaxial (upper) sides of the leaf in cross section (Figure 34). The epidermis contains stomata. The xylem is in the center of the vein with the phloem distributed on both the adaxial and abaxial sides of the bundle. In the center of the leaf lies the mesophyll. The mesophyll

consists of two different cell types. Palisade mesophyll is composed of parenchyma cells. Spongy mesophyll consists of more irregularly shaped parenchyma cells arranged in a loose structure. Most of the plant's photosynthesis takes place in the mesophyll of the leaf.

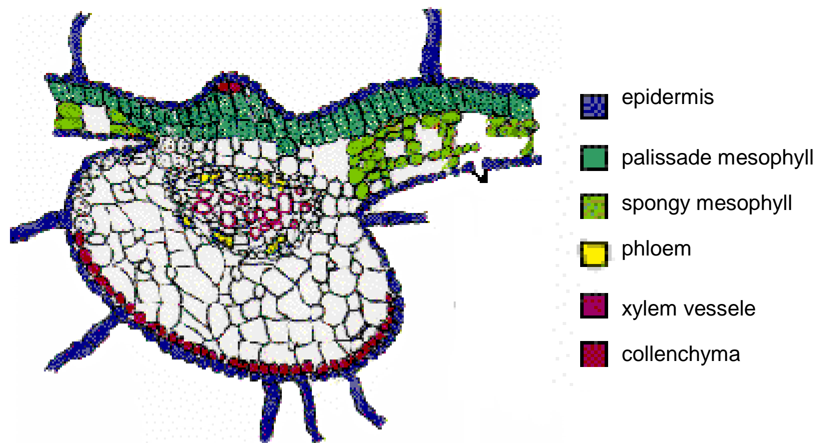


Figure 34. Cross section of a tomato leaf.

VIII.6 Tomato fruit development

The tomato fruit, though commonly classified as a vegetable, is really a fruit, a berry in fact and is composed of an epidermis, a thick pericarp and placental tissues surrounding the seeds. The pericarp is the outer wall of the gynoecium, which is composed of at least two carpels (this number can be much higher in some varieties) (Figure 31).

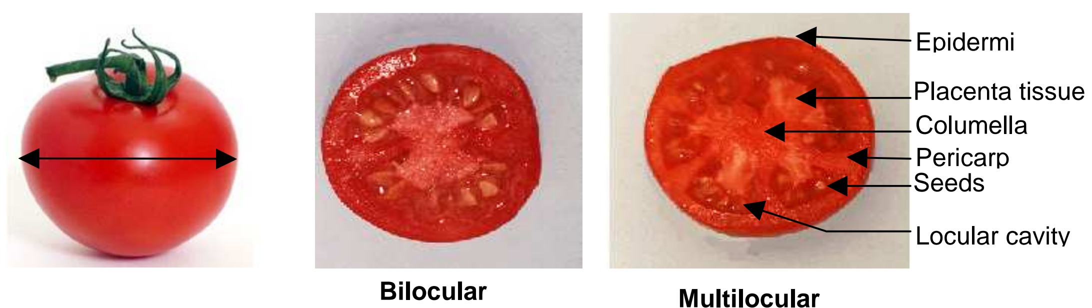
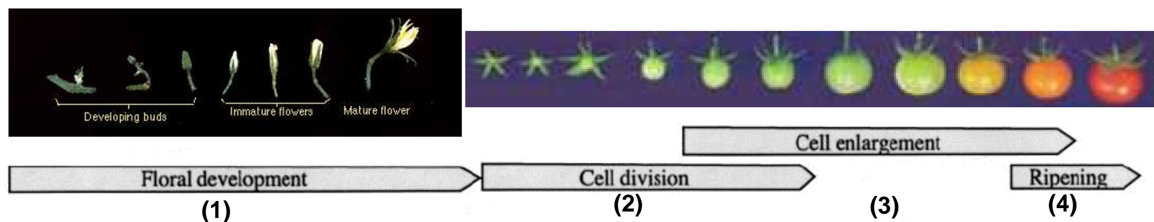


Figure 31. Section of tomato fruit. *The fruit can be either bilocular or multilocular. Tomato fruits exhibit all of the common characteristics of berries. The fruit develops from the ovary of the flower.*

The tomato is fleshy due to the pericarp walls and skin. Finally there are several seeds in each tomato.

There are essentially four stages of development, which are depicted in Figure 32. These are (1) a 2- to 3-weeks period between floral initiation and the production of a mature flower, during which the identity, number, and shape of all floral organs are determined; (2) a period of intensive cell division that begins at anthesis and continues for 2 weeks after fertilization; (3) a period of rapid cell expansion that begins toward the end of the cell division stage and continues until 1 week before the onset of ripening; (4) a ripening phase that initiates after growth has ceased and involves rapid chemical and structural changes that determine fruit aroma, color, texture and biochemical composition (e.g., acids and sugars) but not fruit size and shape.



(Tanksley., 2004)

Figure 32. Stages of Tomato fruit development.

VIII.7 Tomato ripening

Ripe fruit demonstrate a wide range of diversity in form, pigmentation, texture, aroma, flavour and nutrient composition. Fruit of many species undergo modification of cell wall texture, conversion of starch to sugars, increased susceptibility to post-harvest pathogens, alterations in pigment biosynthesis/accumulation and increased levels of flavour and aromatic volatiles during the maturation and ripening processes (Seymour et al., 1993).

As previously outlined, ripening physiology has been classically defined as either 'climacteric' or 'non-climacteric'. Climacteric fruits show a sudden increase in respiration at the onset ripening, usually in concert with increased production of ethylene. Whereas ethylene is typically necessary for climacteric ripening, non-

climacteric fruits do not increase respiration at ripening and often have no requirement for ethylene to complete maturation.

So, climacteric fruits such as tomato are distinguished from non-climacteric by their increased ethylene biosynthesis rates during ripening. This is one of the main reasons that the majority of biochemical research has concentrated on this hormone. Initial molecular studies focused on the isolation of ethylene-regulated genes which include those encoding the ethylene biosynthesis enzymes (Sadenosylmethionine, SAM-synthase, 1-aminocyclopropane carboxylic acid, ACC-synthase and ACC oxidase) (reviewed by Redgwell and Fischer, 2002 Giovanini et al., 2001). It was later demonstrated, using the reverse genetic approach, that delaying ethylene production constituted a successful strategy to extend the shelf-life of fruits (Grierson, 1992). In 2002, Klee group proposed a model for ethylene perception and metabolism. As the receptor also acts as a negative regulator of downstream responses, in the absence of ethylene, receptors actively suppress expression of ethylene responsive genes. Recently, several further ethylene inducible genes have been identified in tomato, including mitochondrial translation elongation factors (Benichou et al., 2003) and CTR-1 (Leclercq et al., 2002).

It seems likely, given the development of micro-array resources for tomato that significant advances will be made in our understanding of ethylene signal transduction and its role in ripening fruit. Examples of this have already been started by using tomato cDNA microarray containing 12000 unique elements encoding 9000 genes covering a range of metabolic and developmental processes (http://bti.cornell.edu/CGEP/CGEP.html) (Fei et al., 2004; Baxter et al., 2005). More recently, a joined effort between four countries (France, Spain, Italy and USA) led to the construction of an oligo-based new generation of tomato chips. A complete list of 70 mer long oligonucleotides was first derived from 27000 individual clones composing the unigene collection of tomato ESTs. Thereafter, 12000 clones were selected for printing in the first version of oligo DNA-chips named EU-TOM1. A Laboratory Information Management Systems (LIMS) for microarrays production and traceability including the set up of a dedicated tomato microarrays database providing access to the gene ID of the EU-TOM1 clones has been installed by the French partner

(http://bioinfo.genopole-toulouse.prd.fr/eusol/base/). The printing of the second set of tomato oligo-based DNA chips (EU-TOM2) has been scheduled within the EU-SOL program for 2007 and will include the remaining tomato unigenes estimated at 15000 clones.

VIII.7.a Revealing the secrets of ripening mutant

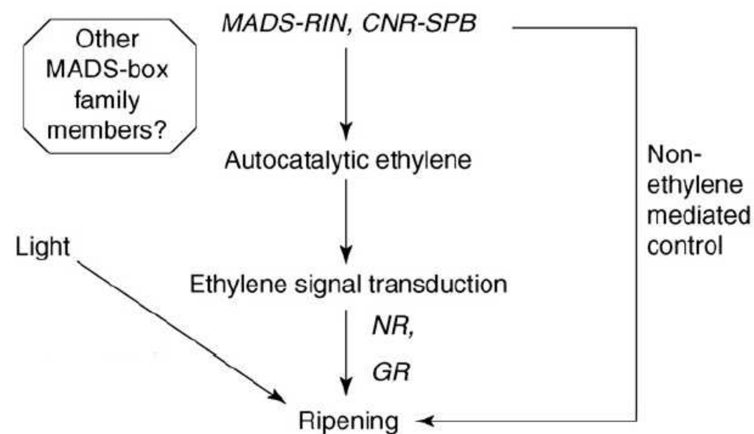
Tomato has proved to be an excellent model system for the analysis of fruit ripening and development, in part due to the availability of well characterized ripening mutants. These include pleiotropic ripening mutations, such as Colorless nonripening (*Cnr*), ripening-inhibitor (*rin*), Never-ripe (*Nr*) and Green-ripe (*Gr*) (Figure 33).

Ripening mutant

(Giovannoni., 2007)

Figure 33. Normal and mutant tomato fruit. *Normal tomato cultivar Ailsa Craig ripe fruit ten days post breaker and equivalent age fruit homozygous for the Never-ripe (Nr/Nr), Green-ripe (Gr/Gr), Colorless non-ripening (Cnr/Cnr) and ripening-inhibitor (rin/rin) mutations.*

Recent cloning of this tomato ripening genes, that were previously known only through mutation, have created new inroads into understanding of the primary ripening control mechanisms, including transcription factors such as those encoded by the RIPENING-INHIBITOR (RIN) MADS-box and COLOURLESS NON-RIPENING (CNR) SPB-box genes, which are necessary for the progression of virtually all ripening processes. These discoveries have also facilitated the elucidation of downstream signal transduction components that impact the hormonal and environmental stimuli that coordinate and modulate ripening phenotypes (Figure 34).



(Giovannoni., 2007)

Figure 34. Summary of ripening control. *The ripening-specific transcription factors MADS-RIN and CNR-SPB are necessary for the induction of ethylene- and non-ethylene-mediated ripening control, as defined by the rin and Cnr mutations, respectively. The ethylene signal is transduced although the ethylene receptor (NR) and the GR protein, which might participate in maintaining receptor-copper homeostasis. These genes were defined by the cloning of the alleles that are responsible for the Nr and Gr mutations, respectively.*

VIII.7.b Potential role on auxin in fruit development and ripening

In comparison with ethylene, very little is known about the role of other hormones in fruit development. The role of auxin has been extensively investigated in other fruits such as strawberry (Manning, 1994) and grape berries (Davies et al., 1997). In tomato, the fact that several expansins encoding genes are expressed during fruit development and that they are regulated by auxins in other plant organs, led to the postulate that auxins are part of the hormonal signalling transduction network controlling cell expansion in tomato fruit (Catala et al., 2000). This hypothesis is further supported by the fact that the auxin concentration in tomato fruits peaks well before the onset of ripening (approximately at 10 DPA) coincident with a higher expression of fruit-specific expansin genes (Gillaspy et al., 1993). In 2003, Balbi and Lomax, by means of characterization of auxin-resistant mutant *dgt (diageotropica)*, have proposed a cross-talk model of auxin responsiveness and ethylene biosynthesis at very early stages of fruit development. In 2005, Wang et al demonstrate, for the first time in our laboratory, that down regulation of auxin transcription factor (IAA9) induce parthenocarpy in the tomato fruit. This finding opens a new route that merits

further investigation to test whether other members of the Aux/IAA family have implications on fruit development and ripening.

CHAPITRE II

Sl-IAA3, a Tomato Aux/IAA at the Crossroads of Auxin and Ethylene Signalling

(Manuscript submitted)

Title: Sl-IAA3, a Tomato Aux/IAA at the Crossroads of Auxin and Ethylene Signalling

Authors: Salma Chaabouni, Corinne Delalande, Brian Jones, Hua Wang, Zhengguo Li, Isabelle Mila, Pierre Frasse, Alain Latché, Jean-Claude Pech, and Mondher Bouzayen

ABSTRACT

Whereas the interplay of multiple hormones is essential for most plant developmental processes, the key integrating molecular players remain largely undiscovered or uncharacterized. We report here that Sl-*IAA3,* a member of the tomato auxin/indole-3-acetic acid (*Aux/IAA*) gene family, is an intersection point between the auxin and ethylene signal transduction pathways. *Aux/IAA* genes encode short-lived transcriptional regulators that mediate auxin responses. Their functions have mostly been defined by dominant, gain-of-function mutant alleles in Arabidopsis. The Sl-*IAA3* gene encodes a nuclear-targeted protein that can repress transcription from auxin-responsive promoters. Sl-*IAA3* expression is controlled by both auxin and ethylene and is regulated on a tight tissue-specific basis. Sl-*IAA3* displays an expression gradient associated with tissues undergoing differential growth. In ethylene-treated etiolated seedlings, the expression of Sl-*IAA3* is restricted to the inner side of the apical hook opposite to that of the tomato *HOOKLESS* gene. Down-regulation of Sl-*IAA3* via an antisense strategy results in auxin and ethylene-related phenotypes including altered apical dominance, lower auxin sensitivity, exaggerated apical hook curvature in the dark and reduced petiole epinasty in the light. The ethylene-related phenotypes in the antisense tomato lines (*AS-IAA3*) reveal new roles for *Aux/IAAs* genes and position Sl-*IAA3* firmly at the crossroads between auxin and ethylene signalling in tomato.

INTRODUCTION

Development in multicellular organisms is a highly complex process that requires the precise coordination of inter and intracellular signalling and responses. Before the molecular era, the regulation of plant developmental processes was most often described as modifications in the hormonal balance, rather than as changes in the level of a single hormone. Over several decades, genetic screens led to tremendous advances in our understanding of the key components of the individual hormone metabolism and response pathways. However, as the understanding of these mechanisms grew it became more apparent that the growth of plant organs is dependent on an intricate orchestration of hormonal and non-hormonal signals (Stepanova et al., 2007; Swarup et al., 2007). Identifying the central players in the interplay between different signaling pathways is critical to unravelling the complex mechanisms underlying the control of plant growth and development. Interactions between ethylene and auxin are among the most frequently addressed in hormonal cross-talk studies and yet little is known about the main actors that take part in this dialogue (Stepanova et al., 2005 and 2007; Chae et al., 2000).

The plant hormone auxin, indole-3-acetic acid (IAA), has long been recognised as being a major regulator of plant growth and developmental processes. It exerts its effects by modulating the expression of downstream genes that in turn regulate a vast array of physiological processes. Recent genetic and molecular studies in Arabidopsis have revealed a crucial intracellular auxin signalling pathway in which a ubiquitin-dependent proteolytic system plays a key role in sensing and transducing the hormone signal to transcriptional programs (Dharmasiri and Estelle, 2004). At the center of the signalling cascade is the ubiquitin-ligase complex, SCF^{TIR1}, which promotes the ubiquitin-dependent proteolysis of a family of transcriptional regulators known as Aux/IAAs in an auxin-dependent manner (Gray et al., 2001). Binding of auxin to the Transport Inhibitor Response1/TIR1, the F-box protein subunit of SCF^{TIR1} or its paralogues AUXIN RECEPTOR F-BOX/AFB1 and AFB3, leads to the degradation of the Aux/IAA class of proteins (Dharmasiri et al., 2005a and 2005b; Kepinski and Leyser, 2005). Degradation of the Aux/IAAs activates the DNA-binding Auxin Response Factors (ARF), whose activities in regulating auxin-responsive genes

are otherwise inhibited by the Aux/IAA proteins (Hagen and Guilfoyle, 2002; Reed, 2001; Liscum and Reed, 2002; Zenser et al., 2001; Tiwari et al., 2001). Aux/IAAs are therefore central to the regulation of auxin-mediated processes. The Arabidopsis genome encodes 29 Aux/IAA proteins (Remington et al., 2004; Overvoorde et al., 2005). Biochemical and genetic studies indicate that they generally function as transcriptional repressors of auxin-regulated genes (Ulmasov et al., 1997; Tiwari et al., 2004; Woodward and Bartel, 2005). *Aux/IAA* genes encode short-lived nuclear proteins characterized by four highly conserved domains (domain I, II, III, and IV), each contributing to the functional properties of the protein. Domain I is responsible for the repression activity of the proteins (Tiwari et al., 2004). Domain II confers the auxin-mediated instability to the proteins (Worley et al., 2000; Ouellet et al., 2001) and domains III and IV are involved in homo- and heterodimerization with other Aux/IAA proteins and the ARFs (Kim et al., 1997; Ouellet et al., 2001; Ulmasov et al., 1997).

Gain-of-function mutations in several *Aux/IAA* genes have pleiotropic effects on plant growth, including altered root formation, apical dominance, stem/hypocotyl elongation, leaf expansion and phototropism/gravitropism. These mutants have been identified from a variety of developmental and auxin-specific genetic screens. The phenotypes are mostly associated with decreased auxin responsiveness. For example, in Arabidopsis the *shy2-2/iaa3*, *axr2-1/iaa7* and *iaa28-1* mutants show decreased apical dominance (Tian and Reed, 1999; Nagpal et al., 2000; Rogg et al., 2001), consistent with a reduced auxin response in these mutants. In a few cases, however, gain-of-function mutants are associated with an enhanced auxin response phenotype. For example, *axr3-1/iaa17-1* mutant plants have increased apical dominance and increased adventitious rooting, indicative of an enhanced auxin response (Leyser et al., 1996). Each of these *Aux/IAA* gain-of-function mutants is caused by a single mutation in domain II that stabilizes the Aux/IAA proteins. Strikingly, with the exception of the *Shy2* mutant that displays subtle modifications (Tian and Reed,1999), none of the Arabidopsis "null mutants" show obvious visible phenotypes, suggesting that highly similar proteins encoded by one or more members of the *Aux/IAA* gene family are capable of performing overlapping functions during plant growth and development (Overvoorde et al., 2005). However, the wide diversity of auxin responses and the tissue specific expression

displayed by members of the gene family suggest that individual Aux/IAAs have precise and distinct functions during normal plant growth and development. We have previously shown that members of the *Aux/IAA* gene family can be regulated by ethylene in the tomato (Jones et al., 2002) and that down-regulation of the tomato Aux/IAA, Sl-*IAA9,* results in a pleiotropic phenotype, including altered leaf architecture and parthenocarpy (Wang et al., 2005). Similarly, Kloosterman et al. (2006) have shown that suppression of St-*IAA2* in potato results in distinctive phenotypes including increased plant height, petiole hyponasty and curvature of growing leaf primordia in the shoot apex. In Arabidopsis, it has been reported that a mutation in an *ARF* reverses the ethylene-related hookless phenotype in the *hls1* mutant (Li et al., 2004). This suggests that auxin related transcription factors have evolved as points of intersection between ethylene and auxin responses.

We report here on the isolation and functional characterization of the tomato *Aux/IAA*, Sl-*IAA3* that defines one of the mechanisms by which auxin and ethylene signaling pathways converge. Both expression data and the analysis of transgenic plants down-regulated for Sl-*IAA3* support the hypothesis that Sl-IAA3 is a key molecular link between ethylene and auxin responses in tomato plants**.**

RESULTS

Isolation and Structure of the Sl-*IAA3* Gene

Sl-*IAA3* and other partial tomato *Aux/IAA* clones were initially isolated from tomato fruit using gene family-specific degenerate primers designed from conserved *Aux/IAA* sequences (Jones et al., 2002). Sl-*IAA3* corresponds to a previously isolated, partial tomato clone named *IAA3* (Nebenführ et al., 2000). Sl-*IAA3*, formerly named DR3, was reported to be ethylene inducible and differentially expressed during fruit ripening (Jones et al., 2002). We isolated the full length Sl-*IAA3* cDNA (U 320812, now available from the Solanaceae Genome Network Database, http://www.sgn.cornell.edu) and determined the transcription start site by 5’ Race-PCR. An open reading frame of 558 bp encoding a putative protein of 185 amino acids was identified. The predicted protein comprises the

four conserved domains (I to IV) characteristic of Aux/IAA proteins and falls into sub-family I of the four Aux/IAA sub-families (Wang et al., 2005). We also isolated a genomic fragment of 2723 bp in order to identify the structure of the tomato Sl-*IAA3* gene. The sequence comprised 1668 bp of promoter and 1055 bp of transcribed sequence composed of three exons and two introns (Figure 1) and matched that of its closest Arabidopsis homologs, At-*IAA3* (AT1G04240) and *IAA4* (AT5G43700) consistent with the phylogenetic analysis (see Supplemental Figure 1 online). The Sl-*IAA3* nucleotide coding and predicted amino acid sequences display 65.8 % and 56 % identity, respectively with At-*IAA3* and 65.4 % and 56.3 % identity, respectively with At-*IAA4*.

Features of the Sl-*IAA3* Promoter

The 1668 bp promoter fragment was analyzed *in silico* using PlantCare software (Lescot et al., 2002). The program identified several putative *cis*-acting elements, including two degenerate auxin-response elements (TGTCNC) at positions -216 and -175, an ethylene-response element ERE (ATTTCAAA) at position -1174, three methyl-jasmonate response elements (CGTCA) at positions -434, -969, and -1018, a heat-response element HSE (AAAAAATTTC) at position -934 and an

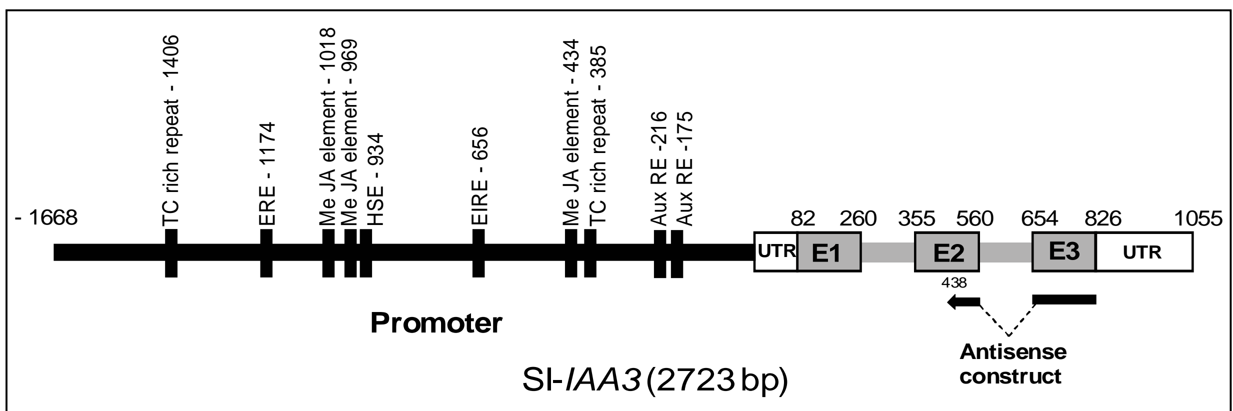


Figure 1. Genomic Structure of the Tomato Sl-IAA3 Gene. *The black portion represents the promoter region, the gray lines the introns, the gray boxes the exons and the white boxes the untranslated regions (UTR). The putative cis-acting elements found in the promoter region are indicated by black bars. The black arrow represents the antisense construct used to generate the silenced lines.*

elicitor-responsive element EIRE (TTCGACC) at position -656 and two TC-rich repeats (ATTTTCTTCA) at positions –385 and -1406 that have been shown to be involved in defence and stress responsiveness (Figure 1).

Sl-*IAA3* Transcripts Are Ubiquitous in all Plant Tissues but Show Higher Accumulation During Fruit Ripening

Quantitative Real-Time RT-PCR (qRT-PCR) performed on different tomato plant tissues revealed Sl-*IAA3* expression in all tissues tested (Figure 2A). Transcripts for the gene were most abundant in red fruit, where they were 6-fold higher than in the reference tissue (stem). This preferential expression in ripening fruit prompted us to investigate Sl-*IAA3* expression throughout the ripening process and in different tomato ripening mutants. In normal fruit, Sl-*IAA3* transcript levels increased during ripening until the orange/red stage and thereafter decreased to the red-ripe stage (Figure 2B). In the ripening and ethylene response-impaired monogenic tomato mutants *rin (ripening inhibitor)*, *nor (non-ripening)* and *Nr (Never-ripe)*, there were substantially lower levels of Sl-*IAA3* transcripts at stages equivalent to mature green and red in wild-type (WT) fruit (Figure 2C), suggesting that the Sl-IAA3 protein is integral to normal fruit ripening. Tomato fruit are climacteric and the majority of processes occurring throughout ripening are triggered by ethylene. To further examine whether the ripening associated expression of Sl-*IAA3* is ethylene-dependent, we assessed the effect of exogenous ethylene treatment on mature green fruit and conversely the effect of 1-MCP (1-methyl cyclopropene), a potent inhibitor of ethylene perception, on breaker (onset of ripening) stage fruit. Five hours of ethylene treatment (50 µl L^{-1}) resulted in almost 11-fold increase in Sl-*IAA3* transcript accumulation in MG fruit (Figure 2D). Conversely, in breaker stage fruit, an overnight treatment with 1-MCP treatment (1 µL L^{-1}) led to a 10-fold reduction in Sl-*IAA3* transcripts (Figure 2E) clearly indicating that ethylene plays an important role in the ripening-associated expression of Sl-*IAA3*. Given that Sl-IAA3 is a presumptive auxin signal response component, its ethylene regulation indicates that the protein is involved in the integration of responses to the two hormones during fruit ripening.

Sl-*IAA3* Transcript Accumulation Is Positively Regulated by Auxin

In Arabidopsis, most *Aux/IAA* genes are auxin inducible (Abel et al., 1994). We used two independent approaches to determine if Sl-*IAA3* is similarly auxin responsive. qRT-PCR analysis of RNA extracted from three-week-old light-grown tomato

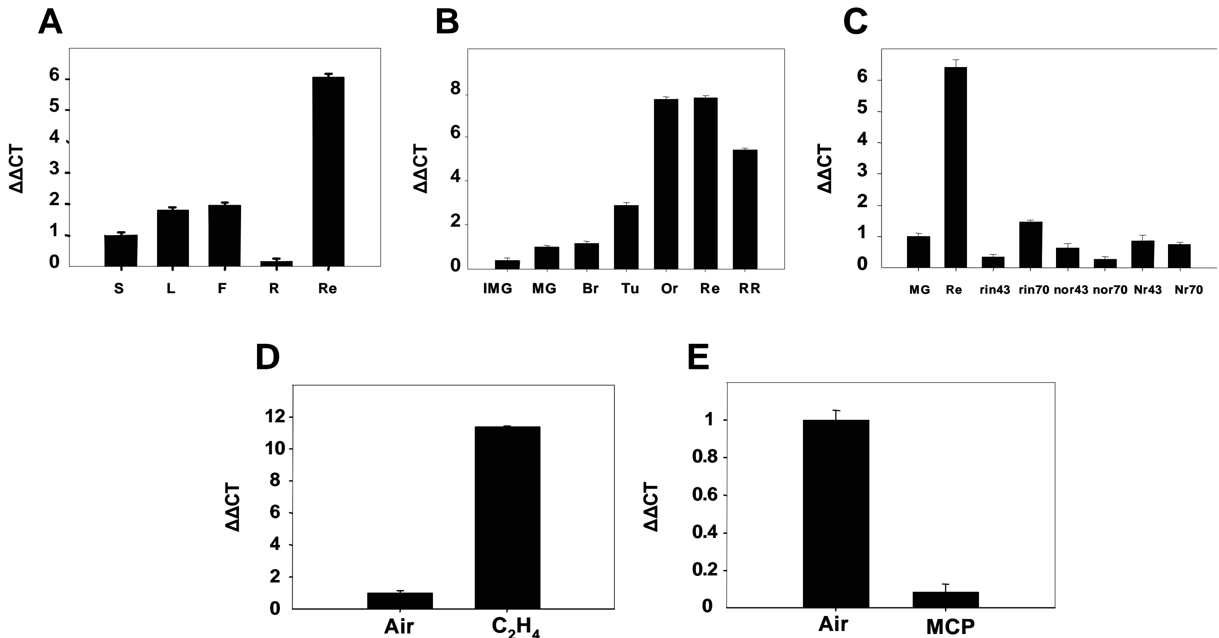


Figure 2. Tissue-Specific and Ethylene-Dependent Expression of Sl-IAA3*. The expression analyses were carried out by quantitative Real-Time RT-PCR using RNA samples extracted from various tomato tissues.* ***A****. Analysis of the level of Sl-IAA3 transcripts in different organs. Sl-IAA3 mRNA accumulation was monitored in stem (S), leaf (L), flower (F), root (R) and red fruit (Re). ΔΔCT on the y axis refers to the fold difference in Sl-IAA3 expression relative to the control tissue (stem) taken as a reference.* ***B****. Expression pattern of Sl-IAA3 during the late stages of fruit development. Immature green (IMG), mature green (MG), breaker (Br), turning (Tu), orange (Or), red (Re) and red-ripe (RR) fruit. ΔΔCT on the y axis refers to the fold difference in Sl-IAA3 expression relative to the MG stage.* ***C****. Expression pattern of Sl-IAA3 in WT and rin, nor and Nr ripening mutants. RNA samples were extracted from fruit collected 43 and 70 days after anthesis, corresponding in the WT to MG and Re stages, respectively. ΔΔCT on the y axis refers to the fold difference in Sl-IAA3 expression relative to the MG stage.* ***D****. Ethylene responsiveness of the Sl-IAA3 gene. RNA samples were extracted from MG fruit treated for 5h with air or with 50 µL L^{-1} ethylene. ΔΔCT on the y axis refers to the fold difference in Sl-IAA3 expression relative to the untreated control fruit.* ***E****. Br fruit treated with 1 µL L^{-1} of 1-MCP for 16 h. ΔΔCT on the y axis refers to the fold difference in Sl-IAA3 expression relative to untreated control fruit. All the expression data presented in Figure 2 correspond to values that are means of 3 replicates ± SE.*

seedlings showed that Sl-*IAA3* transcript levels increased 4-fold after two hours treatment with 20 µM IAA (Figure 3A). Furthermore, in tobacco BY2 protoplasts transfection assays, Sl-*IAA3* promoter (1668 bp)-driven GFP levels increased 4-

fold after auxin (50 µM of 2,4-D) treatment (Figure 3B), clearly indicating that Sl-*IAA3* expression is positively regulated by auxin**.** Since auxin is known to stimulate ethylene production (Abel et al, 1995), we sought to determine whether the auxin-responsiveness of Sl-*IAA3* results from increased ethylene production. Light-grown seedlings were thus treated overnight with 1-MCP (1µL L^{-1}) and then incubated in presence or absence of auxin. qRT-PCR analysis revealed that 1-MCP treatment completely abolished the expression of Sl-*IAA3* gene in the absence of auxin but only partially reduced Sl-*IAA3* transcript accumulation in the auxin-treated plants (Figure 3A). This indicates that the basal expression of Sl-*IAA3* is ethylene-dependent and that the auxin-responsiveness of this gene is not mediated by ethylene.

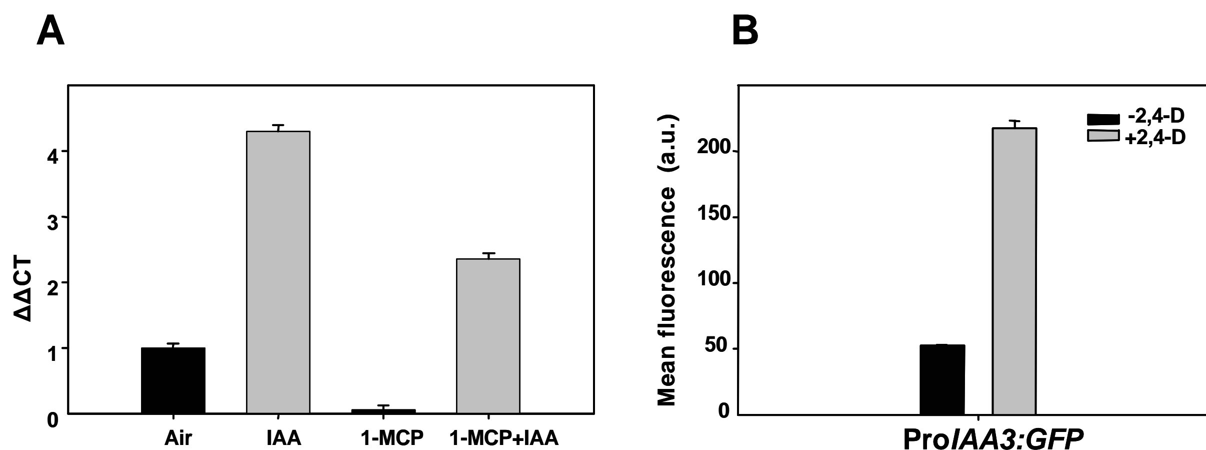


Figure 3. Auxin Responsiveness of the Sl-IAA3 Gene. A*. RT-PCR analysis of Sl-IAA3 transcript levels in RNA samples extracted from three week-old light-grown control and auxin treated (20 µM IAA for two hours) seedlings in presence or absence of 1-MCP, the ethylene perception inhibitor (1 µL L^{-1} 1-MCP applied 16h prior to auxin treatment). ΔΔCT on the y axis refers to the fold difference in Sl-IAA3 transcript levels relative to the non-treated plantlets.* ***B.*** *Auxin responsiveness of the Sl-IAA3 promoter. Tobacco protoplasts were transformed by a chimeric construct consisting of 1668 bp of the Sl-IAA3 promoter fused to the GFP reporter gene (ProIAA3:GFP) and incubated in the presence or absence of 50 µM of 2,4-D. Transformation was performed in triplicate and in each experiment GFP fluorescence was measured by flow cytometry 16 h after transfection. Values are expressed in arbitrary units (a.u.) ± SE.*

Sl-*IAA3* Is Differentially Expressed in Different Plant Organs and Displays Tightly Regulated Tissue-Specific Expression

To gain further insights into Sl-*IAA3* expression, we fused the Sl-*IAA3* promoter to the *GUS* (*β–glucuronidase*) reporter gene (Pro*IAA3:GUS*) and stably introduced it

into tomato plants. GUS activity driven by the Sl-*IAA3* promoter was then assessed in lines homozygous for the chimeric construct. In untreated vegetative tissues, the Sl-*IAA3* promoter drove GUS expression exclusively in the leaf vasculature and root tips (Figure 4A to 4C). However, a brief auxin treatment (20 µM for two hours) of light grown seedlings led to a dramatic increase in GUS expression throughout the roots and shoots (Figure 4E to 4G). In mature green fruit, GUS staining was restricted to a narrow band in the placenta exolayer at the junction between the placenta and pericarp tissues (Figure 4D). Once again,

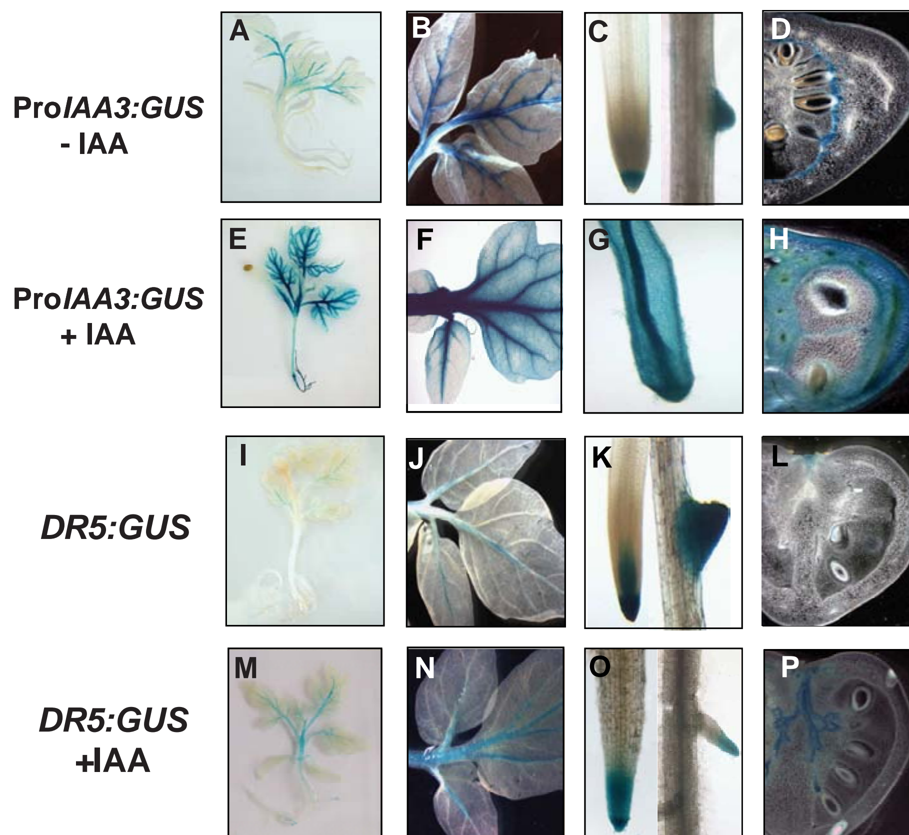


Figure 4. Tissue-Specific Expression of Sl-IAA3 Monitored by GUS Reporter Gene Activity Driven by the Sl-IAA3 Promoter (ProIAA3:GUS). *The construct was stably transformed into tomato and GUS staining was assessed in various tissues of homozygous plants. The expression pattern was assessed in 3-week-old seedlings (**A**), leaves (**B**), roots (**C**) and MG fruit (**D**). **E**, **F**, **G** and **H** correspond to the same tissues treated for 2 hours with 20 µM IAA. **I**, **J**, **K** and **L** correspond to the same tissues expressing the DR5 auxin-responsive promoter fused to the GUS reporter gene (DR5:GUS) used as sensor for active auxin signalling. **M**, **N**, **O** **and** **P** correspond to DR5:GUS treated with 20 µM IAA. The images displayed are representative of at least three independent experiments with n>20 seedlings examined per experiment.*

exogenous auxin (20 µM for two hours) led to an increase in the range of GUS expressing tissues. After hormone treatment, GUS staining was detected throughout the pericarp and columella tissues but remained excluded from the placental tissues (Figure 4H). As a control for auxin responsiveness, we also transformed tomato plants with a construct containing the artificial auxin-responsive promoter, *DR5*, fused to the *GUS* reporter gene (*DR5:GUS*). Interestingly, this synthetic promoter used here as auxin sensor, drove GUS expression in the leaf midrib and root tips (Fig 4I to 4K), but not in the fruit (Figure 4L). Exogenous auxin treatment of *DR5:GUS* plants resulted in enhanced staining in vegetative tissues but in the fruit, expression remained restricted to the vascular tissues (Figure 4M to 4P). These data indicate that the transcriptional control of Sl-*IAA3* is more complex than that of *DR5*, providing evidence that although it is auxin responsive, its tight tissue-specific regulation depends on a variety of factors.

Sl-*IAA3* Encodes a Nuclear Localized Protein that Acts *In Vivo* as a Repressor of Auxin-Responsiveness

We investigated the sub-cellular localization of the Sl-IAA3 protein by expressing in tobacco cell protoplasts the green fluorescent protein (GFP) fused to Sl-IAA3 (Sl-IAA3:GFP). As expected, 35S Cauliflower Mosaic Virus (CaMV) promoter-driven GFP alone was present throughout the cytoplasm (see supplemental Figure 2A online) whereas the Sl-IAA3:GFP fusion protein was localized exclusively in the nucleus (see supplemental Figure 2B online). This nuclear localization is consistent with a transcriptional regulatory function for the native Sl-IAA3 protein.

In order to determine the function of the Sl-IAA3 encoded protein and to address its ability to regulate *in vivo* the activity of auxin-responsive promoters, a DR5-driven GFP reporter construct was used (Ottenschlager et al., 2003). This reporter construct was co-transfected into tobacco protoplasts with an effector construct giving constitutive 35S-driven Sl-IAA3 protein expression. Transient expression experiments using this dedicated "single cell system" revealed that in the absence of the effector construct, DR5-driven GFP expression was enhanced

up to 10-fold by the auxin (2,4-D) treatment (see supplemental Figure 3 online). The presence of 35S-driven Sl-IAA3 in co-transfection assays, however, strongly reduced this auxin induction. A mock effector plasmid containing the 35S promoter but lacking the Sl-*IAA3* coding sequence did not impact the auxin-induction of the DR5 promoter activity (see supplemental Figure 3 online). These data indicate that Sl-IAA3 acts *in vivo* as a repressor of auxin-dependent transcription and is consistent with Sl-IAA3 being a member of the Aux/IAA family.

Sl-*IAA3* Down-Regulation Results in Vegetative Growth Phenotypes

We generated Sl-*IAA3*-suppressed antisense tomato lines (*AS-IAA3*) in order to address the function of the protein *in planta*. Several homozygous transgenic lines corresponding to independent transformation events were obtained and analyzed. Two representative lines (1 and 2) showing 6-fold and 10-fold reductions in Sl-*IAA3* transcript levels, respectively, were selected for further study (Figure 5A). In these lines, down-regulation of Sl-*IAA3* resulted in multiple vegetative growth phenotypes, including a dramatic reduction in apical dominance (Figure 5B and 5C). In determinate WT tomato plants (e.g. MicroTom), lateral shoots develop only after floral transition and their growth is initiated in an apical-basal sequence along the primary shoot axis. The first lateral shoot arises from the last leaf node just below the first inflorescence. By contrast, outgrowth of the axillary shoots in the *AS-IAA3* plants began in the lowest leaf node (Figure 5B) and the number of lateral shoots was increased in the transgenic lines (Figure 5C). The *AS-IAA3* lines also showed a higher frequency of ectopic cotyledons than the WT (Figure 5D and 5E). The frequency of polycotyledon structure reached 25 and 20% in *AS-AA3-1* and *AS-IAA3-2* lines, respectively, compared to only 5% in the WT (Figure 5E).

Sl-*IAA3* Suppression Results in Reduced Auxin Responsiveness

To further investigate the role of the Sl-IAA3 in auxin responses, we assessed the elongation of hypocotyl sections after auxin treatment in WT and *AS-IAA3* lines.

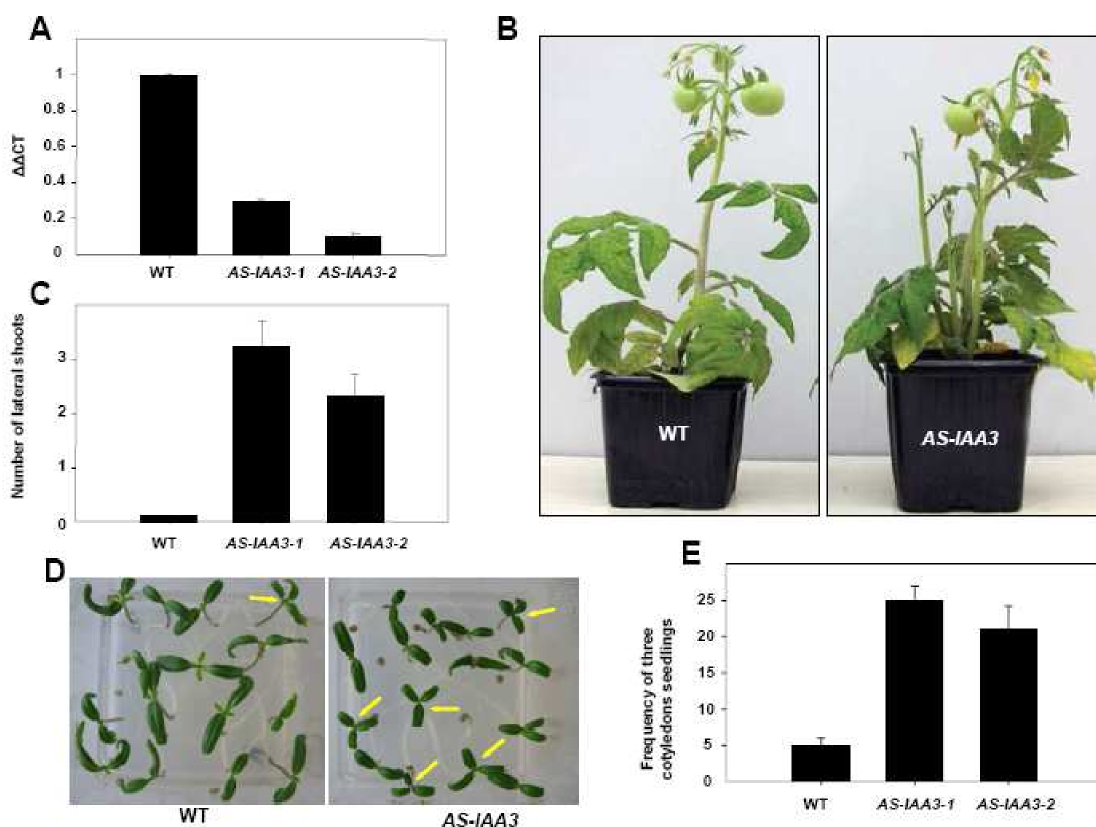


Figure 5. Altered Vegetative Growth Phenotypes in Antisense Sl-IAA3 Plants. A. *Down-regulation of Sl-IAA3 in transgenic tomato plants. The level of Sl-IAA3 transcripts in transgenic antisense lines (1 and 2) was assessed by qRT-PCR. ΔΔCT refers to the fold difference in Sl-IAA3 transcript levels relative to the WT.* ***B.*** *Reduced apical dominance in 7-week-old AS-IAA3 transgenic plants compared to WT plants.* ***C.*** *The number of lateral shoots branching from the first leaf node in WT and AS-IAA3 plants. The data are the mean ± SE of at least 30 plants and are representative of three independent experiments.* ***D.*** *Triple cotyledon phenotype occurring at higher frequency in AS-IAA3 transgenic lines compared to WT. Plants are light-grown in MS medium for 7 days. Three cotyledon structures are indicated by yellow arrows.* ***E.*** *Frequency of triplicate cotyledons occurring in AS-IAA3 and WT seedlings expressed as a % of the total population. Error bars represent mean ± SE of 40 plants.*

After two hours auxin treatment (Figure 6A), the WT hypocotyl segments elongated more than the *AS-IAA3* plants at all auxin concentrations tested, indicating that Sl-*IAA3* suppression reduces sensitivity to auxin *in planta*. To further investigate this, we examined the effects of the auxin transport inhibitor, N-1-napthylphthalamic (NPA) on the growth of WT and *AS-IAA3* seedlings. NPA treatment is known to alter the endogenous auxin gradient, leading to an over-accumulation of auxin in the root apical meristem which results in an inhibition of cell division and root growth. When grown in the presence of 1μM NPA, there was a marked reduction of primary root elongation and a complete suppression of lateral root formation in the WT seedlings (Figure 6B and 6C). In the absence of

NPA, 19-day-old *AS-IAA3* seedlings are not morphologically different to the WT (Figure 6B). By contrast, NPA only weakly affected primary root growth in *AS-IAA3* plants, although lateral root formation was inhibited similarly to the WT (Figure 6Band 6C). Leaf emergence was also strongly inhibited in NPA-treated WT seedlings, but not in the *AS-IAA3* plants (arrow Figure 6B). Once again, these data are consistent with reduced auxin sensitivity in the antisense lines. Other auxin-related processes like hypocotyl and root gravitropism were not affected in

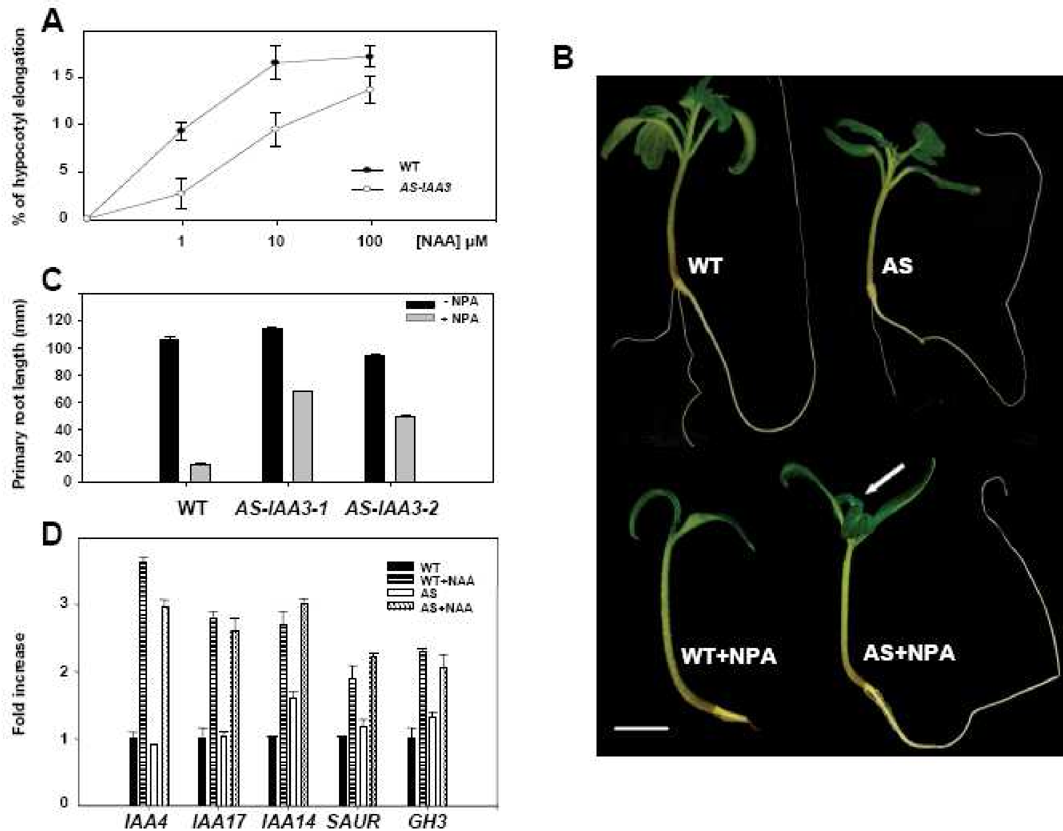


Figure 6. Auxin-Associated Phenotypes of Sl-IAA3 Down-Regulated Lines. A. *Auxin dose-response in hypocotyl segments. 8 mm hypocotyl fragments cut from 3-week-old light grown seedlings were incubated for 2 h in a solution containing the indicated concentration of NAA. Elongation is given as percentage increase in final length over the initial length. The results are representative of data obtained with two independent AS-IAA3 lines and with two replicates for each line. Standard errors are indicated (n ≥ 25).* ***B.*** *Effect of NPA treatment on the development of light-grown WT and AS-IAA3 seedlings. 19-day-old WT and AS-IAA3 tomato seedlings were grown in the presence or absence of 1 µM of NPA. The scale bar indicates 10 mm* ***C.*** *Primary root length upon NPA treatment of light-grown WT and AS-IAA3 lines. Primary root length was assessed following NPA treatment. Error bars represent mean ± SE (n ≥ 60).* ***D****. Expression of early-auxin-response genes in AS-IAA3 and WT lines. Expression analysis of Sl-IAA4 (SGN-U316052), Sl-IAA14 (SGN-U318434), Sl-IAA17 (SGN-U323974), SAUR (SGN-U318031) and GH3 (SGN-U319351) genes were carried out by qRT-PCR in WT and AS-IAA3 hypocotyl fragments after auxin treatment (1µM NAA for 2h). The data are expressed in fold increase with respect to non treated tissues taken as reference values.*

IAA3-down-regulated lines (see supplemental Figure 4 online). In addition, no alteration in root growth and vascular formation was observed in *AS-IAA3* lines. We therefore investigated typical molecular responses to auxin by assessing the expression of three *Aux/IAA* genes (Sl-*IAA4,* Sl-*IAA14,* Sl-*IAA17*) and two other primary auxin-responsive genes, *SAUR* and *GH3*. RNA samples isolated from *AS-IAA3* and WT hypocotyls incubated 2 h in presence or absence of 1 µM NAA were used in qRT-PCR experiments to monitor transcript accumulation for the five genes. Neither the basal, nor the auxin responsive transcript levels were altered by the down-regulation of Sl-*IAA3* (Figure 6D). It is important to point out that Sl-*IAA4,* Sl-*IAA14* and Sl-*IAA17* were selected among the *Aux/IAA* genes because of their high sequence homology to Sl-*IAA3* (See supplemental Table1 online). Therefore, since their expression remained unchanged in the *AS-IAA3* plants it is most likely that Sl-*IAA3* was exclusively directly targeted by the antisense strategy.

Sl-*IAA3* Suppression Results in Modified Ethylene Sensitivity

The ethylene responsiveness of Sl-*AA3* prompted us to examine the role of the encoded protein in two classical ethylene response processes, epinastic petiole curvature in light-grown plants and the formation of an apical hook in etiolated seedlings. Tomato leaf petioles typically curve downwards in response to exogenous ethylene (Kazemi et al., 1974). To investigate the impact of the down-regulation of Sl-*IAA3* on this epinastic response, light-grown WT and *AS-IAA3* tomato plantlets were treated with exogenous ethylene (50 µL L^{-1}) for 16 h. The angle of the petioles was then measured in the first and second leaf-nodes as indicated in Figure 7B. In WT, ethylene treatment led to a leaf angle of 100°. By contrast in *AS-IAA3* lines 1 and 2, the leaf angle after treatment was 87° and 75°, respectively (Figure 7A and Table 1), indicating an alteration in the ethylene-induced epinastic response in the *AS-IAA3* plants. In the absence of ethylene treatment, both WT and *AS-IAA3* lines display the same leaf angle (Table 1). One of the most striking phenotypes in the *AS-IAA3* seedlings is the exaggerated apical hook formation in the absence of exogenous ethylene (Figure 7C). Exaggeration of the apical hook is one of the hallmarks of the classical ethylene triple response (Ecker, 1995). To better characterise this phenotype we first

defined different grades of hook formation ranging from stage 0, corresponding to total absence of the hook, to stage 7 corresponding to maximal exaggerated hook (Figure 8A). Treatment of seedlings with 1-MCP abolished apical hook formation

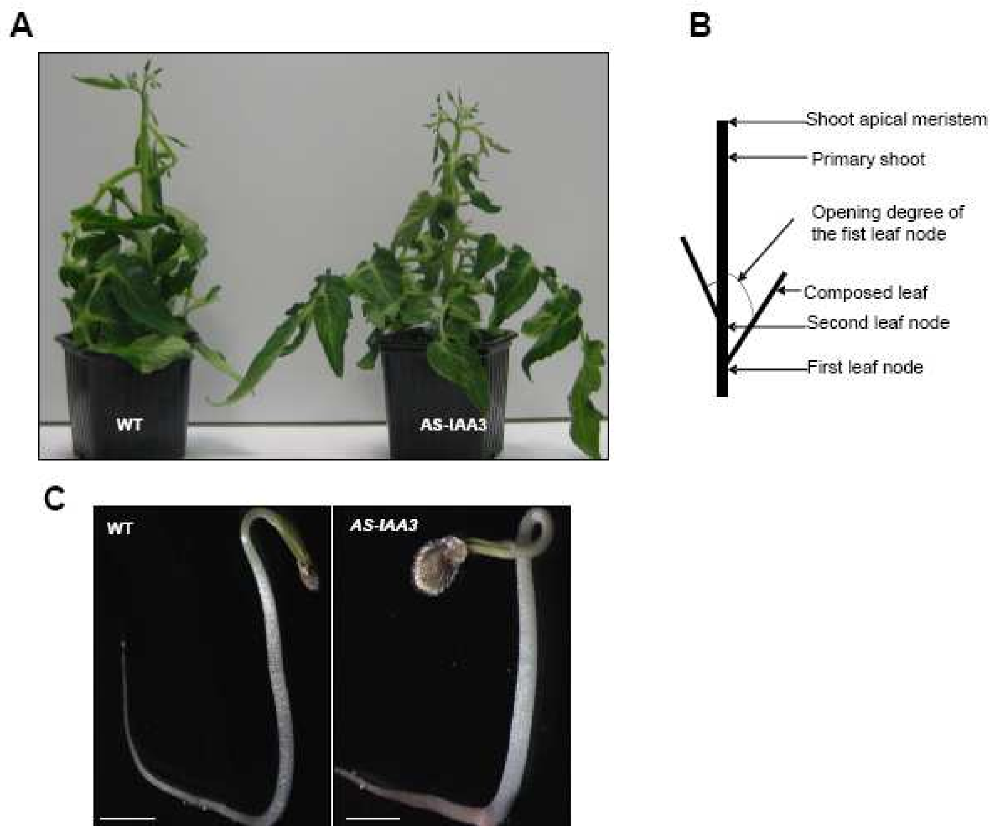


Figure 7. Ethylene Associated Phenotypes of AS-IAA3 Lines. A*. Petiole epinasty in WT and AS-IAA3 plants in response to ethylene. Five-week-old light-grown plants were placed in airtight chambers for 16 h in the presence of 50 µL L^{-1} ethylene.* ***B****. Diagram depicting the position of the first and the second leaf node in tomato plants.* ***C****. Hook curvature in 5-day-old WT (left panel) and AS-IAA3 (right panel) etiolated seedlings. The scale bar indicates 5 mm.*

in both the antisense and WT lines (Figure 8B). In air-grown seedlings, in the absence of exogenous ethylene, most *AS-IAA3* seedlings displayed hook curvature corresponding to stage 4 (60%) and stage 3 (35%), while of wild type plants exhibited mainly stage 1 (60%) and stage 2 (35%) hook curvature (Figure 8C). Adding exogenous ethylene (0.1 µL L^{-1}) increased hook curvature to stage 3 and 4 for wild type and to stages 4 and 5 for *AS-IAA3* (Figure 8D). Under 1 µL L^{-1} exogenous ethylene, 90 % of WT seedlings displayed hook curvature from stage 5 and 6 and 80% of *AS-IAA3* lines exhibited hook curvature from stage 6 and 7 (Figure 8E). These data indicate that the exaggerated apical hook curvature

phenotype of the *AS-IAA3* plants requires active ethylene signalling and that transgenic lines are more responsive to ethylene.

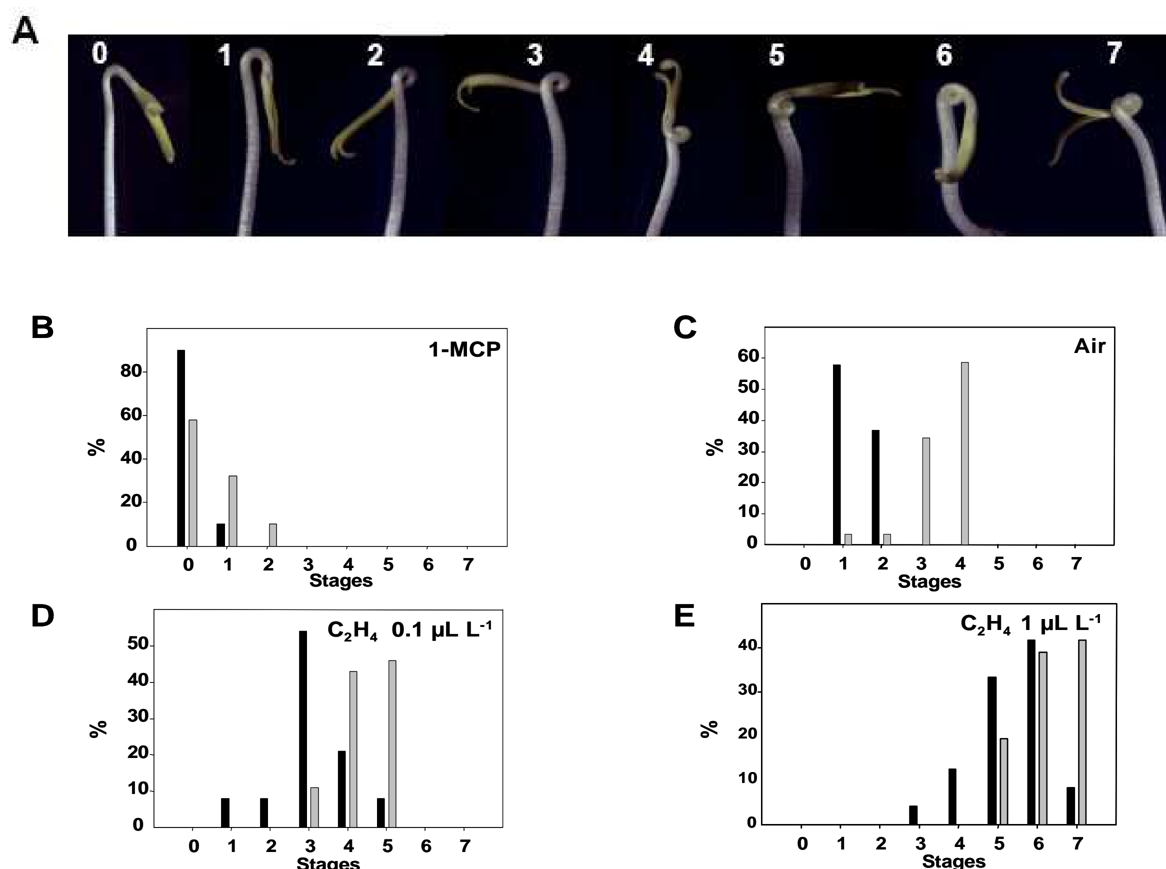


Figure 8. Increased Ethylene Sensitivity of AS-IAA3 Lines. A. *Eight stages of hook formation have been defined (stage 0 to 7) in etiolated tomato seedlings treated with different concentrations of ethylene (0 to 1 µL L^{-1}).* ***B to E.*** *Proportion of WT (black bars) and AS-IAA3 (grey bars) plants corresponding to the eight stages of hook formation upon treatment with (**B**) 1-MCP (1µL L^{-1} for 16 h), (**C**) air or (**D** and **E**) exogenous ethylene (0.1 and 1µL L^{-1}).*

The Expression of Sl-*IAA3* Is Tightly Regulated in Apical Hook and Epinastic Petiole upon Ethylene Treatment

To further investigate the role of Sl-*IAA3* in apical hook formation and epinastic response, we analyzed the expression pattern of this gene in the corresponding tissues of transgenic tomato lines expressing the *GUS* reporter gene driven by the Sl-*IAA3* promoter (Pro*IAA3:GUS*). In the absence of exogenous ethylene, there was no detectable GUS staining in the apical hook of dark-grown seedlings. By contrast, after 48 hours ethylene treatment (10 µL L^{-1}), a strong band of GUS staining was observed on the inner surface of the apical hook. We also analysed the expression of the *GUS* reporter gene driven by the DR5 promoter in order to

determine whether ethylene treatment alters the expression pattern of this auxin sensor chimerical gene. Ethylene treatment did not affect the *DR5*:GUS staining pattern in the hook suggesting that the ethylene induction of Sl-*IAA3* was not mediated through increased auxin levels in the treated tissue (Figure 9A). In epinastic petioles treated with ethylene, the expression of Pro*IAA3:GUS* was restricted to the upper side of the leaf nods while no expression was detected in untreated non-epinastic petioles (Figure 9B). These data indicate that the expression of Sl-*IAA3* is associated with tissues undergoing differential growth, both in etiolated seedlings and in epinastic petioles.

Table 1. *Altered Petiole Epinastic Response in AS-IAA3 Plants in Response to Ethylene. Petiole opening degree of the first and the second leaf node was measured before and after ethylene treatment in WT and AS-IAA3 plants. The data are means ± SE of at least 36 plants and are representative of three independent experiments*

	Petiole opening degree	
	Air	C_2H_4
WT	70.8°± 2.8	100°± 4.46
AS-IAA3-1	70.1°± 3.5	87°± 4.31
AS-IAA3-2	72.2°± 1.8	75°± 2.87

The Sl-*IAA3* and Sl-*HOOKLESS* Genes Are Expressed on Opposite Sides of the Apical Hook

In Arabidopsis, At-*HOOKLESS1* (At-*HLS1*) is a key regulator of apical hook formation that has been proposed by Lehman et al. (1996) to integrate ethylene and auxin signalling during hook formation and maintenance in dark-grown seedlings. The Arabidopsis *hls1* mutant showed no differential growth in the apical region of the hypocotyl even after ethylene treatment. We isolated a putative tomato ortholog of At-*HLS1* and showed that it fully rescues the Arabidopsis *hls1-1* mutant phenotype (see supplemental Figure 5 online). Accumulation of Sl-*HLS* transcripts is not altered in the *AS-IAA3* plants (Figure11D) suggesting that the exaggerated hook formation in the transgenic lines does not involve an alteration in Sl-*HLS* expression. To further investigate

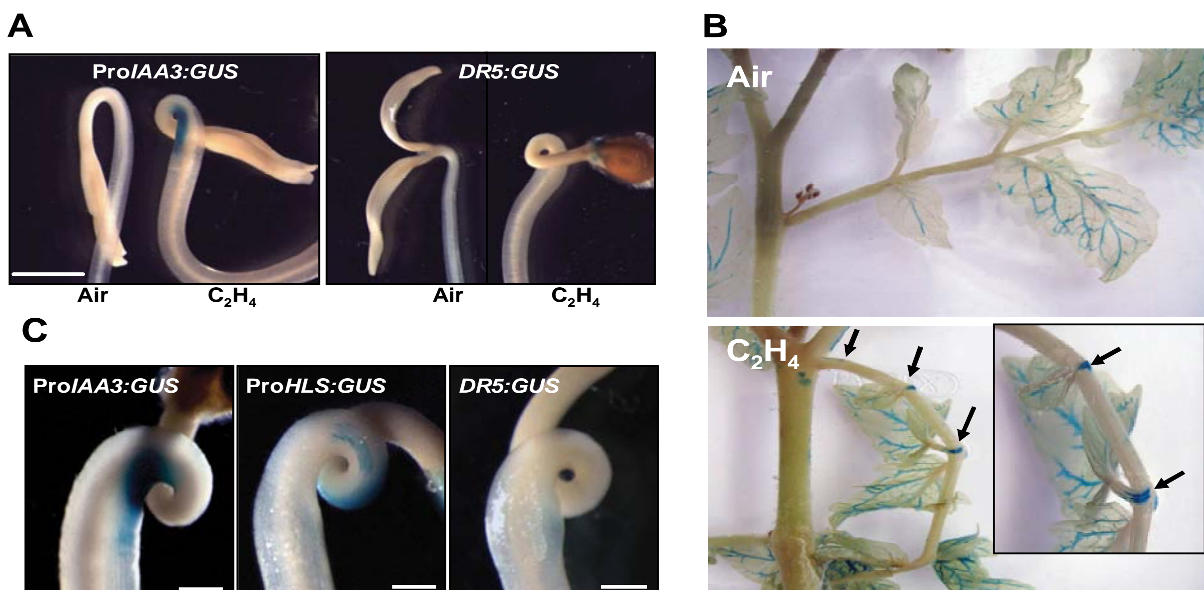


Figure 9. Expression of ProIAA3:GUS and ProHLS:GUS Associated with Differential Growth during Hook Formation and Epinastic Response. A. *Tissue-specific expression of ProIAA3:GUS and DR5:GUS in etiolated seedlings. Etiolated seedlings expressing the GUS reporter gene driven either by the Sl-IAA3 or DR5 promoter were dark grown on MS media for five days and then treated with air or 10 µL L^{-1} ethylene for 48 hours. The left panel shows the ethylene-dependent GUS staining in the apical hook of transgenic tomato plants expressing the ProIAA3:GUS construct. The right panel shows GUS staining in the DR5:GUS transformed plants used for detection of active auxin signalling in the hook. Bars = 5 mm* ***B.*** *Expression pattern of ProIAA3:GUS in epinastic and non-epinastic petioles. Six-week-old light-grown plants were placed in airtight chambers for 16 h in the absence (upper panel) or presence of 50 µL L^{-1} ethylene (lower panel). The arrows indicate the expression of GUS in the leaf nodes of the petiole.* ***C.*** *Comparative expression patterns of ProIAA3:GUS (left panel) and ProHLS:GUS (middle panel) in the apical hook. The right panel shows the expression of GUS driven by DR5. Bars = 1 mm. Etiolated seedlings expressing the GUS reporter gene driven either by the Sl-IAA3, Sl-HLS or DR5 promoter were dark grown on MS media for five days and then treated with 10 µL L^{-1} ethylene for 48 hours. The images displayed are representative of at least three independent experiments with n >30 seedlings examined per experiment.*

potential interactions between Sl-*HLS* and Sl-*IAA3* in controlling hook formation we analyzed the spatial expression of the Sl-*HLS* and Sl-*IAA3* in the apical hook by native promoter-reporter constructs (Figure 9C). We isolated a 1.3 kb fragment of the Sl-*HLS* promoter and fused it to the *GUS* reporter gene. Etiolated seedlings expressing this construct were treated with ethylene. Remarkably, Pro*HLS*: GUS staining was restricted to the outer side of the hook curvature, whereas the Sl-*IAA3* promoter drove GUS staining exclusively on the inner side. These data suggest that Sl-IAA3 acts as a repressor of auxin/ethylene-mediated cell

elongation on the inner surface of the apical hook and/or conversely that Sl-HLS1 is involved in promoting cell elongation on the outer surface.

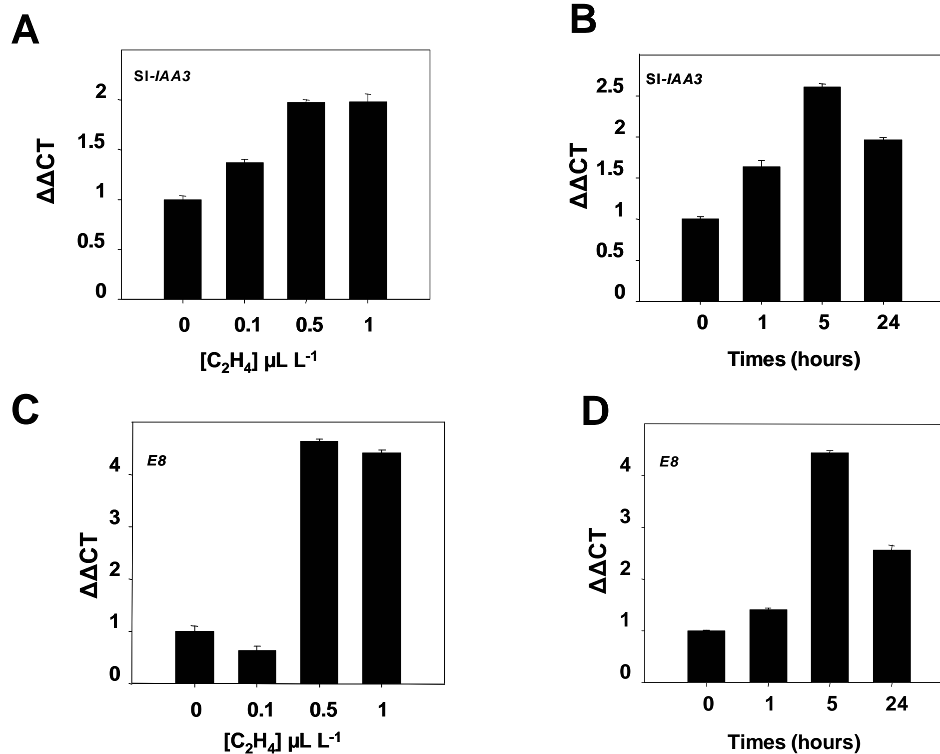


Figure 10. Characterization of Ethylene Regulation of Sl-IAA3. *Ethylene dose response (**A**) and time course (**B**) of Sl-IAA3 induction in 5-day-old dark-grown WT seedlings. Expression of the ethylene-responsive gene E8 was used as control for hormone treatment (**C** and **D**). ΔΔCT on the y axis refers to the fold difference in Sl-IAA3 expression relative to untreated seedlings. The data presented correspond to mean values of 3 replicates ± SE.*

To further characterise the ethylene regulation of Sl-*IAA3* we determined the ethylene dose-response using 5-day-old dark-grown wild type seedlings. qRT-PCR analysis revealed that Sl-*IAA3* transcript accumulation reached a maximum at 0.5 µL L^{-1} (Figure 10A), the same saturating concentration was found for the previously characterised ethylene-responsive gene, *E8* (Figure 10C). A study of the time-course of ethylene induction (performed with 0.5 µL L^{-1}) also indicated that Sl-*IAA3* transcript accumulation mimicked that of *E8* and reaching a maximum after 5 hours treatment and then decreasing at 24 hours (Figure 10B and 10D).

Down-regulation of Sl-*IAA3* Specifically impacts the Expression of Selected Auxin and Ethylene Transcription Factors

To provide mechanistic insights into how Sl-IAA3 functions to bring about the observed phenotypes, we attempted to identify putative target genes controlled by Sl-IAA3. Taking into account that down-regulation of Sl-*IAA3* mainly resulted in auxin and ethylene-related phenotypes, we analysed the expression of transcription factors known to mediate auxin and ethylene responses. The expression of tomato *Aux/IAA* (14), *ARF* (10) and *ERF* (12) genes was

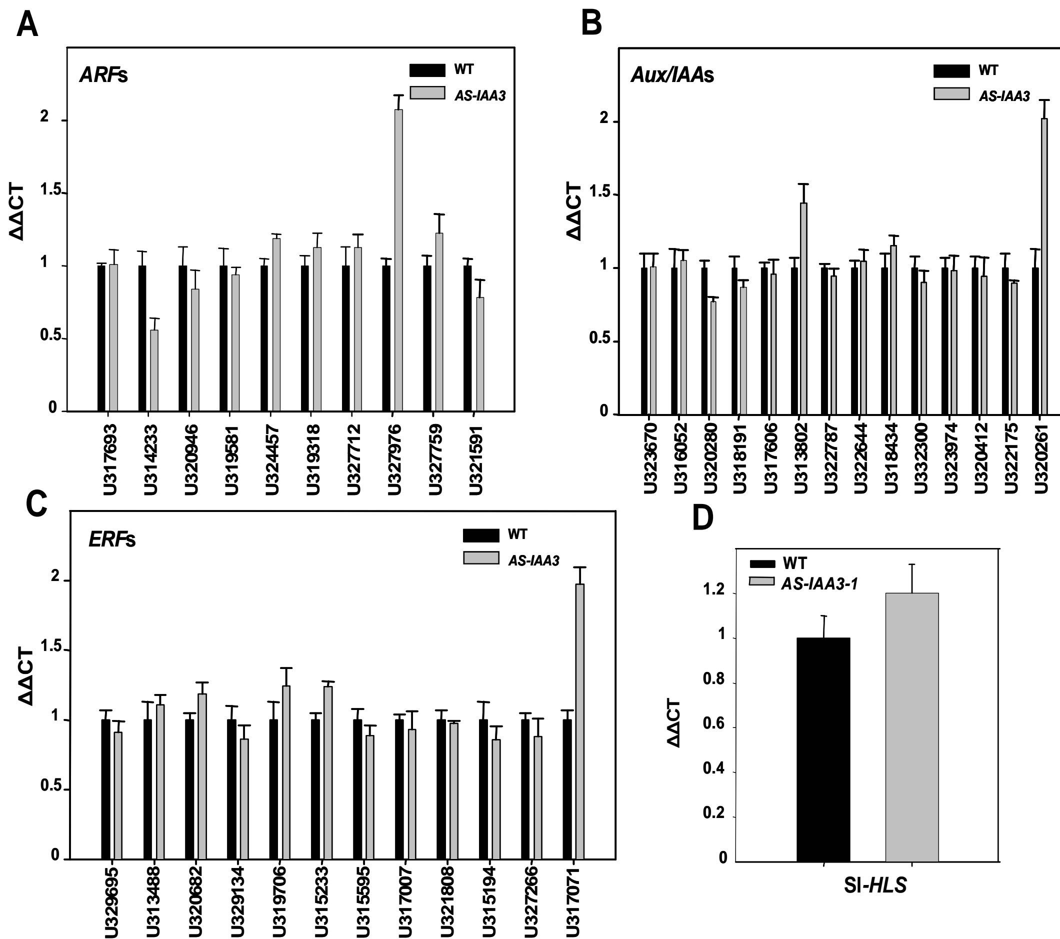


Figure 11. Impact of Sl-IAA3 Down-regulation on the Expression of Auxin and Ethylene Response Genes. *The expression of members of the ARF (**A**), Aux/IAA (**B**), and ERF (**C**) gene families of transcription factors as well as the Sl-HLS gene **(D)** was assessed by qRT-PCR in 5-day-old dark-grown WT and AS-IAA3 seedlings. The gene names correspond to the SGN annotation. Primers used are listed in Supplemental Table 2. ΔΔCT on the y axis refers to the fold difference in expression of each gene relative to that in WT seedlings taken as reference tissues. The data presented correspond to mean values of 3 replicates ± SE.*

assessed by qRT-PCR in 5-day-old dark-grown wild type and AS-*IAA3* seedlings (Figure 11).

While most of the genes showed similar expression in wild type and transgenic lines, there was a clear down-regulation of the putative tomato ortholog of Arabidopsis *ARF2* (SGN-U314233) and conversely a significant up-regulation of the *ARF8* (SGN-U327976) transcript (Figure 11A). The down-regulation of *ARF2* observed in the transgenic lines is in keeping with the data reporting that the hookless phenotype of *hls1* Arabidopsis mutants can be partially reversed by a loss-of-function mutation in the *ARF2* gene (Li et al., 2004). Notably, the expression of *IAA29* (SGN-U320261) and *Pti4* (SGN-U317071), a tomato *ERF* gene, were also significantly up-regulated in the transgenic lines (Figure 11B and 11C) indicating that down-regulation of Sl-*IAA3* altered the expression of specific auxin and ethylene transcriptional mediators.

DISCUSSION

Aux/IAA proteins are critical components of the auxin response. In Arabidopsis, dominant gain-of-function mutations in individual *Aux/IAAs* have provided telling insights into the roles played by the various family members in eliciting specific auxin responses. We show here that Sl-IAA3, a tomato Aux/IAA, is an integral component of both auxin and ethylene response pathways. Indeed, transcripts for the gene accumulate in response to both of the hormones and its down-regulation results in auxin- and ethylene-related phenotypes. Phenotypic responses to Sl-*IAA3* down-regulation include alterations to the classical auxin regulated processes of apical dominance and hypocotyl elongation, and to the typical ethylene responses such as apical hook formation in etiolated seedlings and leaf epinasty in light-grown plants.

Sl-*IAA3* and a number of other partial tomato *Aux/IAA* clones were initially isolated from fruit tissues (Jones et al., 2002). The Sl-*IAA3* gene has strong sequence and structural similarities with its putative Arabidopsis orthologs, At-*IAA4* and At-*IAA3.* An Arabidopsis mutant for At-*IAA4* has an insertion in the first exon but shows no obvious growth phenotype (Overvoorde et al., 2005). In fact, although loss-of-function mutations have been identified in Arabidopsis for several *Aux/IAA* genes, the only phenotypes reported are subtle changes in the

shy2 mutant (Tian and Reed, 1999). Interestingly, the expression of At-*IAA3,* one of the putative orthologs of tomato Sl-*IAA3,* was shown to be altered in *Shy2* mutant. Double or triple mutants of closely related *Aux/IAA* genes, such as *iaa8-1/iaa9-1* or *iaa5-1/iaa6-1/iaa19-1* also exhibit WT phenotypes, indicating extensive functional redundancy among Arabidopsis Aux/IAA family members (Overvoorde et al., 2005). Arabidopsis gain-of-function *Aux/IAA* mutants result from genetic changes that lead to alterations in amino acids in the highly conserved domain II. This stabilizes the proteins and results in a variety of gene-specific auxin-related phenotypes (Reed, 2001). We have previously shown that down-regulation of a tomato *Aux/IAA* gene, Sl-*IAA9*, resulted in pleiotropic phenotypes including altered leaf architecture and parthenocarpic fruit, consistent with a pivotal role for auxin in tomato fruit set and leaf morphogenesis (Wang et al., 2005). In the present study we show that the down-regulation of Sl-*IAA3* (*AS-IAA3*) also leads to well defined phenotypes in transgenic tomato lines. We were able to rule out that the observed changes might result from a lack of specificity of our antisense strategy by verifying that the expression of closely related *Aux/IAA* genes was not altered in the *AS-IAA3* transgenic lines. These data strongly support the hypothesis that different members of the Aux/IAA family are involved in distinctive developmental processes.

Sl-*IAA3* Mediates Auxin-Dependent Gene Transcription and Auxin-Associated Phenotypes

Aux/IAA genes were originally identified based on their rapid induction by auxin in etiolated soybean (*Glycine max*) and pea (*Pisum sativum*) tissues (Walker and Key, 1982; Theologis et al., 1985). Many Arabidopsis auxin responsive genes contain the canonical auxin response elements (*AuxRE*), TGTCTC or GAGACA in their promoters (Guilfoyle and Hagen, 2007). Our *in silico* search led to the identification of two degenerate *AuxRE* elements in the Sl-*IAA3* promoter that may be responsible for the auxin responsiveness observed in this study (Figure 1 and Figure 3).

Sl-*IAA3* transcript levels varied dramatically among the different tomato tissues. The highest and lowest levels were found in the fruit and roots, respectively. Analyses of tomato lines expressing the Sl-*IAA3* promoter fused to the GUS

reporter gene revealed that basal levels of expression were spatially restricted within organs. In the root, Sl-*IAA3*-driven *GUS* expression was restricted to the root cap and lateral root meristems. In the leaves it was restricted to the vasculature, and in the fruit in a narrow band defining the junction between placenta and pericarp. Interestingly, the precise tissue-specific expression patterns were abolished by exogenous auxin. Auxin treatment led to GUS staining throughout the whole pericarp in fruit and to all parts of the leaves and roots. These auxin responsive expression patterns are in agreement with previous data (Jones et al., 2002) but differed considerably from those seen with the artificial auxin-responsive promoter, *DR5* (Figure 4). This suggests that a combination of promoter elements, including the ethylene-responsive element (ERE), contribute to the maintenance of the precise tissue-specific pattern of Sl-*IAA3* expression.

In most cases in Arabidopsis, *Aux/IAA* gain-of-function mutations are associated with phenotypes reminiscent of reduced auxin responsiveness (Tian et al., 2002; Nagpal et al., 2000; Rogg et al., 2001). Arabidopsis Aux/IAAs have been shown to repress the *DR5*-driven transcription (Ulmasov et al., 1997; Tiwari et al., 2001). This repression is thought to occur via interactions between the Aux/IAA proteins and their DNA-binding ARF partners (Guilfoyle and Hagen, 2007). As we were able to show that Sl-IAA3 also has the capacity to repress the activity of *DR5 in vivo,* we hypothesized that the down-regulation of Sl-*IAA3* would reduce the level of auxin-responsive gene repression and ultimately lead to enhanced auxin responses. Unexpectedly, the *AS-IAA3* lines have many phenotypes consistent with a reduced auxin sensitivity (Figure 8). This suggests that, even though *in vivo* assays showed that Sl-IAA3 has the capacity to repress auxin-responsive gene expression, *in planta* the protein seems to act as a positive regulator of auxin responses. One possible explanation for this apparent discrepancy is that *in planta*, Sl-IAA3 may repress the expression of some negative regulators of auxin responses. Two *ARFs* (*ARF2* and *ARF8*) and one *Aux/IAA* (*IAA29*) that were differentially regulated in the *AS-IAA3* lines, may contribute to the reduced auxin-responsiveness.

Ethylene Related Expression and Phenotypes

We have previously shown that the accumulation of Sl-*IAA3* transcripts is enhanced by ethylene treatment in mature green fruit (Jones et al., 2002). In this work, we further characterized the dynamics of the ethylene responsiveness of the gene. A time-course analysis of *Sl-IAA3* transcript accumulation in response to ethylene revealed a similar pattern to that shown by the well-characterized ethylene-responsive gene, *E8* (Lincoln et al., 1987). The highest levels of Sl-*IAA3* transcripts in tomato plants were found in the fruit at the orange/red stages of ripening. In tomato, autocatalytic ethylene production leads to high levels of endogenous ethylene during fruit ripening (Lelievre et al., 1997). In breaker stage fruit, when autocatalytic ethylene is already actively driving the ripening process, the ethylene inhibitor, 1-MCP, sharply reduced Sl-*IAA3* transcript levels. There was also a dramatic reduction in Sl-*IAA3* transcript levels in the tomato mutants *rin*, *nor* and *Nr* that lack the capacity to respond to autocatalytic ethylene and to undergo normal ethylene-regulated ripening processes (Figure 2). Together, these data strongly suggest that this presumptive auxin response element is ethylene-responsive and integral to ethylene-regulated fruit ripening. In untreated fruit, the Sl-*IAA3* promoter drove GUS expression strictly at the boundary between the placenta and pericarp tissues. These observations suggested that down-regulation of Sl-*IAA3* in transgenic lines may have resulted in a fruit ripening phenotype. Nevertheless, no changes were observed in the fruit ripening of antisense lines including the timing of the onset, levels of climacteric ethylene production and pigment accumulation examined in our study. Though we cannot exclude the possibility that other ripening aspects may have been altered, our data suggest that either there is some functional redundancy or that residual levels of Sl-IAA3 were sufficient to drive the fruit processes that rely on the protein. The data do, however, indicate that Sl-IAA3 lies at the crossroads of the auxin and ethylene responses during tomato fruit ripening. Trainotti et al. (2007) have recently demonstrated a network of interactions between auxin and ethylene during ripening in peaches. Our data show that modification of the auxin response is one of the suite of ethylene driven processes that together constitute climacteric fruit ripening.

Two other phenotypes in the *AS-IAA3* lines, the exaggerated apical hook formation and reduced epinasty, indicated that Sl-IAA3 is involved in physiological responses to ethylene. An exaggerated apical hook is formed in etiolated

seedlings in response to exogenous ethylene. This process forms the classical ethylene triple response together with reduced hypocotyl and root elongation (Bleecker et al., 1988; Ecker, 1995). An apical hook is formed during early seedling growth to protect the fragile shoot meristem from damage as the seedling grows through the soil (Darwin and Darwin, 1896). A complex pattern of coordinated cell elongation is needed to establish and maintain the apical hook structure (Silk and Erickson, 1978). The involvement of both ethylene and auxin in this differential cell elongation has been demonstrated through the analysis of ethylene and auxin signalling mutants that are altered in the process of hook formation (Lehman et al., 1996; Li et al., 2004). In Arabidopsis, mutants that are defective in ethylene perception and signalling, such as *etr1-1, ein2, ein3*, do not form an exaggerated hook in response to ethylene treatment. By contrast, the constitutive ethylene response mutant, *ctr1*, develops an exaggerated hook in the absence of ethylene (Guzman and Ecker, 1990; Kieber et al., 1993). Auxin promotes hypocotyl cell elongation and is unequally distributed in the apical hook (Schwark and Schierle, 1992)**.** The *axr1* mutant, which is altered in auxin responses, lacks a normal apical hook and the inhibition of auxin transport disrupts formation of the hook (Lincoln et al., 1990). Clearly, the apical hook is established and maintained by interplay between ethylene and auxin. The exaggerated apical hook phenotype in the *AS-IAA3* lines provides direct evidence that Sl-IAA3 is important in physiological processes that rely on both auxin and ethylene. Active ethylene signalling is essential for the appearance of the exaggerated hook phenotype since blocking ethylene perception with 1-MCP prevents hook formation in the *AS-IAA3* plants. Noteworthy, apart from the exaggerated hook, other aspects of the triple response like hypocotyl elongation and thickening and root elongation were not altered in *AS-IAA3* lines indicating that Sl-IAA3 is rather involved in differential growth processes. Ethylene treatment of etiolated seedlings increased the Pro*IAA3:GUS* expression in the inner surface of the apical hook (Figure 9). Likewise, Pro*IAA3:GUS* staining was also clearly delimited in epinastic petioles suggesting that the ethylene-induced gradient of Sl-*IAA3* expression is involved in the differential growth associated with both apical hook formation and the petiole epinastic response. Whereas, down-regulation of Sl-*IAA3* resulted in an exaggerated ethylene-response of etiolated seedlings, it conferred reduced ethylene sensitivity in light-grown plants. The ability of

ethylene to induce opposite growth responses in the dark and in the light have been previously described (Smalle et al., 1997) and could explain the seemingly contradictory phenotypes displayed by *AS-IAA3* plants in the seedlings and petioles. In keeping with this complex regulation of Sl-*IAA3*, the ethylene-induced expression of this gene in light-grown plants was found in the upper side of epinastic petioles, opposite to the pattern observed in the hook of etiolated seedlings.

Arabidopsis plants with a loss-of-function mutation in *HLS1* are unable to form an apical hook even in the presence of ethylene (Lehman et al., 1996). A mutation that reverses the *hls1* phenotype has been identified and was found to encode the auxin response factor, *ARF2* (Li et al., 2004). Interestingly, the putative tomato ortholog of *ARF2* is down-regulated in the *AS-IAA3* lines suggesting that the process of hook formation may require an interplay between HLS1, IAA3 and ARF2. We isolated Sl-*HLS*, a tomato homolog of the Arabidopsis *HLS1* gene which was able to rescue the Arabidopsis *hls1* mutant, indicating that it encodes a functional HOOKLESS protein. In contrast to the Pro*IAA3:GUS,* Pro*HLS:GUS* expression was restricted to the outer side side of the hook (Figure 9). This suggests that Sl-IAA3 and Sl-HLS regulate cell elongation on opposite sides of the hook. Sl-*IAA3* and Sl-*HLS* genes provide therefore tissue-specific markers for the inner and outer sides of the apical hook, respectively, and the corresponding promoters could be useful to target the ectopic expression of transgenes to a specific side of the hook. The previous model proposed by Li et al. (2004) postulates that ARF2 acts downstream of HLS1. Real-time RT-PCR data indicated that the expression of Sl-*HLS* is not altered in the *AS-IAA3* plants suggesting that Sl-IAA3 and Sl-HLS may act in parallel pathways both of them involving ARF2 as a downstream component. On the other hand, we cannot ruled out that Sl-*HLS* may act upstream of Sl-*IAA3* to down-regulate its expression which might explain why Sl-*IAA3* is not expressed in the upper side of the hook where the expression of Sl-*HLS* is high.

The altered apical dominance found in the *AS-IAA3* lines was also observed in the antisense Sl-*IAA9* plants (Wang et al., 2005). Unlike Sl-IAA9, however, Sl-IAA3 has distinct roles in ethylene-related responses. By revealing that a number of transcription factors from the ARF (Sl-*ARF2* and Sl-*ARF8*), Aux/IAA (Sl-*IAA29*) and ERF (*Ethylene Response Factor Pti4*) families are regulated by Sl-IAA3, our

study provides important clues into how Sl-IAA3 functions to bring about some of the observed phenotypes. While continued effort is required to gain a more complete understanding of the hormonal dialogue mediated by Sl-IAA3, the data described here confirm that Aux/IAA proteins have both distinct and overlapping roles and reveal that these proteins can be integral auxin as well as ethylene response elements.

METHODS

Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum* cv MicroTom) plants were grown under standard greenhouse conditions. The conditions for the culture chamber room are as follows: 14-h-day/10-h-night cycle, 25/20°C day/night temperature, 80% hygrometry, 250 µmol $m^{-2}s^{-1}$ intense luminosity. Seeds were first sterilized, rinsed in sterile water and then sown in recipient Magenta vessels containing 50 mL of 50% Murashige and Skoog (MS) culture medium added with R3 vitamin (0.5 mg L^{-1} thiamine, 0.25 mg L^{-1} nicotinic acid and 0.5 mg L^{-1}pyridoxine), 1.5% (w/v) sucrose and 0.8% (w/v) agar, pH 5.9.

Plant Transformation

To generate *AS-IAA3* transgenic plants, the forward 5'-AACAA GACTCAGCTCCTGCACC-3' and reverse 5'-CATCACCAACAAGCATCCAATC-3' primers were used to amplify a partial Sl-*IAA3* clone (Figure 1). This 297 bp fragment was then cloned into the pGA643 binary vector in the antisense orientation under the transcriptional control of the cauliflower mosaic virus 35S (35S CaMV) promoter and the nopaline synthase (Nos) terminator. Transgenic plants were generated by *Agrobacterium tumefaciens*–mediated transformation according to Jones et al. (2002) and transformed lines were selected as in Wang et al. (2005). All experiments were carried out using homozygous lines from F3 or later generations.

Isolation of the Sl-*IAA3* Genomic Clone

Genomic DNA was extracted from tomato leaf tissue using a DNA extraction Kit (Promega, Lyon, France) and was treated with RNase for 10 min at 37℃. PCR reactions were performed on genomic DNA using primers designed from the cDNA sequence. The amplified fragments were cloned and fully sequenced. Comparative analysis between the genomic clone and cDNA sequences allowed the delimitation of introns and exons.

Isolation of the Sl-*IAA3* Promoter

The Universal Genome Walker Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) was used to isolate the Sl-*IAA3* gene promoter region. Each tomato genomic DNA aliquot was digested with four restriction enzymes, DraI, EcoRV, PvuII and StuI. Adaptor DNA which contained two primer-binding sites for AP1 and AP2 primers provided by the Genome Walker Kit was linked to both ends of the restricted tomato DNA fragment at 16℃. Two pri mers, AP1 (5'-GTAATACGACTCACTATAGGGC-3') and AP2 (5'-ACTATAGGGCACG CGTGGT-3') paired with two Sl-*IAA3* gene specific antisense primers were used for PCR amplification. The tomato genomic DNA fragment with adaptors at both ends was used as a template for the amplification of the promoter region. The PCR product was cloned into the pGEMT-easy vector (Promega) and fully sequenced. The Sl-*IAA3* promoter was then fused to the *GUS* reporter gene in the plp100 binary vector (Szabados et al., 1995) and used for stable tomato transformation. DNA sequences were analyzed with BLAST network services at the National Center for Biotechnology Information (Altschul et al., 1997) and by PlantCARE (Lescot et al., 2002).

Transient Expression Using a Single Cell System

For nuclear localization of the Sl-IAA3 fusion protein, the coding sequence of Sl-*IAA3* was cloned as a C-terminal fusion in frame with GFP (Green Fluorescent Protein) into the pGreen vector (Hellens et al., 2000) and expressed under the control of the 35S CaMV, a Cauliflower Mosaic Virus promoter. Protoplasts for

transfection were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells according to the method described previously (Leclercq et al., 2005). Protoplasts were transfected by a modified polyethylene glycol method as described by Abel and Theologis (1994). Typically, 0.2 mL of protoplast suspension (0.5×10^6) was transfected with 50 µg of sheared salmon sperm carrier DNA and 10 µg of either 35S-IAA3:GFP or 35S:GFP (control) plasmid DNA. Transfected protoplasts were incubated 16 h at 25°C and analyzed for GFP fluorescence by confocal microscopy. Light micrographs and fluorescence images are merged to illustrate the different location of the two proteins.

For co-transfection assays, the coding sequence of Sl-*IAA3* was cloned into the pGreen vector and expressed under the control of the 35S CaMV promoter. Aliquots of protoplasts (0.5×10^6) were transformed either with 10 µg of the reporter vector alone containing the *DR5* synthetic auxin-response element fused to the *GFP* reporter gene (gift from Prof. K. Palme, Freiburg, Germany) or in combination with 10 µg of the effector plasmid, allowing the constitutive expression of the Sl-IAA3 protein. Transformation assays were performed in three independent replicates. After 16 h incubation in the presence or absence of 2,4-D (50 µM), GFP expression was analyzed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA). For each sample, 100 to 1000 protoplasts were gated on forward light scatter and the GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the population of cells above the background threshold (set arbitrarily based on a zero DNA transformed control, so that all control cells fall below this threshold). Data were analyzed using Cell Quest software. All transient expression assays were repeated at least three times with similar results.

Auxin and Ethylene Treatment

For auxin dose–response experiments, 8 mm long hypocotyl segments were cut from three-week-old light grown plantlets and were immediately floated in sucrose/MES buffer (1% sucrose [w/v] and 5 mM MES/KOH, pH 6.0). After 2 h pre-incubation, the hypocotyl segments were randomly distributed to fresh buffer solutions with or without NAA (0, 1, 10, 100 µM) and were measured following 2 h of incubation. For NPA treatment, the seeds were sown on MS medium

containing 1 µM NPA and the phenotypes affecting root and leaf growth were observed on young 19-day-old seedlings. For qRT-PCR expression studies, 21-day-old tomato seedlings were harvested and treated with auxin (20 µM IAA for two hours) in presence or absence of 1-MCP (Agrofresh, USA), the ethylene perception inhibitor (1 µL L^{-1}) applied 16h prior to auxin treatment. The tissues were then immediately frozen in liquid nitrogen and stored at -80℃ until RNA extraction. For GUS analysis, 21-day-old tomato seedlings and sections of mature green fruit (Vibratom, Leica VT 1000 S, Vetzlar, Germany) were incubated for 2 h in 50% MS buffer with or without 20 µM IAA. Tissues were then immediately incubated on GUS staining buffer. Ethylene treatments were performed for 5 h in sealed glass boxes. Tomato fruit at the mature green (MG) stage were treated with 50 µL L^{-1} ethylene and control fruits were exposed to air alone. 1-MCP (1 µL L^{-1}) was applied to fruit at breaker stage (Br) for 16 h at room temperature. Following treatment, the tissues were immediately frozen in liquid nitrogen and stored at -80℃ until RNA extraction. Ethylene treatment (10 µL L^{-1}) was also performed on 5-day-old etiolated Pro*IAA3:GUS* transformed seedlings. Non-treated seedlings grown under the same conditions as for the control. The samples were immediately incubated on GUS buffer. Ethylene treatment of light grown plants was performed by sealing the WT and *AS-IAA3* plants in airtight chambers and injecting ethylene to a final concentration of 50 µL L^{-1} for 16 h.

Histochemical GUS Analysis

Transgenic lines expressing the *GUS* reporter gene under the control of either the Sl-*IAA3* promoter (Pro*IAA3:GUS*), the Sl-*HLS* promoter (Pro*HLS:GUS*) or the *DR5* synthetic promoter (*DR5:GUS*) were incubated at 37℃ for 5 to 15 hours with *GUS* staining solution containing 100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Triton and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid to reveal GUS activity. Following GUS staining, samples were washed several times with a graded ethanol series to extract chlorophyll.

Assessment of Apical Hook Curvature

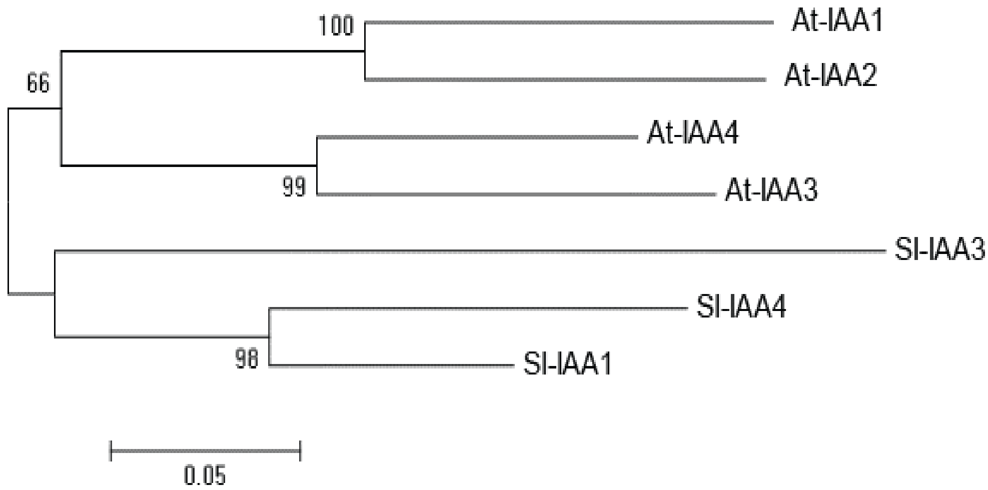
Sterilized seeds were placed on MS agar medium plates and left in the dark for 2 days at 4℃. The vernalized seeds were then placed at 25℃. The level of apical curvature was assessed on 5-day-old dark-grown seedlings using a scale ranging from stage 0, corresponding to total absence of hook, to stage 7 corresponding to maximal exaggerated hook.

RNA Extraction and Quantitative RT-PCR

RNAs were extracted from various tomato tissues according to Zegzouti et al. (1999). DNase-treated RNA (2 µg) was then reverse-transcribed in a total volume of 20 µl using the Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Quantitative Real-Time PCR was performed using cDNAs corresponding to 2.5 ng of total RNA in a 10 µl reaction volume using the SYBR Green PCR Master Mix (PE-Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900HT sequence detection system. PRIMER EXPRESS software (PE-Applied Biosystems) was used to design gene-specific primers. The primer sequences are listed in supplemental Table 2. *Actin* was used as a reference gene with constitutive expression in various tissues. Real-Time PCR conditions were as follow: 50℃ for 2 min, 95℃ for 10 min, then 40 cycles of 95℃ for 15 s and 60℃ for 1 min and finally one cycle at 95℃ for 15 s and 60℃ for 15 s. For all Real-Time RT-PCR experiments, two biological replicates were made and each reaction was run in triplicate. For each sample, a Ct (threshold constant) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the Sl-*Actin-51* as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct value for Sl-*IAA3* gene was normalized to the Ct value for Sl-*Actin-51* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.

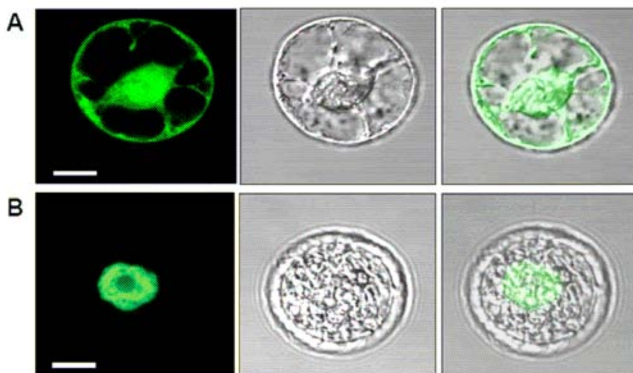
Supplemental data

Supplemental Figure 1.



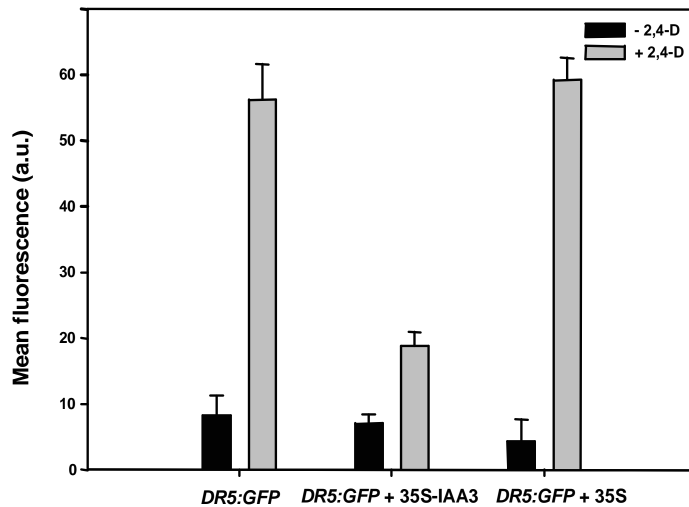
Supplemental Figure 1. Evolutionary Relationship of Sl-IAA3 and the Most Closely Related Tomato and Arabidopsis Aux/IAA Proteins. *The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.02850951 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 156 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al, 2007).*

Supplemental Figure 2.



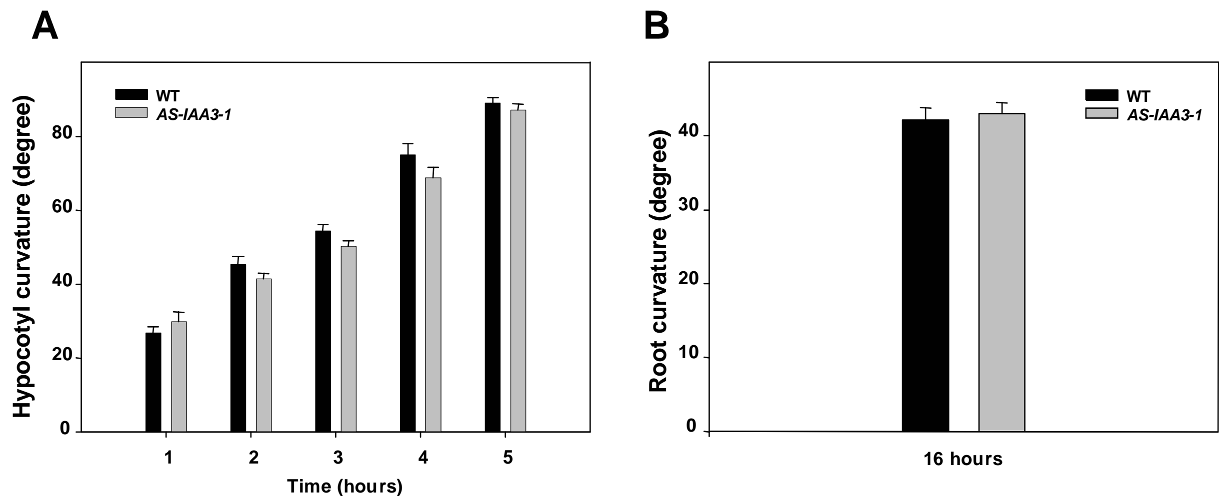
Supplemental Figure 2. Subcellular Localization of Sl-IAA3 Protein. *Sl-IAA3:GFP fusion protein was transiently expressed in BY-2 tobacco protoplasts and sub-cellular localization was analyzed by confocal laser scanning microscopy. The merged pictures of the green fluorescence channel (left panels) and the corresponding bright field (middle panels) are shown (right panels).* ***A.*** *Control cells expressing GFP alone.* ***B.*** *Cells expressing the Sl-IAA3:GFP fusion protein. The scale bar indicates 10 µm.*

Supplemental Figure 3.



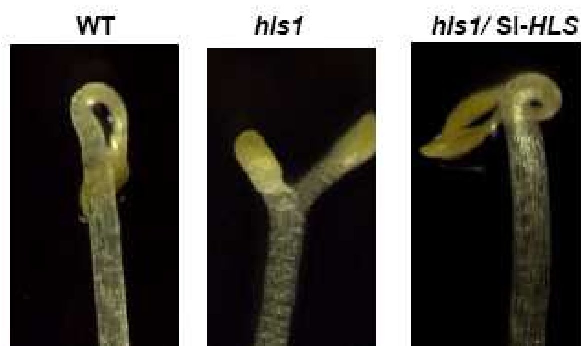
Supplemental Figure 3. Sl-IAA3 Protein Represses the In Vivo Activity of DR5. *Tobacco protoplasts were transformed either with the reporter construct (DR5:GFP) alone or with both the reporter and effector constructs (35S-IAA3) and incubated in the presence or absence of 50 µM 2,4-D. GFP fluorescence was measured 16 h after transfection. A mock effector construct lacking Sl-IAA3 was used as a control for the co-transfection experiments. Transformations were performed in triplicate. Mean fluorescence is indicated in arbitrary unit (a.u.) ± SE.*

Supplemental Figure 4



Supplemental Figure 4. Gravitropic response of hypocotyls and roots in wild type and antisense plants. *Seedlings grown for 4 days in darkness on vertically held plates were turned 90°, and the angle of the hypocotyls (**A**) and roots (**B**) curvature was measured at the indicated time. Values shown represent the mean ± SE of two independent experiments, in which 20 seedlings was measured.*

Supplemental Figure 5.



Supplemental Figure 5. Reversion of the Arabidopsis hls1 Mutant Phenotypes by Complementation with the Sl-HLS tomato hookless gene. *Ectopic expresssion of Sl-HLS in hls1 mutant restores the normal hook formation in 3-day-old etiolated seedlings treated with 1µL L^{-1}ethylene. Right panel: wild type (WT); middle panel: hookless mutant (hls1); left panel: hookless mutant expressing the tomato Sl-HLS gene (hls1/Sl-HLS).*

Supplemental tables

Table 1. Percentage Identity of the Antisense Region Relative to the other Members of Tomato *Aux/IAAs* Family.

Target genes	% of identity
Sl-*AA3*	100
SGN-U323670	79
SGN-U316052	74
SGN-U323974	67
SGN-U318434	65
SGN-U317606	65
SGN-U332300	64
SGN-U330168	64
SGN-U322175	64
SGN-U318191	63
SGN-U313802	63
SGN-U320280	62
SGN-U322787	59
SGN-U320412	56
SGN-U322644	56
SGN-U322499	54
SGN-U320261	54

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The Hookless Gene
A Key Developmental Actor and a Putative Integrator of Hormones and Light Signaling?

CHAPITRE III

HOOKLESS1, an Integrator of Multiple Signaling Pathways, Involved in Glucose and De-etiolation Toxicity/Sensitivity in Arabidopsis

(Manuscript in preparation)

Title: *HOOKLESS1*, an Integrator of Multiple Signaling Pathways, Involved in Glucose and De-etiolation Toxicity/Sensitivity

Authors: Salma Chaabouni, Alain Latche, Jean-Claude Pech, and Mondher Bouzayen

ABSTRACT

Plants need to adjust most of their physiological and developmental processes to constantly changing environmental conditions. With only a handful of plant hormones known, the large array of responses is probably achieved by a combinatorial mechanism of interactions between hormones and other signals. The extention of the hypocotyl has proved to be an excellent system for studing such signal interplay in the regulation of growth and developmental responses. The Arabidopsis *hookless* (*HLS*) gene wich is essential for apical hook formation, is regulated by a network of interacting factors including light and plant hormones. Herein, we report the discovery of new phenotypes associated with *hls1* mutation. We describe a marked bleaching phenotype in *hls1* mutant that had first been germinated in the dark before transfer to white light. The *hls1* mutation also confers an enhanced tolerance to glucose and to elevated ABA concentrations. Moreover, *hls1* seedlings display agravitropic growth in the dark but not in the light. These results indicate that the *HLS1* gene is in the cross-road of multiple signalling pathways including ethylene, auxin, ABA, light and sugar.

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Introduction

During germination of dicotyledonous plants, the shoot emerges from the seed with a hook-shaped structure that protects the apical meristem and first leaves as the seedling pushes through the soil. This apical hook is maintained until the seedling reaches the light, at which time, the hypocotyl straightens. The apical hook is formed by differential cell elongation on opposite sides of the hypocotyl. The growth rate in the outer (convex) side of the hypocotyls exceeds that of the inner (concave) side resulting in hypocotyl bending (Silk and Erikson., 1978). The formation and maintenance of apical hook has been studied extensively and it was suggested that the apical hook establishment is a developmental process driven by multiple hormone cross-talk where ethylene seems to play prominent role. Alterations in the response of dark-grown seedlings to ethylene, the so-called "triple response" were used to isolate a collection of ethylene-related mutants in Arabidopsis thaliana (Guzman and Ecker, 1990). Arabidopsis mutants that are defective in ethylene perception, e.g., *etr1-1, ein2, ein3*, etc., are not able to form a hook in response to ethylene treatment (Roman et al., 1995), whereas the *Constitutive Ethylene Response* mutant *ctr1* develops exaggerated hook even in the absence of ethylene (Guzman and Ecker, 1990; Kieber et al., 1993). Moreover, exogenous ethylene exaggerates the apical hook (Lehman et al., 1996), confirming that the process of hook formation is at least partially dependent on ethylene. Mutants that failed to display the apical hook in the absence of ethylene, *HOOKLESS* (*his1*), exhibited reduced ethylene production. In the presence of exogenous ethylene, hypocotyl and root of etiolated his1-1 seedlings were inhibited in elongation but no apical hook was observed. HLS1 has been proposed to integrate ethylene and auxin signalling during apical hook formation of the Arabidopsis seedlings (Lehman et al., 1996). In addition, *hls1* mutation alters the spatial expression pattern of two primary auxin response genes (SAUR and Aux2-11/IAA4). In 2004, Li et al. pinpoint definitely HLS1 as a key integrator of auxin and ethylene pathway by identifying hookless suppressors as an Auxin Response Factor (ARF2).

On the other hand, auxin treatment or inhibition of auxin transport disrupts apical hook formation (Lehman et al., 1996). Consistent with this results, some

auxin mutants such as *axr1* (Lincoln et al., 1990) *hls3* (king et al., 1995) and *yucca* (Zhao et al., 2001) lack normal apical hook.

An additional level of complexity to the role of HLS1 was added when the requirement for gibberellins in hook maintenance and hook exaggeration upon ethylene treatment was demonstrated (Achard et al., 2003, Vriezen et al., 2004). The role of brassinosteroids (BRs) in hook development is longstanding, as BR mutants such as *cbb1*, *det2* and *cpd*, which are defective in the synthesis of BRs, are constitutively photomorphogenic and thus lack the characteristic hook (Chory et al., 1991, Kauschmann et al., 1996, Szekeres et al., 1996). More recently, it was shown that *HLS1* acts on the auxin-ethylene interaction rather than at the level of BRs (De Grauwe et al., 2005).

In addition to ethylene and auxin, light is another critical regulator of apical hook development. Opposite from apical hook formation and maintenance, light of various wavelengths causes rapid apical hook opening (Liscum and Hangarter, 1993; Rubenstein, 1971), which is part of the photomorphogenesis process. All constitutive photomorphogenic mutants (*cop/det/fus*) completely lack apical hooks (Chory and Peto, 1990; Hou et al., 1993; Kwok et al., 1996). Ethylene and light were shown to affect differential cell growth by modulating the auxin-response factor (ARF2) in a HLS1-dependent manner (Li et al., 2004).

Recently, Ohto et al. (2006) showed that *hls1* mutant is hypersensitive to sucrose in terms of the expression of sugar responsive genes in mature leaves. Furthemore, IAA repression of some sugar-induced gene expression was less pronounced in *hls1* than in the wild type, thus suggesting that the negative effect of auxin on sugar signalling may be mediated by HLS1.

We report here the discovery of new phenotypes associated with *hls1* mutation indicating that the *HLS1* gene is in the cross-road of multiple signalling pathways including ethylene, auxin, ABA, light and sugar.

RESULTS

hls1 Mutant Shows De-etiolated Phenotype

During the course of examination of *hls1* mutant, we found that it commonly shows an interesting phenotype related to greening upon light exposure of

etiolated seedlings (Figure 1). We then examined this phenotype more extensively and found that 100% of 4-day–old etiolated *hls1* seedlings fail to green even after being exposed 48 hours to white light which lead to the death of the plants and not a single individual can be rescued following this treatment (Figure 1A). In comparison, following the same treatment (4 days in the dark) 72% of WT seedlings open green cotyledons and survive (Figure 1B). Extending the etiolating period to 5 and 6 days reduces the proportion of plant survival to 50% and 26%, respectively. Hence, we conclude that beside its role in hook formation and maintenance, *HLS1* gene product is essential for cotyledon greening and subsequent leaf emergence upon white light treatment.

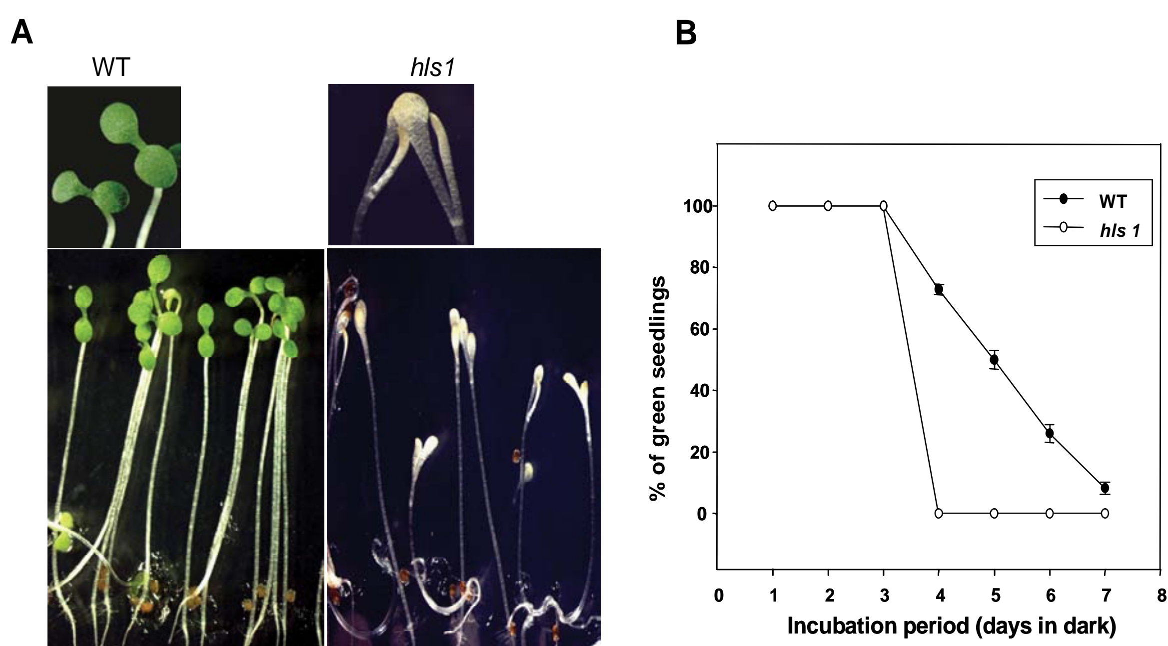

Figure 1: hls1 mutant displays de-etiolated phenotype. *Wild type and hls1 seeds were sown on plates containing MS medium and kept at 4℃ for 48h. The germinating seeds were kept in the dark at 25℃ for a varying number of days, then exposed to white light with a fluence rate (250 µmol.m-2.s-2) for 48 h.* ***A.*** *Wild-type and hookless1 mutant seedlings subjected to the greening experiment following 4 days in the dark.* ***B.*** *the result in panel A were quantitatively expressed by examinating a large number of seedlings (n>30). Each seedling was assessed as to whether it was green or still yellow.*

The *hls1* Mutation Confers Enhanced Tolerance to Glucose

Glucose has recently been shown to act as a regulatory molecule in higher plants. During the last years, the characterisation of glucose insensitive (*gin*) and glucose oversensitive (*glo*) mutants revealed extensive and ultimate connections

between glucose and plant hormone signalling pathway. Because ethylene has been shown to interfere with glucose sensitivity, we tested the glucose sensitivity of the *hls1* mutant. When grown in the presence of 6.5% of glucose, WT seedlings undergo complete arrest of their development leading to death, in contrast to *hls1* mutant which develop green and expanded cotyledons (Figure 2). Subsequently, the *hls1* mutant, but not the WT seedlings, develop true leaves and continue to grow in the presence of 6.5% of glucose. Insensitivity to glucose repression of cotyledon and shoot development in WT could be induced by exogenous ethylene treatment and likewise constitutive ethylene overproducers or constitutive ethylene response mutants display lower sensitivity to glucose (Zhou et al., 1998). Ethylene treatment prevents glucose toxicity in WT but also enhance tolerance to glucose of *hls1* mutant (Figure 2). In presence of 6.5 % glucose, ethylene-treated WT seedlings have small, curved dark green cotyledons (Figure 2E), while *hls1* seedlings grown in the same condition display shorter hypocotyls (3 times, data not shown), longer petioles (data not shown), more expanded and light green cotyledons (Figure 2I-F).These data suggest that

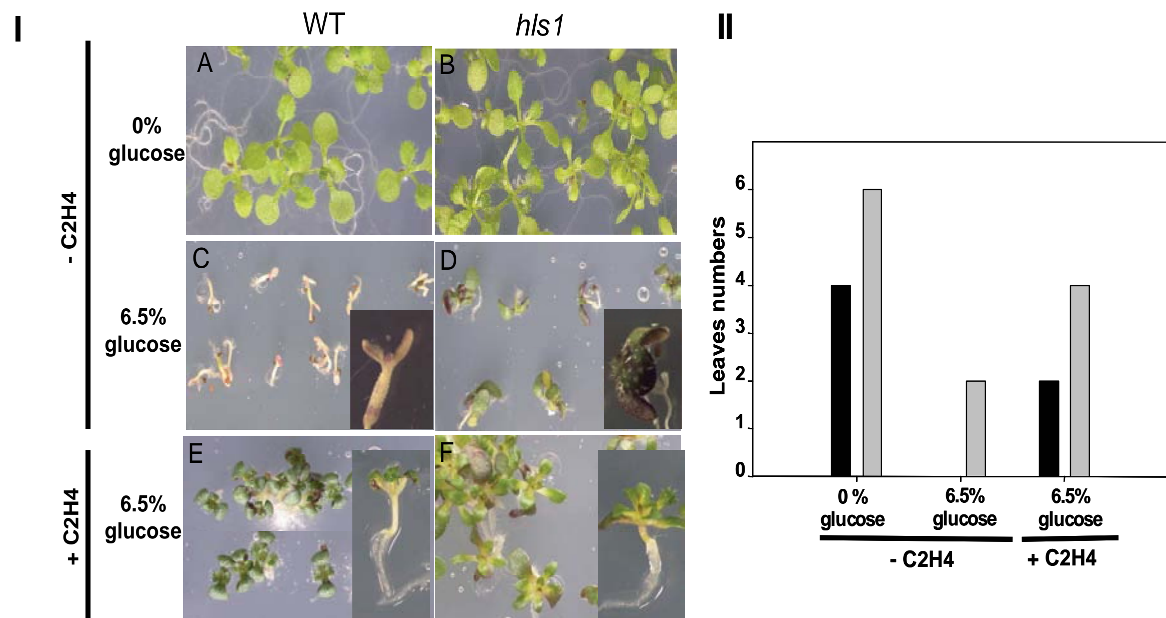

Figure 2. hls1 Mutation Enhances Tolerance to Glucose. I. *WT (**A**, **C**, **E**) and hls1 (**B**, **D**, **F**) seedlings were grown in the absence (**A**, **B**) or presence (**C**, **D**, **E**, **F**) of 6.5% glucose. Seedlings were then grown in air (**A**, **B**, **C** and **D**) or in the presence of 30 µL L^{-1} ethylene (**E** and **F**). **II.** The Number of green and expanded leaves was assessed in seedlings described in panel **I.** A large number of seedlings (n>50) were used and the data presented are representative of four replicates.*

hls1 mutation and ethylene have additive effect to overcome the glucose-induced developmental arrest.

hls1 Mutant is Tolerant to Elevated ABA Concentrations

The characterization of Arabidopsis *gin5 and gin 6* mutants uncovered that an increase in ABA levels is involved in the glucose signalling pathway leading to a decrease in gene expression and developmental arrest (Arenas-Huertero et al., 2006). We therefore tested whether ABA response is altered in *hls1* mutant using seed germination as ABA sensitivity test. When grown in presence of ABA (100 nM), germination of WT seeds is strongly inhibited while *hls1* mutant germinate successfully (Figure 3). However, at higher concentrations seed germination was inhibited in both WT and *hls1* etiolated seedlings indicating that *hls1* mutation enhances tolerance to ABA glucose whithout conferring resistance to this hormone. This result suggests that ABA may act trough *HLS1* in the glucose signalling pathway.

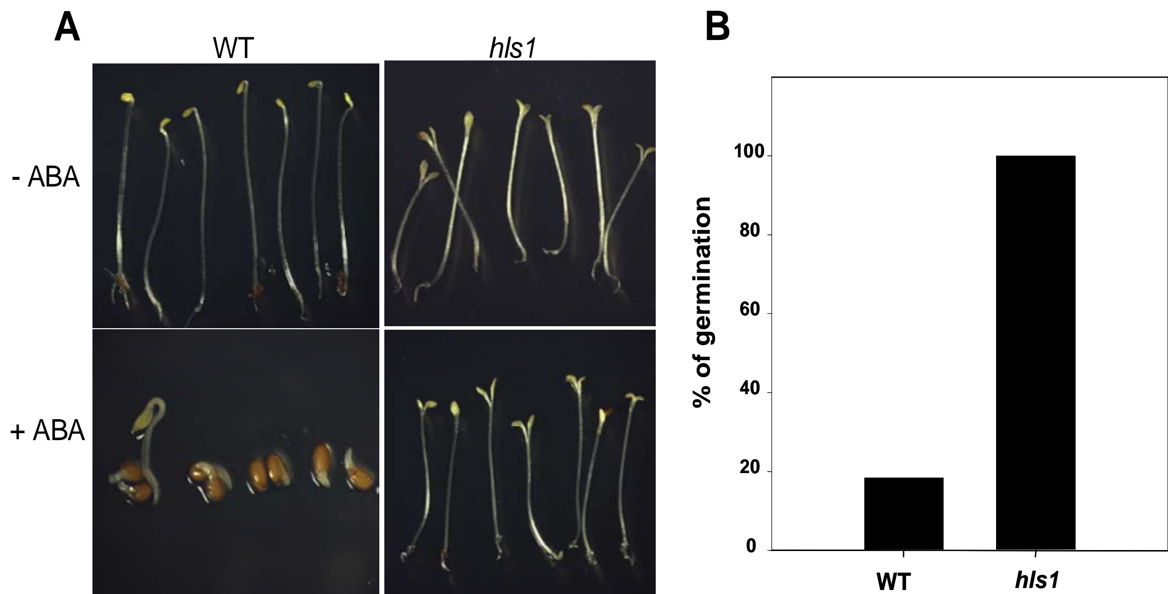

Figure 3. Arabidopsis hls1 Mutant Displays Higher Tolerance to ABA-Mediated Inhibition of Seed Germination. A. *Wild type and hls1 mutant seeds were kept 48h at 4℃ and then germinated in the dar k 5 days in darkness in the absence or presence of 100 nM ABA.* ***B.*** *The Number of germinated seed giving rise to seedlings was assessed. In each experiment a large number of seeds (n>50) was used and the data presented are representative of four independent replicates.*

hls1 Etiolated Seedlings Display Agravitropic Growth

Arabidopsis plants display typical gravitropism, with roots grown toward and shoots growing away from the gravity center. (Bullen et al., 1990; Evens, 1991; Okada and Shimura, 1992). Noteworthy, this gravitropic growth pattern is altered in etiolated *hls1* seedlings where both the roots and the shoots grew in all directions on agar plates (Figure 4). Interestingly, light grown *hls1* mutants display normal gravitropic growth indicating that *hls1* mutation alters gravitropism only in the absence of light.

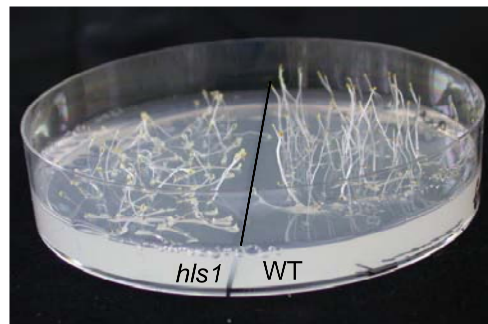

Figure 4. The Arabidopsis hls1 mutant shows altered gravitropic growth. *3-day-old seedlings of wild type (right side) and hls1 (left side) grown on MS media in the dark.*

DISCUSSION

The *HOOKLESS1* gene has been shown to be essential for cell elongation and differential growth in hypocotyl of dark grown Arabidopsis seedlings. Here, we describe new phenotypes associated with the *hls1* mutation that position the *HLS1* gene at the crossroads of multiple signalling controlling a multitude of devlopmental growth processes including light, gravitropic, glucose and hormone resposnes. The role of *HLS1* gene in integrating ethylene, auxin and light signalling has been first shown upon the identification of an auxin response factor, ARF2, as *HLS1* repressor that reverses many of the developmental phenotypes observed in *hls1* plants.

Upon reaching the soil surface, the seedlings undergo a marked developmental transition termed de-etiolation trigged by light, involving coordinate

inhibition of the hypocotyl elongation, unfolding of the hook, stimulation of cell expansion, activation of chloroplast development and chlorophyll accumulation. In preparation of this transition, seedlings accumulate a precursor of chlorophyll (protochlorophyllde) to permit rapid assembly of photosynthetic machinery. Accumulation of this intermediate in excess can result in photooxidative damage because light absorbed by these free molecules can be dissipated as reactive oxygen or free radicals (Reinbothe et al., 1996; Op den Camp et al., 2003). Therefore, coordinate regulation of the chlorophyll biosynthetic pathway and the capacity of enzymatic conversion of protochlorophyllde to chlorophyll is particularly critical during the deetiolation process. In this work, we described for the first time a marked bleaching phenotype in *hls1* mutant that had first been germinated and grown in the dark for several days before been transferred to white light. Uncontrolled over-accumulation of protochlorophyllde in etiolated seedlings was proposed as major cause of the failure to green in many *pif* (*photochrome interacting factor 1*) mutants (huq et al., 2004). This phenomenon may also operate in the case of *hls1* mutant where de-etiolation leads to seedlings death. We suggest that *HLS1* gene may contribute to the regulation of

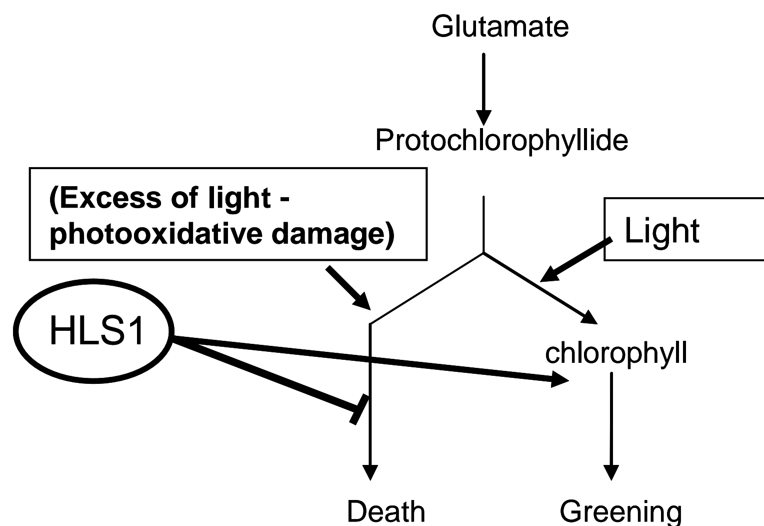

Figure 5: Proposed view of hypothetical linkage between regulation of chlorophyll synthesis and HLS1 involvement during early photomorphogenesis. The process of chlorophyll synthesis is depicted schematicaly and the hypothetical implication of *HLS1* gene in promoting greening or preventing cell death is shown.

chlorophyll biosynthesis or chloroplast development and hence prevent the accumulation of excess protochlorophyllde in prolonged darkness. Further experiments will be needed to test this hypothesis. This hypothesis is supported by the fact that *COP3*, an allelic mutant of *hls1* was shown to be involved in light-regulated seedling development mediated trough the phytochrome system (hou et al., 1993).

Interestingly, light signal regulation plant morphogenesis can be over ridden by metabolic signals such as the availability of glucose released from photosynthesis. For example, in Arabidopsis the availability of abundant glucose can be sensed during germination and can exert a profound influence resulting in seedlings developmental arrest (Jang and Shenn, 1997). Although the underlying mechanisms of this glucose-inducible developmental arrest are mostly unknown, a previous analysis of the *gin1* mutant has revealed an antagonistic role of ethylene on sugar signalling pathway (Zhou et al., 1998). Based on the physiological characterization of *hls1* mutants, we show in the present study that loss-of-function mutation of the *HLS1* gene prevents glucose repression of germination, cotyledon greening and expansion and true leaf development. These data suggest that HLS1 gene may act as positive regulator of glucose-induced repression of seedling growth in light conditions.

A central role of ABA in plant sugar signalling emerged from the analysis of Arabidopsis *gin5* and *gin6* mutants. It has been proposed that exogenous glucose increases the expression of ABA biosynthesis and signalling genes and that this glucose-specific accumulation of ABA appears to be essential for glucose signalling (Arenas-Huertero., et al., in 2000). Surprisingly, the addition of ABA in the medium of dark grown seedlings inhibit germination of wild type seeds but has not effect on *hls1*. This result suggests that *HLS1* gene participates glucose signalling downstream of the induction of ABA (Figure 6).

A molecular link between ethylene and glucose signalling has been established trough the analysis of EIN3 proteins (Yanagisawa et al in 2003).

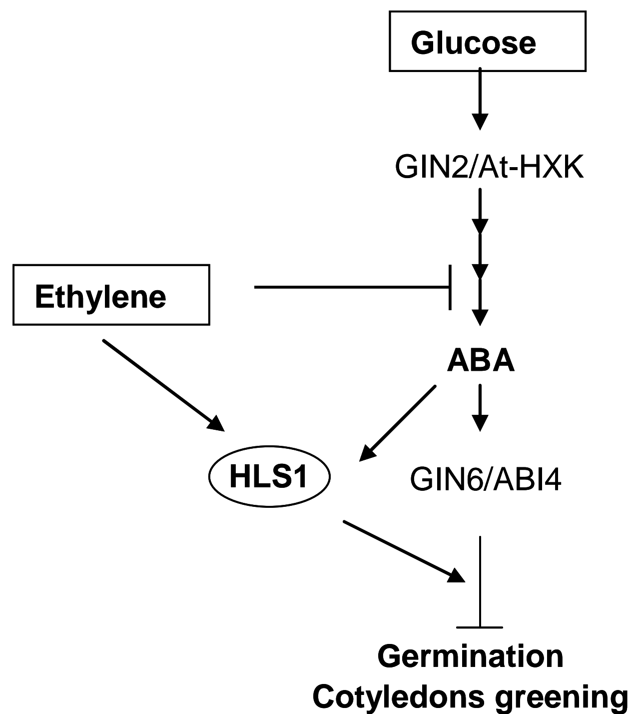

Figure 6. *A model for the role of HLS1 in enhancing glucose sensitivity compatible with ethylene being a repressor of glucose sensitivity*

Glucose enhances the the proteasome-mediated degradation of the nuclear EIN3 protein, while ethylene enhances its stability. Considering that HLS1 is homologue to diverse class of N-acetyltransferase, it is possible that HLS1 could be implicated in the regulation of protein stability of EIN3 (Ohto et al., 2006). Strikingly, expression analysis performed in this study indicate that *HLS1* gene is not regulated by glucose at the transcriptional level and that *gin1*, *ctr1*, *etr1* mutations do not affect the accumulation of *HLS1* transcript in presence or absence of glucose (data not shown). We conclude that HLS1 implication in the glucose signalling pathway may be at the post transcriptional level.

Taken together, these data clearly indicate that *HLS1* gene is an integrator of many signalling pathways including ethylene, light, glucose, as well as gravitropic response. Efforts are currently being directed towards uncovering the molecular mechanisms by which HLS1 impacts all these singling pathways.

METHODS

Plant Material

hls1-1 in the Columbia ecotype was obtained from the Nottingham Arabidopsis Stock Center. Arabidopsis (Columbia) was always used as control. Plants were grown under standard green house condition.

Greening Test

Sterilized seeds were put on Murashige and Skoog agar medium plates and placed at 4℃ for 2 days. After the seeds were kept at 25℃ in the dark for a number of days. They were then exposed to white light with a fluence rate ($\mu mol.m^{-2}.s^{-2}$) for 48 h. Each seedling was then assessed visually as to whether it was green or still yellow. The experiments were done independently more than 2 times.

Glucose Sensitivity

The glucose-resistant phenotype was scored by growing sterilized seeds on Murashige and Skoog agar medium containing 6.5 % (w/v) filter-sterilized Glc. After 2 days at 4℃, plates were placed in light in a sealed box containing either air or 10 $\mu L\ L^{-1}$ ethylene. Evry 2 days, the boxes were opened to allow a renewal of the atmosphere and put back either with air or 10 $\mu L\ L^{-1}$ ethylene. The experiment was stopped after 10 days of culture.

ABA Treatment

ABA (Lomon, bio) was prepared as a 10 mM stock in NAOH and diluted into appropriate concentrations (100 nM). ABA sensitivity tests were done in darkness using agar medium supplemented with 100 nM of ABA and scored after 5 days after sowing.

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CHAPITRE IV

Isolation and Functional Characterization of Tomato *Hookless* Genes by Complementation of Arabidopsis *hookless 1* mutant

(Manuscript in preparation)

Title: Isolation and Functional Characterization of Tomato *Hookless* Genes by Complementation of Arabidopsis *hookless 1* mutant

Authors : Salma Chaabouni, Alain Latche, Jean-Claude Pech, and Mondher Bouzayen

ABSTARCT

The apical hook of dark-grown dicotyledonous plants results from asymmetric growth of its inner and outer side of the upper part of the hypocotyl. This protective structure prevents damage of the shoot apical meristem and the young leaves as the seedlings pushes through the soil. *HOOKLESS (HLS1)* of Arabidopsis was recognised as an ethylene response gene whose product is required for hook formation. Tow hormones, ethylene and auxin are through to be involved in regulating apical hook formation. We cloned two cDNA from tomato, Sl-*HLS1* and Sl-*HLS2*, and showed that the encoded proteins are functional homologs to At-HLS1. Both Sl-*HLS1* and Sl-*HLS2* complement the *hls1* mutation. Ectopic ewpression of both tomato genes were able to restaure wild type phenotypes such as de-etiolation, enhanced tolerance to glucose and to elevated ABA concentrations and agravitropic growth. The genomic clones of Sl-*HLS1* and Sl-*HLS2* showed similar structure with two introns and three exons. While these data indicate complete functional redundancy between the two tomato HLs genes, only the expression of Sl-*HLS2* is enhanced by ethylene and auxin. The expression of Sl-*HLS2* gene, assessed by Sl-*HLS2* promoter-driven GUS revealed its up-regulation by ethylene restricted to the outer side of the apical hook. These data is in agreement with the important role of *HLS* gene in the integration of multiple signalling pathways.

INTRODUCTION

Nearly all processes of plant growth and development are regulated by more than one single phytohormone. The apical hook of dark-grown Arabidopsis seedlings has been studied extensively as a developmental process driven by multiple hormone cross talk. In Arabidopsis, the apical hook is formed 24h after germination and is maintained for about 4 days by a process of differential growth (Ecker, 1995; Raz and Ecker, 1999). It was suggested that in nature, the apical hook protects the shoot meristem when the germinating seedling protrudes trough the soil (Goeschl et al., 1967). Once the seedling emerges into the light, the apical hook opens, the leaves expand, and the photosynthetic apparatus differentiates. Physiological and genetic evidence shows that ethylene is involved in regulating closure and maintenance of the apical hook (kang and Ray, 1969b; Guzman and Ecker, 1990). Mutations in ethylene perception or signalling alter the normal hook formation or maintain. For example, the constitutive ethylene responsive mutant *ctr1* develops exaggerated hooks even in the absence of ethylene (Guzman and Ecker, 1990; Kieber et al., 1993).

Lehman et al., 1996 characterized hookless mutants in Arabidopsis and suggest a requirement for an asymmetric distribution of the auxin signal in the hook formation. Li et al., 2004 identified suppressor mutations of *hls1* that was allelic with loss-of-function mutations of the auxin response transcription gene, ARF2. These results showed that ethylene- and light-regulated differential cell elongation in the hypocotyls of dark-grown seedlings was maintained by regulation of ARF2 in a HLS1-dependant manner. Recently, we showed also that At-*HLS1* is an integrator of many cross-road including light, glucose and ABA signalling pathway (Ohto et al., 2006; chapitre III).

Here we report the cloning of two functional homologs of Arabidopsis *HLS1* from the tomato (Sl-*HLS1* and Sl-*HLS2*). We show their ability to complement all the phenotypes associated with the *hls1* mutation, and investigate the regulation of this two tomato hookless gene by auxin and ethylene in different tomato tissues.

RESULTS

Isolation and Structural Analysis of the tomato Sl-*HLS1* and Sl-*HLS 2* Genomic Clone

Two tomato EST (BE 434512; BG 734949), available in the database (www.tigr.org) showed significant homology to Arabidopsis *HLS1* gene. Extention by 5' and 3' RACE allowed the isoaltion of the full coding sequence indicating that the two EST correspond to the same gene. Further screening led to the isolation of another tomto homolog of the hookless gene that was not represented in the databases. Therefore, in contrast to Arabidopsis where hookless is encoded by a single gene, two different cDNA clones were isolated, amplified and sequenced from tomato leaves. These two genes, named Sl-*HLS1* and Sl-*HLS2,* contain an open reading frame of 1242 bp and 1254 bp, respectively, encoding putative proteins of 413 and 417 amino acids, respectively. The predicted Sl-HLS1 and Sl-HLS2 proteins share significant homology with HLS1 of Arabidopsis (66.7% and 67.6%, respectively) and similarly comprise a putative N-acetyltransferase domain. The Sl-*HLS1* and Sl-*HLS2* genomic clones have been subsequently obtained by PCR amplification using tomato genomic DNA as template.

At-*HLS1*

Sl-*HLS1*

Sl-*HLS2*

Figure 1. Genomic Structure of the Tomato Sl-HLS1 and Sl-HLS2 Genes*. Black portions represent the exons, white portions represent the introns and gray portions represent the untranslated region of the Arabidopsis At-HLS1genes and the tomato Sl-HLS1 and Sl-HLS2 genes.*

Comparison of genomic and cDNA sequences allowed the delineation of intron and exon positions. Both At-*HLS1* and Sl-*HLS1* and Sl-*HLS2* have 3 exons and 2 introns (Figure1) indicating a conserved structural organisation between the two

plant species. Moreover, the size of exons was perfectly conserved between the two species. The size of Sl-*HLS1* and Sl-*HLS2* introns are quite different from those of At-*HLS1* but the overall size of the tomato coding sequences is very close to that of At-*HLS1* (1212 bp). Such high conservation of genomic structure is indicative of conserved function.

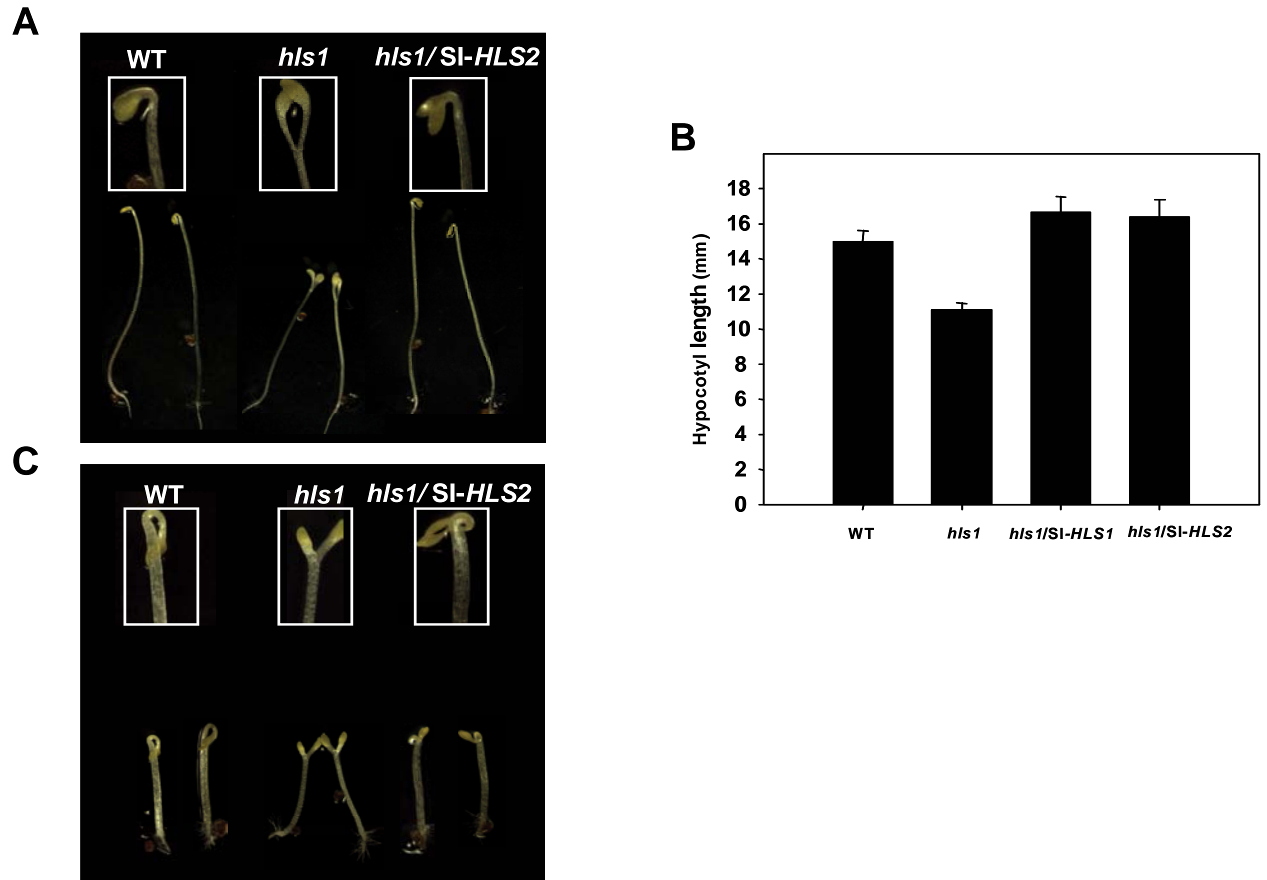

Figure 2. Recovery of Hook Formation via Complementation of Arabidopdis hls1 Mutant with Sl-HLS1 and Sl-HLS2. *Complemented lines display wild type apical hook in air-grown (**A**) and ethylene treated seedlings (**C**). Hypocotyl length of dark grown seedlings were measured at 4 days post-germination on air (**B**). The experiments were performed with homozygous transfomed lines and the data are mean ± SE of two independent replicates.*

Reversion of the Arabidopsis *hls1* Mutant Phenotypes by Complementation with Sl-HLS1 and Sl-HLS2

Expresssion of Sl-HLS1 and Sl-HLS2 in hls1 Mutant Restores Normal Hook Formation

To assess the functional significance of both tomato hookless genes, the arabidopsis *hls1* mutant was transformed with Sl-*HLS1* and Sl-*HLS2* in sens

orientation under the control of 35S cauliflower mosaic virus promoter. Among over twenty independent transgenic lines that display complemented phenotypes, three lines for each gene were used for further physiological analysis. In order not to increase the size of the figures the results corresponding to only one line are shown being representative of the two other lines. While no differential growth in the apical hook was observed in *hls1* mutant, 3-day-old dark-grown seedlings transformed with either Sl-*HLS1* or Sl-HLS2 develop an apical hook similar to that of wild type Arabidopsis plants both in absence or in presence of exogenous ethylene (Figure 2A, C). Since *hls1* seedlings showed a reduced elongation of the hypocotyls in darkness, hypocotyl length was assessed in WT and complemented etiolated seedlings. The data presented in Figure 2B indicate that the reduced hypocotyl elongation in *hls1* mutant is fully restored by ectopic expression of *Sl*-HLS1 and *Sl*-HLS2 tomato genes.

Recovery of Normal Greening Phenotype of hls1 Mutant Complemented with Sl-HLS1 and Sl-HLS2

We showed previously that *hls1* Arabidopsis seedlings that first and grown in the dark for several days and then transferred to white light undergo a marked bleaching leading to the plant death (Chapitre III). Complementation of *hls1*

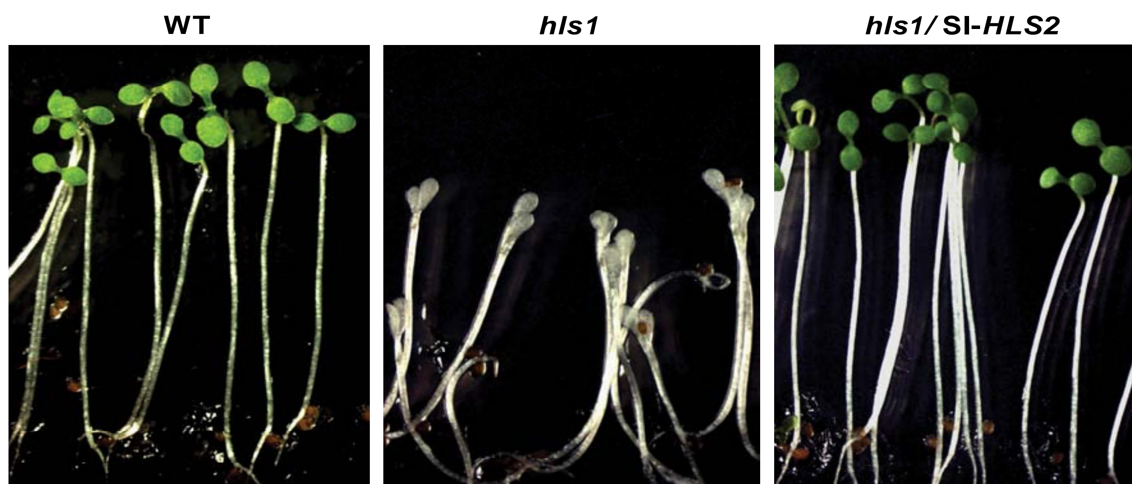

Figure3. Recovery of the Greening Phenotype in Sl-HLS1 and Sl-HLS2 Complemented Lines. *4-day-old etiolated seedlings of wild type (WT), hls1 mutant (hls1) and Sl-HLS2 complemented (hls1/Sl-HLS2) lines were kept 48 hours on white light with a fluence rate (250 µmol.m^{-2}.s^{-2}) on culture room.*

mutants with tomato Sl-*HLS1* and Sl-*HLS2* resulted in complete recovery of the greening phenotype and rescued the complemented lines from death (Figure 3). These indicated that beside their role in hook formation, and like At-*HLS1* gene, both tomato hookless genes are required in the greening upon white light treatment.

Sl-HLS1 and Sl-HLS2 Expression Restores Glucose Sensitivity to the hls1 Mutant

Wild-type seedlings of Arabidopsis undergo growth arrest when cultivated in light in the presence of 6% (w/v) glucose whereas *hls1* mutant is capable to grow in the presence of up to 6.5 % (w/v) glucose (Figure 4 A). Complementation of *hls1* with *Sl-HLS1* and *Sl-HLS2* resulted in recovery of glucose-induced growth

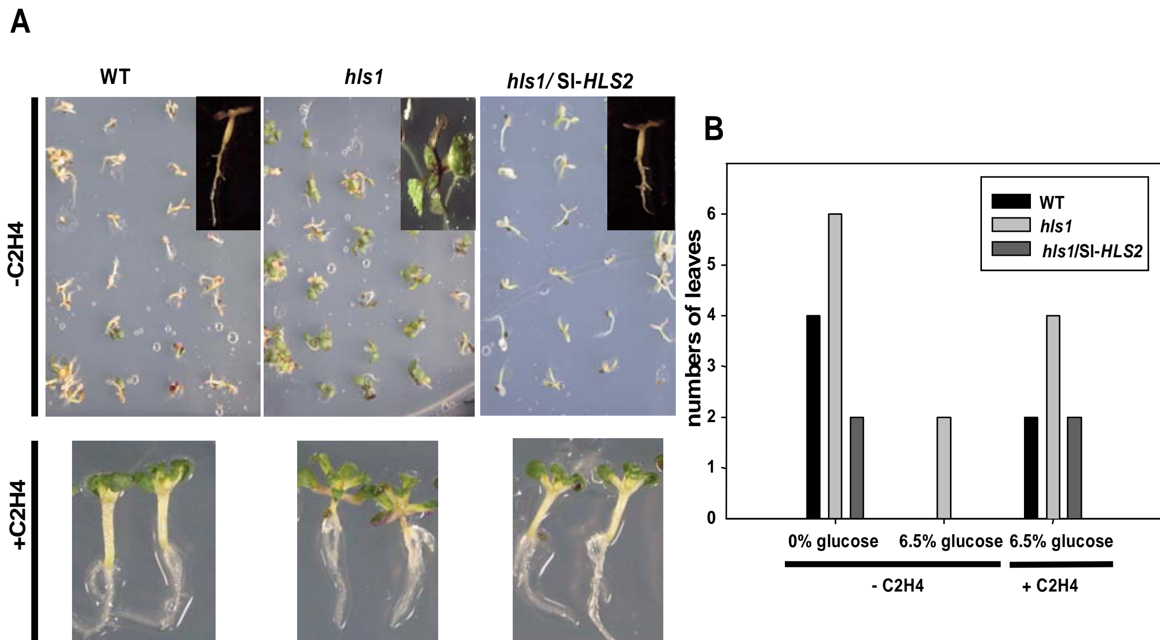

Figure 4. Glucose sensitivity of hls1 Mutant Complemented with Sl-HLS1 and Sl-HLS2*. *A. *Wild type, hls1 mutant and complemented lines were light-grown on MS medium containing 6.5% (w/v) glucose during 12 days in the presence of air or 10 µM of ethylene. For each line, 50 seedlings were grown and the data presented are representative of more than tree replicates.* ***B.*** *Leaf differenciation was assessed by counting the number of leaves emerging in each seedling lines.*

inhibition similar to that shown by the wild type (Figure 4 A). It is known that exogenous ethylene allows seedlings to overcome glucose-induced inhibition of growth. Accordingly, when seedlings were supplemented with 10 µL.L^{-1} ethylene,

all lines including wild-type control, escape glucose-induced growth inhibition (Figure 4A). Upon ethylene treatment, the complemented seedlings show long hypocotyls, small and curved dark green cotyledons similar to wild type seedlings. This is in contrast with *hookless* mutant that show short hypocothyls, expanded and light green cotyledons and longer petioles (Figure 4A). Assseesing leaf differenciation in 12-day-old plantlets indicated that in absence of glucose, *hls1* mutant bears higher number of developing leaves than wild type whereas in complemented lines there were twice less leaves than wild type (Figure 4B). Glucose treatment completely prevents leaf developemnt in wild type and complemented lines while it significantly reduced the number of leaves in *hls1* mutant (Figure 4B). In the presence of glucose, ethylene treatment enhanced leaf development in all three types of lines. These data indicate that hookless genes are required for glucose-induced developmental arrest but their overexpression does not seem to enable overcaming the antagonistic effect of ethylene on seedling growth development.

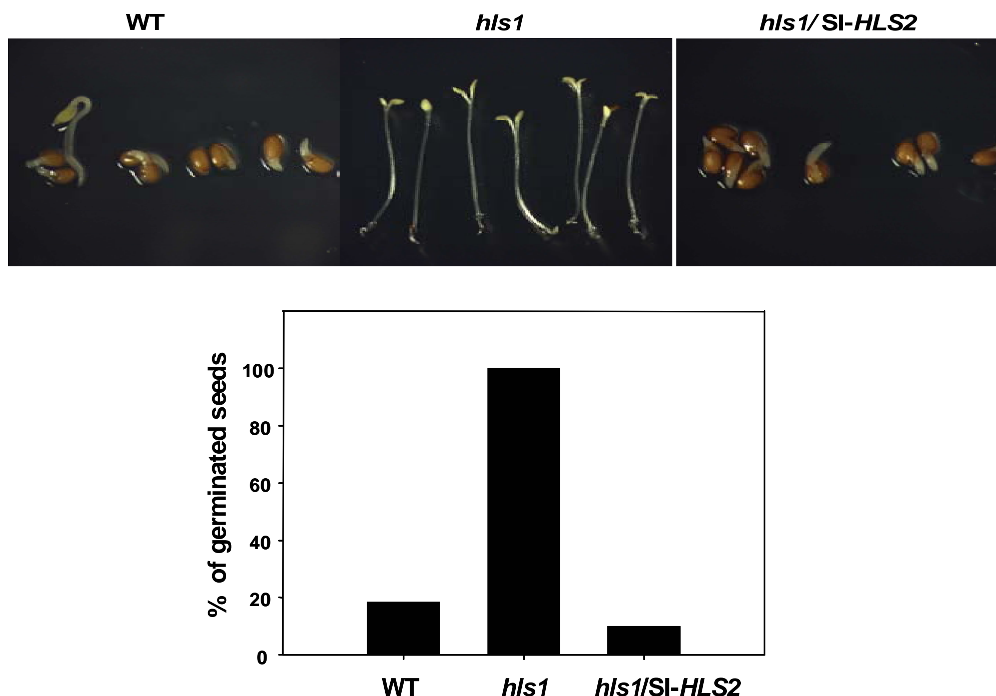

Figure 5. Effect of ABA on seed germination of Sl-HLS1 and Sl-HLS2 Complemented Lines. A. *Etiolated seedlings of WT, hls1, and tomato-complemented hls1 mutant were grown in presence of 100mM of ABA.* ***B.*** *The result in panel A were quantitatively expressed by examinating a large number of seedlings (n>50).*

Sl-HLS1 and Sl-HLS2 Expression Restores Normal ABA Response

Taking into account that hls1 mutantant is altered in ABA response, we tested the complemented lines for ABA-dependent developmental response. In the presence of 100 nM of ABA, 100% of *hls1* mutant seeds underwent normal germination and subsequent growth while germination of WT seeds (18 % of germinated seeds) was strongly inhibited (Figure 5). Transgenic *hls1* lines complemented with Sl-*HLS1* and Sl-*HLS2* genes, display even stronger inhibition of seed germination (10% of germinated seeds) than WT in the presence of ABA. These data confirm the implication of hookless genes in ABA signalling.

Recovery of Normal Gravitropic Response of the hls1 Mutant Complemented with Sl-HLS1 and Sl-HLS2

We reported previously that both the root and the shoot of etiolated *hls1* seedlings have agravitropic growth (Chaabouni et al. 2008, ChapitreIII). Expression of Sl-*HLS1* and Sl-*HLS2* in *hls1* mutant was capable of restoring normal seedling gravitropism (Figure 6).

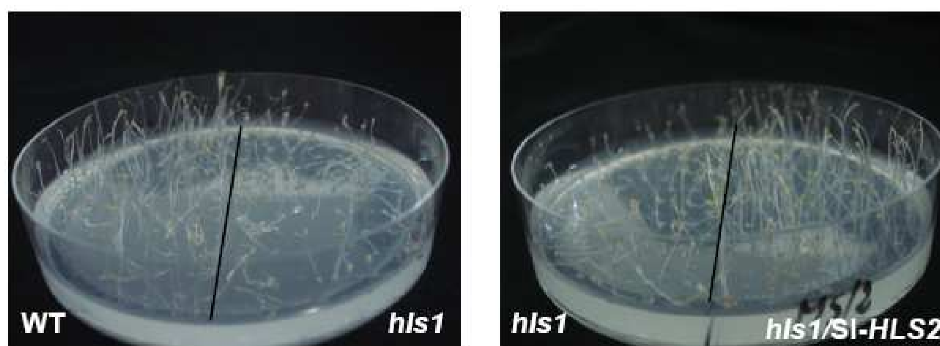

Figure 6. Arabidopsis hls1 Mutant Complemented with Sl-HLS1 and Sl-HLS2 recover Normal Gravitropic Response. *3-day-old seedlings of wild type (WT), hls1 mutant (hls1) and hls1 complemented lines (hls1/Sl-HLS2) grown on MS media in the dark.*

Phenotypes Associated with Overexpression of Sl-*HLS1* and Sl-*HLS2* in wild type Arabidopsis

To examine the effects of over-expression of the hookless gene on various developmental growth processes, wild type Arabidopsis plants were transformed

with a tomato Sl-*HLS1* and Sl-*HLS2* genes under the control of 35S promoter. More than thirty independent transformed lines were identified for each gene that were cheked for their high level of Sl-*HLS1* and Sl-*HLS2* mRNA accululation (data not shown). Figure 7A shows that in the absence of ethylene, complemented etiolated seedling lines did not display exagerated apical hook curvature compared to wild type. We then established a dose response curve of

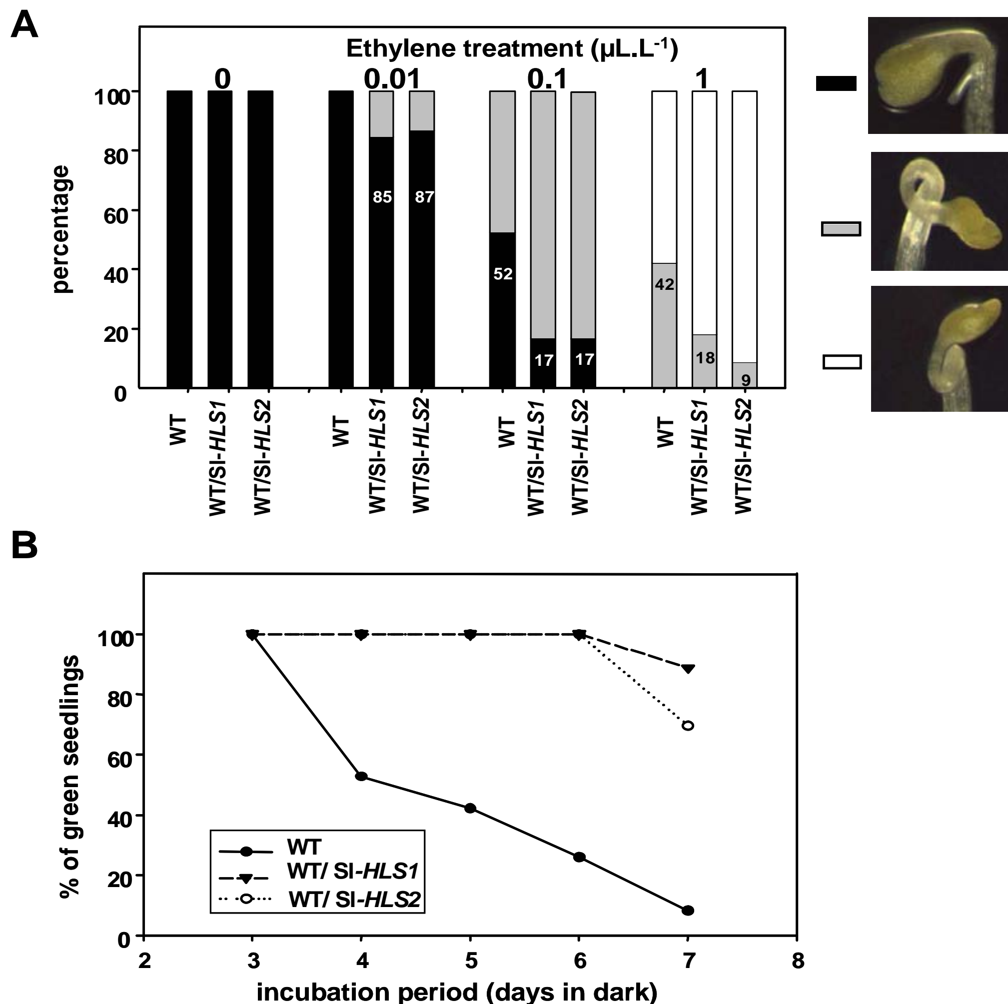

Figure 7. Over-expression of Tomato Sl-HLS1 and Sl-HLS2 Genes in Wild type Arabidopsis Confers Hypersensitivity to Ethylene. A. *The effect of ethylene on the apical hook curvature of overexpressed lines comaped to the wild type was estimated visually and a tree scale range were used: begining of hook formation, full hook, and exagerated hook. The data are mean of two independent experiments.* ***B.*** *A greening phenotype of seedlings overexpressing tomato hookless gene in Arabidopsis****.*** *Etiolated seedlings of WT and overexpressed tomato hookless plants were kept 48 hours on white light after being in the darkness for a number of days. Seedlings (n>50) were assessed as to whether they were green or still yellow.*

hook curvature in response to exogenous ethylene treatment. After 5 days of dark-growth and 24h of ethylene treatment (0.1 µL.L^{-1}), more than 80% of the overexpressing seedlings displayed a full hook whereas only 48% of wild type did (Figure 7A). This indicates that overexpression of tomato hookless genes in wild type Arabidopsis confers hypersensitivity to ethylene.

Sl-*HLS1* and Sl-*HLS2 o*verexpressing lines displayed a clear greening phenotype. After 6 days growth in the dark 100% of the overexpressing lines opened green cotyledons while under the same conditions 85 % wild type control seedlings failed to green (Figure 7B).

In glucose assay, we showed previously that complementation of *hls1* with Sl-*HLS1* and Sl-*HLS2* resulted in recovery of glucose–induced growth inhibition similar to that shown by wild type. We carried out a comparison between the hookless overexpressing lines and wild type plants at different concentrations of glucose. No overt difference in cotyledon development was found in the absence

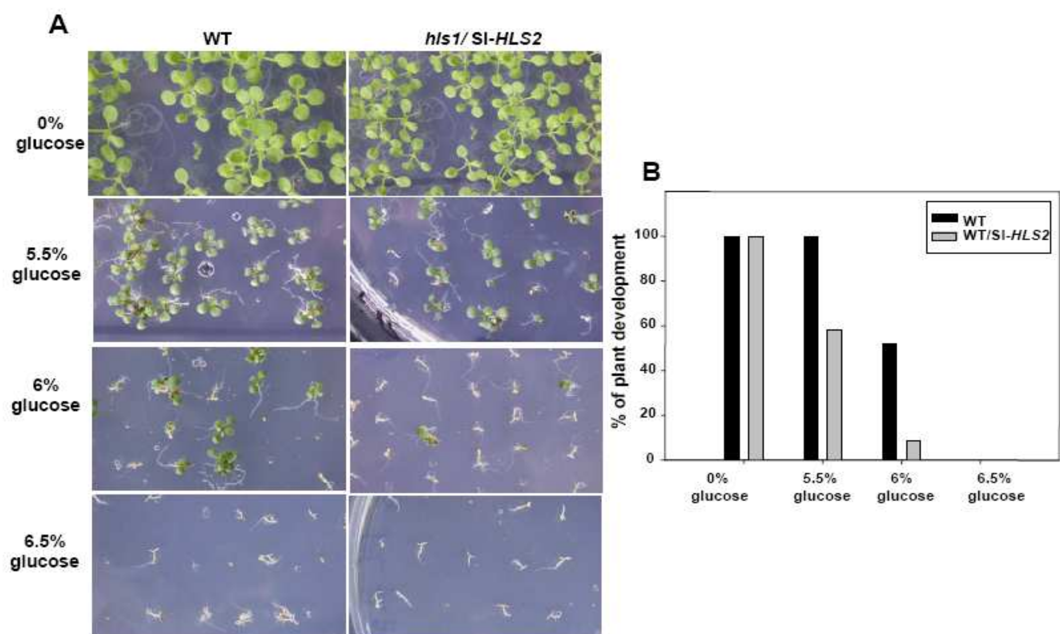

Figure 8. Over-expression of Tomato Sl-HLS1 and Sl-HLS2 Genes in Arabidopsis Confers Hypersensitivity to Glucose. A. *seeds of WT and overexpressed hooklees lines were germinated and grown on MS medium containing 0%, 5.5%, 6 or 6.5% glucose for 10 days.* ***B.*** *The Numbers of green seedlings in panel* ***A*** *were quantitatively expressed by examinating a large number of seedlings (n>30).*

of exogenous glucose (Figure 8A). However, in the presence of 5.5% glucose, 55% of Sl-*HLS2* overexpressing lines displayed developmental arrest while 100% of wild type seedlings underwent normal growth. At 6% glucose, up to 91.3% of Sl-*HLS2* overexpressing lines displayed growth inhibition compared to only 55 % in wild type (Figure 8B). Similar data were obtained with Sl-*HLS1* overexpressing lines indicating that both tomato genes play important role in germination and suncsequent cotyledon and true leaves development.

Expression Pattern and Regulation of Sl-*HLS1* and Sl-*HLS2* in Tomato

Sl-HLS2 Gene Expression Is Regulated by Ethylene and Auxin in Tomato

RNA samples extracted from various tissues of tomato plants were analyzed for assessing the level of Sl-*HLS1* and Sl-*HLS2* mRNA accumulation using real-time quantitative PCR. Sl-*HLS1* and Sl-*HLS2* transcripts were detected in all tissues examined but showed the highest levels in ripening fruit tissue (Figure 9A and B). Sl-*HLS1* and Sl-*HLS2* transcript levels increased from immature green throughout breaker stage. In vegetative tissues, the highest levels of Sl-*HLS1* and Sl-*HLS2* transcript accumulation were found in leaves.

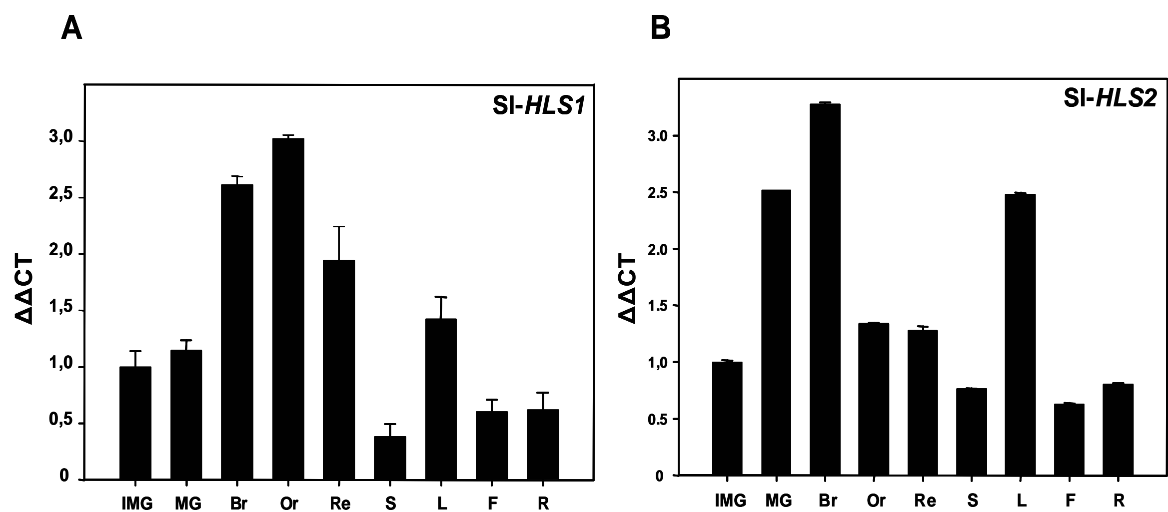

Figure 9. Tissue-specific Expression of Sl-HLS1 and Sl-HLS2 in Tomato. *The levels of transcripts were addressed by real-time quantitative PCR. Sl-HLS1 and Sl-HLS2 mRNA accumulation was monitored in reproductive tissues including immature green (IMG), mature green (MG), breaker fruit (Br), Orange (Or), Red (R), Flower (F), and in vegetative tissue including Stem (S), leaf (L), and Roots (R). ΔΔCT on the y axis refers to the fold difference in the Sl-HLS1* ***(A)*** *and Sl-HLS2* ***(B)*** *expression relative to the immature green fruit.*

In Arabidopsis and pea, ethylene enhanced the expression level of At-*HLS1* and Ps-*HLS1* respectiely in the apical hook (Lehmanet al., 1996; Du et al., 2001). To study the expression of tomato *HLS* in 5-day-old etiolated seedlings, different tissues were isolated from the seedlings as indicated in Figure 10A. Sl-*HLS* mRNA was detected in all tissues harvested (data not shown). Ethylene-induced expression of Sl-*HLS2* was observed only in the hook of etiolated seedlings after 5 hours of treatment (Figure 10C). Auxin has also been implicated in regulating apical hook closure through the *HLS1* gene in Arabidopsis (Li et al., 2004). Sl-*HLS2* transcript is highly accumulated upon auxin treatment in the cotyledons and in the hook of dark-grown seedlings, however, auxin had no effect on the level of Sl-*HLS2* expression in other tissues tested (Figure11E).

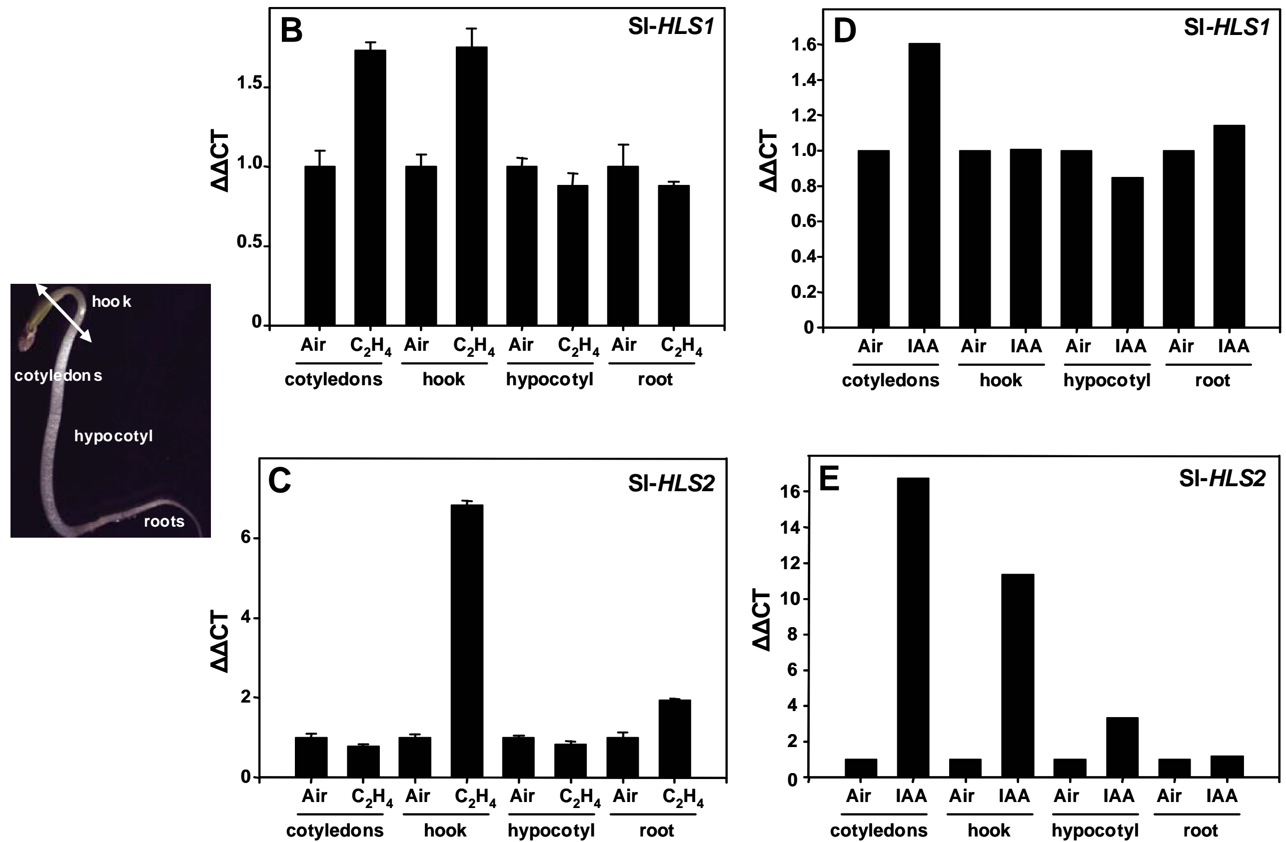

Figure10. Ethylene and auxin regulation of Sl-HLS1 and Sl-HLS2 genes in different tissues of etiolated tomato seedlings. A. *Four days etiolated seedlings of tomato.Cotyledons, hook, hypocotyls and roots were harvest for RNA extraction and analyzed for level of Sl-HLS1 (**B,D**) and Sl-HLS2 (**C,E**) using real time quantitative PCR. **X** ppm of ethylene was applied on seedlings during 5 hours (**B,C**). Auxin treatment (100µM of IAA) was applied by spraying seedlings during **X** hours (**D,E**). **B.** ΔΔCT on the y axis refers to the fold difference in the Sl-HLS1 (**A**), and Sl-HLS2 (**B**) expression relative to air-treated cotyledons, hook, hypocotyl and root, respectively.*

The level of Sl-*HLS1* transcripts accumulation was hunderds of times lower than that of Sl-HLS2 in all tissues tested (data not shown). Moreover, no significant differential regulation of this gene after ethylene and auxin treatment could be observed in any tissues (Figure10 B-D). This prompted us to further focus our interest on the characterization of Sl-*HLS2* promoter.

To better characterize the dynamics of ethylene responsiveness of Sl-*HLS2* and particularly at the onset of ripening, we examined the Sl-*HLS2* transcript accumulation on mature green fruit upon ethylene treatment, and breaker fruit upon MCP treatment. In tomaot fruit, Sl-*HLS2* gene did not display ethylene regulation as revealed by treatment of mature green fruit by ethylene or by 1-MCP, the inhibitor of ethylene perception (data not shown). This result suggests that Sl-*HLS2* is ethylene inductible only in the hook of etiolated seedlings and not in tomato fruit tissues.

The Expression of Sl-*HLS2* Is Tightly Regulated in Tomato Plant Tissues

The tomato Sl-*HLS2* genomic clone contains a 1.298 bp fragment upstream of the transcription site corresponding to the promoter region that is likely to harbor

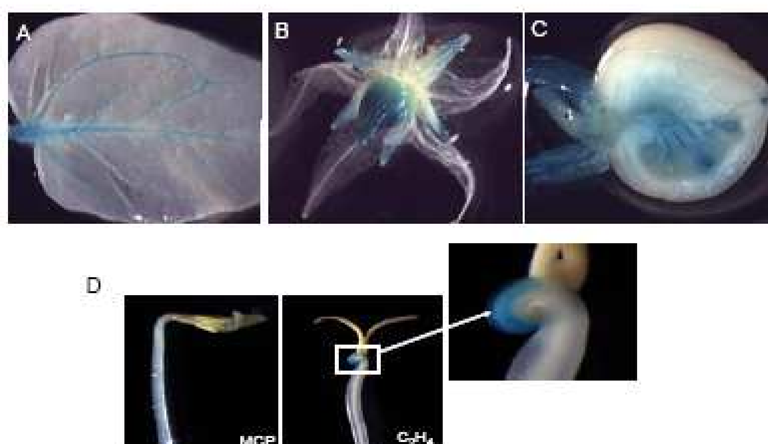

Figure12: Expression pattern of HLS2 revealed by the expression of the GUS reporter gene driven by the pHLS2 promoter. *The GUS staining is analysed in leaves (A), flowers (B), mature green fruit (C) and etiolated seedlings (D).*

most regulatory elements necessary for driving the regulated transcription of the gene. To address the tissue-specific expression of the Sl-*HLS2* gene in *planta*, the corresponding promoter was fused to the *GUS* reporter gene and stably transformed into tomato plants (Pro*HLS2:GUS)*. *GUS* expression, assessed in homozygous tomato lines, was mainly associated with leaf vascular tissues, flowers (specially anthers and sepals), seeds and placenta of mature green fruit (Figure 12 A-C). Since real time PCR revealed that Sl-*HLS2* gene is regulated by ethylene only on the hook (Figure 10C), we further explored its ethylene responsiveness by analysing in Pro*HLS2:GUS*-expressing etiolated seedlings. Figure 13 revealed that upon 5 hour of ethylene treatment (10 μLL^{-1}), GUS activity in the seedlings is tightly restricted to the outer side of the apical hook.

DISCUSSION

Lehman et al (1996) identified the Arabidopsis *hls1* mutant on the basis of its failure to form an apical hook. The corresponding gene was cloned and its derived amino acid sequences were found to have similarity to N-acethyltransferases. Li et al (2004) identified that auxin reponse factor (ARF2) is a repressor of *hls1* mutation, and showed that light and ethylene control the differential cell elongation in hypocotyls of dark-grown seedlings by regulating the expression of ARF2 in a hookless dependant manner.

We have cloned two cDNA from tomato with high similarity to the Arabidopsis *HLS1* genes. These two cDNAs Sl-*HLS1* and Sl-*HLS2* encode proteins that possess the same putative N-acetyktransferase domain than Arabidopsis *HLS1*. The functional equivalence of the Arabidopsis HLS1 and the two tomato hookless proteins in causing hook formation was confirmed by complementation of the Arabidopsis *hls1* mutant with both Sl-*HLS1* and Sl-*HLS2*. We reported previously (Chaabouni et la 2008, Chapter III) the discovery of new phenotypes in *hls1* mutatant indicating that the *HLS1* gene is in the cross-roads of multiple signalling including light, sugar, abscisic acid and gravitropism. These phenotypes have also been restored by the overexpression of Sl-*HLS1* and Sl-*HLS2* in *hls 1* mutant.

The isolation of the Sl-*HLS1 and* Sl-*HLS2* genomic clones showed that their structural organization was very well conserved when compared with the Arabidopsis *HLS1* gene. Both are composed of tree exons and two small introns. The availability of a well-characterized Arabidopsis *hls1* mutant offered a unique opportunity to investigate the predicted function of tomato hookless proteins through heterologous expression. By reversing the absence of hook formation in etiolated Arabidopsis *hls1* seedlings to a largely normal ethylene-responsive phenotype, we demonstrated that Sl-*HLS1* and Sl-*HLS2* genes encode a functional protein. These data strongly support the hypothesis that components of the hookless signaling pathways are conserved between tomato and Arabidopsis.

Lehman et al. (1996) have shown that At-*HLS1* is ethylene inducible. In accordance, Sl-*HLS2* mRNA accumulation was up regulated in the hook of etiolated tomato seedlings especially in the outer side of the hook (Figure 10 and 13). No significant ripening-related expression or ethylene-regulation has been detected in tomato fruit tissues. However, In Arabidopsis, sequence analysis of the *HLS1* promoter revealed the presence of an ethylene response element, a GCC box. This DNA sequence element confers ethylene responsiveness to a minimal promoter in transgenic plants (Ohme-takagi and Shinshi, 1995). In *silico* analysis of Sl-*HLS2* promoter identified an ATTTCAAA ethylene-response element but not a canonical GCC box.

Auxin has also been implicated in regulating apical hook closure (Kang and Ray, 1996a; Lehman et al., 1996; Li et al., 2004). The precise relationship between auxin and ethylene in hook formation started to be elucidated, and the model supported by Li et al. (2004) suppose that one of the role for At-*HLS1* is to inhibit the Auxin Response Factor, ARF2, leading to enhanced differential growth and exaggerated hook curvature. In addition to its ethylene regulation, Sl-*HLS2* gene shows auxin-incuced-expression in the cotyledons and in the hook of dark-grown seedlings. This result favours the hypothesis that Sl-*HLS2* regulation by ethylene and auxin is an important step in the process of hook formation. Interestingly, the software failed to identify any of the canonical auxin-response elements (TGTCTC) in the Sl-*HLS2* promoter sequence.

Based on the physiological characterization of *hls1* mutants, it was discovered that *HLS1* gene may act to prevent the bleaching phenotype in prolonged darkness. Arabidopsis *hls1* mutant lines complemented by Sl-*HLS1* and Sl-*HLS2*

genes show normal de-etioleted phenotype (Figure 3). Moreover, overexppression of tomato hookless genes promote normal opening of green cotyledons even after prolonged time in the dark, under which conditions the wild type control seedlings failed to green (Figure 7B). These data strongly support the implication of hookless gene in normal greening process.

It has been shown previously that At-*HLS1* is a positive regulator of the glucose repression of germination, cotyledon greening and expansion and true leaf development. Complemented Sl-*HLS1* and Sl-*HLS2* plants, like wild type seedlings, undergo growth arrest when cultivated in the presence of 6.5% of glucose. Tomato overexpressed plants show hypersensitivity to glucose. This result further supports the evidence that HLS gene plays an important role in germination and development of cotyledons and true leaves.

Although a number of hookless like sequences from Arabidopsis and other plant spicies are available in the gene database, there is no experimental evidence regarding their putative N-acetyl transferase activity. We have identified two cDNA, Sl-*HLS1* and Sl-*HLS2* that show high similarity to Arabidopsis *HLS1*. Sl-*HLS2* is much more abundant than Sl-*HLS1* and display a differential expression pattern in etiolated seedlings upon hormones treatments. Although the protein products of both cDNAs complement the *hls1* mutant of Arabidopsis in all phenotype tested, it's not clear whether Sl-*HLS1* and Sl-*HLS2* exert the same function in tomato seedlings.

In conclusion, the identification of two *HLS* genes from tomato and the demonstration that their encoded proteins are functional homologs of the Arabidopsis HLS protein confirm the importance of HLS in the regulation in the hook closure, greening, glucose and ABA signalling pathway. Elucidating the mode of action of HLS will, therefore, help to explain the mechanism controlling formation of the apical hook and managing different signalling pathway.

METHODS

Plant Material

Arabidopsis transformation

hls1-1 in the Columbia ecotype were obtained from the Nottingham Arabidopsis Stock Center. Wild type (Columbia ecotype) was used as control. Plants were grown under standard green house condition. *Agrobacterium tumefaciens*-mediated transformation was carried out using the pGreen 2935SOMCaMVT (Dr julie cullimore, INRA toulouse) binary vector according to Bird et al. (1988). The sense construct was generated by cloning the full *Sl-HLS1* and *Sl-HLS2* open reading frame under the transcriptional control of the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator (Sl-*HLS1* F 5' ATGATGGCGGT TAATGAACAAGTGAG3'; Sl-*HLS1* R 5' TTAAAATTCTCTGGGATCAACAAAGAT AG; Sl-*HLS2* F 5' ATGGTGGAGAATGGTGATTTGGTTGTGTCG 3'; Sl-*HLS2* R 5' T TAGACTTCTCTAG GATCAACAAATATAGAAAG3'). The transformation protocol of *hls1* Arabidopsis mutant was as described by Leclerc et al. (2002). The selection of putative transformants was done on a 70 mg L−1 kanamycin-containing agar medium.

Tomato transformation

Tomato (*Solanum lycopersicum* cv MicroTom) plants were grown under standard greenhouse conditions. For growth in chamber rooms, the conditions are as follow: 14 h day/10 h night cycle, 25/208C day/night temperature, 80% hygrometry, 250 µmolm−2 s−1 intense luminosity.

To generate an antisense *Sl-HLS1 (AS-HLS1) and* antisense *Sl-HLS2 (AS-HLS2)* transgenic plants, forward and reverse primers were used to amplify a specific partial clone of each gene (*AS-HLS1* F 5'CCGGGTCAAAATCTCAAAATCCG 3'; *AS-HLS1* R 5'GCGGATTCGTTAAGAACTCGTTG3'; *AS-HLS2* F 5' TCGGGTCCGGGTATCTA GTCG 3'; *AS-HLS2* R CGGATGAACCAAGAAATCTTCTAC. Moreover, a highly conserved sequence between *HLS1* and *HLS2* was used to downregulate both of these genes. We called this construction AS-HLS1+2. The primers used were AS-HLS1+2 (F 5' ATGGTGGAGAATGGTGATTTGGTTG and R 5'GCGGCTTAGCTG

AATCAGAGCTG3'). For overexpressing lines, the same sense construct (S-*HLS1*) (from ATG to the Stop codon) generated for Arabidopsis *hls1* complementation was introduced into tomato plants. Agrobacterium tumefaciens-mediated transformation of tomato plants was carried out according to Jones et al. (2002) and transformed lines were selected as in Wang et al. (2005).

Cloning of Sl-*HLS1* and Sl-*HLS2*

Two tomatos *EST, homologue to Arabidopsis hookless 1 gene, were found in tigr database. The lacked parts of each EST were filled in by 5' and 3' r*apid amplification of cDNA ends (RACE) according to the manufacturer's instructions (Clontech, BD SMART RACE cDNA Amplification Kit). Two cDNA was then isolated, amplified, and sequenced. We refer them as *Sl-HLS1* and *Sl-HLS2.*

Isolation of the Genomic Clones

Genomic DNA was extracted from 1 g of ground tomato (*Lycopersicon esculentum*) leaves using DNA extraction Kit (Promega). An RNase treatment was done at 37℃ for 10 min. A pair of primers was chosen based on the cDNA sequence, and PCRs were performed on the genomic DNA. The amplified fragments were cloned and fully sequenced. Comparative analysis between the genomic clone and cDNA sequences allowed the delimitation of introns and exons.

Isolation of Sl-*HLS2* Promoter

The Universal Genome Walker Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) was used to isolate *the Sl-HLS2* gene promoter region.The tomato genomic DNA fragment with adaptors at the ends was used as a template for the amplification of the promoter region. We used AP1 (5' GTAATACGACTCACTATAGGGC 3') and AP2 (5' ACTATAGGGCACGCGTGGT 3') primers provided by the Genome Walker kit, and two specific antisens primers for our gene SP1 (5'AAGATAGGAAGCGGCTTAGCTGAATC3') and SP2 (5' CTGTTTTT GAC ACCACTCCTCGAGAG3'). The generated PCR product was cloned, fully sequenced and analyzed by PlantCARE, (Lescot et al., 2002). This

promoter is then fused to Gus reporter gene in binary vector (plp100) and stably transformed in tomato lines as indicated before.

Hormones Treatments

Ethylene treatments were performed for 5 h in 25 L sealed glass boxes. 5-days etiolated seedlings were treated with 10 ppm of ethylene. Tomato fruits at the Immature green (IMG), mature green (MG) and breaker (Br) stage were treated with 50 µl.l.1 ethylene. Control seedlings and fruits were exposed to air alone. Auxin treatment was applied during 2 hours on dark-grown seedlings at 25℃. Auxin (IAA) solutions (100µM) were made up in 0.05% ethanol and were adjusted to ph 6 with dilluate NaOH. To disturb seedlings as little as possible, IAA solutions were sprayed onto intact plants as a fine mist. After treatments, different tissues were immediately frozen in liquid nitrogen and stored at -80℃ until RNA extraction. For etiolated seedlings, different tissues corresponding to cotyledons, hook, hypocotyls and root were harvest as indicated in figure 11.

Greening, Glucose and ABA Test

These tests were applied as indicated in ChapitreIII.

RNA Extraction and Quantitative RT-PCR

RNA from etiolated seedlings was isolated using the Qiagen kit (RNaeasy Plant Mini kit) according to the manufacturer's instructions. For all the other tissues, RNA was extracted by the phenol–chloroform method according to Zegzouti et al. (1999). DNase-treated RNA (2 mg) was then reverse-transcribed in a total volume of 20 µl using the Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed using cDNAs corresponding to 2.5 ng of total RNA in a 10 µl reaction volume using the SYBR Green PCR Master Mix (PE-Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900HT sequence detection system. PRIMER EXPRESS software (PE-Applied Biosystems) was used to design gene-specific primers. The following hookless-specific primers were used:

Sl-*HLS1* F (5'AAGAGGCTGTGGAGGAACAATC3')

Sl-*HLS1* R (5'GGAAAGTTTAGTGAAAACA GGAAGGT3')

Sl-*HLS2* F (5'CCTATACCGCCGCCGATACT3')

Sl-*HLS2* R (5'ACC GAGATTGAGAGGGTTGTTG3')
Sl-*Actin* F (5'TGTCCCTATTTA CGAGGGTTATG C3')
Sl-*Actin* R (5'CAGTTAAATCACGACCAGCAAGAT3').
Actin was used as a reference gene with constitutive expression in various tissues.

For Sl-*HLS1 and* Sl-*HLS2*, the optimal primer concentration was 900nM and 50 nM respectively. For *Actin* the primers were used at 50nM concentration. Real-time PCR conditions were as follow: 50℃ for 2 min, 95℃ for 10 min, then 40 cycles of 95℃ for 15 s and 60℃ for1 min, and fina lly one cycle at 95℃ for 15 s and 60℃ for 15 s. For all real-time PCR experiment s, two biological replicates were made and each reaction was run in triplicate. For each sample, a Ct (threshold constant) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the Sl-*Actin-51* (accessionNo.Q96483) as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct value for Sl-*HLS1 and* Sl-*HLS2* gene was normalized to the Ct value for Sl-*Actin-51* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.

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CONCLUSIONS GENERALES ET PERSPECTIVES

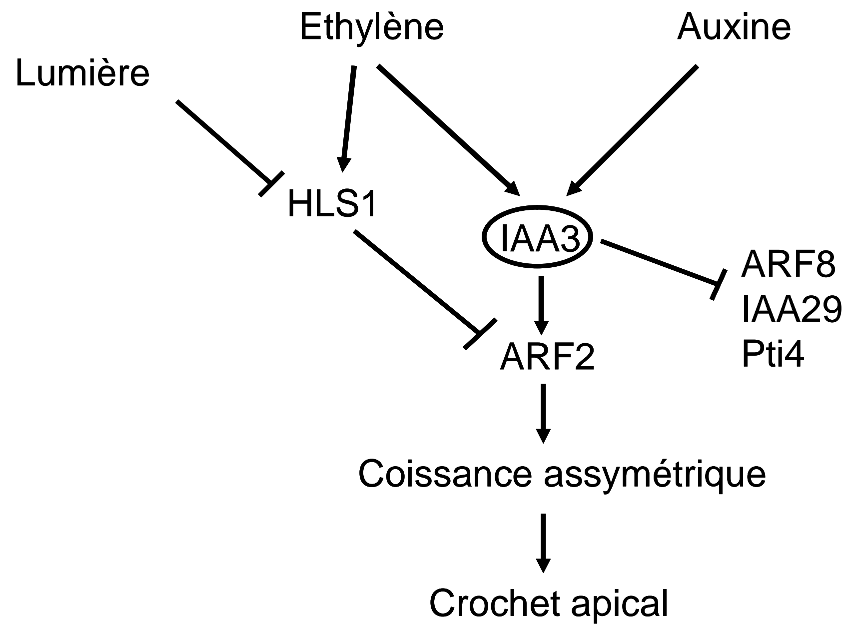
Le projet de recherche réalisé dans le cadre de cette thèse a permis de caractériser deux gènes, Sl-*IAA3* et SL-*HLS,* qui sont à la croisée de plusieurs voies de signalisation. En participant à l'intégration nécessaire de plusieurs signaux endogènes et exogènes, il a été montré que ces gènes permettent la régulation fine et dirigée de nombreux processus de développement. De ce point de vue, ces gènes contribuent, parmi d'autres, à l'adaptation des plantes à leur milieu en activant les réponses appropriées au contexte environnemental.

Le gène Sl-*IAA3* qui code pour un régulateur transcriptionnel permet l'intégration des voies de signalisation de l'auxine et de l'éthylène. L'étude menée a montré son rôle dans les processus de croissance différentielle (differential growth) et apporté un premier éclairage sur son mode d'action lors de la formation du crochet apical. En effet, la croissance différentielle des tissus du crochet semble être le résultat d'un jeu d'expression décalée entre les gènes Sl-*IAA3* et Sl-*HLS* qui s'attribuent des territoires opposés et exercent une régulation inverse de l'élongation cellulaire dans les faces interne et externe du crochet. L'expression exclusive du gène Sl-*IAA3* dans une partie déterminée du pétiole semble également intervenir dans la réponse épinastique, un autre exemple de croissance différentielle. Leurs profils d'expression localisés et contrastés font de Sl-*IAA3* et Sl-*HLS* deux gènes marqueurs pouvant être utilisés pour visualiser les tissus en état d'élongation. Une analyse à grande échelle par une approche transcriptomique de l'expression génique dans les lignées sous-exprimant Sl-*AA3* permettra d'identifier les réseaux de gènes dont la régulation dépend de Sl-IAA3. Ces données permettront de repérer les acteurs potentiels agissant de concert avec Sl-IAA3 au cours de la formation du crochet mais également dans d'autres processus comme la dominance apicale ou le développement du fruit. En effet, le profil d'expression finement régulé du gène Sl-*IAA3* dans le fruit et sa faible

expression dans les mutants de maturation *rin nor* et *Nr*, suggèrent fortement un rôle potentiel de ce gène dans l'intégration de signaux éthylène et auxine dans le fruit. Il en est de même du gène Sl-HLS qui présente un profil d'expression qui permet d'envisager sa participation au contrôle de l'avènement de la maturation. L'analyse des lignées transgéniques ou mutants EMS altérées dans l'expression des gènes Sl-*IAA3* et Sl-*HLS* permettra d'évaluer leur impact réel sur la maturation des fruits. Par ailleurs, sachant que la régulation prépondérante des protéines Aux/IAA au niveau post-traductionnel, l'analyse de l'expression du gène Sl-*IAA3* au niveau protéique devient nécessaire car susceptible d'apporter un éclairage nouveau sur son mode d'action. La production d'anticorps dirigés contre la protéine Sl-IAA3 est maintenant envisagée qui permettront de suivre son accumulation tissulaire. Aussi, la connaissance des partenaires directs de la protéine Sl-IAA3 pourrait fournir des clés importantes quant au mécanisme d'action de ce gène. Il s'agit en particulier d'identifier parmi les facteurs de transcription de type ARF, les partenaires naturels des Aux/IAA, ceux qui dimérisent avec la protéine Sl-IAA3. Une approche double hybride ou une stratégie de co-immunoprécipitation seraient envisagées dans ce cas.

En ce qui concerne le gène hookless, en dépit des nombreux travaux réalisés par différents groupes et ceux réalisées au cours de la présente thèse, la fonction de la protéine codée par le gène *HOOKLESS* demeure à ce jour inconnue. Les tentatives menées ici pour explorer la fonction enzymatique de la protéine HLS se basant son homologie avec la famille des N-acétyl-transférases, sont restées infructueuses. Cet effort se poursuit actuellement par la caractérisation des profils métaboliques comparés des mutants *hookless* de sur et sous-expression qui vise à fournir des indications sur les voies métaboliques et les substrats potentiels utilisés par cette protéine. Cette analyse métabolique cherche également à vérifier si la mutation *hookless* affecte les niveaux de certaines hormones telle que l'auxine. Enfin, la disponibilité croissante de ressources nouvelles de génétique inverse telles que les populations de TILLING permettra d'isoler des variants alléliques des protéines Sl-IAA3 et Sl-HLS qui fourniront des outils originaux pour explorer leurs différents rôles.

Les avancées acquises au cours de ce travail de thèse ajoutées aux connaissances disponibles dans la littérature permettent de proposer un modèle précisant le niveau d'intervention du gène Sl-*IAA3* et son positionnement par rapport au gène Sl-HLS lors du contôle de la mise en place du crochet apical.



Modèle d'intégration des signaux éthylène, auxine et lumière dans la croissance différentielle au niveau des plantules étiolées. Le processus de formation du crochet apical nécessite une interaction entre les gènes *HLS1*, *IAA3* et *ARF2*. Sl-*IAA3* et Sl-*HLS* agissent probablement à travers des voies parallèles qui de croisent au niveau de ARF2.

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