The Control of Auxin Homeostasis Through The Regulation of *IAMT1* by DELLA Proteins

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Valencia, July 2014

Alas I am here, where it all started, I've been to the end of my journey and back again. My biggest achievement is this, that although life looks messy and chaotic, if you translate it into a world of pathways and molecules, patterns emerge and you start to understand why things are the way they are. So why am I here now? Simply because of you -*all of you*- every single effecter in my pathway lead me to my final homeostasis. So how can I acknowledge all of you when I simple aspire to be like you.

To ***Dr. Miguel Á. Blázquez***, a director, a teacher, and forever a good friend. Ever before we first met, you believed in me when you had no reason to. I can never acknowledge what you've done for me, it is far beyond words. You taught me that a good scientist is the one who is effective in his work but a great scientist is the one who listens to his inner instinct. I will always remember that and I will always remember when you told me that you would feel proud if people said one day that you were my director, well I can tell you this, I will forever be proud that I was your student.
I will always aspire to be like you.

To ***Dr. David Alabadí***, a director, a teacher, and forever a good friend. I still remember that first day we met, when you picked me up from the airport and took me to the beach for a coffee, the way you passionately spoke about science made me love what I will do ever before I started doing it. I will always admire that day, you enlightened me and were by my side all the time. You never gave up on me and were always my support.
I will always aspire to be like you.

To ***Dr. Javier Gallego-Bartolomé***, a college, a mentor, and forever a good friend. You taught be that separating yourself apart from others, and thinking outside the box is what makes a scientist brilliant. Thank you, I am forever in your debt.
I will always aspire to be like you in that way.

To ***Dr. Antonella Locascio***, a college, and forever a good friend. You showed me that being the best at what you do earns you the respect you deserve. For that I thank you.
I will always aspire to be like you in that way.

To ***Dr. Eugenio Minguet***, a college, and forever a good friend. I will miss our discussions, you are a model for all of us. You showed me that being systematic in my experiments reflects on my personality as a scientist and that things should always be done right.
I will always aspire to be like you in that way.

To ***Dr. Nora Alicia Marín de la Rosa***, a college, and forever a good friend. You should me that consistency and hard work always pay off, for that I thank you.

I will always aspire to be like you in that way.

To ***Juan Camilo Álvarez Mahecha*** and ***Amelia Felipo Benavent***, I thank you for all the great times, on and off the bench, for all the small talks, silly laughs and coffee breaks. We shall meet again but until then I leave you with this quote for *Hakim Sanai* ***"This too, shall pass".***

To ***Rodrigo***, ***Cris***, ***Fede***, ***Noel***, ***Laura*** and ***Jorge***, Thank you for all the good and bad times. It has been a fun journey.

To ***Prof. Juan Carbonell*** "El Guru", ***MD Gomez, Cristina Urbez*** and ***Maria Angeles,*** You have been a family for me. Thank you, all of you for the best times. My experience in the **IBMCP** would not have been the same without you.

To the four labs and their members, ***Miguel Perez, Pako, Jose Leon, Alejandro Ferrando, Toni Granell and Diego,*** for all members of the **IBMCP**, administrators, technicians and everyone who aided me in my journey, Thank you.

To my collaborators, ***Stephan Pollmann*** (CBGP, Madrid) and ***Michael Holdsworth*** (Nottingham, UK) for you aid and patience.

For my jury, ***Cristina Ferrandiz***, ***Juan Carlos del Pozo*** and ***Rishikesh Bhalerao*** thank you for being a part of my journey. It is an honor to be judged by you.

For my family, ***Atef , Mariam, Ali, Ashraf*** and ***Samer***. This, all this, would've been impossible without you. I can never wish for a better family than you. From the deepest corners of my soul, thank you, I love and miss you all so much. We shall meet again under the bluest sky, it is time for this wanderer to come home where he belongs. I will come back, wherever I am, whoever I am, a wanderer, worshiper, lover of leaving. Ours is not a caravan of despair. I am coming back, even if I have broken my vow a thousand time. I am coming back, you are the cradle of life.

For my friends, spread all around the globe, thank you.

...And the best for last,

For ***Nada Mohamad*** , how can I thank you; I've never met two lovers who thanked each other. But if it must be let it be the way me and you want it to be. Let it be the beauty within us. There are hundreds of ways to kneel and kiss your hands but while words are a pretext, it is the inner bond that draws one person to another, not words. I will close my mouth put down my pen and speak to you in a hundred silent ways...you simply complete me.

To ***Honey*** and ***Bunny***, in one word, I love you, you deserve nothing less.

I attribute my success to this: I never gave or took any excuse.

Florence Nightingale

SUMMARY

The plant hormones gibberellins (GAs) and auxin display overlapping activities in the regulation of multiple developmental processes, including the differential growth that mediates the response to tropic stimuli and the formation of the apical hook. Several mechanisms have been proposed that explain the interaction between these two hormones, such as the regulation of auxin transport by GAs, and the regulation of GA biosynthesis by auxin. GAs are known to exert their action at the transcriptional level by promoting the degradation of DELLA proteins, which in turn interact with numerous transcription factors and modulate their activity. We have identified *INDOLE-3-ACETIC ACID METHYLTRANSFERASE 1* (*IAMT1*) as one of the earliest target genes upregulated after conditional expression of the DELLA protein GAI in *Arabidopsis thaliana*. In this Thesis, we have addressed two main issues: (1) the contribution of IAMT1 to auxin homeostasis and its biological relevance; and (2) the molecular mechanism by which DELLAs are able to induce the expression of *IAMT1*.

Using combinations of *iamt1* loss-of-function mutants and reporter lines for auxin accumulation and activity, we have found that IAMT1 activity is essential for proper generation and maintenance of the auxin gradients that underlie differential growth. According to our results, the role of IAMT1 would be to restrict polar auxin transport especially during the response to tropic stimuli, preventing excessive auxin accumulation in the responding tissues, and IAMT1 exerts this function, at least in part, by inhibiting the expression of the *PIN* genes, encoding auxin efflux carriers.

Regarding the regulation of *IAMT1* expression by DELLAs, dissection of the promoter, *in silico* analysis of putative DELLA partners, and molecular genetic analysis of reporter lines has allowed us to identify two mechanisms with different relevance depending on the environmental conditions, and through different *cis* elements. In etiolated seedlings, DELLA proteins are recruited by DORNRÖSCHEN (DRN) to the *IAMT1* promoter to induce *IAMT1* expression. In the light and in a temperature-dependent manner, DELLA proteins inhibit the DNA-binding activity of PHYTOCHROME-INTERACTING FACTOR4 (PIF4) and BRI1 EMS-SUPPRESSOR1(BES1), which act as repressors of *IAMT1* expression.

The work presented here highlights how GAs may affect local accumulation of auxin, being particularly relevant in processes that involve differential growth.

Las hormonas vegetales giberelinas (GAs) y las auxinas solapan en la regulación de múltiples procesos de desarrollo, incluyendo el crecimiento diferencial que media la respuesta a los estímulos trópicos y la formación del gancho apical. Se han propuesto varios mecanismos que explicarían la interacción entre estas dos hormonas, como la regulación del transporte de auxinas por GAs, y la regulación de la biosíntesis de GAs por auxinas. Por otra parte, se sabe que las GAs ejercen su acción a nivel transcripcional a través de la degradación de las proteínas DELLA, que a su vez interaccionan con numerosos factores de transcripción modulando su actividad. En el laboratorio hemos identificado a *INDOLE-3-ACETIC ACID METHYLTRANSFERASE 1* (*IAMT1*) como uno de los genes diana que se inducen de forma más rápida tras la expresión condicional de la proteína GAI, una de las cinco DELLAs de *Arabidopsis thaliana*. En esta Tesis hemos abordado dos cuestiones: (1) la contribución de IAMT1 a la homeostasis de auxinas y su relevancia biológica; y (2) el mecanismo molecular por el que las DELLAs inducen la expresión de *IAMT1*.

Empleando combinaciones de mutantes de pérdida de función en *IAMT1* con construcciones testigo para la acumulación y la actividad de auxinas, hemos encontrado que la actividad de IAMT1 es esencial para la correcta generación y el mantenimiento de los gradientes de auxina que subyacen al crecimiento diferencial. De acuerdo con nuestros resultados, el papel de IAMT1 sería el de restringir el transporte polar de auxinas especialmente durante la respuesta a los estímulos trópicos, impidiendo la acumulación excesiva de auxinas en los tejidos de respuesta. IAMT1 ejercería dicha función, al menos en parte, a través de la inhibición de la expresión de los transportadores codificados por los genes *PIN*.

Respecto a la regulación de la expresión de *IAMT1* por las DELLAs, la disección del promotor, el análisis *in silico* de los posibles interactores de DELLAs, y el análisis molecular de líneas testigo nos han permitido identificar dos mecanismos con distinta relevancia dependiendo de las condiciones ambientales, y a través de elementos *cis* diferentes. En plántulas etioladas, las proteínas DELLA son reclutadas por DORNRÖSCHEN (DRN) al promotor de *IAMT1* para inducir su expresión. En la luz, y de forma dependiente de temperatura, las proteínas DELLA inhiben la capacidad de unión al DNA de PHYTOCHROME-INTERACTING FACTOR4 (PIF4) y BRI1 EMS-SUPPRESSOR1 (BES1), que actúan como represores de la expresión de *IAMT1*.

El trabajo aquí presentado pone de manifiesto cómo las GAs pueden afectar de forma local a la acumulación de auxinas, y que este mecanismo tiene especial relevancia durante procesos de crecimiento diferencial .

Les hormones vegetals com les giberellines (GAs) i les auxines regulen diversos processos comuns, com és el creixement diferencial que es produeix en resposta a estímuls tròpics o durant el desenvolupament del ganxo apical. S'han proposat diversos mecanismes per tal d'explicar la interacció entre estes dos hormones, com la regulació del transport d'auxines per GAs, i la regulació de la biosíntesi de GAs per auxines. Per altra banda, les GAs actuen a nivell transcripcional promovent la degradació de les proteïnes DELLA, que interaccionen físicament amb nombrosos factors de transcripció regulant la seua activitat. Al laboratori, hem identificat a *INDOLE-3-ACETIC ACID METHYLTRANSFERASE 1* (*IAMT1*) com a un dels gens diana de GAI, una de les cinc proteïnes DELLA d'*Arabidopsis thaliana*. En esta tesi hem abordat dos qüestions: (1) la contribució d'IAMT1 a la homeostasi d'auxines i la possible rellevància biològica; i (2) el mecanisme pel que les DELLA regulen l'expressió d'*IAMT1*.

Mitjançant l'ús de mutants de pèrdua de funció d'IAMT1 amb construccions testic per l'acumulació i acció d'auxines, hem trobat que IAMT1 és important per la correcta generació i manteniment dels gradients d'auxines que determinen el creixement diferencial. IAMT1 sembla restringir el transport polar d'auxines, especialment durant les respostes tròpiques, impedint la acumulació excessiva d'auxina. Al menys en part, esta acció és inhibint l'expressió dels gens dels transportadors *PIN*.

La dissecció del promotor d'*IAMT1*, l'anàlisi *in silico* dels interactors de DELLA i l'anàlisi de línies testic ens han premés identificar un parell de mecanismes per la regulació del promotor per part de les DELLA que poden tindre diversa rellevància depenent de les condicions ambientals i a través de dos elements *cis* diferents. En plàntules etiolades les proteïnes DELLA son reclutades al promotor d'*IAMT1* mitjançant la proteïna DORNRÖSCHEN (DRN) per induir la seua expressió. En la llum i de forma depenent de la temperatura, les DELLA reprimeixen la capacitat d'unir DNA de PHYTOCHROME-INTERACTING FACTOR4 (PIF4) i BRI1 EMS-SUPPRESSOR1 (BES1), que reprimeixen l'expressió d'*IAMT1*.

En este treball mostrem com les GAs afecten de forma local la acumulació d'auxines, i que este mecanisme te especial rellevància durant processos de creixement diferencial.

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INTRODUCTION

I.1 Phytohormones

The term “phytohormones” was first introduced by Frits Went and Kenneth Thimann in 1937 to define small chemical messengers present at very low concentrations in plants and able to affect the plant life cycle from seed to seed, either locally or by moving to target tissues. We often refer to the five classical hormones, which were identified in the early to the mid 20th century. They are auxin cytokinins (CKs), ethylene, gibberellins (GAs), and abscisic acid (ABA). Within the past 50 years, several other plant growth regulators have been identified that meet the criteria that define hormones in a broad sense: brassinosteroids (BRs), salicylates, jasmonates and strigolactones. It is possible that there still exist unidentified hormones.

Not all plant cells respond to hormones, but those that do are programmed to respond at specific points in their growth cycle. Plants need hormones at very specific times during growth and at specific locations. They also need to disengage the effects that hormones exert, when they are no longer needed. The production of hormones occurs often at sites of active growth within the meristems before cells have fully differentiated. Frequently, they move to other parts of the plant, where they cause an immediate effect; or they can be stored in cells to be released later. Although the physiological function and effect of hormones have been studied for decades, certain aspects of their molecular mode of action, their homeostasis, signaling and transport are still not fully understood. As explained below, GAs constitute an excellent study model to investigate how plants integrate environmental and endogenous information to modulate plant growth, and their signaling activity has become the main focus of this thesis.

I.2 Gibberellins

Although GAs did not become known to American and British scientists until the 1950s, they had been discovered much earlier by Japanese scientists. Rice farmers in Asia had long known of a disease that makes the rice plants grow tall but prevents seed production. In Japan this disease was called the "foolish seedling" disease or *bakanae*. Plant pathologists investigating the disease found that the elongation was induced by a chemical secreted by a fungus that had infected the plants. This chemical was isolated from filtrates of the cultured fungus and called *gibberellin* after *Gibberella fujikuroi,* the name of the fungus. All known gibberellins are diterpenoid acids that are synthesized by the terpenoid pathway in plastids and then modified in the endoplasmic reticulum and cytosol until they reach their biologically-active form. Since their discovery, GAs have been found to influence and control many aspects of plant development. GA deficient mutants show impaired germination (Ogawa M, 2003), photomorphogenic in darkness, (Alabadi et al., 2004; Achard P, 2006) impaired growth of roots and aerial parts (Fu X, 2003), late flowering (Wilson et al., 1992; Silverstone et al., 1997; Blazquez et al., 1998), retarded growth of floral organs (Yu H, 2004), male sterility and deficiency in pollen development (Goto and Pharis, 1999), impaired fruit development (Singh DP, 2002) and increased tolerance to biotic and abiotic stress (Navarro L, 2008).

I.3 Gibberellin signaling

The importance of GAs for plants can be seen by the phenotype of GA-deficient mutants. Accordingly GA mutants can be classified in 3 different groups (Figure I.1):

(1) GA-sensitive mutants such as *ga1*, a mutant lacking *ent*-kaurene synthetase, an enzyme in the GA biosynthesis pathway. *ga1-3* mutants are characterized by impaired germination, severe dwarfism, dark green leaves and late flowering; moreover, the mutant flowers exhibit impaired petal and stamen development and are male sterile. All these phenotypes can be reverted to the wild-type phenotype by the application of exogenous GAs (Peng J, 1999).

(2) GA-insensitive mutants, including dwarf mutants whose phenotype cannot be reverted to wild type by the application of GAs. Such mutants include *gibberellic acid-insensitive* (*gai; Arabidopsis thaliana*), *D8* (*Zea mays*) and *Rht-B1b/Rht-D1b* (*Triticum aestivum*) (Koornneef, 1985; Harberd, 1989; Peng, 1997). The various mutant alleles involved act in a genetically dominant fashion and encode active mutant products with an altered function that reduces the response to GAs.

(3) Slender mutants, exhibiting exaggerated growth: tall and slim, they resemble wild-type plants treated with saturating levels of GAs. such mutants include *slender rice1* (*slr1*, *Oryza sativa*) and *della* loss-of-function mutants in Arabidopsis (Ikeda A, 2001; Feng S, 2008).

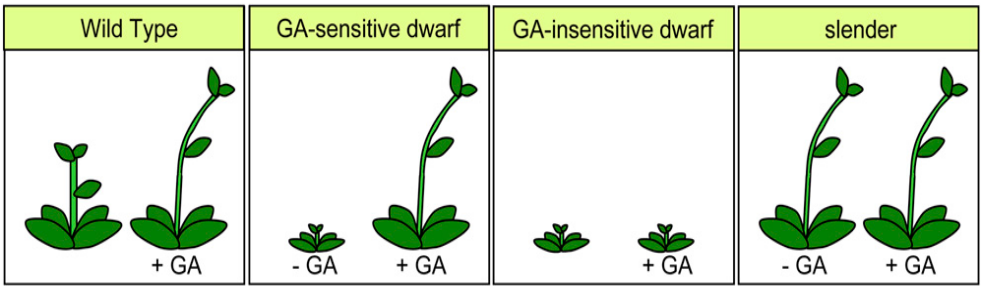

Figure I.1 Schematic representation of the three classes of GA mutants. Adapted from Harberd, N et al, 2009.

Studies related to these mutants, especially the slender mutants, confirmed the early proposition (Brian, 1957) that GA works as an "inhibitor of an inhibitor" , in a signaling mechanism that is known today as the "GA-GID1-DELLA pathway".

I.4 DELLA proteins

DELLA proteins are negative regulators of gibberellin signaling that act immediately downstream of the GA receptor. DELLAs are a subset of the plant-specific GRAS family of putative transcription factors. They are a key intracellular repressors of GA responses (Peng, 1997; Ikeda A, 2001). Like all GRAS proteins, DELLAs share a conserved C-terminal GRAS domain that is involved in transcriptional regulation and is characterized by two leucine heptad repeats (LHRI and LHRII) and three conserved motifs, VHIID, PFYRE and SAW (Achard, 2013). DELLAs are distinguished from the rest of the GRAS family by a specific N-terminal sequence containing two conserved domains: the DELLA domain (hence the name) and the TVHYNP domain (Figure I.2).

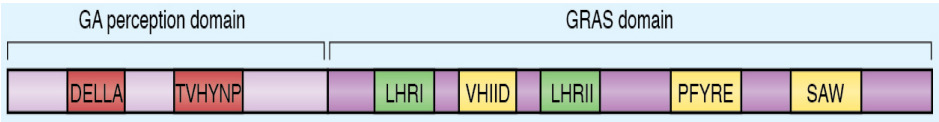

Figure I.2 Schematic representation of the DELLA domains.
Adapted from Davière and Achard 2013.

The number of genes encoding DELLA proteins varies between species: whereas rice contains a single DELLA gene, *SLR1* (Ikeda A, 2001), in Arabidopsis DELLAs are encoded by five genes: *GAI, REPRESSOR OF GA1-3 1 (RGA), RGA-LIKE 1 (RGL1), RGL2* and *RGL3* (Peng, 1997). Although all five DELLA in Arabidopsis share the same basic domains, they seem to be involved in different mechanisms (Table I.1).

Table I.1: DELLAs involvement in Arabidopsis development.

DELLAs	Functions	References
GAI, RGA, RGL1, RGL2, RGL3	Hypocotyl and root elongation, chlorophyll biosynthesis, Sesquiterpene biosynthesis, pathogen defense, seed maturation and GA-JA crosstalk	(de Lucas et al., 2008; Feng S, 2008; Gallego-Bartolome et al., 2010; Hou, 2010; Cheminant, 2011; Feurtado, 2011; Hong, 2012; Yang et al., 2012)
GAI, RGA, RGL1, RGL3	GA-BR crosstalk	(Bai, 2012)
GAI, RGA, RGL1	Seed germination	(Zhang, 2011)
GAI, RGA, RGL2	Fruit patterning	(Arnaud, 2010)
GAI, RGA	GA-ET crosstalk, apical hook development	(An, 2012)
RGA, RGL2	Cotyledon expansion	(Gallego-Bartolome et al., 2010)
RGA	Floral transition	(Yu, 2012)

It has been proposed, based on promoter-swapping studies, that the functional diversification of the different DELLA proteins in Arabidopsis is the result of subfunctionalization probably due to changes in their regulatory sequences (Gallego-Bartolome et al., 2010).

Studies with RGA-GFP (Green Fluorescent Protein) fusion in Arabidopsis showed that DELLA proteins accumulate in the nuclei of cells with low GA concentration, but they suffer rapid destabilization in the presence of GAs (Silverstone AL, 2001). Moreover, GA-induced degradation is absolutely dependent on the DELLA motif, so that *DELLA* mutant alleles lacking this motif (such as *gai-1* and *rga-Δ17*) are stable even in the presence of GAs (Peng, 1997; Dill A, 2001).

I.5 GA-GID1-DELLA mechanism and DELLA degradation

Despite the initial evidence that GAs would be perceived by a plasma membrane receptor (Lovegrove, 1998), the characterization of the GA-insensitive dwarf *gid1* mutant in rice led to the discovery of the GA receptor, GID1 (Ueguchi-Tanaka, 2005). *GID1* encodes the only soluble nuclear GA receptor in rice, but Arabidopsis contains three: *GID1A*, *GID1B* and *GID1C*, all of which exhibit overlapping function (Nakajima, 2006). Crystallization of GID1 revealed that it contains a GA-binding pocket and a flexible N-terminal extension. Upon the binding of bioactive GAs, a conformational change is induced in the N-terminal extension to cover the GA pocket (Shimada, 2006; Murase, 2008). This closing of the pocket allows DELLAs are recognized by the GID1 receptor through the DELLA and TVHYNP regions, thus forming the GA-GID1-DELLA complex (Figure I.3) (Griffiths, 2006; Ueguchi-Tanaka, 2007; Willige, 2007). The discovery of the GA-GID1-DELLA complex and the observation that the levels of RGA-GFP decrease after the addition of GA (Silverstone AL, 2001) pointed out that GA might be causing the degradation of DELLA through the GA-GID1-DELLA complex. For this to occur, the F-box proteins encoded by the Arabidopsis *SLY1* or the rice

GID2 are necessary components that become incorporated to the complex. These F-box proteins recruit the SCF (SKP1, CULLIN, F-BOX) E3 ubiquitin-ligase complex, which catalyze the attachment of polyubiquitin chains to target proteins for their subsequent degradation by the 26S proteasome (Lechner, 2006). Based on yeast-interaction assays, the formation of the GA-GID1-DELLA complex has been proposed to induce conformational changes in the GRAS domain of DELLA that enhance recognition between the VHIID and LHRII motifs of DELLA and the F-box protein SLY1/GID2 (Hirano, 2010). In turn, the $SCF^{SLY1/GID2}$ complex promotes the ubiquitination and subsequent destruction of DELLAs by the 26S proteasome, thereby relieving their growth-restraining effects (McGinnis, 2003; Sasaki, 2003). Thus, GA promotes growth by inducing the proteasome-dependent destabilization of DELLA proteins (Figure I.3).

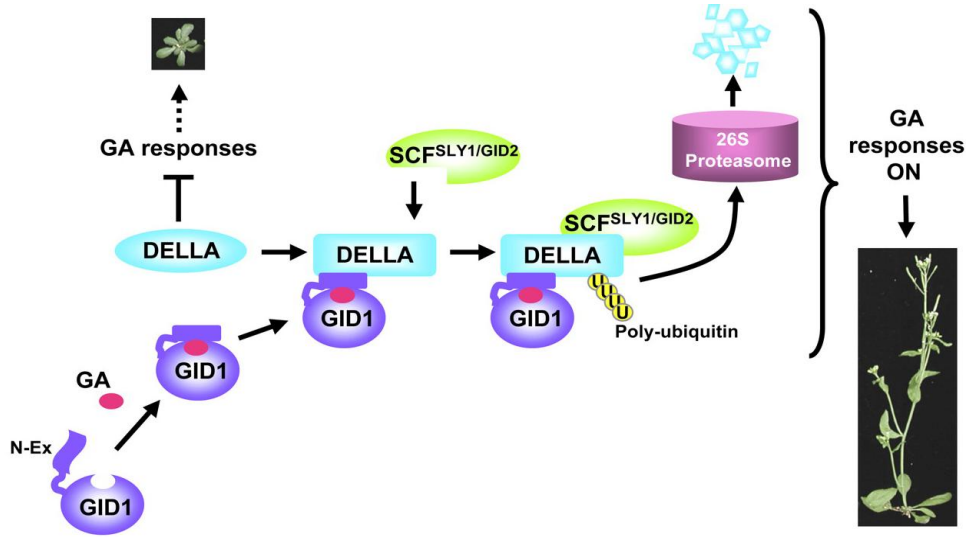

Figure I.3 GA-GID1-DELLA complex and DELLA degradation.
Adapted from Tai-ping Sun 2010.

It is clear that DELLA proteins have an important role in integrating multiple environmental and hormonal signals to coordinate plant growth

and development which is necessary for improving the chances of survival and reproductive success. These signals act at multiple points within the GA signaling pathway to control the levels of growth repressing DELLA proteins. However, we are only just beginning to understand some of the molecular events that control these processes, and it is likely that there are many unknown interactions yet to be uncovered. Whilst the importance of DELLA targeted degradation has been most extensively studied, it has recently become apparent that the transcriptional regulation of the DELLA genes also has a role in controlling GA responsiveness and subsequent plant growth and development (Oh et al., 2007). It also appears likely that the post-translational modifications of DELLA proteins, including phosphorylation or *O*-Glc-NAc modification, may also provide a mechanism for regulating their growth-repressing activities (Olszewski et al., 2010). Another major gap in our knowledge of the GA-DELLA signaling pathway is understanding of how DELLA proteins control the myriad of growth response through their regulation of transcription since the localization of DELLA proteins in the nucleus serves as a strong evidence for its transcriptional activity (Silverstone et al., 1998; Dill A, 2001) and yet there exist no evidence for the binding of these proteins to DNA. It is now believed that DELLAs interact with many different transcription factors to regulate the diverse growth processes that are controlled by GA signaling. This discovery suggests that these transcription factors also represent important nodes for the integration of multiple signals.

I.6 Interaction between the GA-GID1-DELLA module, environmental pathways and other phytohormones.

It is well established now that GAs regulate growth and other processes through modulating gene expression. Taking into consideration that all changes in gene expression in response to GAs are mediated by DELLA proteins (Cao D, 2006) and the fact that DELLAs are nuclear proteins (Silverstone AL, 2001) which contain a C-terminus that is similar to other GRAS proteins that regulate transcription like SHORTROOT (SHR) and SCARECROW (SCR) (Levesque MP, 2006; Cui H, 2007), though there is no evidence for direct interaction between DELLA proteins and DNA *in vitro* it is most probably that DELLA proteins interact with other DNA-binding transcription factors to modify their activity.

Considering that DELLAs interact with several signaling elements present in other hormonal and environmental pathways (de Lucas et al., 2008; An, 2012; Gallego-Bartolome et al., 2012; Hong, 2012), it is fair enough to look at DELLAs and the GA-GID1-DELLA module as internodes in the signaling of GAs with environmental pathways and other hormones on several levels.

Indeed, one level of crosstalk mechanism is through the DELLA-transcription factor (TF) interaction where the TF serves as a signaling element in another pathway. For instance, two recent studies investigating the interaction between light and GA signaling in controlling hypocotyl elongation in *Arabidopsis* have demonstrated that DELLA proteins interact with the basic helix-loop-helix (bHLH) transcription factors PIF3 and PIF4 (de Lucas et al., 2008; Feng et al., 2008). This interaction blocks their activity in upregulating the expression of downstream response genes which are necessary for promoting hypocotyl elongation. Essentially, in the

absence of GA, DELLA proteins block hypocotyl elongation by sequestrating these transcription factors. When bioactive GA is produced in response to environmental conditions that promote hypocotyl elongation, DELLAs are degraded relieving the repression on PIF3/4 and allowing them to promote the expression of genes necessary for this growth response.

In the same sense, the GA and BR signaling pathways are integrated by the crosstalk between DELLA proteins and BRASSINAZOLE RESISTANT1 (BZR1), a transcription factor that triggers the expression of BR-responsive genes. DELLA proteins are able to repress the activity of BZR1 (Gallego-Bartolome et al., 2012). In addition, manipulation of GA signaling indicates that DELLAs may reduce the abundance of protein phosphatase 2A, thereby promoting the accumulation of the phosphorylated and inactive form of BRZ1 (Li et al., 2012).

A second level of crosstalk is depicted in the ability of DELLAs, through the activity of a TF, to regulate the expression of an important element in the signaling or synthesis of the other hormone. Such is the case of crosstalk between GA and ABA, DELLA proteins induce the transcript levels of *XERICO* gene, an ubiquitin E3 ligase involved in ABA metabolism (Zentella R, 2007). The crosstalk between GAs and ethylene falls in the same category, the expression of *ACS5/ETO2* and *ACS8* which encode key ethylene biosynthetic enzyme, is sustained by GAs. In particular, GAs promote DELLA degradation allowing PIF5 to bind to the promoter of *ACS8* and activating its expression (Gallego-Bartolome et al., 2011c). A similar regulation exists between GAs and auxin, DELLAs are able to repress the expression of *INDOLE-3-ACETIC ACID INDUCIBLE19/MASSUGU2* (*IAA19/MSG2*), which encodes a member of

the Aux/IAA family of proteins that negatively regulate auxin signaling (Gallego-Bartolome et al., 2011b).

Finally, the last level of crosstalk comprises the effect of several hormones on GA homeostasis or DELLA expression. Auxin affects GA production by positively regulating the expression of GA biosynthetic genes, especially *GA20ox and GA3ox* (O'Neill and Ross, 2002; Wolbang et al., 2004). Cytokinin seem to have a repressing effect of GA homeostasis, cytokinin is able to induce the expression of the gene encoding the GA-deactivating enzyme GA2ox (Jasinski et al., 2005). Other hormones are able to regulate the expression and stability of DELLA proteins. ABA treatments causes an accumulation of DELLAs in roots (Achard P, 2006). Ethylene inhibit RGA degradation in root-cell nuclei in response to GA (Weiss and Ori, 2007).

Since this thesis will be dealing with a likely new level of regulation between GAs-auxin, a detailed review of auxin homeostasis/signaling and GA-auxin crosstalk will be summarized below.

I.6.1 Gibberellin and Auxin

Recent studies have showed that the GAs and auxin pathways regulate one another on different levels. Taking in mind that GAs and auxin work in synergetic manner to regulate plant development especially in differential growth, root development, response to temperature, the transition from photomorphogensis to skotomorphogenesis and meristem development, that and the fact that using anti-GA antibodies shows that GAs and auxin co-localize at least in roots upon response to gravity (Lofke et al., 2013) points to the direction that GAs and auxin might have higher levels of regulation. Addressing these levels would help us understand even more how these two hormones interconnect to assure the proper plant development.

I.6.1.1 Auxin

Auxin was the first hormone to be studied in plants. In 1881, Sir. Charles Darwin published "The Power of Movement in Plants" where he hypothesized that there would be some kind of signal generated in the coleoptile tips which travels to the elongation zone and causes the shaded side to grow more than the non-shaded part (Darwin, 1880). Based on this idea, Frits Went identified in 1926 the diffusible growth-promoting factor from the tip of oat (*Avena sativa*) coleoptiles and named it auxin. Indole-3-acetic acid (IAA) was determined as the principal natural auxin in the mid-1930s, and since then IAA has always been associated with every aspect of plant growth and development.

I.6.1.2 Auxin Biosynthesis and Conjugation

Auxin biosynthesis is a complex process in plants that occurs in the aerial parts of the plants and in the roots (Ljung K, 2001; Ljung K, 2005). Biochemical and genetic analysis have showed that two pathways exist for IAA biosynthesis: tryptophan (Trp) dependent and Trp-independent (Woodward AW, 2005) reviewed extensively recently (Mano and Nemoto, 2012). The Trp-dependent auxin biosynthesis pathway is categorized into four parts depending on their intermediates, which include the indole-3-acetaldoxime (IAOx) pathway, the indole-3-pyruvic acid (IPA) pathway, the tryptamine (TAM) pathway, and the indole-3-acetamide (IAM) pathway (Figure I.4). The IAOx pathway is catalyzed by cytochrome P450 monooxygenase (CYP79B2/B3 and CYP71A13) which converts Trp to IAOx and IAN respectively. This pathway was identified in glucosinolate producing plant species (Brassicaceae species specific) (Rosquete et al., 2012). IAM pathway was well studied in bacterial system, and it is also

conserved in plants. In this pathway, Trp is converted to IAM and with the help of indole-3-acetamide hydrolase, which is encoded by AMI1 gene IAM, is then converted to IAA (Mano Y, 2010). IPA pathway is the main auxin biosynthesis pathway in Arabidopsis (Mashiguchi K et al., 2011). In this pathway, Trp is converted to IPA with the help of Trp aminotransferase encoded by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) gene. Downstream to TAA1 and IPA acts the YUC proteins which are flavin monooxygenase-like proteins encoded by YUCCA (YUC) gene family. YUC family acts at a rate-limiting step in the IPA pathway and facilitates conversion of IPA to IAA (Mashiguchi K et al., 2011). In TAM pathway tryptophan decarboxylase (TDC) converts Trp to TAM; this pathway is thought to convert TAM to IAOX (Mashiguchi K et al., 2011). Any deficiency in these intermediates or Trp causes various developmental defects during organogenesis. In addition to Trp-dependent pathway in plants, a Trp-independent pathway also operates simultaneously, which was discovered after the study of Trp biosynthetic mutants, and it was found that they can also synthesize IAA without using Trp intermediates, but the detailed pathway is still to be elucidated.

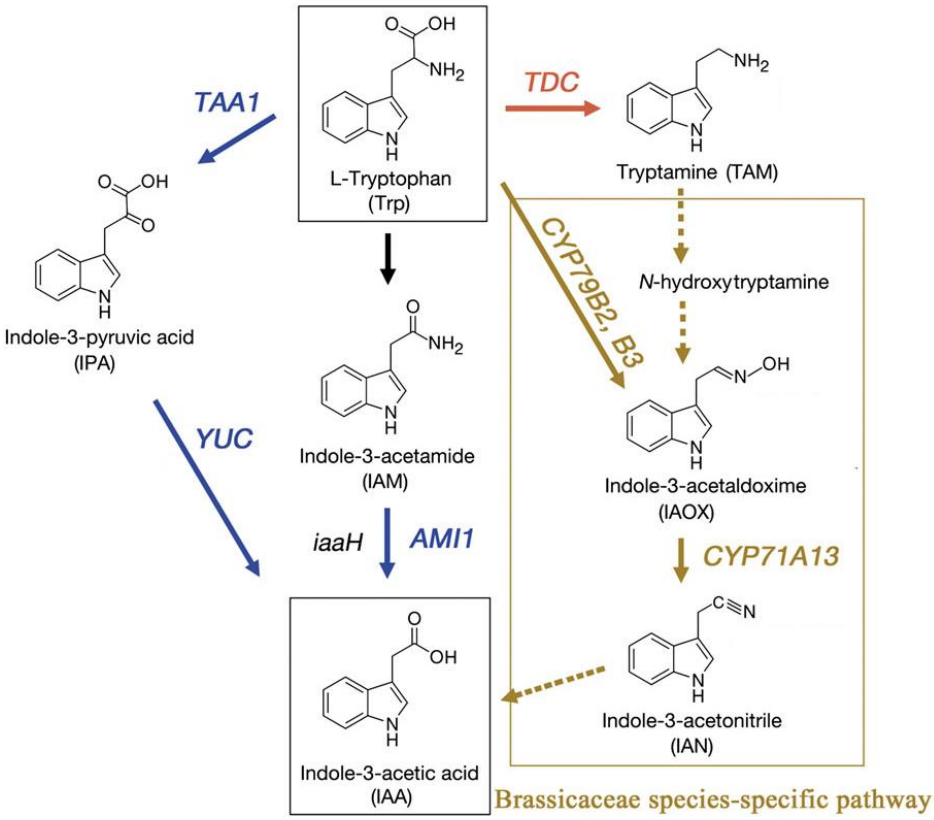

Figure I.4 Presumptive pathways for IAA biosynthesis in plants.
Blue arrows indicate steps for which the gene and enzymatic function are known in the tryptophan-dependent IAA biosynthetic pathway. Red arrows indicate TAM pathway. Mustard-colored arrows indicate the Brassicaceae species-specific pathway. Black arrows indicate steps for which the gene(s) and enzymatic function(s) are unknown. Dashed mustard-colored arrows indicate steps for which the gene and enzymatic function(s) remain poorly understood.
Adapted from Mano and Nemoto, 2012.

IAA can have several fates: it can be directly used by the cell, it can be transported to different tissues to exert its function or it can be conjugated to different molecules. The main purpose of IAA conjugates seems to be storage, although increasing evidence has been found concerning the activity of the conjugates. In general, IAA conjugates can be classified into three groups (Weijers D, 2009), depending on their final form: IAA-amino acid conjugates, IAA-sugar conjugates, or methylated IAA (MeIAA)

(Figure. I.5). Conjugation with amino acids is catalyzed by the activity of GH3 enzymes (Staswick et al., 2005; Park et al., 2007b; Park et al., 2007a). The products of this conjugation include irreversible conjugations that are degraded eventually, such as IAA-Asp and IAA-Glu (Tam YY et al., 2000; Kowalczyk M, 2001; Rampey RA et al., 2004), but other IAA-amino acid conjugates seem to occur in a reversible manner such as IAA-Phe, IAA-Ala and IAA-leu (Le Bail A et al., 2010) and are thought to be important for the regulation of IAA homeostasis. Interestingly, IAA-Trp has been attributed a biological activity, as an inhibitor of IAA-induced growth responses. For instance, IAA-Trp caused agravitropic root growth in seedlings (Staswick PE et al., 2005).

IAA can be also conjugated to sugars such as glucose although the clear function of IAA-Gluc is not yet proved, and it is thought to regulate auxin homeostasis (Tam YY et al., 2000; Kowalczyk M, 2001). The conjugation of IAA to IAA-Glu is catalyzed by the activity of the UDP-glucosyl transferase UGT84B1, while the conversion of IAA-Glu back to IAA is carried out by an IAA-glucose hydrodalse (Jackson RG et al., 2001; Zubieta C et al., 2003).

Finally, MeIAA is produced by the activity of at least the IAA methyl-transferase enzyme encoded by the gene *IAMT1* (Zubieta C et al., 2003), while the conversion back to free IAA *in vitro* is through the effect of methyl esterases, one of which is encoded by the *MES17* gene (Yue Yang, 2008).

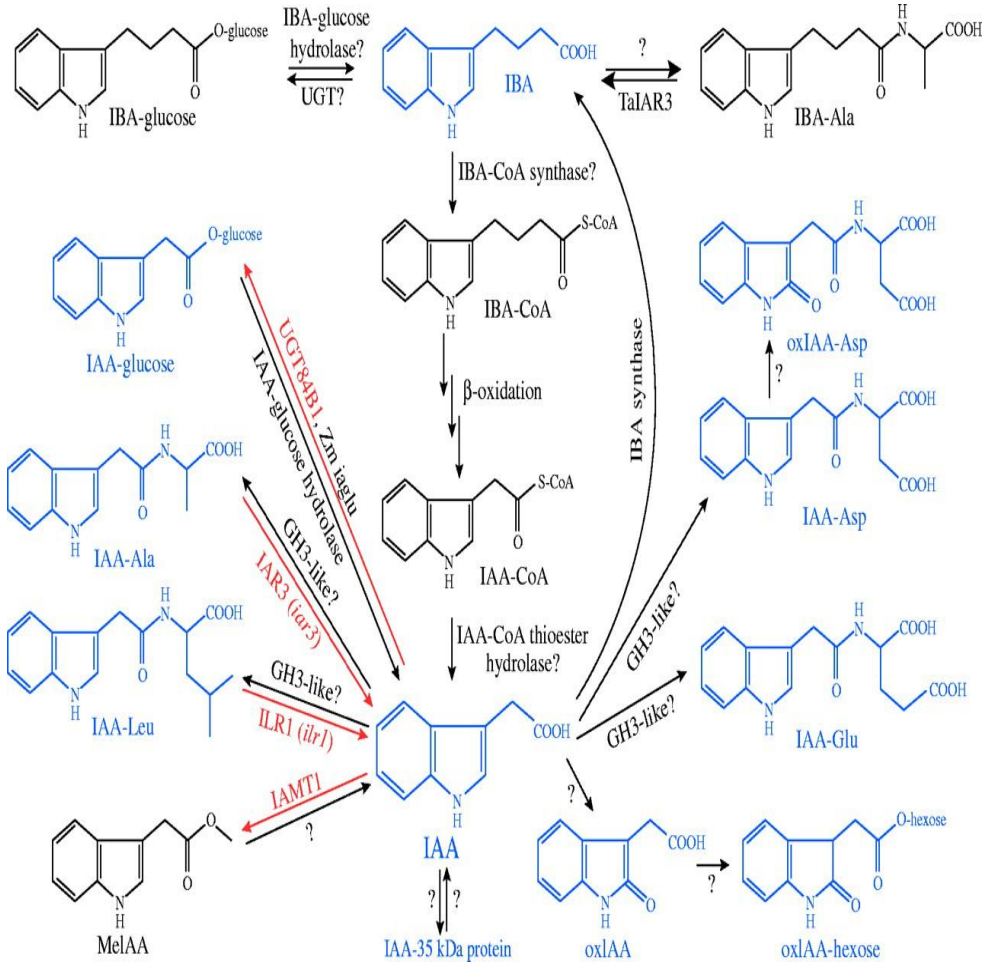

Figure I. 5 Potential pathways of IAA metabolism.
Compounds quantified in Arabidopsis are in blue, enzymes for which the Arabidopsis genes are cloned are in red, and Arabidopsis mutants are in lower-case italics. Suggested conversions for which plant genes are not identified are indicated with question marks. A family of aminohydrolases that apparently resides in the ER lumen can release IAA from IAA conjugates. ILR1 has specificity for IAA-Leu (B Bartel, 1995), whereas IAR3 prefers IAA-Ala (Davies RT, 1999). Arabidopsis UGT84B1 esterify IAA to glucose (Jackson RG et al., 2001); the enzymes that form and hydrolyse IAA-peptides have not been idtenified. IBA is likely to be converted to IAA-CoA in a peroxisomal process that parallels fatty acid β-oxidation to acetyl-CoA (Bartel B, 2001). IAA can be inactivated by oxidation (oxIAA) or by formation of non-hydrolysable conjugates (IAA-Asp and IAA-Glu). IAA-amino acid conjugates can be formed by members of the GH3/JAR1 family (Paul E. Staswick, 2002). OxIAA can be conjugated to hexose, and IAA-Asp can be further oxidized (Östin A, 1998). IAMT1 can methylate IAA (Zubieta, 2003), but whether this activates or inactivates IAA is

not known. IBA and hydrosable 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; the wheat (Ta) enzyme TaIAR3 hydrolyses IBA-Ala (Campanella JJ, 2004).
Adapted from (Woodward AW, 2005).

I.6.1.3 Auxin transport and signaling

For proper plant development, a well-defined transport of auxin is required throughout the system. Various studies have shown that auxin transport is necessary for lateral root development, vascular differentiation, embryonic axis formation, tropisms, and phyllotaxy (Woodward AW, 2005). The site of auxin synthesis and its place of action are separated, and thus, to reach its destination auxin is translocated in a directional manner. Auxin translocates in the plant through two distinct modes. One is from the source to sink, which is faster, non-polar, and passive. In this type of transport, auxins are loaded in mature phloem of biosynthetically highly active tissue and unloaded again in sink tissues, such as the root (Marchant A, 2002). The other is polar auxin transport (PAT), which is slower and more controlled. Correct plant patterning does not only depend on the rates of auxin synthesis and its catabolism, but also on cells' capacity to retain or release auxin. Among the multiple proteins involved in auxin transport, the PIN-FORMED (PIN) family of polarly localized auxin-efflux carriers (Figure I.6) localized in the plasma membrane are the best studied ones. In Arabidopsis there are eight *PIN* genes (*PIN1-8*) and there are orthologs throughout the whole plant kingdom. Auxin influx, on the other hand, is facilitated by the transporters encoded by *AUXIN RESISTANT1* (*AUX1*) and *LIKE AUX1* (*LAX*) *1*, *2*, and *3* (Peer WA, 2011). Other well-characterized auxin carriers include MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (MDR/PGP) transporters, some of which facilitate

auxin efflux while others mediate auxin influx, and members of "B" subclass of plant ATP-binding cassette (ABC) membrane transporters, which function as ATP-dependent amphipathic anion carriers during cellular auxin efflux (Cho et al., 2012).

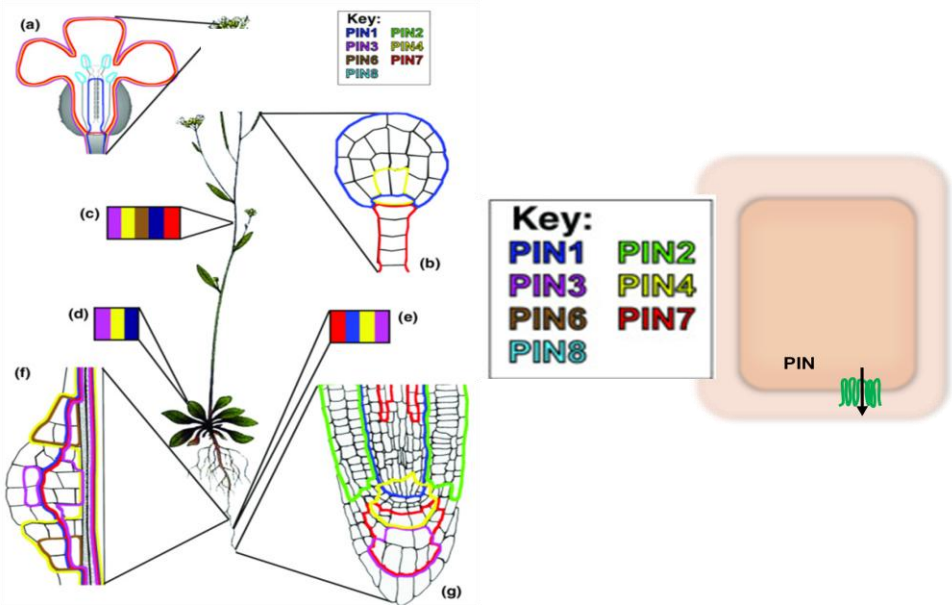

Figure I.6 localization of the PINs throughout Arabidopsis plants.
Adapted from Křeček , P et al., 2009.

Every event related to auxin biosynthesis, metabolism, and transport together collaborate to maintain an optimum auxin level so that a proper auxin signaling and its downstream effects can ensure optimal plant development.

The physiological response of specific cells to auxin can be controlled transcriptionally and non-transcriptionally. The non-transcriptional auxin signaling machinery is not yet well characterized, and AUXIN BINDING PROTEIN (ABP1) is considered an auxin receptor involved in this branch of auxin signaling. ABP1 mediates rapid cell expansion by directly binding to auxin and activating membrane potential and ion channels, which affect

structural changes in cell wall and cytoskeleton (Paque et al., 2014). ABP1 also regulates the clathrin-mediated endocytosis of PIN proteins from the plasma membrane (Hayashi, 2012). The auxin signaling machinery that mediates the transcriptional response is triggered by auxin binding to the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and AUXIN SIGNALING F BOX (AFB) protein receptors. They are integral components of the SKP1/CULLIN1/F-BOX PROTEIN $(SCF)^{TIR1/AFB}$ complex (Kepinski S, 2005). The transcriptional regulators of the AUXIN/INDOLE-3-ACETIC ACID AUX/IAA family then bind to TIR1 through their domain II, and this interaction is enhanced by auxin acting as molecular glue (Lau S et al., 2008). The SCF ligase consists of four subunits Skp1, Cullin, F-box protein, and RING protein Rbx1. The Cullin1 (CUL1) protein acts as the scaffold for Skp1 and Rbx1, whereas F-box protein is attached to Skp1 (Hayashi, 2012). Auxin henceforth promotes Aux/IAAs ubiquitination by TIR1/AFBs E3-ubiquitin ligases and subsequent degradation by the proteasome (Kepinski S, 2005) thus releasing the transcription factors AUXIN RESPONSE FACTOR (ARFs) from the repression exerted by AUX/IAA (Figure I.7) (Gray et al., 2001).

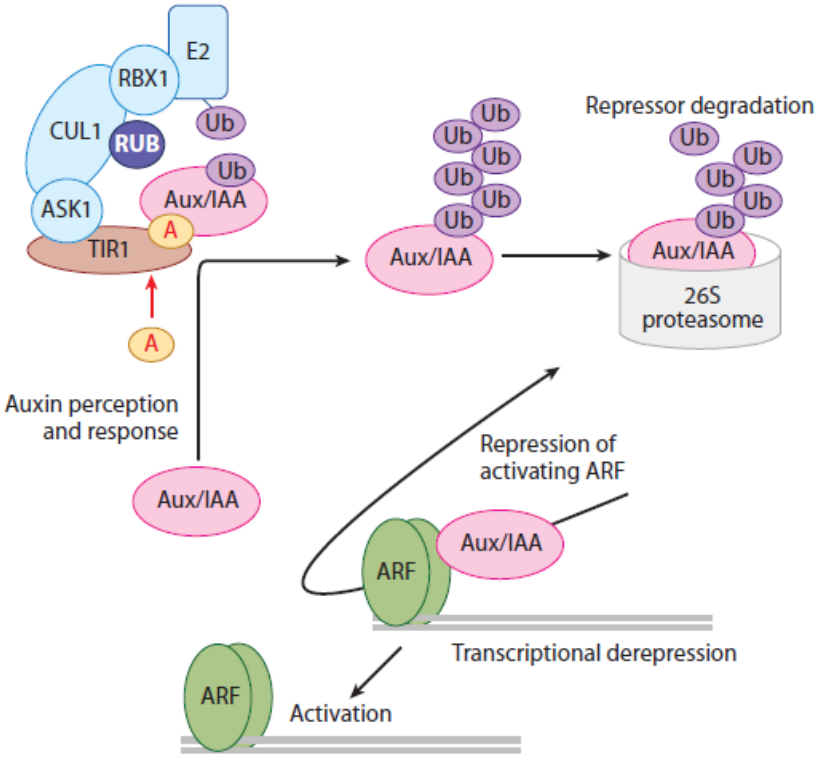

Figure I.7 Mechanism of Auxin signaling and transcription regulation.
A: AUXIN; **TIR1**: TRANSPORT INHIBITOR RESPONSE 1; **AUX/IAA**: AUXIN/INDOLE-3-ACETIC ACID; **ASK1, CUL1, RBX1, E2 ligase, RUB**: subunits of the SCF ligase; **ARF:** AUXIN RESPONSE FACTOR.
Adapted from Keithanne Mockaitis and Mark Estelle 2008.

I.6.1.4 Tools in auxin research

Many of our recent insights into auxin action are a consequence of our ability to examine auxin accumulation, action, and transport at the cellular level. The traditional and most precise method for IAA quantification is tissue extraction followed by gas chromatography–mass spectrometry. Until recently this method was unable to provide cell-specific information, but a refinement has been developed in which cells can be isolated from Arabidopsis roots and sorted based on their expression of cell type–specific

green fluorescent protein (GFP) constructs. Using this method, the auxin concentration of pools of homogenous cell types has been measured by gas chromatography–mass spectrometry, greatly refining our knowledge of auxin concentrations throughout the root and confirming the presence of a significant auxin maximum at the quiescent center of the root. Auxin-specific antibodies also have been used with varying degrees of success to analyze auxin distribution using immunolocalization methods. Recently characterized auxin synthesis inhibitors will help explore auxin's roles as well as help define the auxin biosynthetic pathway in different tissues.

Auxin responses have been examined using two artificial sensors. The first one uses a synthetic auxin responsive promoter called *DR5*, fused to a reporter gene (*GUS*) encoding β-glucuronidase (which cleaves a colorless substrate to produce a blue precipitate) or to GFP (Nakamura et al., 2003). As this sensor monitors transcription of auxin responsive genes, it is termed an "output sensor". A second sensor, called DII-VENUS, a constitutively expressed protein consisting of a nuclear-localized yellow fluorescent protein (YFP) fused to an amino acid sequence that promotes proteolysis in the presence of auxin, the degron domain II of Aux/IAA protein. At high auxin levels, the fluorescent protein is degraded, leading to non-fluorescent regions against a background of fluorescence. Often, the spatial patterns conferred by the DR5 and DII-VENUS reporters are complementary.

An important tool for examining patterns of auxin movement (from which some rough approximation of auxin levels can be inferred) comes from the study of auxin transport proteins (Brunoud et al., 2011). Auxin movement through tissues is conferred to a large extent by the polar distribution of auxin influx and efflux proteins. For example, examining localization in

neighboring cells of auxin efflux proteins of the PIN family gives an approximate picture of the direction of local auxin transport within a tissue. Using this method to monitor living tissues over time, very dynamic changes in PIN protein orientation have been revealed, as well as the important roles of auxin and auxin transport during development. Finally, computer models and simulations based on experimental data replicate and predict patterns of auxin accumulation and support the interpretation that auxin has a key role in developmental patterning.

I.6.2 Gibberellin and Auxin crosstalk

It has always been clear that GAs and auxin affect overlapping developmental processes. For instance, both hormones seem to participate in the signals that trigger early fruit development in response to fertilization in many different species, including pea (*Pisum staivum*) and Arabidopsis (van Huizen et al., 1995; Vivian-Smith and Koltunow, 1999), as well as in the promotion of organ expansion, such as the Arabidopsis hypocotyl (Smalle et al., 1997; Collett et al., 2000) and pea stems (Ross and O'Neill, 2001), or in the promotion of Arabidopsis root growth (Fu XD, 2003). Although it is possible that these two hormones regulate these processes independent from one another it is possible that the output of these actions depends on a specific combination of both rather on the independent activities of each. This is evident in the synergetic induction by GAs and auxin on the pericarp growth in pea fruits (Ozga and Reinecke, 1999), or hypocotyl and stomata development, for which GAs are absolutely required, but their action is modulated by auxin and ethylene (Saibo et al., 2003), GA stimulation of root elongation seem also to require auxin (Fu XD, 2003).

Extensive studies revealed that the ability of GAs and auxin to dependently control developmental processes is derived from the fact the GAs and auxin exhibit high levels of crosstalk. In fact, this crosstalk can be observed by the fact that these two hormones are able to control the biosynthesis, transport and signaling of one another. The DELLA dependent regulation of auxin biosynthesis can be seen through the ability of GAI to regulate the expression levels of the *YUC3* gene (Gallego-Bartolome et al., 2011a), an enzyme in the biosynthesis reactions of IAA. GAs apparently are able to control the levels of auxin present by regulating its transport. In the presence of DELLAs the levels of the PIN transporters seem to decline (Lofke et al., 2013). GAs are also crucial to maintain auxin signaling, the levels of the auxin induced genes such as *IAA5*, *IAA6* and *IAA19* decreases upon the accumulation of DELLAs (Figure I.8)(Gallego-Bartolome et al., 2011a).

In the same sense, auxin seem to have developed its own network of control over the different steps of GA pathway (Figure I.9). For instance, ARFs are able to regulate GA levels by inducing the expression of enzymes in the GA biosynthesis pathway, such as $GA20_{ox1}$ and $GA3_{ox1}$ (O'Neill and Ross, 2002). On the other hand, IAA plays a role in regulating GA signaling by decreasing DELLA levels (Fu XD, 2003).

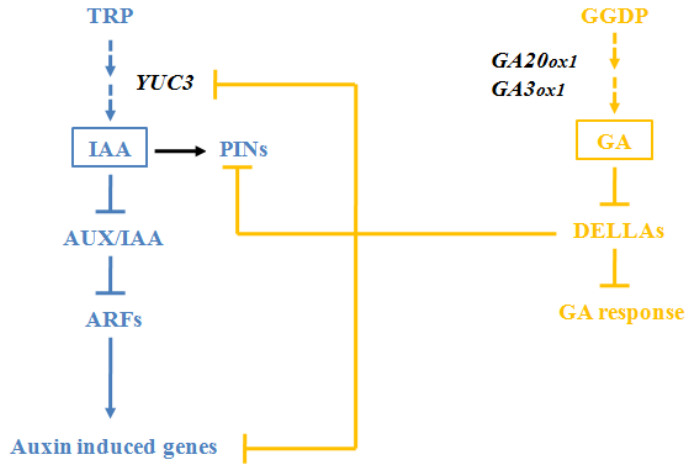

Figure I.8 A schematic representation of the GA pathway regulating IAA synthesis, transport and signaling.

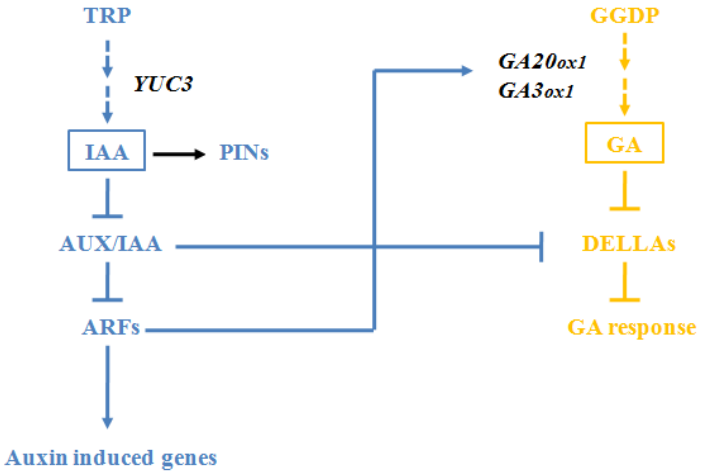

Figure I.9 A schematic representation of the IAA pathway regulating GA synthesis and signaling.

It has long been recognized that auxin is critically and fundamentally important in the life of a plant and that its localized synthesis, transport, and response underlie plant growth and development. With each new tool in our inquiry toolbox, auxin researchers have pushed forward our understanding of these complex phenomena. Thanks to genomic sequence information

from many plants, along with the powerful gene discovery approaches from Arabidopsis genetics, we now know the identity of many of the genes that contribute to auxin biosynthesis, transport, perception, and downstream signaling responses and some of the ways these genes respond to each other and other signaling pathways. Our ability to image auxin flow and response in vivo has shown us the phenomenally dynamic auxin distribution patterns that coordinate development.

I.6.3 A possible new mechanism: regulation of IAA methylation by GAs

Recently, new data from our laboratory has suggested the existence of a previously unsuspected point of regulation of auxin homeostasis by GAs. In a search for new direct transcriptional targets of DELLA proteins, the *IAMT1* gene was one of the most strongly up-regulated in response to conditional activation of gai-1:GR (Gallego-Bartolome et al., 2011a). The up-regulation of *IAMT1* by DELLAs was confirmed with other mutants that accumulate DELLA proteins and, more importantly, it still occurred in *GAI::gai-1-GR* seedlings supplemented with cycloheximide and dexamethasone, indicating that the up-regulation happened without the requirement for the synthesis of any additional protein (i.e., *IAMT1* is a direct target for DELLAs) (Gallego-Bartolome et al., 2011a). This Thesis has been devoted to the study of the physiological relevance of IAA methylation, and its regulation by GAs as a possible mechanism to control IAA homeostasis and/or IAA action during development.

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OBJECTIVES

The observation that *IAMT1* expression is strongly upregulated by DELLA proteins has led us to hypothesize that this mechanism may be relevant for the interaction between GAs and auxin in the control of certain aspects of plant development. Therefore, the two main objectives pursued in this thesis are:

1. Unveiling the developmental processes in which IAA methylation is physiologically relevant.

   This will be approached by procuring *iamt1KO* mutants and examining their phenotype in those aspects related to auxin action, in particular differential growth.

2. Identifying the molecular mechanism by which DELLAs regulate the expression of *IAMT1*.

   The main approach will consist in the analysis of serial deletions of the *IAMT1* promoter to identify the responsible *cis* elements, and the corresponding DELLA interactors.

IAMT1 is a carboxyl methyltransferase with a high specificity towards IAA (Zubieta, 2003). Structural, biochemical and phylogenetic analysis showed that IAMT1 is an evolutionary ancient member of the SABATH family (Zhao et al., 2008), a family of methyltransferases first described in 2003 (D'Auria JC et al., 2003). The SABATH family includes methyltransferases with different targets (including hormones) such as salicylic acid, benzoic acid, jasmonic acid, farnesoic acid, and gibberellic acid, apart from IAA (Zhao et al., 2008). Interestingly, the rice genome contains 41 members of the *SABATH* gene family, while Arabidopsis contains only 24 members, with only one ortholog for *IAMT1* in each species (Zhao et al., 2008).

The relevance of the SABATH family is reflected by the conjugates they produce (Table II.1). Methyl-salycilate and methyl-benzoate, products of SALICYLIC ACID METHYLTRANSFERASE (SAMT) and BENZOIC ACID METHYLTRANSFERSE (BAMT) respectively, are important components of floral scents, also produced by vegetative organs in response to environmental challenges (Chen F, 2003). JASMONIC ACID METHYLTRANSFERASE (JMT) and its product methyl-jasmonate (MeJA) seem to be involved in plant defense (Seo et al., 2001), and GIBBERELLIC ACID METHYL TRANSFERASE 1 and 2 produce methyl-GA, whose function is still unknown (Varbanova et al., 2007).

IAMT1, on the other hand, seems to be involved in leaf development. Over expressing IAMT1 leads to dramatic hyponastic leaf curvature phenotype, while plants with low *IAMT1* mRNA levels due to the expression of interfering RNA (iRNA) display an epinastic leaf phenotype (Qin et al., 2005).

Table II.1: Different hormone methyltransferases, their targets and possible functions.

Substrate	Enzyme	Product	Relevance	Reference
Salicylic acid (**SA)**	SAMT	MeSA	Component of floral scents.	(Chen F, 2003)
Benzoic acid **(BA)**	BAMT	MeBA	Component of floral scents	(Chen F, 2003)
Jasmonic acid **(JA)**	JMT	MeJA	Plant defense	(Seo et al., 2001)
Farnesoic acid **(FA)**	FAMT	MeFA	unknown	(Zhao et al., 2008)
Indole-acetic-acid **(IAA)**	IAMT	MeIAA	Leaf development	(Qin et al., 2005)
Gibberellic acid **(GA)**	GAMT	MeGA	unknown	(Varbanova et al., 2007)

IAMT1 seems to share high levels of similarity with other methyl transferases such as *SAMT*, *GAMT1*, *GAMT2*, and *JMT* (Zhao et al., 2008; Zhao et al., 2009; Zhao et al., 2012). Therefore, it is likely that the severely defective phenotype observed in iRNA lines for *IAMT1* is in fact caused by simultaneous inactivation of more than one methyl transferase (Qin et al., 2005). We thus decided to isolate *bona fide iamt1* knockout (*iamt1KO*) mutants among the public collections of T-DNA insertion lines.

III.1 Characterization of *iamt1* mutants

Genetic analysis shows that *IAMT1* exists in two different isoforms AT5g55250.1 and AT5g55250.2 (Figure III.1). At5g55250.1 encodes a protein with 386 amino acids while At5g55250.2 encodes a protein with 348 amino acids, the alternative splicing that generate AT5g55250.2

removes the 5' UTR region (1-90 bp). Although alternative splicing results in different variant sizes, both variants contain the SAM dependent carboxyl methyltransferase domain.

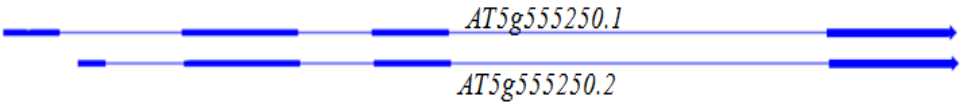


Figure III.1 schematic representation of the two isoforms of *IAMT1*.
Thick bars represent exons, thin bars represent introns.

In this thesis two T-DNA lines have been used: SALK_072125 in Columbia background (Col-0), henceforth termed *iamt1-1*, that contains an insertion in the third exon; and GT_5_41946 in Landsberg *erecta* (L*er*) background, henceforth termed *iamt1-2*, that contains an insertion in the fourth exon (Figure III.2).

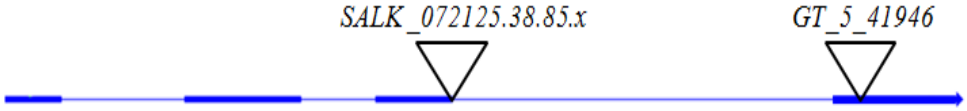


Figure III.2 schematic representation of the two insertion sites of *iamt1-1* and *iamt1-2*.

The T-DNA insertion line *iamt1-1* is located at the end of the third of the four exons, and results in a truncated transcript of around 800 bp (Qin et al., 2005). The amino acids deleted in this protein such as Leu-226, Leu-242 and Phe-243 form the carboxyl bearing substrate binding pocket in IAMT1, together with Phe-158, Val-326 and Phe-346 (Fig. III.3)(Zhao et al., 2008). Moreover the presence of Gly-226 (absent in the truncated IAMT1 variant of *iamt1-1*) is essential to create the space needed for the recognition and binding of the indole ring in IAA (Zubieta, 2003). The absence of these highly essential amino acids in the truncated variant produced in *iamt1-1* implies that it is unable to recognize, bind nor methylate IAA.

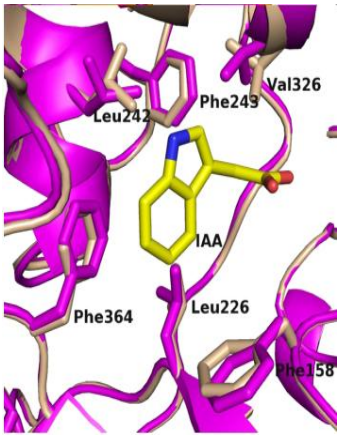


Figure II.3: The binding of IAA (yellow) to *IAMT1* (magenta) and the amino acids needed for the completion of the interaction and conjugation.
Adapted from Zhao *et al.*, 2008.

Homozygous lines with the *iamt1-1* and *iamt1-2* alleles were selected using two sets of oligos flanking the insertion sites and one extra oligo in the insertion itself for genotyping (Figure III.4A).

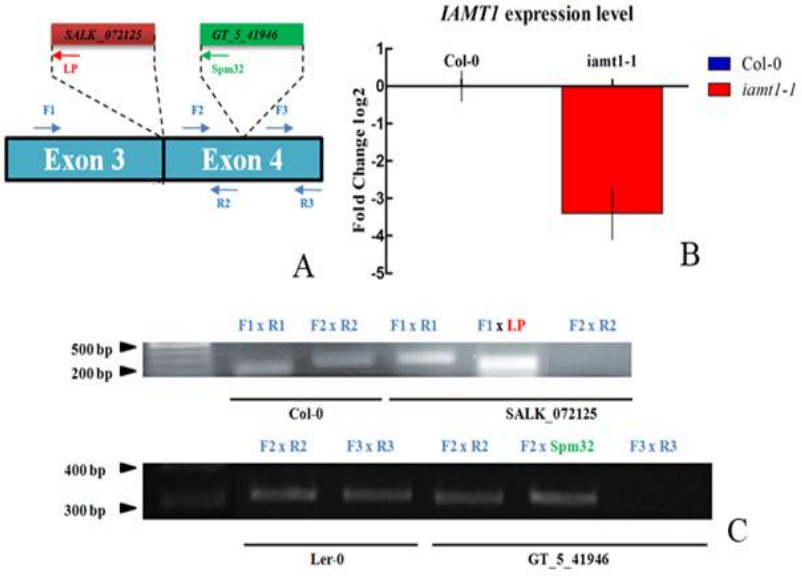


Figure III.4: Genotyping*iamt1-1* and *iamt1-2* and measuring their transcript levels.

(A): Position of the transposons of *iamt1-1* in the third exon and of *iamt1-2* in the fourth exon and the position of the genotyping primers; (B): Transcript level of *IAMT1* in Col-0 and *iamt1-1*; (C) SQ-PCR for *iamt1-1* and *iamt1-2* with primers flanking the insertion sites and universal primers in the transposons.
Error bars represent the variation of three technical repeats for 3 biological replicates.

Our results show that while all PCR reactions using combinations (F1-R1, F2-R2 and F3-R3) in wild type plants result in the corresponding amplicons; the combination of F1-R1 and F1-LP resulted in PCR products for the SALK_072125 line; and F2-R2 did not (Figure III.4C). On the other hand, F2-R2 and F2-Spm32 result in PCR amplification from *iamt1-2*, while F3-R3 did not (Figure III.4C). Furthermore, transcript analysis in *iamt1-1* and *iamt1-2* by RT-qPCR showed decreased levels of *IAMT1* expression (Figure III.4B, Suppl. III.1). These results indicate that neither *iamt1-1* nor *iamt1-2* are able to produce a full cDNA.

To confirm that the insertions in *IAMT1* rendered an inactive enzyme, MeIAA levels were quantified in wild-type and *iamt1-1* mutant seedlings. MeIAA measurements showed that *iamt1-1* indeed have lower levels of MeIAA in light-grown and etiolated seedlings compared to Col-0 (Figure III.5). The presence of low levels of MeIAA in *iamt1-1* might be due to the existence of non-specific methyltransferases that are able to conjugate free IAA.

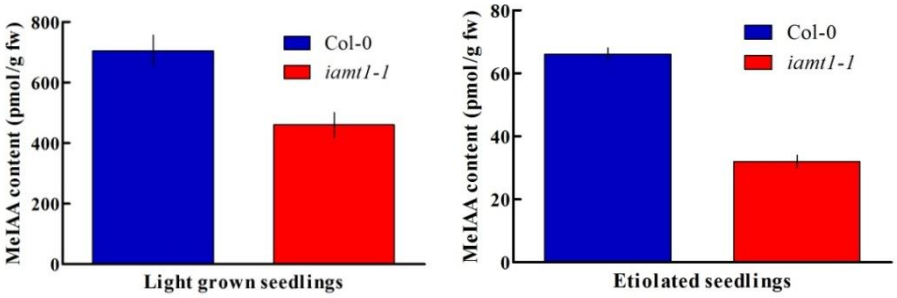


Figure III.5 MeIAA content in Col-0 compared to *iamt1-1* of light grown (left) and etiolated seedlings (right).
Error bars represent the variation of 3 different measurements.

III.2 IAMT1 and differential growth

To investigate the biological relevance of IAMT1 in plant development, we examined several auxin-related processes affecting aerial development in the *iamt1KO* mutants, such as hypocotyl growth, the formation of the apical hook in etiolated seedlings, and gravitropism.

Both *iamt1* mutants showed longer hypocotyls in darkness compared to wild type, probably due to faster germination, since the mutants did not undergo the initial lag phase observed in the wild type (Figure III.6, Suppl. III.2).

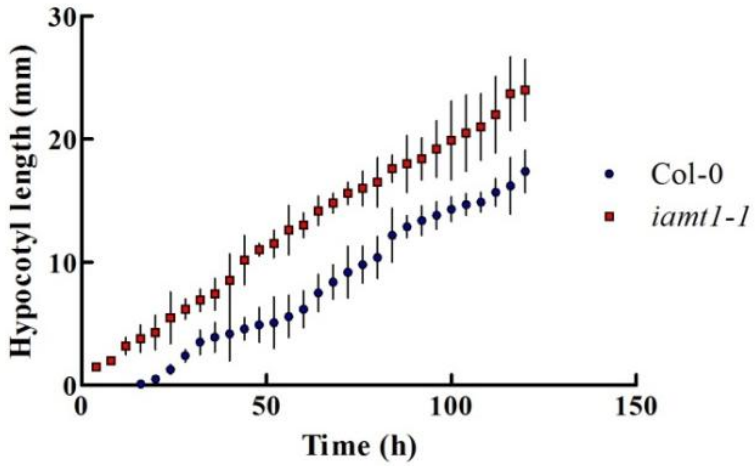


Figure III.6 Growth rate of *iamt-1* mutant compared to wild type in etiolated seedlings.
Seeds were sown on MS and subjected to a 6-h light treatment to promote germination, after which their growth was monitored in darkness with Infrared (IR)-cameras. Error bars represent SD (n=15-20 seedlings).

The apical hook is a developmentally regulated structure that appears in dicotyledonous seedlings when seeds germinate buried in the soil. It

protects the shoot apical meristem and cotyledons from damage while the seedling is pushing upwards seeking for light, and it is formed by differential cell expansion between both sides of the upper part of the hypocotyl. Its apparent simplicity and the fact that it is dispensable when seedlings are grown in vitro have converted the apical hook in one of the favorite experimental models to study the regulation of differential growth. The involvement of hormones –especially auxin—in this process was manifested already in the early studies. Remarkably, a gradient of this hormone across the hook curvature is instrumental to complete its development, similar to what has been proposed for other processes involving the bending of an organ, such as tropic responses. In agreement with this, other hormones—mainly gibberellins and ethylene—and the light, regulate in a timely and interconnected manner the auxin gradient to promote hook development and its opening, respectively (Figure III.7).

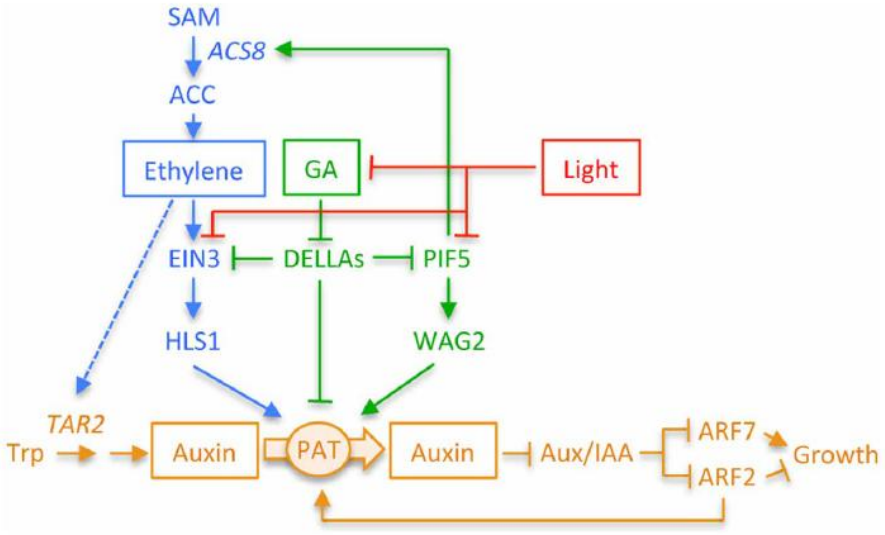


Figure III.7 Diagram depicting the interactions between GAs, ethylene, and light signaling pathways in *Arabidopsis* and how they modulate the auxin response in the apical hook.
Reviewed by Abbas *et al.*, 2013.

Apical hook development undergoes three different phases: the formation phase (characterized by the cotyledons bending to form a 180 degree structure), the maintenance phase (characterized by elongation of the hypocotyl as the seedlings protrude through the soil in search for light), and the opening phase (in which the apical hook unfolds to allow the cotyledons to expand)(Abbas et al., 2013).

To evaluate whether *iamt1KO* mutants have defects in hook development, seedlings were grown on vertical plates after germination, and images were taken every half an hour. The apical hook angle was measured using imageJ software. While *iamt1-1* and *iamt1-2* had no obvious difference in hook formation or in maintenance phases compared to the respective wild-type seedlings, the opening phase seemed to be faster in both mutants: while wild type seedlings required almost 6 days to completely unfold the hook, *iamt1-1* and *iamt1-2* were able to do that in only 4 days (Figure III.8, Suppl. III.3).

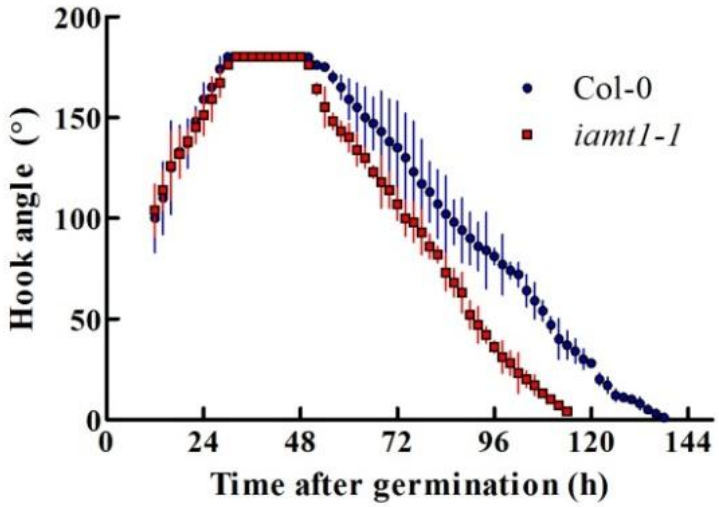


Figure III.8 Kinetics of apical hook development of the *iamt1-1* mutant in correspondence to the wild type ecotype.

Seeds were sown on MS and subjected to a 6-h light treatment to promote germination, after which hook angle was monitored in darkness with IR-cameras. Error bars represent SD (n=15-20 seedlings).

In addition to a faster growth rate and a faster opening phase of the apical hook, *iamt1* mutants seem to have additional defects in differential growth, and specifically in gravitropic reorientation. Darwin was the first to describe that plants can sense their environment and orient themselves for optimal growth and development (Darwin, 1880). Among the signals that promote a tropic response in plants, gravity is unique in that it is constant and unidirectional. In addition, it generally induces the underground tissues to bend toward the signal, and the aerial parts against the stimulating vector. Like in other tropisms, when plants perceive a change in their position relative to the gravity vector, they respond by differential growth on either side of the affected organ (Esmon et al., 2005), and several hormones have been involved in the control of these responses. Among them, auxin is instrumental because it forms a lateral gradient in response to the stimulus and thus establishes the framework for the differential growth (Rashotte et al., 2000; Esmon et al., 2006). The differential response to auxin on either side of an organ has been shown to depend on the correct functioning of polar auxin transport and activity of auxin efflux carriers (Friml et al., 2002).

Plants were grown in darkness on vertical plates for three days, then the plateswereturned90 degrees and the bending response was monitored. Our results shows that the hypocotyls of*iamt1* mutants were largely impaired in gravitropic reorientation, and remained in a partially horizontal state (Figure III.9, Suppl. III.4).

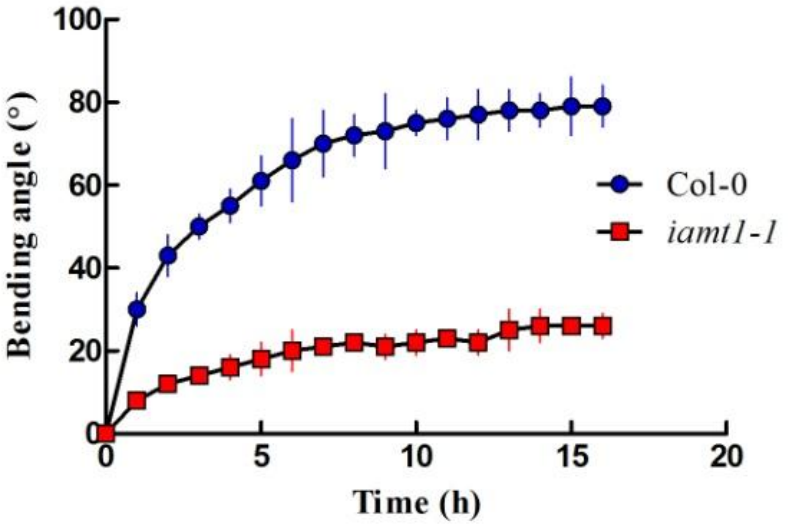


Figure III.9 Kinetics of gravitropic reorientation of the *iamt1-1* mutant in correspondence to the wild type ecotypes.
Seedlings were grown for 3 days in darkness on vertical plates. At time zero, the plates were turned 90º and gravitropic reorientation was monitored with IR-cameras. Error bars represent SD (n=15-20 seedlings).

III.3 IAMT1 and IAA levels

Since differential growth is generated and maintained through an auxin gradient, it is reasonable to suggest that the defects displayed by the *iamt1* mutants with respect to differential growth are due to increased levels of IAA. To test this hypothesis, we quantified IAA levels in 3-day-old light- and dark-grown seedlings. IAA measurements did not show any significant difference in IAA content in whole seedlings (Figure III.10). Considering that Me-IAA is usually only 2% of the free IAA, this result is not striking. There are at least two possibilities to reconcile these results with the defective differential growth observed in the *iamt1* mutants; (1) the mutants could indeed have local auxin changes, not detectable in whole extracts; or

(2) Me-IAA itself presents a potent –and yet unknown– biological activity that antagonizes IAA action.

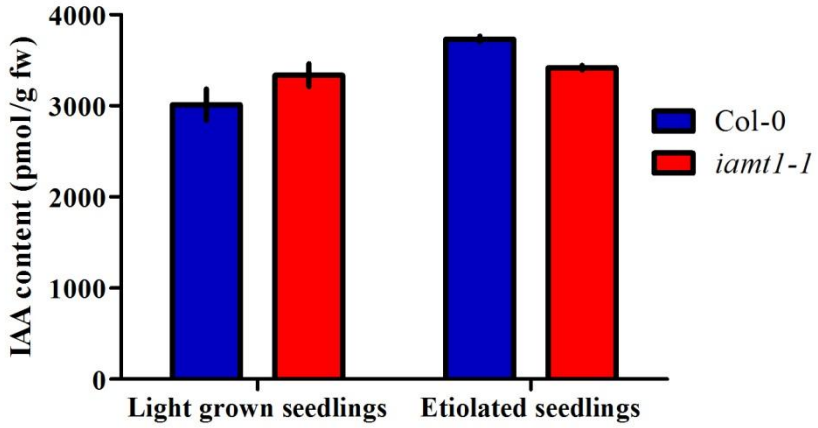


Figure III.10 IAA content in Arabidopsis seedlings
Error bars represent SD (n=3 biological replicates).

III.4 Local defects in auxin accumulation in the *iamt1* mutants

To check the possibility that the *iamt1* mutant provokes altered distribution of auxin without dramatically affecting the overall levels, we decided to introduce the *DR5rev*::*GFP*, *DR5*::*GUS* (Sabatini et al., 1999; Friml et al., 2003a) and DII-VENUS (Brunoud et al., 2011) auxin reporters into the mutant background and examine their localization during hook development and gravitropic reorientation –the two processes that we have found to be defective in the *iamt1* mutants.

As previously reported, the activity of auxin is stronger in the inner (concave) than in the outer (convex) side of the apical hook of wild-type seedlings, based on *DR5*::*GFP* examination (Figure III.11). Importantly, the intensity of the GFP signal was much higher in the *iamt1-1* mutant,

although a clear gradient was still visible, at least at the maintenance phase. This result is in agreement with the wild-type phenotype of *iamt1* hooks up to that phase, and also indicates that the lack of IAA methylation indeed affects the accumulation of auxin or its downstream signaling events.

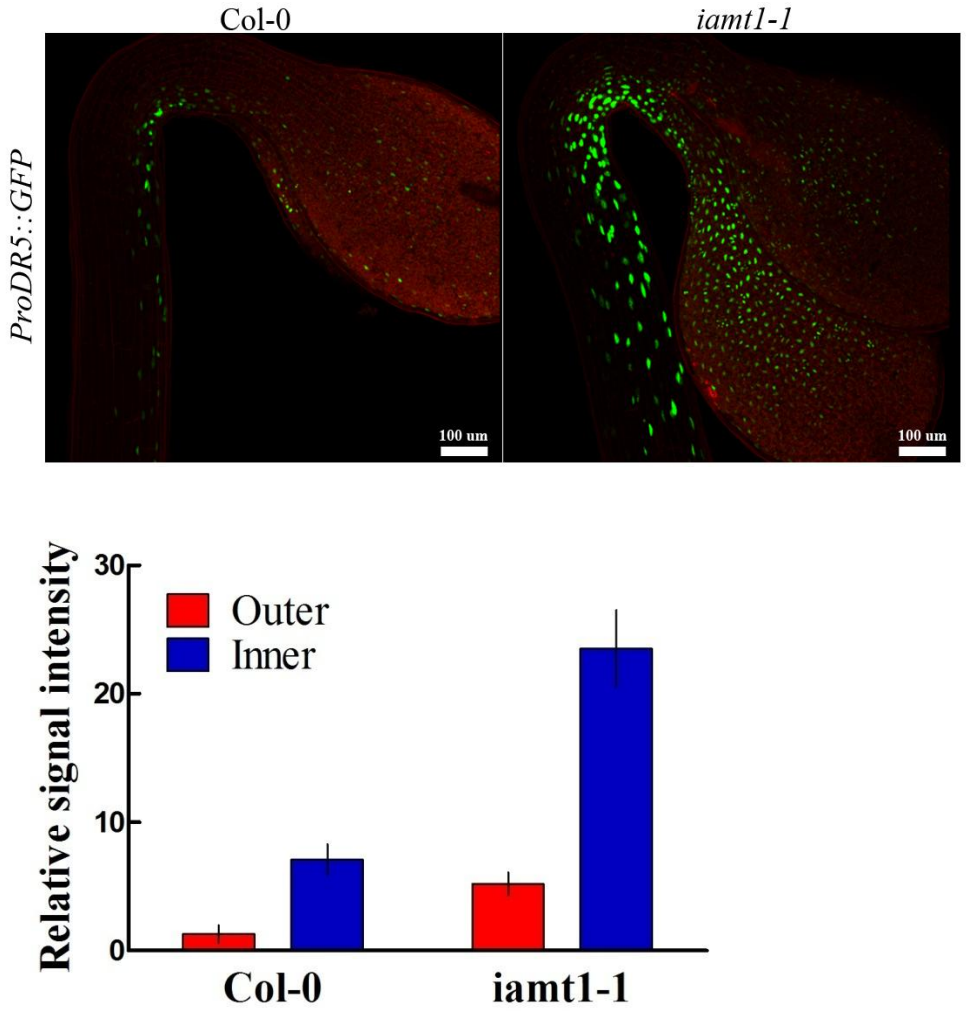


Figure III.11 Expression levels and magnitude of *DR5::GFP* in Col-0 and *iamt1-1*.
The graph shows the average of the intensity of the GFP fluorescence in either side of the hooks, analyzed from the confocal images with ImageJ software. Error bars represent SD (n>10).

On the other hand, examination of *DR5::GUS* along hook development revealed that auxin activity was higher in the *iamt1* mutant in all phases, and the quicker opening of the hook in the mutant was paralleled by the disappearance of the auxin gradient through expansion of auxin action towards the outer side of the hook (Figure III.12).

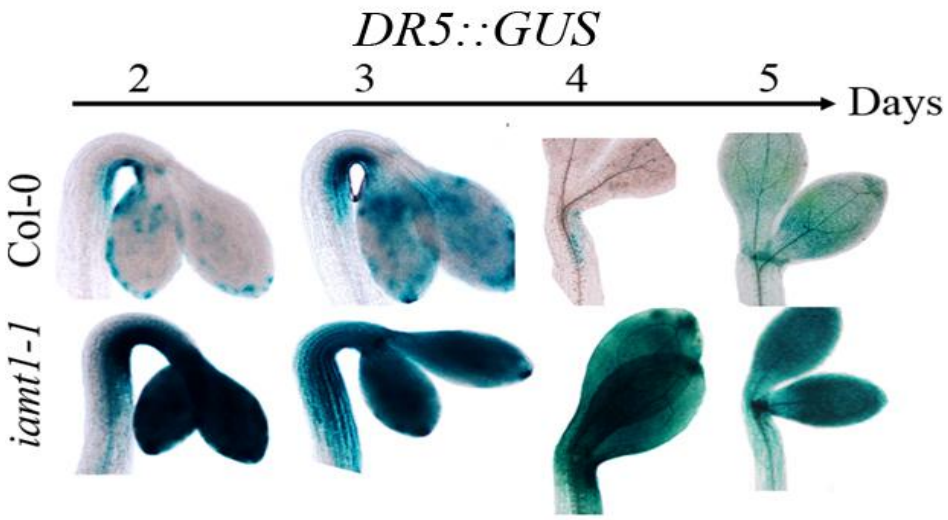
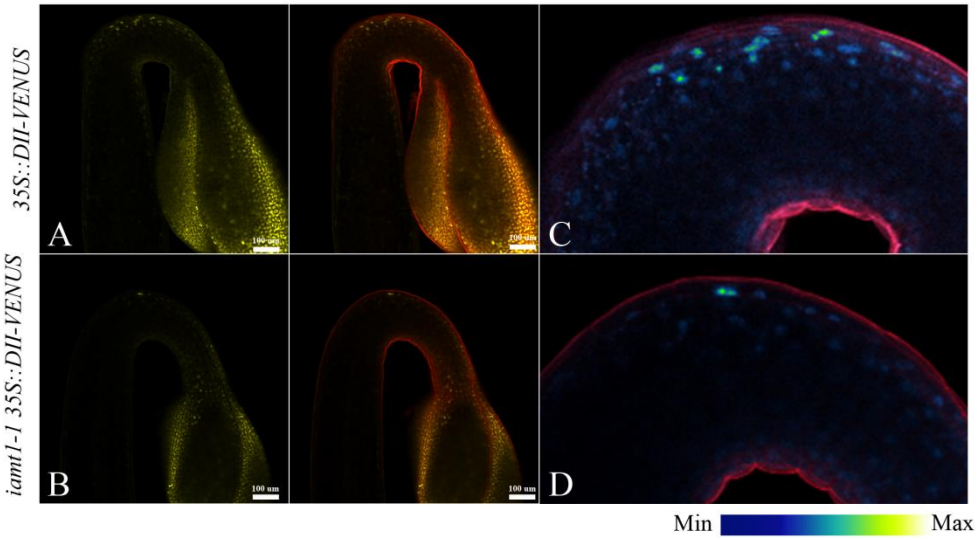


Figure III.12. Expression of *DR5::GUS* in the *iamt1* mutant.

The expression of the DR5 reporters very likely reflects the accumulation of auxin, rather than an effect of *iamt1* on auxin signaling, because equivalent results were observed in the hooks of *iamt1-1 35S::DII-VENUS* seedlings (Figure III.13).



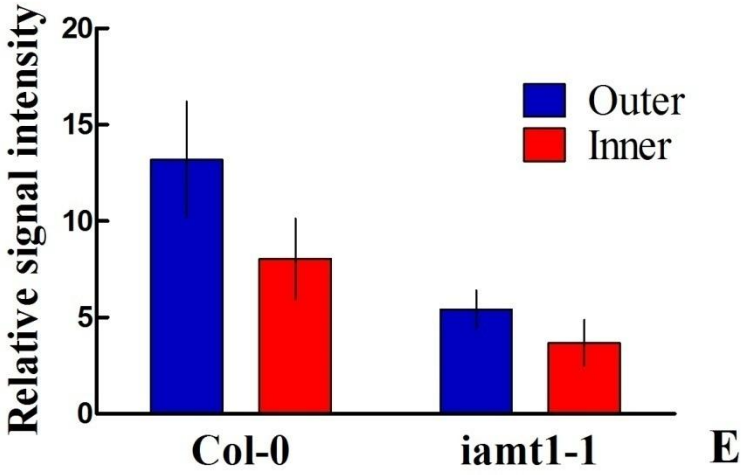


Figure III.13 Localization of*35S::DII-VENUS* in apical hooks of the *iamt1* mutant.
(A,B) localization of DII-VENUS in the apical hook of Col-0 and *iamt1-1*; (C,D) a close-up on the intensity and localization of DII-VENUS in the apical hook of Col-0 and *iamt1-1*; (E) The graph shows the average of the intensity of the VENUS fluorescence in either side of the hooks, analyzed from the confocal images with ImageJ software. Error bars represent SD (n>10).

In a similar way, the expression of *DR5*::*GFP* and DII-VENUS during gravitropic reorientation also supported the view that the *iamt1* mutant

causes excessive accumulation of auxin on both sides of the hypocotyl, preventing the gradient to be formed. First, *DR5*::*GFP* signal was stronger in the hypocotyls of *iamt1* mutant seedlings even before reorientation (Figure III.14A,B,E,F), and, although the signal increased upon reorientation in both the wild type and the mutant hypocotyls, a clear difference was observed between the upper and lower sides of the gravistimulated hypocotyls in the wild type, but not in the mutant (Figure III.14C,D,E,F). Second, the pattern of DII-VENUS accumulation was perfectly complementary to that of *DR5*::*GFP*, indicating that the hypocotyls of *iamt1* mutants are defective in the formation of the auxin gradient upon reorientation (Figure III.15).

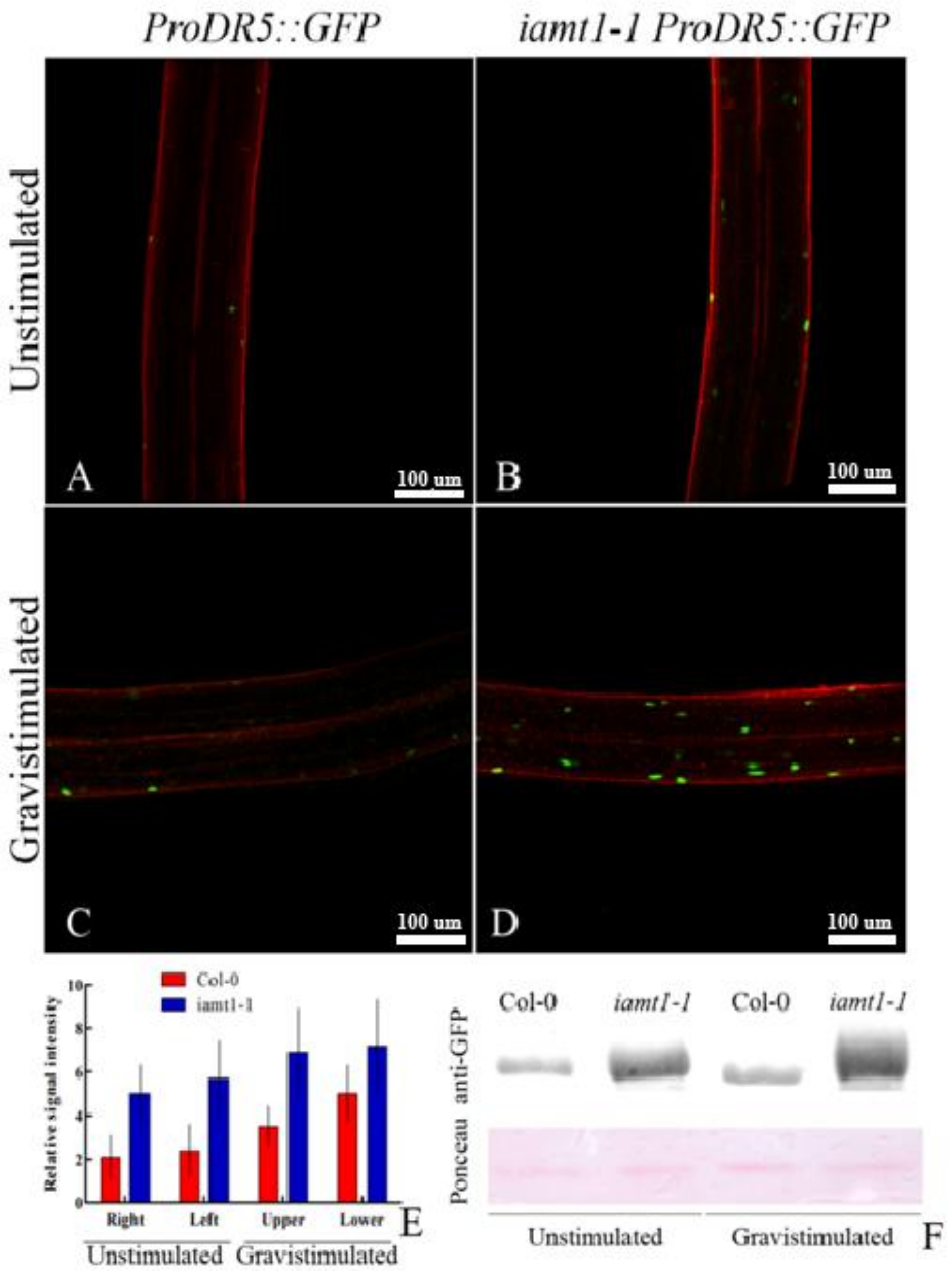
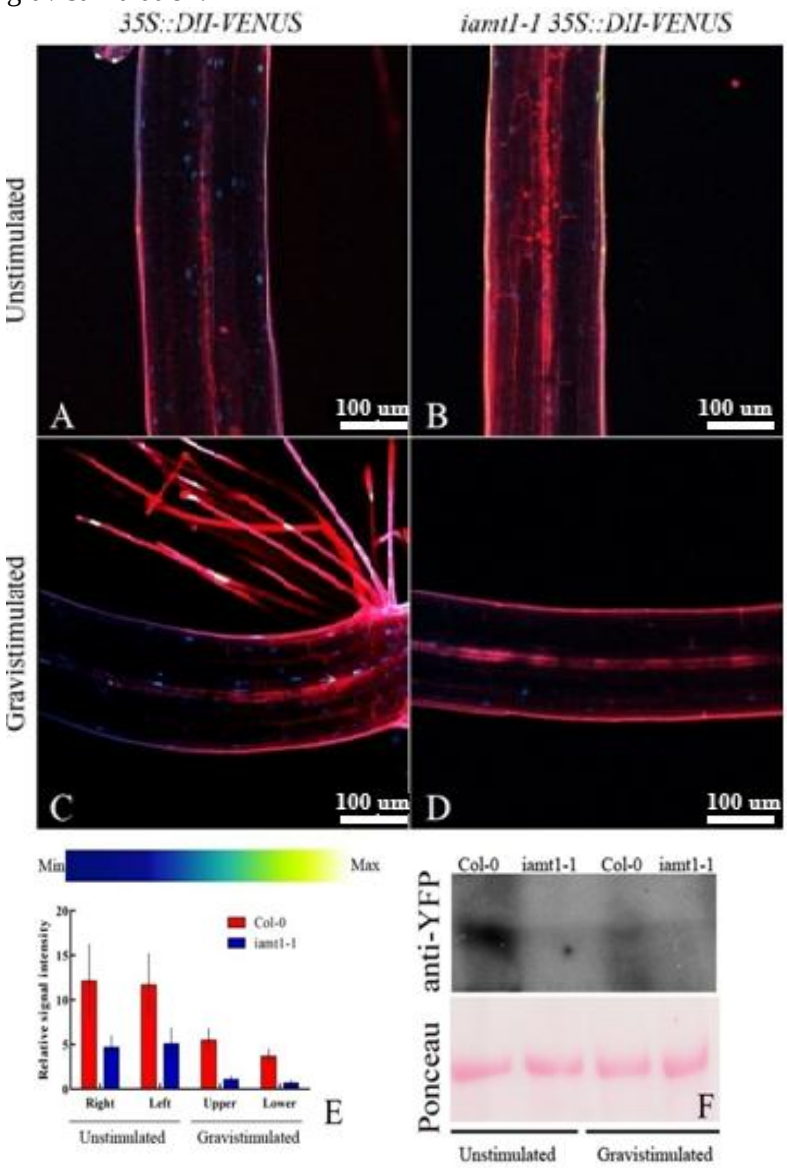


Figure III.14 Expression pattern and magnitude of *DR5::GFP* in Col-0 and *iamt1-1* in three days old unstimulated and 6 hours gravistimulated seedlings. (A, B) Unstimulated three day old Col-0 and *iamt1-1* seedlings respectively showing the expression of GFP in the hypocotyl; (C, D) Gravistimulated Col-0 and *iamt1-1* after 6 hours or orientation showing GFP gradient in Col-0 and the absence of the gradient in *iamt1-1*; (E) the average of the intensity of the GFP

fluorescence in either side of the hypocotyl, analyzed from the confocal images with ImageJ software. Error bars represent SD (n>10); (F) detection of GFP protein levels using anti-GFP antibodies in Col-0 and *iamt1-1* before and after gravistimulation.



III.14 Expression pattern and magnitude of *35S:DII-VENUS* in Col-0 and *iamt1-1*.

(A, B) Unstimulated three day old Col-0 and *iamt1-1* seedlings respectively showing the expression of DII-VENUS in the hypocotyl; (C, D) Gravistimulated Col-0 and *iamt1-1* after 6 hours or orientation showing the DII-VENUS gradient in Col-0 and the absence of the gradient in *iamt1-1*; (E) the average of the

intensity of the GFP fluorescence in either side of the hypocotyl, analyzed from the confocal images with ImageJ software. Error bars represent SD (n>10); (F) detection of VENUS protein levels using anti-GFP antibodies in Col-0 and *iamt1-1* before and after gravistimulation.

These observations allowed us to conclude that the typical phenotypes of faster apical hook opening and defective gravitropic response in the aerial part of the *iamt1* mutant plants can be due to high levels of auxin and not only auxin deficiency.

III.5 *IAMT1* expression patterns

Since the defects shown by *iamt1* mutants are associated to local changes in auxin distribution in aerial parts of seedlings, we wondered if IAMT1 would be expressed in the same tissues where the defects are observed. A 1.2 Kb fragment of the *IAMT1* promoter seems to contain all the relevant regulatory regions for the expression of *IAMT1*, considering that the expression of the *IAMT1* cDNA fused to GFP (*IAMT1-GFP*) under the control of this promoter fragment was able to rescue the gravitropism defects of the *iamt1* mutant (Figure III.15).

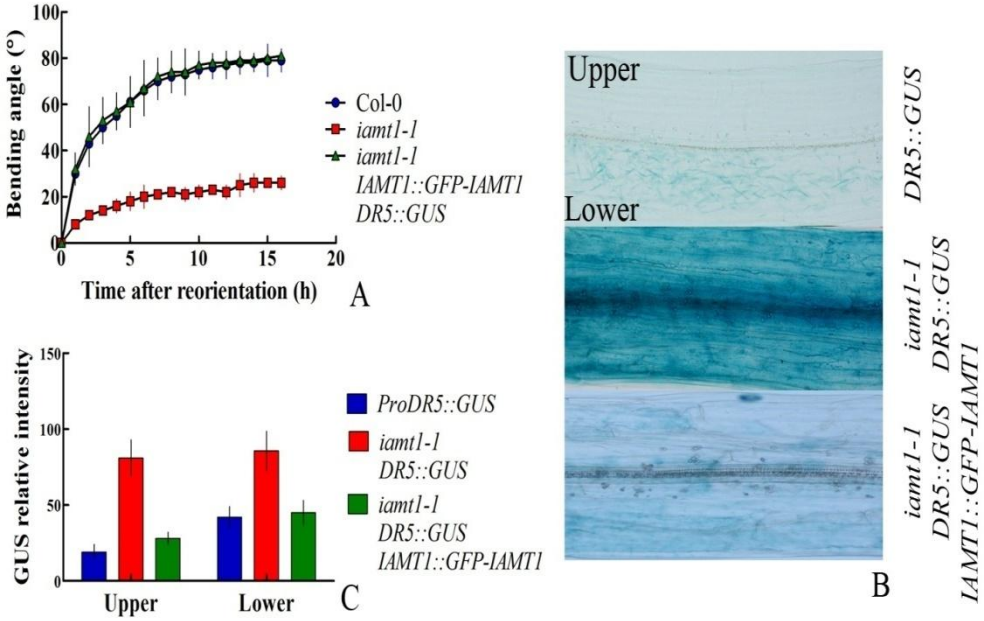


Figure III.15 Rescuing the phenotype of *iamt1-1* by the transformation of *ProIAMT1::GFP-IAMT1.*
(A) The bending kinetics of the recuperation of the agravitropic phenotype of *iamt1-1* through the transformation by *ProIAMT1:GFP-IAMT1*, Error bars represent SD (n=15-20 seedlings); (B) GUS staining and the recuperation of the auxin gradient in *iamt1-1* by the presence of *ProIAMT1:GFP-IAMT1*; (C) the average of the intensity of the GUS staining in either side of the hypocotyl, analyzed from the confocal images with ImageJ software. Error bars represent SD (n>10).

Therefore, this 1.2-Kb fragment of the *IAMT1* promoter was fused to the β-glucuronidase gene (GUS) and the pattern of expression was examined. The *IAMT1::GUS* transgenic lines generated displayed an expression pattern in light-grown seedlings similar to what has been previously described, visible mainly as a uniform signal in cotyledons, hypocotyls and the top part of the roots (Qin et al., 2005)(Figure III.16A). On contrary, the expression of the promoter of *IAMT1* in etiolated seedlings was restricted to the stomata of cotyledons (Fig. III.16B).

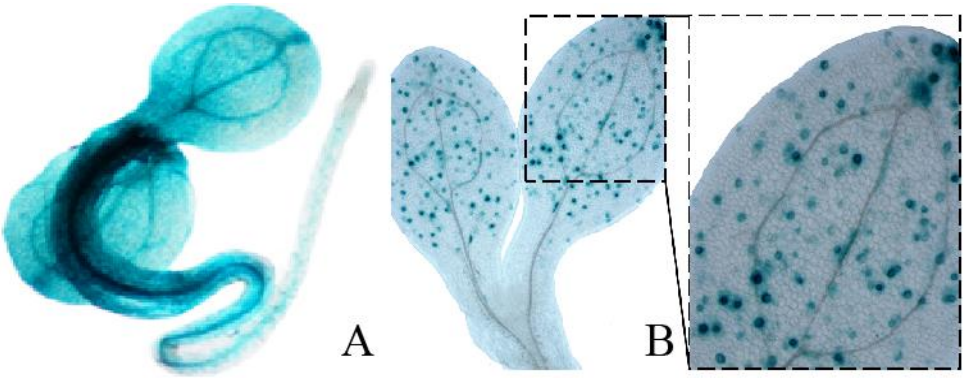


Figure III.16 Expression pattern of *IAMT1::GUS* in 3-day-old seedlings.
(A) Light grown seedlings; (B) Etiolated seedlings.
Seedlings were stained for 3 hours.

Since *iamt1* loss-of-function mutants have increased IAA signaling and defects in reorientation in response to gravity, we checked if the expression pattern of *IAMT1* changes after gravistimulation. Indeed, expression of *IAMT1* expanded to all areas of the cotyledons of gravistimulated seedlings (Figure III.17A). Moreover, the expression of *IAMT1* appeared in the bending zone of the reorienting hypocotyl, forming a gradient with higher level at the lower side, where auxin normally accumulates under these circumstances (Figure III.17B). Interestingly, the levels of *IAMT1* transcript increased after 2 hours of gravistimulation and peaked at around 6 hours only to go decrease again after that (Fig. III.18).

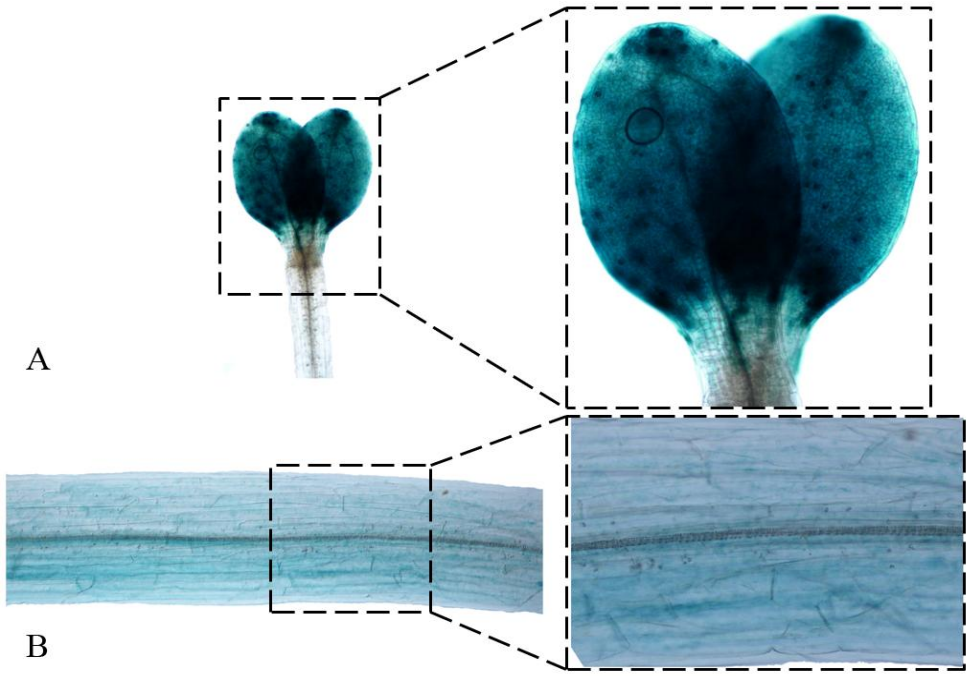


Figure III.17 Expression of *pIAMT1::GUS* after 6 hours of gravistimulation.
(A) Expression of *pIAMT1::GUS* in the cotyledons; (B) Expression of *pIAMT1::GUS* in the hypocotyl after 6 hours of gravistimulation, a gradient of expression is formed with stronger expression being at the lower side of the hypocotyl facing the gravity vector.
Seedlings were stained for 3 hours.

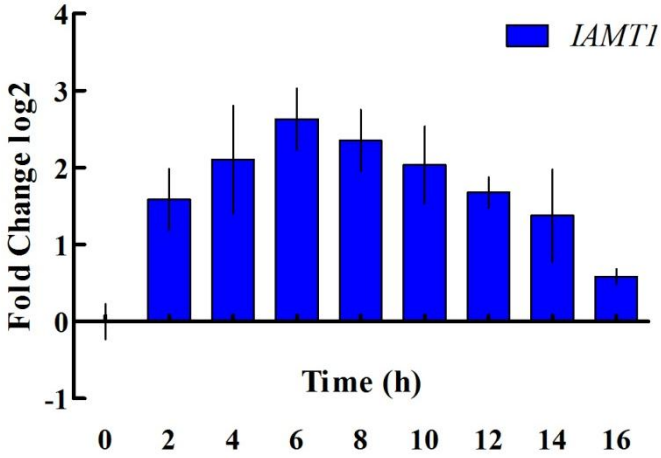


Figure III.18 Effect of gravistimulation on *IAMT1* expression.

Three-day-old etiolated Col-0 seedlings were grown vertically then gravistimulated. Tissue was collected at the indicated time after gravistimulation and expression was monitored by RT-qPCR. Values are log ratio between the gravistimulation and the control. Data represent mean and the standard error of the mean from three independent biological replicates. Data from each biological replicate consisted in three technical replicates that were averaged and normalized.

The expression pattern of *IAMT1* is in agreement with a role for IAA methylation in the generation and maintenance of the auxin gradients during differential growth. However, it is not clear from the GUS images if IAA methylation is required in the tissue that serves as a source for auxin, or in the responding tissue, or in both. Gravity is known to be perceived by hypocotyls through the positioning of amyloplasts inside the endodermal cells (Tasaka et al., 1999; Blancaflor and Masson, 2003; Driss-Ecole et al., 2003), which determines the localization of PIN3 and other PIN transporters (Friml et al., 2002; Rakusova et al., 2011) to redirect the auxin flux to the lower side of the reorienting hypocotyl. Therefore, to know if IAMT1 was differentially required in the endodermis, we expressed *GFP-IAMT1* under the control of the endodermis-specific *SCARECROW* (*SCR*) promoter, and the epidermis-specific *MERISTEM LAYER1* (*ML1*) promoter as a control. As shown in Figure III.18, expression of *IAMT1* from the *SCR*, but not the *ML1* promoter, was able to restore the formation of the *DR5::GUS* gradient and, accordingly, to suppress the defects in gravitropic reorientation of the *iamt1* mutant.

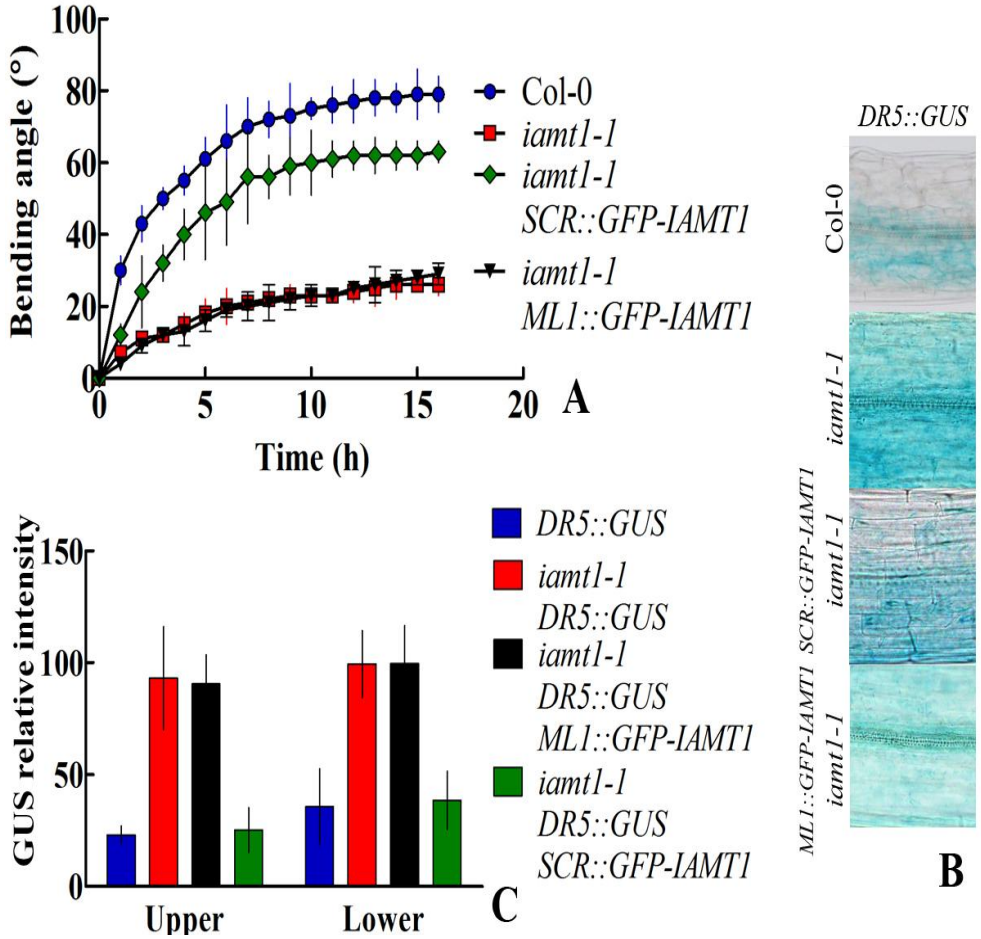


Figure III.18 Expression of IAMT1 under the tissue specific promoter *SCARECROW* and *MERISTEM LAYER1* in *iamt1-1* background.
(A) Bending kinetics and gravitropic reorientation of Col-0, *iamt1-1* seedlings and tissue specific expression lines *SCR::GFP-IAMT1* and *ML1::GFP-IAMT1* showing the ability of *SCR:GFP-IAMT1* to rescue the agravitropic phenotype of *iamt1-1* but not *ML1::GFP-IAMT1*, Error bars represent SD (n=15-20 seedlings) (B) The reformation of auxin gradient in *ProDR5::GUS* in *iamt1-1* through the expression of *SCR::GFP-IAMT1* but not *ML1::GFP-IAMT1*; (C) the average of the intensity of the GUS staining in either side of the hypocotyl, analyzed from the confocal images with ImageJ software. Error bars represent SD (n>10).

Altogether, these results suggest that IAA methylation is necessary in the cell types that perceive gravity for the generation of the auxin gradient.

III.6 Regulation of polar auxin transport by IAMT1

Given that loss of IAMT1 function does not affect auxin levels dramatically, but alters the generation of proper auxin gradients in differential growth processes, a likely possibility is that IAA methylation is necessary to adjust auxin transport. To test this hypothesis, plants were grown vertically for 3 days in darkness and parafilm was placed between the growth medium and the cotyledons (Fig. III.19), after which ^{3}H-IAA was added on the cotyledons of wild type and *iamt1* mutants. Three hours later, the lower half of the hypocotyls was collected, and ^{3}H activity was measured. Since the appearance of labeled IAA in the lower half of the hypocotyl was abolished by increasing concentrations of N-(1-Naphthyl)phthalamic acid (NPA), our results clearly show that polar auxin transport (PAT) through the etiolated hypocotyl was enhanced in the *iamt1* mutant (Fig. III.19).

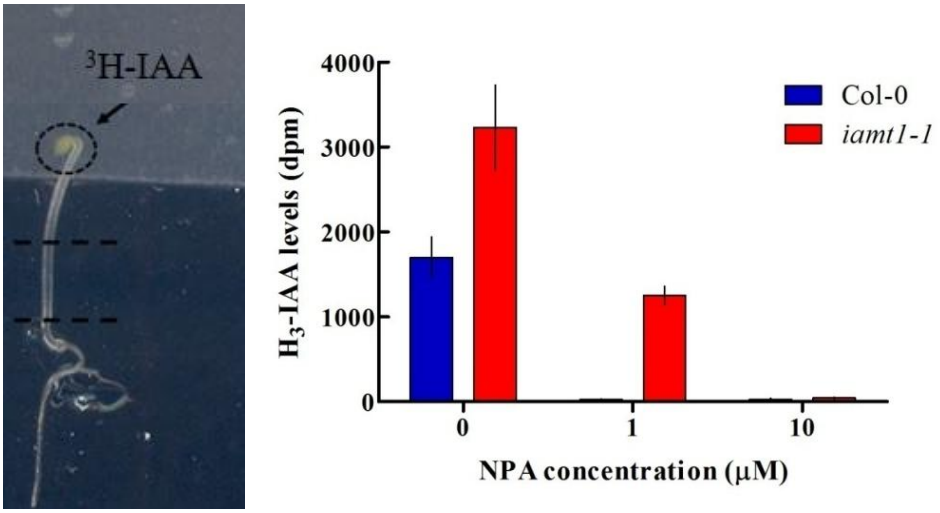


Figure III.19 Polar auxin transport through etiolated hypocotyls.
PAT was measured by applying 15000 dpm of ^{3}H-IAA on the cotyledons of seedlings growing on a bed of parafilm and collecting the lower half of the hypocotyl. The graph shows the levels of collected ^{3}H -IAA in Col-0 and *iamt1-1*

with different concentrations of NPA. Error bars represent SD (n=3 biological replicates).

To investigate if the observed defect in IAA transport in *iamt1* correlates agravitropic phenotype of the mutant, we measured PAT in wild-type and mutant seedlings after gravistimulation by the application of ^{3}H-IAA to the cotyledons, incubation for 3 hours before orienting the plate 90 degrees, and then collecting the lower half of the hypocotyls 6 hours later. Interestingly, PAT was enhanced in the wild type after reorientation, but this increase was even higher in the *iamt1* mutant (Fig. III.20).

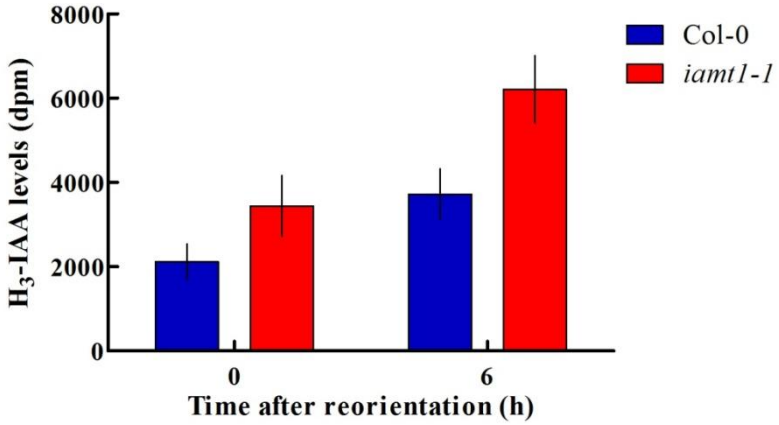


Figure III.20 ^{3}H-IAA transport in Col-0 and *iamt1-1* before and after gravistimulation.
Error bars represent the variation 3 different measurements.

These results suggest that there may be a causal connection between the increased PAT in the *iamt1* mutant and its agravitropic phenotype. To investigate this possibility, we assayed the capacity of wild-type and mutant seedlings to reorient in the presence of the PAT inhibitor, NPA. As expected, the gravitropic reorientation of the hypocotyls of wild-type seedlings was gradually reduced with increasing doses of NPA (Figure III.21; Supp III.4). However, low doses of NPA promoted the reorientation

of *iamt1* seedlings, until a high concentration of 10µM NPA was reached, when reorientation was also abolished (Figure III.21; Supp. III.5). The confirmation that a reduction of PAT alleviates the agravitropic phenotype of the *iamt1* mutant suggests that PAT restriction by IAA methylation is an important element in the generation of lateral auxin gradients.

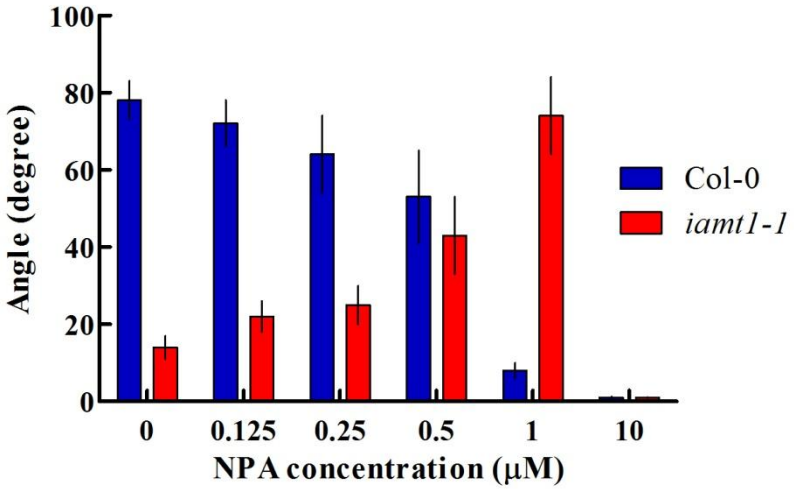


Figure III.21 Final angle of gravitropic reorientation of Col-0 and *iamt1-1* with different concentrations of NPA;
Bars represent SD (n=15-20 seedlings).

III.7 Regulation of *PIN* gene expression by IAMT1

What is the mechanism by which IAMT1 regulates PAT? Given that auxin has been proposed to regulate its own transport at the transcriptional level (Peret et al., 2013), it can be hypothesized that IAA methylation interferes with this regulation. To test this hypothesis, we measured the transcript levels of *PIN1*, *PIN2*, *PIN3* and *PIN7* in 3-day-old etiolated seedlings of wild-type and *iamt1* mutants, and found that the levels of these *PINs* is were indeed higher in *iamt1* mutants (Figure IIII.22; Supp. III.6).

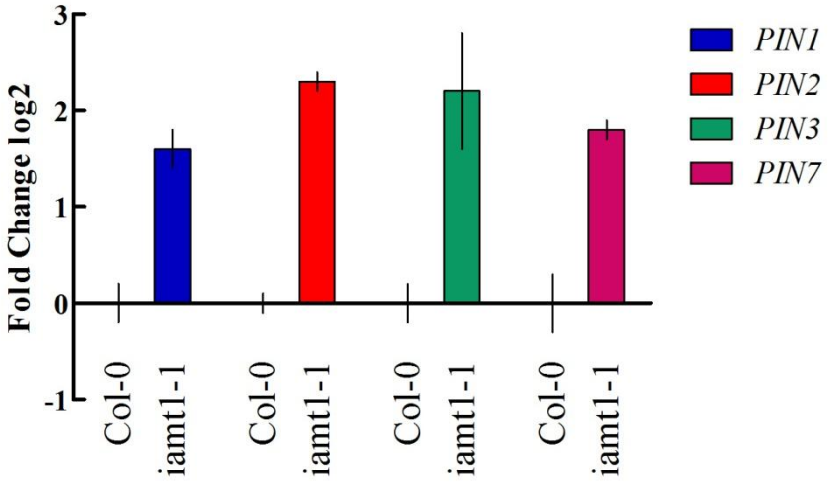


Figure III.22 Effect of the *iamt1* mutation on *PIN* gene expression.
Tissue from three-day-old etiolated Col-0 and *iamt1-1* seedlings was collected and expression of PIN1,2,3 and 7 was monitored by RT-qPCR. Values are log ratio between the gravistimulation and the control. Data represent mean and the standard error of the mean from three independent biological replicates. Data from each biological replicate consisted in three technical replicates that were averaged and normalized.

Transcriptional regulation of *PIN* genes by IAMT1 may be physiologically relevant for the formation of auxin gradients, since gravistimulation provoked not only an increase in PAT in wild-type seedlings (Figure III.22), but also an increase in the expression of all *PIN* genes tested, albeit with different kinetics (Figure III.23). More importantly, the expression of *PIN* genes in the *iamt1* mutant followed the same transient induction upon reorientation as in the wild type, but always with a much higher level (Figure III.23).

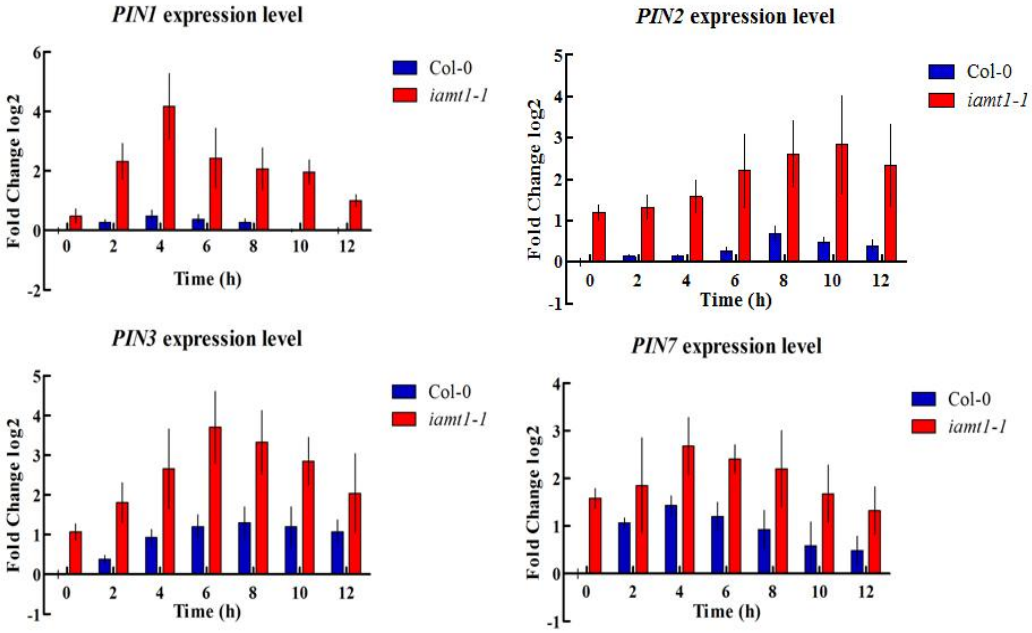


Figure III.23 *PIN1, 2, 3* and *7* expression levels after gravistimulation in Col-0 and *iamt1-1*.
Three-day-old etiolated Col-0 and *iamt1-1* seedlings were grown vertically then gravistimulated. Tissue was collected at the indicated time after gravistimulation and expression was monitored by RT-qPCR. Values are log ratio between the gravistimulation and the control. Data represent mean and the standard error of the mean from three independent biological replicates. Data from each biological replicate consisted in three technical replicates that were averaged and normalized.

To visualize the increase of expression of the *PIN*s, we introduced the reporter constructs *PIN2*::*GUS* (Friml et al., 2003b) and *PIN3*::*GFP-PIN3* (Blilou et al., 2005) in the *iamt1-1* background. As shown in Figure III.24 the GUS expression of *PIN2*::*GUS* in unstimulated hypocotyls showed increased intensity and expression level in *iamt1-1* than in the wild type (Fig. IIII.24), and gravistimulation provoked an expansion of the domain of expression to the cells adjacent to the central vascular cylinder, both in the upper and in the lower sides (Figure III.25). A very similar expression pattern and behavior was observed for PIN3::GFP-PIN3 (Figure III.26)

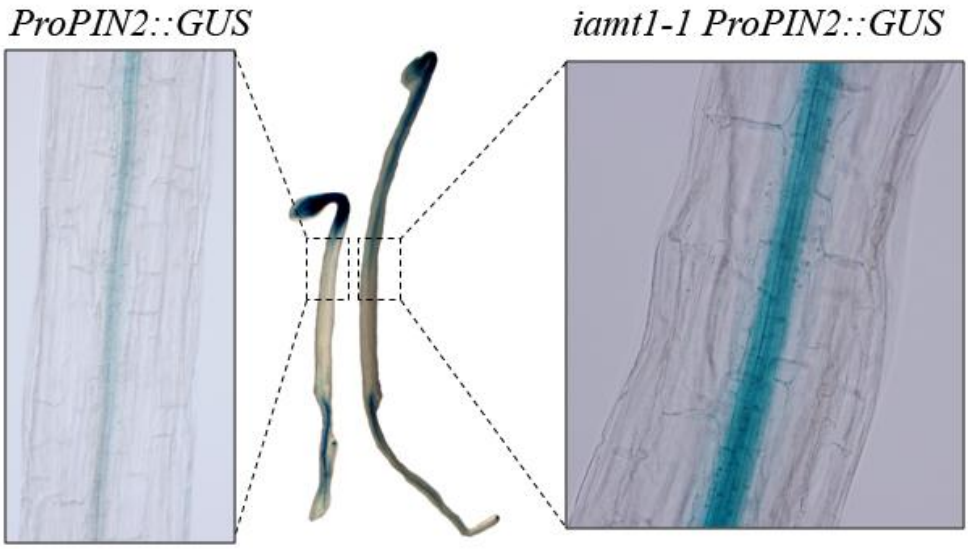


Figure III.24 *PIN2::GUS* expression in Col-0 and *iamt1-1* in 3 days old etiolated seedlings.

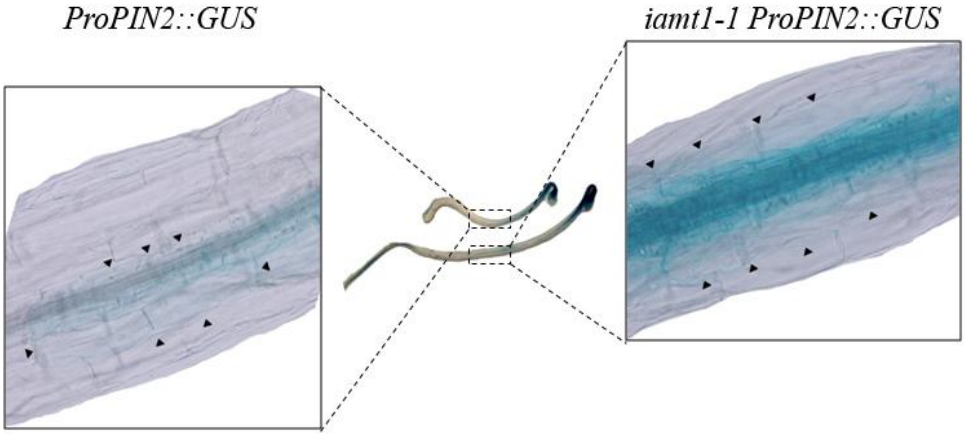


Figure III.25 *PIN2::GUS* expression in Col-0 and *iamt1-1* in 3 days old seedlings after 6 hours of gravistimulation.
Arrows represent the limits of expression.

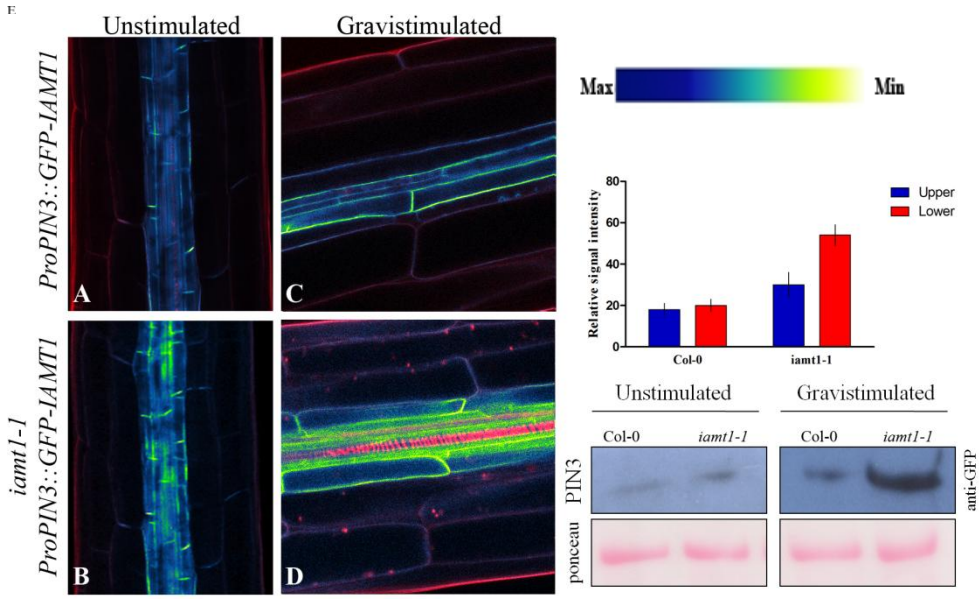
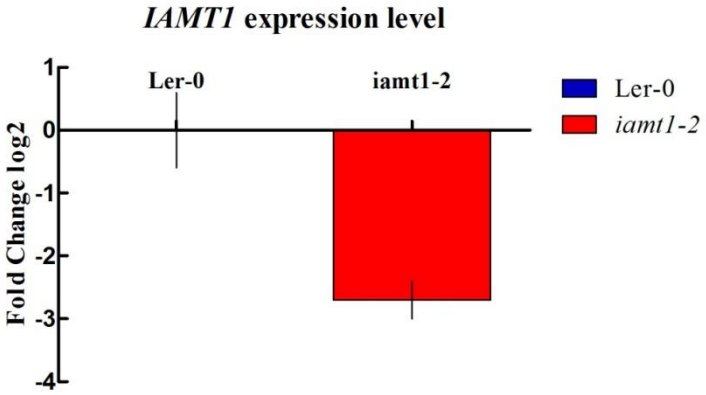


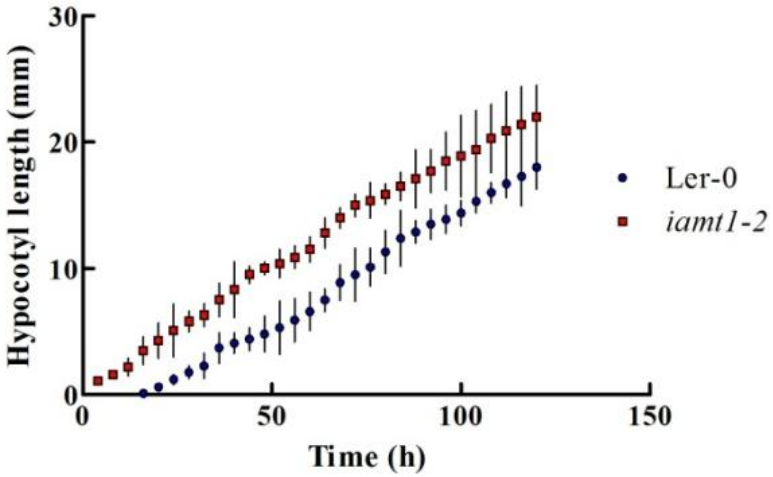
Figure III.26 The expression and localization of *PIN3::PIN3-GFP* in Col-0 and *iamt1-1*.

(A,B) Expression of *PIN3::GFP-PIN3* in Col-0 and *iamt1-1* in 3 days old seedlings; (C,D) expression of *PIN3::GFP-PIN3* in Col-0 and *iamt1-1* in 3 days old seedlings after 6 hours of gravistimulation; (E) The graph shows the average of the intensity of the VENUS fluorescence in either side of the hooks, analyzed from the confocal images with ImageJ software. Error bars represent SD (n>10); (F) The levels of PIN3-GFP in Col-0 and *iamt1-1* before and after 6 hours of gravistimulation.

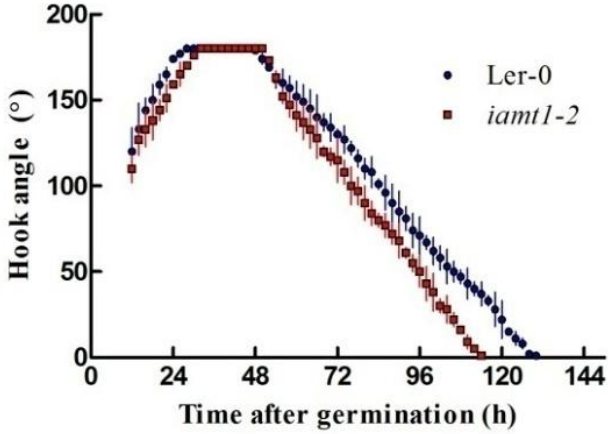
III.8 Supplementary



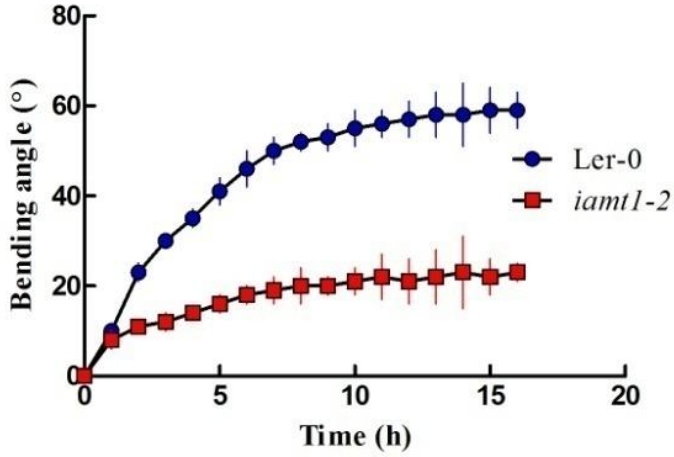
Supplementary III.1 level of *IAMT1* transcription L*er*-0 and *iamt1-2*.
error bars represent the variation of three technical replicates of three different biological replicates.



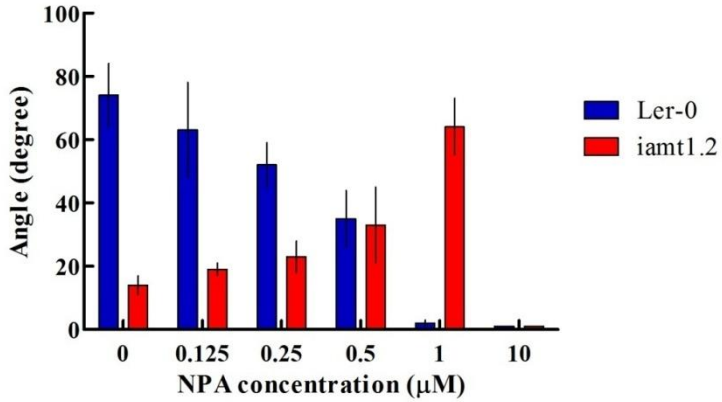
Supplementary III.2 Growth rate of *iamt-2* mutant compared to wild type in etiolated seedlings.
Seeds were sown on MS and subjected to a 6-h light treatment to promote germination, after which their growth was monitored in darkness with Infrared (IR)-cameras. Error bars represent SD (n=15-20 seedlings).



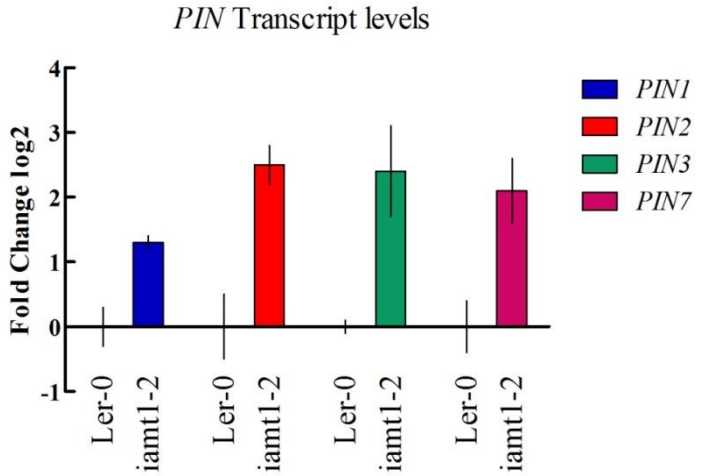
Supplementary III.3 Kinetics of apical hook development of the *iamt1-2* mutant in correspondence to the wild type ecotype.
Seeds were sown on MS and subjected to a 6-h light treatment to promote germination, after which hook angle was monitored in darkness with IR-cameras. Error bars represent SD (n=15-20 seedlings).



Supplementary III.4 Kinetics of gravitropic reorientation of the *iamt1-2* mutant in correspondence to the wild type ecotypes.
Seedlings were grown for 3 days in darkness on vertical plates. At time zero, the plates were turned 90º and gravitropic reorientation was monitored with IR-cameras. Error bars represent SD (n=15-20 seedlings).



Supplementary III.5 Final angle of the gravitropic reorientation of L*er*-0 and *iamt1-2* on MS supplied with different concentrations of NPA.
error bars represent the measurement of at least 15 to 20 seedlings.



Supplementary III.6 Transcript levels of *PIN1,2,3* and *7* in Ler-0 and *iamt1-2* seedlings.
errors bars represent the variation of three technical replicates of three different biological replicates.

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RESULTS 1: IAMT1 AND IAA HOMEOSTASIS

IAMT1 is a direct target of DELLA's transcriptional regulation (Gallego-Bartolome et al., 2011). For instance, its expression is strongly induced after the accumulation of the dominant version of GAI (gai-1) in response to a heat shock in the line *ProHS::gai-1*, in the dominant mutants *gai-1* and *rga-Δ17*, and in wild type plants treated with the GA biosynthesis inhibitor paclobutrazol (PAC) (Gallego-Bartolome et al., 2011). DELLAs are able to transmit multiple environmental signals to control plant growth and development by interacting with multiple target proteins that control gene transcription (Zentella R, 2007; de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolome et al., 2012). The idea that DELLA proteins regulate transcription was originally based on nuclear localization and protein homology (Harberd et al., 1998). Based on recent studies, DELLAs control gene transcription through protein interaction with specific transcription factors (TFs) (Daviere and Achard, 2013). Interestingly, chromatin immunoprecipitation (ChIP) experiments showed that the Arabidopsis DELLA protein RGA localizes to the promoter regions of several early DELLA-regulated genes (Zentella R, 2007). Since all of these genes were GA down-regulated and DELLA up-regulated, it was hypothesized that DELLA functions as a transcriptional activator of repressors of GA signaling. That the DELLA domain required to activate transcription in a yeast one-hybrid assay is also required for functioning as a negative regulator of stem elongation in rice provides circumstantial evidence in favor of this hypothesis (Hirano et al., 2012). Since DELLAs showed no more than 3.5-fold enrichment at any early DELLA-regulated promoters, it was proposed that DELLAs act through interaction with DNA-binding proteins (Zentella R, 2007). Recently, it has been demonstrated that DELLA proteins are recruited to the target promoters through interaction

with specific TFs, for instance ABI3/ABI5 (Lim et al., 2013) or IDDs (Yoshida et al., 2014). Besides this mode of action in which DELLAs act as co-activators of transcription at the target promoters, DELLAs also act by sequestering TFs away from promoters by physical interaction (Daviere and Achard, 2013). In this Chapter we aimed to define the mechanism through which DELLAs regulate the expression of the *IAMT1* gene, in particular (i) to identify the TF or TFs that mediate this regulation, (ii) to define how DELLAs influence the TF's activity, and (iii) to determine the physiological relevance of the regulation of *IAMT1* by DELLAs.

IV.1 IAMT1 mediates the influence of DELLA proteins on auxin homeostasis

A prediction if the transcriptional regulation of *IAMT1* by DELLAs is relevant to control auxin homeostasis, is that auxin levels should diminish in response to increased DELLA levels, and that an *iamt1* mutant should be resistant to this effect.

To test this hypothesis, we visualized endogenous auxin levels indirectly by using the newly developed auxin sensor DII-VENUS (Brunoud et al, 2012). In agreement with previous results, DII-VENUS signal was low at the root tip indicative of high auxin levels (Figure IV.1) (Brunoud et al., 2012). However, its signal was below the detection limit in the *iamt1-1* background in the root tip but not the elongation zone (Supp. IV.1), suggesting that local auxin levels are higher in the mutant. Interestingly, treatment of seedlings with PAC caused a strong increase in DII-VENUS signal, suggesting auxin levels were diminished. These results indicate that DELLA activity influences the auxin pool at the root tip. Importantly, when

the effect of PAC on DII-VENUS was assayed in the *iamt1* background, signal was not affected. These results indicate that the regulation of auxin homeostasis by DELLAs is dependent on IAMT1 activity, likely through the transcriptional regulation of its gene.

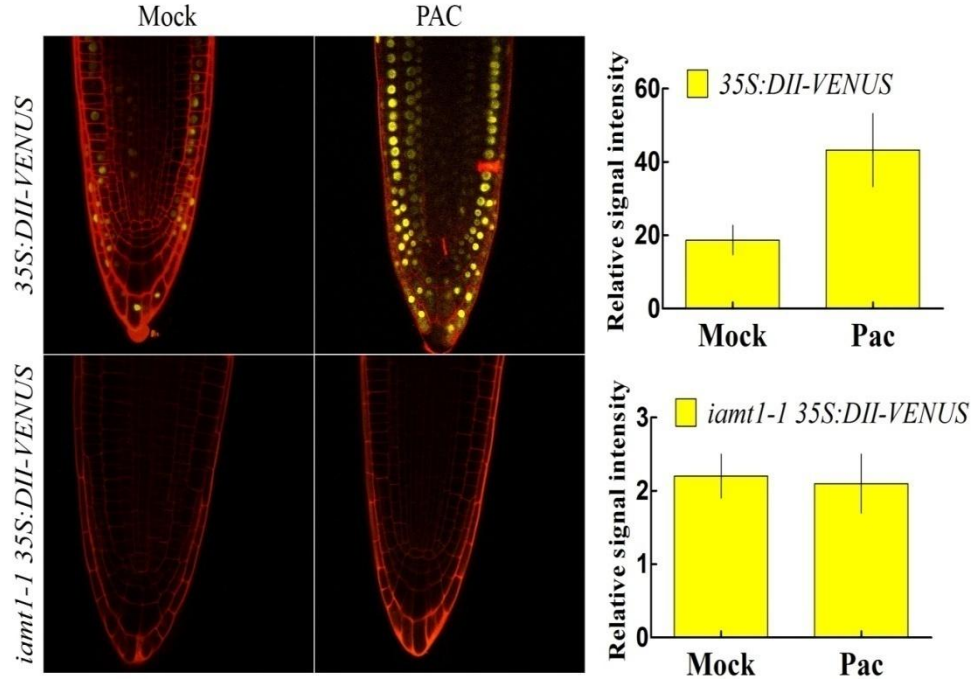


Figure IV.1 The effect of PAC treatment on the roots of *35S::DII-VENUS* and *iamt1-1 35S::DII-VENUS.*
(Left) wild type and *iamt1-1* seedlings were incubated with MS supplied with or without 0.4 µM pac; (right) the graph shows the average of the intensity of the VENUS fluorescence in roots with or without PAC treatment, analyzed from the confocal images with ImageJ software. Error bars represent SD (n>10).

IV.2 Identification of DELLA binding sites

Due to the growing range and variety of interactors (Locascio et al., 2013), DELLAs are able to regulate target genes through multiple *cis* elements, which makes it impossible to predict which TF mediates the regulation of *IAMT1* just by analyzing its promoter sequence. To start approaching this problem we chopped the promoter of *IAMT1* in two regions, deletion 1

extending from -1.5 Kb to -1 and deletion 1R extending from -2.7 Kb to -1.2 Kb and fused to a minimal 35S CaMV promoter. The two deletions were then cloned into the transient expression vector pGREENII 0800-LUC (Hellens et al., 2005) to control the luciferase (*LUC*) gene (Figure IV.2).

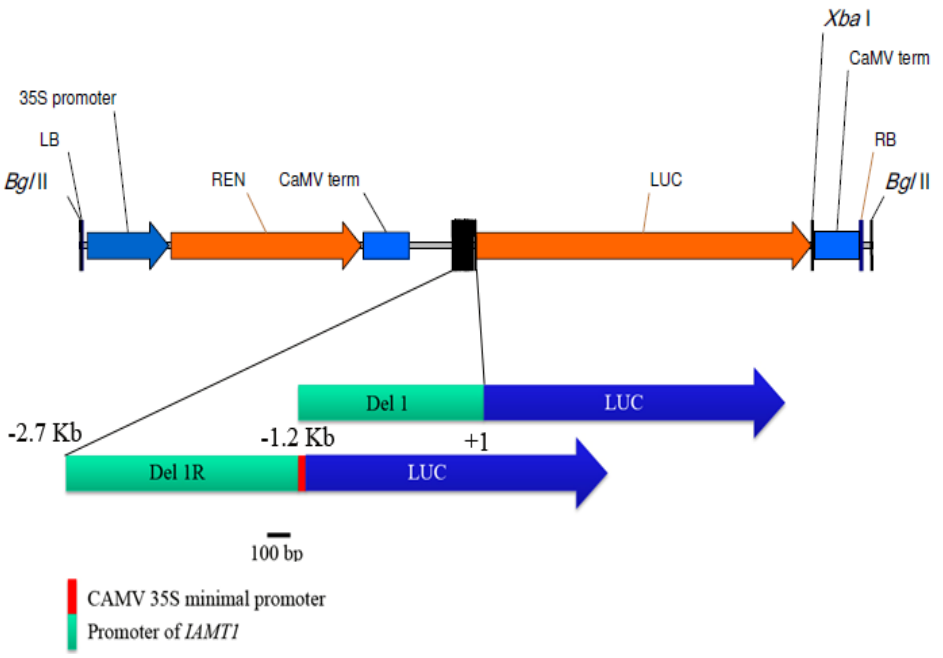


Figure IV.2 Cloning the deletions of the promoter of *IAMT1* in the T-DNA region of the transient expression vector pGreenII 0800-LUC.

Constructs were expressed in *Nicotiana benthamiana* leaves with or without GAI-TAP Our results showed that only Del 1 is inducible by GAI- (Figure IV.3), these results allowed us to narrow down the areas of responsiveness to GAI-TAP to the first 1.2 Kb of the promoter of *IAMT1*.

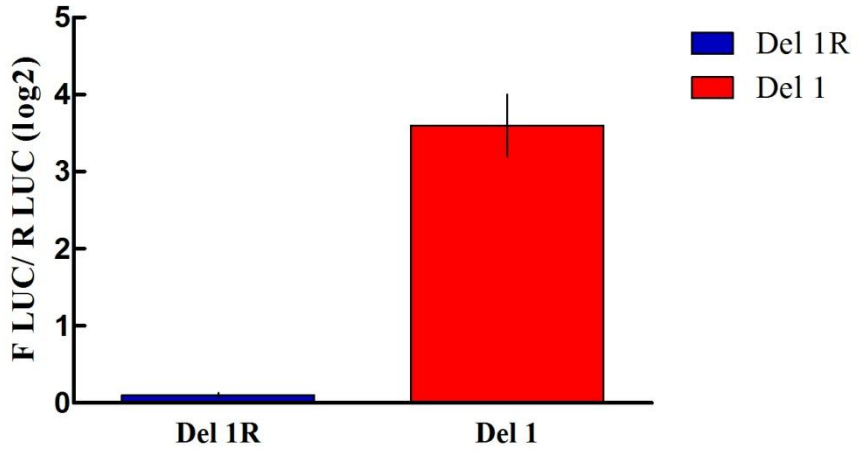


Figure IV.3 Induction levels of two deletions of the promoter of *IAMT1* by GAI in *Nicotiana benthamiana* leaves.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

Due to its relatively large size, Del 1 was subjected to further deletions (Figure IV.4).

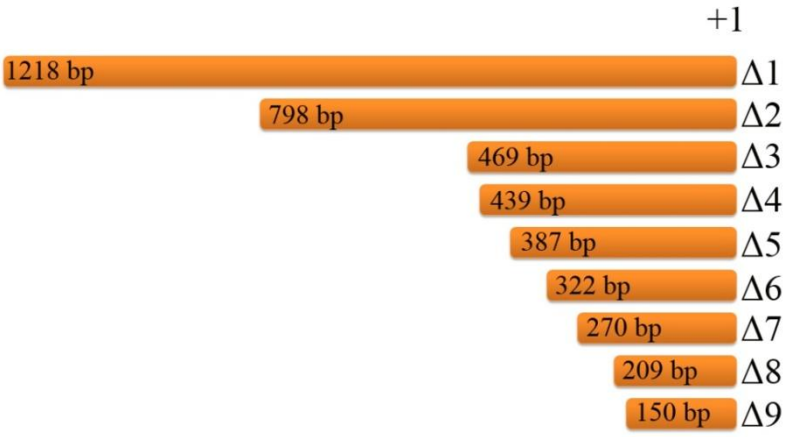


Figure IV.4 Successive deletions of the promoter of *IAMT1*.

In general, the successive deletions of the promoter caused a decrease in the response to GAI-TAP (Figure IV.5).

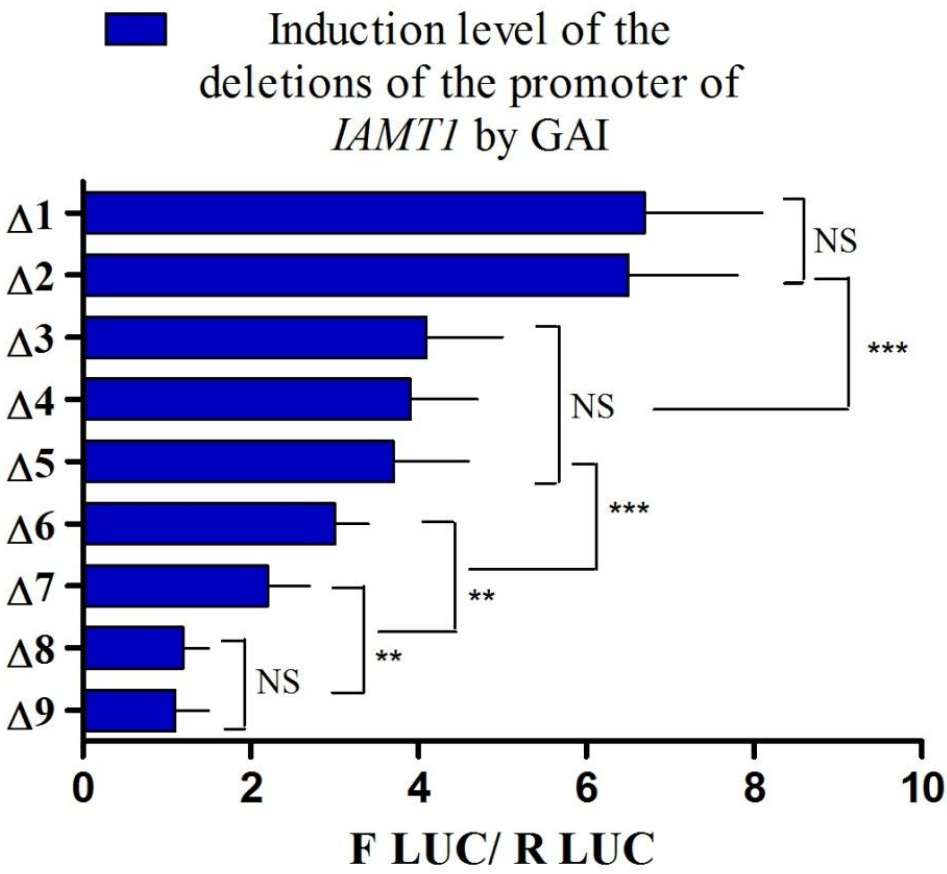


Figure IV.5 Transient expression assay in Nicotiana leaves showing the responsiveness of the deletions of the promoter of *IAMT1* to GAI.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

Deletions 1 and 2 showed no difference in response to GAI-TAP. Then, the decrease in response observed for del 3 is maintained in del 4 and 5 to gradually decrease in del 6 to 8. This result showed that the regions that might respond to GAI-TAP are located between deletions 2 and 3 and 5 and 8.

We prepared an additional set of constructs to test the relevance of these two regions for the regulation of the promoter by DELLAs. (Figure IV.6).

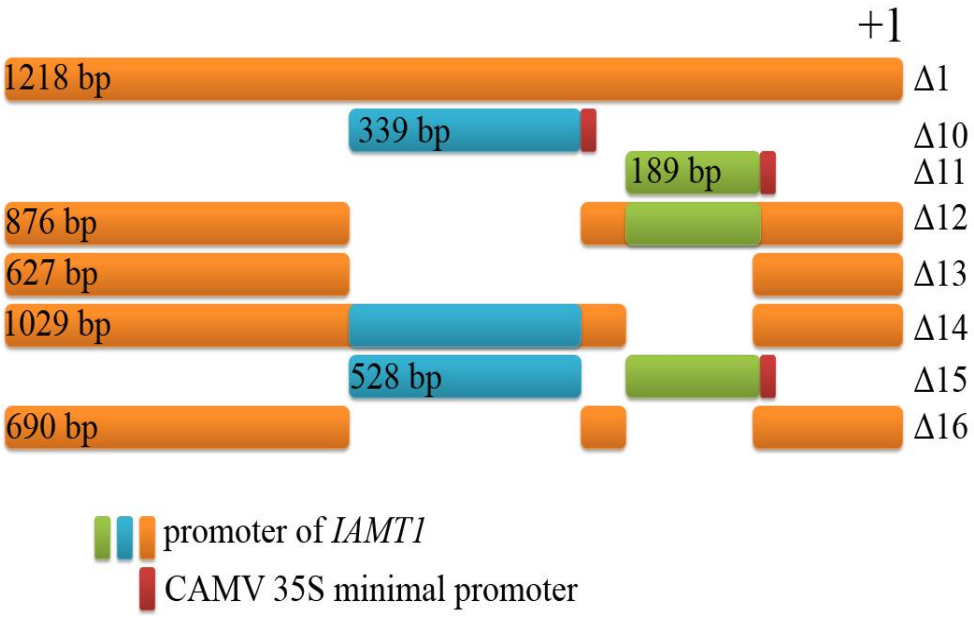


Figure IV.6 Isolation of the putative regions of the promoter of *IAMT1* that respond to 35S::TAP-GAI.

Results in Figure IV.7 show that deletions 10 and 11, containing the GAI responsive sites, and deletion 15 containing a fusion of both regions were able to respond to GAI. In turn deletions 12 and 14, containing only one putative site, were also responsive to GAI. On the other hand, deletions of the promoter containing neither of the putative sites were completely irresponsive to induction by GAI. These results indicate that the promoter fragments needed to regulate the expression of *IAMT1* by DELLAs are located in two distinct regions, one located between -209 and -387 and another located between -469 and -854, and that those regions are sufficient to grant DELLA-responsiveness.

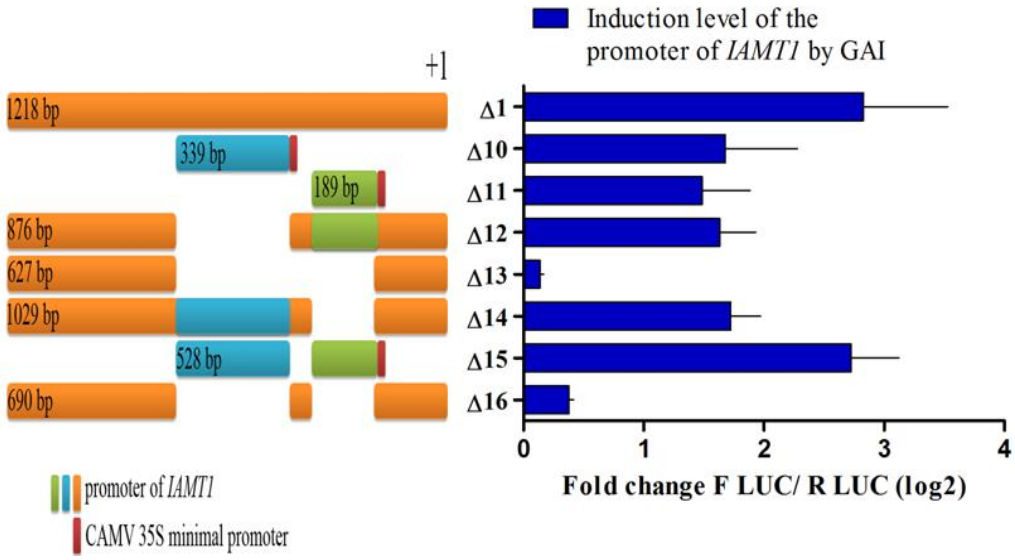


Figure IV.7 Transient expression assay in *Nicotiana* leaves showing two putative sites in the promoter of *IAMT1* that are induced by GAI.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

IV.3 *In silico* analysis of the promoter of *IAMT1*

In an attempt to identify putative TFs that regulate the expression of *IAMT1*, we scanned the two regions that respond to GAI-TAP for known regulatory regions that could eventually serve for the binding of GAI interacting TFs. For this purpose we used the promoter searching tool ELEMENT (http://element.mocklerlab.org/element_jobs) (Nemhauser et al., 2006). When we compared the *cis* elements returned by the program with those elements overrepresented in GAI targets (Gallego-Bartolome et al., 2011), we found out that deletion 10 contained at least 8 different types of *cis* elements while deletion 11 contained 4 types (Figure IV.8).

Logo	*cis* element	Number of CIS elements in deletion 10	Number of CIS elements in deletion 11
	**AAAG** Dof binding site	**1**	**-**
	**Unknown**	**1**	**2**
	**AAAG** Dof binding site	**1**	**-**
	**TATAbox**	**2**	**-**
	**Unknown**	**4**	**-**
	**Unknown**	**11**	**6**
	**CA_6G** (CArG-like) MADS binding site	**4**	**1**
	**TATAbox**	**7**	**1**

Figure IV.8 Logos from *cis* elements in deletions 10 and 11.
Adapted from Gallego-Bartolome et al., 2011a.

The high number of cis elements in deletions 10 and 11 make it dificult to use this information to readily identify the TF that mediates regulation by DELLAs. In an attempt to solve this dilemma, we followed a bottom-up approach to narrow down possible TFs. We used public microarray data present in GeneMANIA tool (http://genemania.org/) (Mostafavi et al.,

2008) and looked for co-expressed genes, co-localized genes, their genetic and physical interactors, and proteins that share domains with IAMT1. We generated a list of 165 genes (Supp. Table IV.2), out of which 102 were anotated as TFs. Surely the 102 TFs we found may not all be important for the the regulation of *IAMT1*. To narrow down this number we compared this list of TFs with those that interact with GAI, either reported in the literature or found in our laboratory (Marín de la Rosa, unpublished) after the screening by yeast 2-hybrid (Y2H) of a library containing ca. 1200 TFs from Arabidopsis (Castrillo et al., 2011) . This comparison allowed us to identify 6 TFs that interact with GAI and that are co-expressed with *IAMT1* (Figure IV.9).

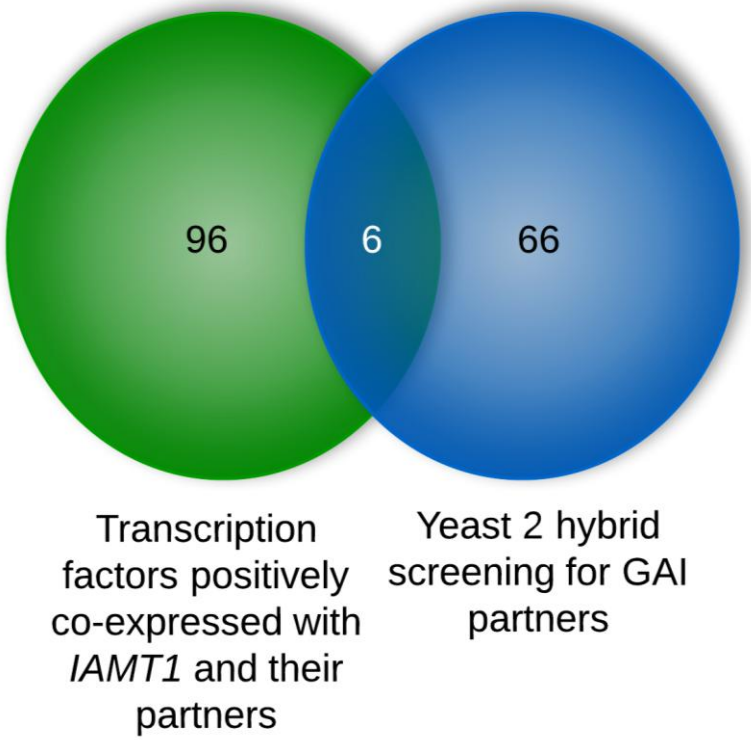


Figure IV.9 Venn diagram showing the transcription factors positively correlated with IAMT1 (green), GAI partners from the yeast-2-hybrid, and the 6 common between the two groups.

The 6 transcription factors identified are summarized in table IV.1

Table IV.1The name and the AGI code of the 6 GAI partners co-expressed with IAMT1.

Name	AGI code
TCP14	AT3G47620
ANAC42	AT2G43000
ANAC59	AT3G29035
bHLH18	AT2G22750
DRN	AT1G12980
AGL86	AT1G31630

IV.4 DORNRÖSCHEN, a possible co-activator of *IAMT1*'s expression

Our *in silico* analysis and our candidate elimination approach allowed us to identify 6 TFs that could eventually mediate the regulation of *IAMT1* by GAI. Next, we tested whether these TFs were able to bind to and to regulate expression from the promoter of *IAMT1*. For that purpose we used the CDS of all six candidates –taken from the REGIA collection (REgulatory Gene Initiative in Arabidopsis)(Valencia et al., 2002)- and fused them to the Herpes simplex virion particle protein 16 (VP16) activation domain (Babb et al., 2001). The VP16 fusions were co-expressed with deletion 1 in leaves of *N. benthamiana*. Despite the presence of VP16, 5 out of 6 of the putative TFs were not able to induce the expression of *IAMT1*. Nonetheless, one TF, DORNRÖSCHEN (DRN) was able to induce the expression (Figure IV.10).

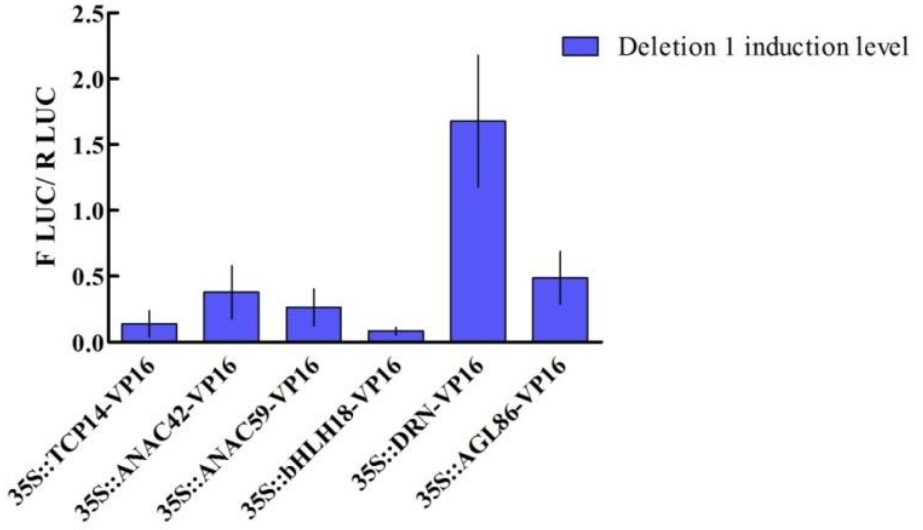


Figure IV.10 Transient expression assay in Nicotiana leaves showing the induction levels of the deletion 1 of the promoter of *IAMT1* by the 6 candidates.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

DRN, or ENCHANCER OF SHOOT REGENERATION (ESR1), is an AP2/EREBP domain containing protein (Matsuo and Banno, 2008), involved in the development and differentiation of embryos (Chandler et al., 2007). Early studies showed that *DRN* transcript levels increases after plant incubation with cytokinin (Banno et al., 2001), and that is also a transcriptional target for auxin signaling through the activity of MONOPTEROS (MP), an auxin response factor. 50% of the *drn-1* loss-of-function mutants exhibit abnormal hypophyseal cell divisions and lack basal embryo organization in addition to defective cotyledon development phenotypes at incomplete penetrance, including monocotyledonous seedlings, seedlings with partially fused cotyledons, tricots or various tricot fusion combinations. The inappropriate cotyledon development leads to alterations of leaf phyllotaxy (Chandler et al., 2007). These phenotypes are

presumed to be auxin related, since the double loss-of-function mutant *drn-1 drnl* (DRN like) leads to pin-like phenotype with no cotyledons (Chandler et al., 2007). Interestingly, it was found that the *drn-1* mutant exhibits disturbance in the localization of PIN1 (Chandler et al., 2007).

DRN is a transcriptional activator (Matsuo and Banno, 2008), and since GAI is an activator of *IAMT1* it might be possible that GAI work as co-activator together with DRN to regulate *IAMT1*.

To check whether DRN is able to activate the transcription of *IAMT1* without the aid of VP16, *N. benthamiana* leaves where co-infiltrated with 35S::DRN-VP16 or 35S::DRN and deletion 1 of the promoter of *IAMT1*. Indeed, our results show that DRN is able to induce the expression of *IAMT1* without the aid of the transcriptional activator VP16 indicating that it acts as a transcriptional activator also in the context of the *IAMT1* promoter (Figure IV.11).

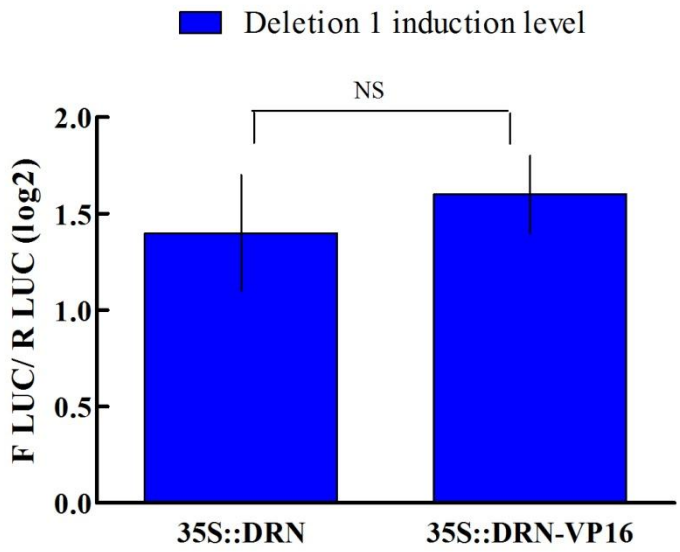


Figure IV.11 Transient expression assay in Nicotiana leaves showing the activation ability of DRN on the promoter of *IAMT1* with and without the aid of the transcriptional activator VP16.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

IV.4.1 Molecular interaction between DRN and GAI

Although DRN was identified as a GAI partner in a Y2H screening, we reconfirmed it (Figure IV.12).

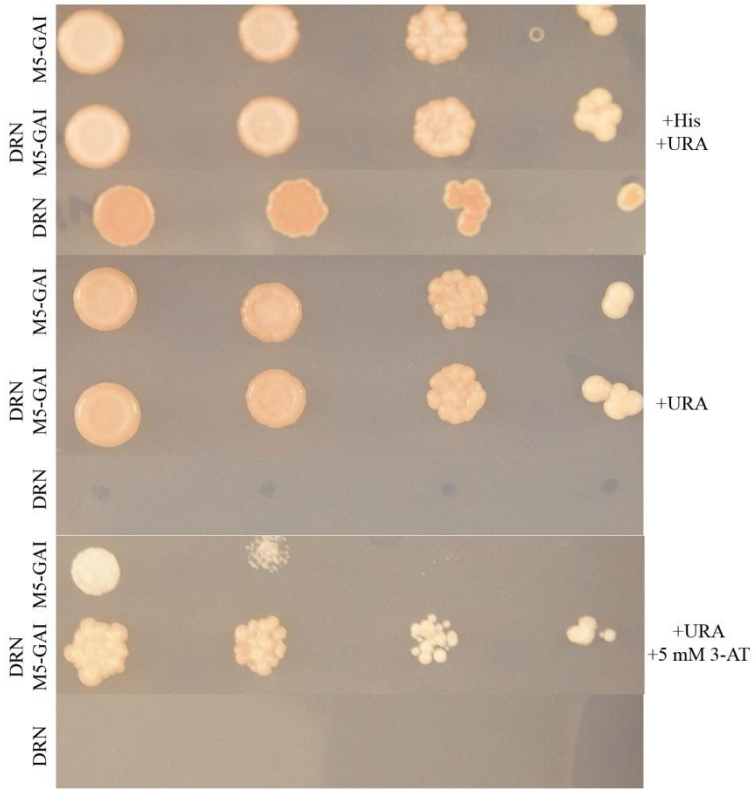


Figure IV.12 Yeast two hybrid interaction between GAI and DRN.

While our positive control (+ Histidine) grew, only cells containing both proteins survived in the absence of histidine and the presence of 5mM 3-AT (3-Amino-1,2,4-triazol), the competitive inhibitor of the yeast HIS3 reporter protein. The interaction between GAI and DRN *in planta* was verified as shown by bimolecular fluorescence complementation (BiFC)

assays in *N. benthamiana* leaves. Nuclear fluorescence caused by the reconstruction of YFP was observed in nuclei of epidermal cells of leaves that co-expressed YFC-DRN and YFN-GAI, whereas fluorescence in nuclei of control leaves that co-expressed either YFC-DRN and YFN or YFC and YFN-GAI was below detection limits (Figure IV.13).

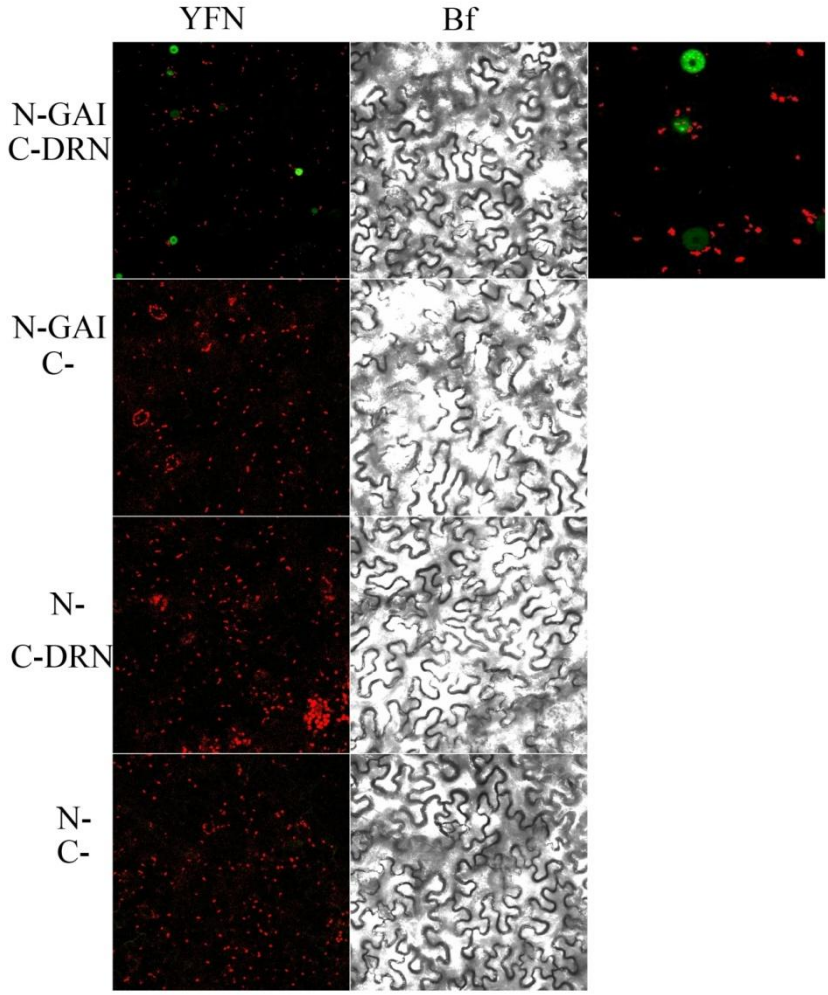


Figure IV.13 Bimolecular fluorescence complementation (BiFC) between YFN-GAI and YFC-DRN.

IV.4.2 Identification of DRN binding sites in the promoter of *IAMT1*

To test if the region of the promoter of *IAMT1* induced by GAI is also induced by DRN, we checked the presence of known AP2 binding sites in these two regions. We found out that both regions contain several repeats of different variants of the binding sites (Figure IV.14).

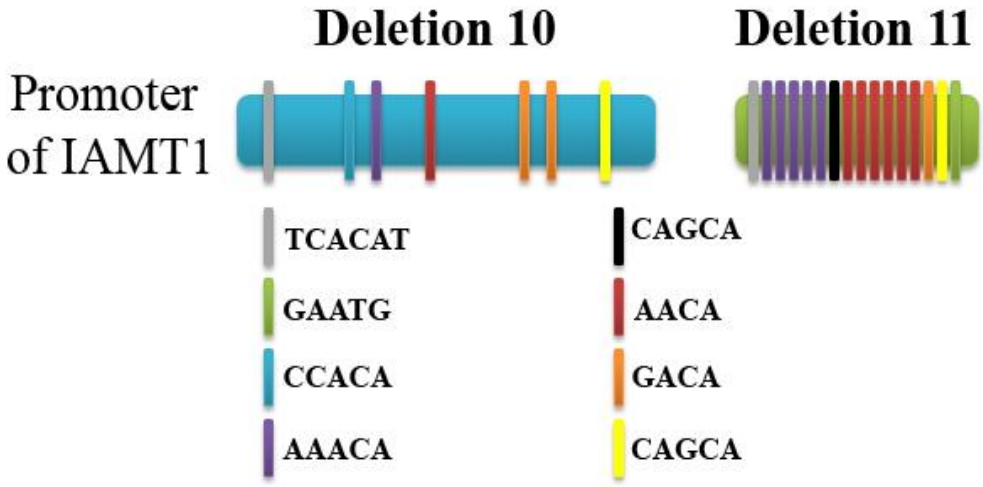


Figure IV.14 over-represented AP2 binding *cis* elements in deletions 10 and 11 in the promoter of *IAMT1*.

To test which deletion DRN binds to activate *IAMT1*, we co-infiltrated different deletions of the promoter with DRN. While deletion 1 (containing both GAI responsive sites) was responsive to DRN, deletion 10 representing one of the GAI binding site (339 bp) was irresponsive, as is deletion 14. Deletion 10 representing the second binding site (189 bp) and deletion 12 responded to DRN just like deletion 1 (Figure IV.15).

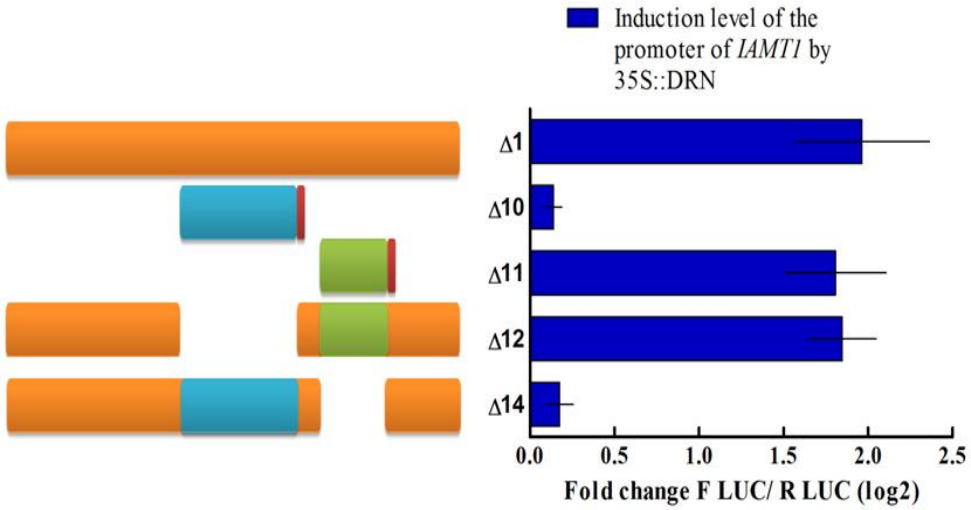


Figure IV.15 Transient expression assay in Nicotiana leaves showing the induction by DRN of the expression of *IAMT1* through binding to CIS elements found in deletion 10.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

Next we tested whether this transcriptional regulation by DRN is aided by GAI. For this purpose we co-infiltrated *N. benthamiana* leaves with low doses of DRN and GAI that are unable to induce the expression of *IAMT1* by themselves (Figure IV.16). Our results show that only in the simultaneous presence of GAI and DRN the LUC activity was increased from deletion 1 and deletion 12, but not from deletion 14 (Figure IV.16). These results suggest that DRN activates the promoter of *IAMT1* aided by GAI through a region contained in deletion 11.

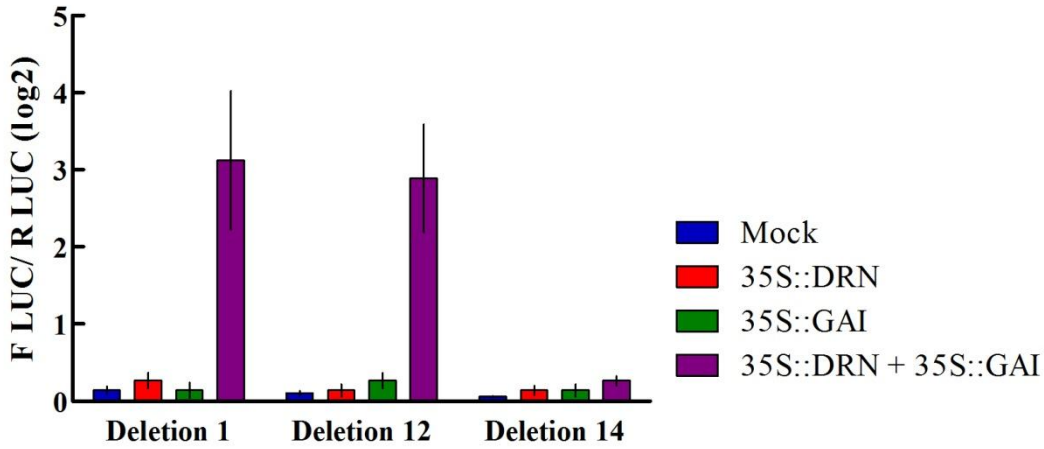


Figure IV.16 Transient expression assay in Nicotiana leaves showing the induction levels of different deletions of the promoter of *IAMT1* in Nicotiana benthamiana leaves infiltrated with the promoters Mock, DRN and GAI.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

IV.5 PIF4 and BES1, possible repressors of *IAMT1's* expression

The GAI transcriptional activation of *IAMT1* is through two regions, one located between -209 and -387 upstream the promoter and another located between -469 and -854. DRN mediates GAI effect on IAMT1 promoter through the region located between -209 and -387. In order to indentify putative transcriptional regulators that would recognize the region between -469 and -854 we used again the candidate approach, but this time we looked for genes that are negatively co-expressed with *IAMT1*, following that idea that DELLAs function as repressors-of-repressors. For that end we used the Expression Angular tool in http://bar.utoronto.ca/welcome.htm and set up the search criteria for genes that are negatively correlated with *IAMT1*.

The number of genes found was 3292 (due to the large number only the first 100 genes are represented in Suppl. IV.3). Given the large number found and since there existed no option to choose the transcription factors from these genes we decided to decrease the number of genes by using the matrix found in CressExpress (http://cressexpress.org/) to look for negatively correlated genes. The number of genes found was around 4293 (only the first 100 are represented in Suppl. IV.3). The two databases were cross referenced with the yeast two hybrid screening for GAI partners and the result was 2 transcription factors (Figure IV.17)

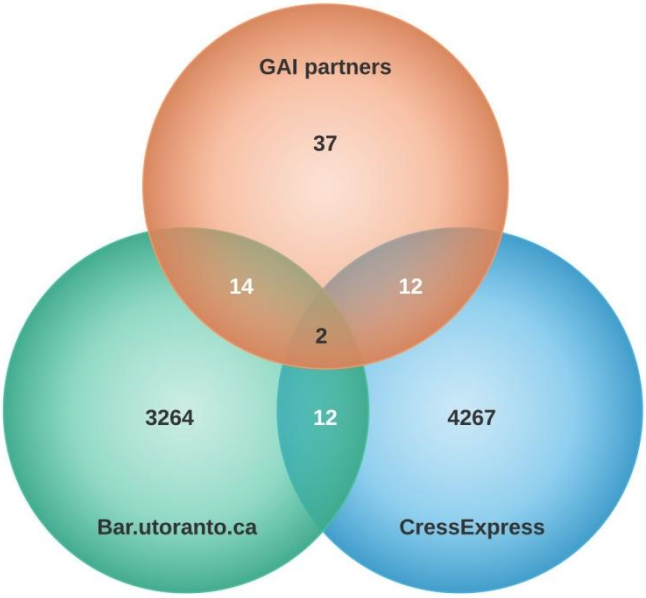


Figure IV.17 Venn diagram showing the comparison between the number of genes negatively correlated with IAMT1.
(Red) GAI partners, (Green) genes correlated with IAMT1 using BAR Expression Angular. (Blue) genes correlated with IAMT1 using CressExpress.

A closer look at the 2 common transcription factors, BES1 and PIF4, provides an insight on the possible regulator of *IAMT1*.

Our results show that BES1 alone represses *IAMT1* transcription, while GAI is able to counteract this effect according to the reported inhibition that DELLAs exert on the activity of BES1/BZR1 upon interaction (Li et al., 2012; Zhiponova et al., 2014). Similar results were obtained with PIF4, according to published results (de Lucas et al., 2008) (Figure IV.18).

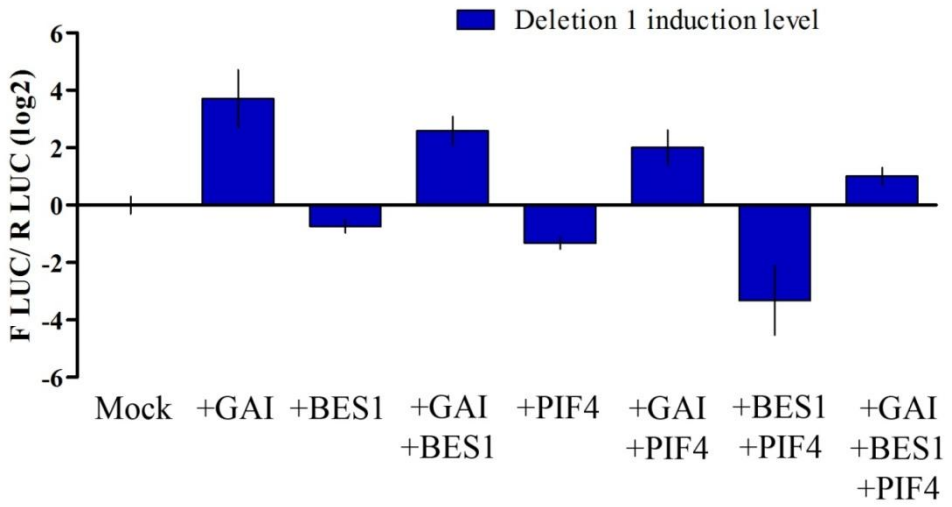


Figure IV.18 Transient expression assay in Nicotiana leaves showing the induction levels of the deletion 1 of the promoter of *IAMT1* by GAI, PIF4 and BES1.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

The combination of BES1 and PIF4 strongly decreased the expression of reporter LUC, while GAI still could counteract this effect (Figure IV.18). To check if the strong repression by the combination of BES1 and PIF4 was due to a complex formed by those two TF, as reported, or an additive effect of the repression, we infiltrated *N. benthamiana* leaves with low concentrations of BES1 and PIF4 that have no effect on the promoter. Our results show that while low concentrations BES1 or PIF4 alone were unable

to repress *IAMT1* promoter, expressing them together greatly repressed it, and again this effect was counteracted by GAI (Suppl. IV.4).

To localize the region of the promoter bound by PIF4 and BES1, both TFs were co-infiltrated with the different deletions of the promoter in the absence or presence of GAI. Our results show that BES1 and PIF4 are able to repress the activity of the promoter of *IAMT1* only in the presence of the region locate between -469 and -854 (blue)(Figure IV.19); this repression can be saved by the co-expression of GAI.

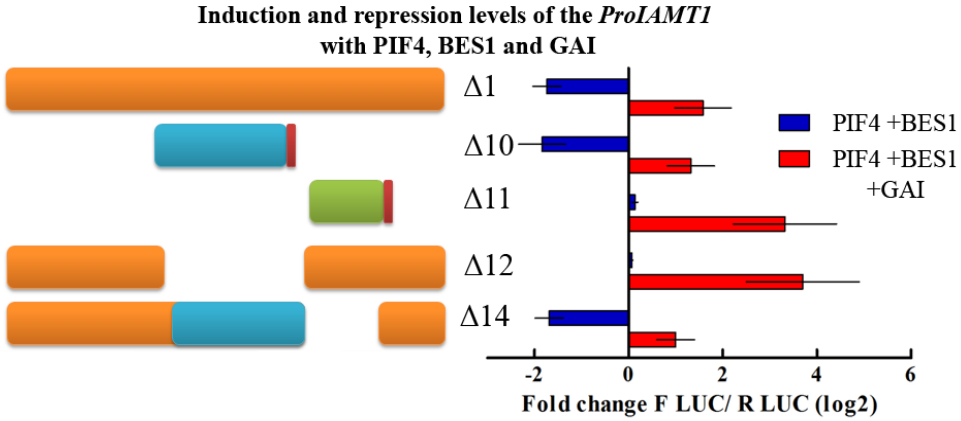


Figure IV.19 Transient expression assay in Nicotiana leaves showing the repression of the promoter of *IAMT1* through PIF4 and BES1 and the activation through GAI.
Error bars represent variation of measurements of three technical replicates of three biological replicates.

PIF4 has emerged as a key regulator of elongation in response to external signals, such as temperature and light, as well as internal signals, including GA and the circadian clock (Nozue et al., 2007; Lorrain et al., 2008). PIF4 is known to bind a conserved motif termed G-box (Sun et al., 2013). A close scan of the sequence of the region located between -469 and -854 for G-boxes using the ELEMENT tool (http://element.mocklerlab.org/motif_finders) allowed to identify two boxes

with the core sequence CATGCA suggesting that PIF4 binds the promoter of *IAMT1* through these motifs.

In summary, our results suggests that GAI is able to activate the expression of *IAMT1* by repressing PIF4 and BES1, which act in a region located -469 and -854 in the promoter of *IAMT1*, and at the same time aiding DRN to activate the promoter by binding to a region located in -209 and -387. This dual role for GAI raises questions about the conformation state GAI exhibits that allows it to act as a co-activator and a repressor of different TFs.

IV.6 Regulation of *IAMT1*'s expression through DELLA in *Arabidopsis thaliana*

Recently, a microarray experiment comparing gene expression between DRN expressing cells vs non-expressing cells in embryos showed that the levels of *IAMT1* is induced by almost 11-fold in DRN-expressing cells (Cole et al., 2013), supporting our hypothesis of *IAMT1* being a direct target activated by DRN. To study the regulation of *IAMT1* by DRN, BES1 and PIF4 in Arabidopsis plants, we used a loss-of-function *drn-1* mutant (Chandler et al., 2007) and 3 DRN inducible lines from the TRANSPLANTA collection (TPT_DRN) (Coego et al., 2014), a dominant version of BES1, *bes1-1D* (Yin et al., 2002) as well as a loss-of-function *pif4-101* (Niwa et al., 2009) and an over expresser *35S::PIF4* (de Lucas et al., 2008). RT-PCR showed that the transcript levels of *IAMT1* were strongly reduced in 3 days old etiolated *drn-1* seedlings, although PAC can still partially induce the levels of *IAMT1* (Figure IV.22). On the other hand, over expression of DRN through the estradiol inducible lines caused an

increase in the expression of *IAMT1* that was enhanced by PAC treatments (Figure IV.22, Suppl. III.5).

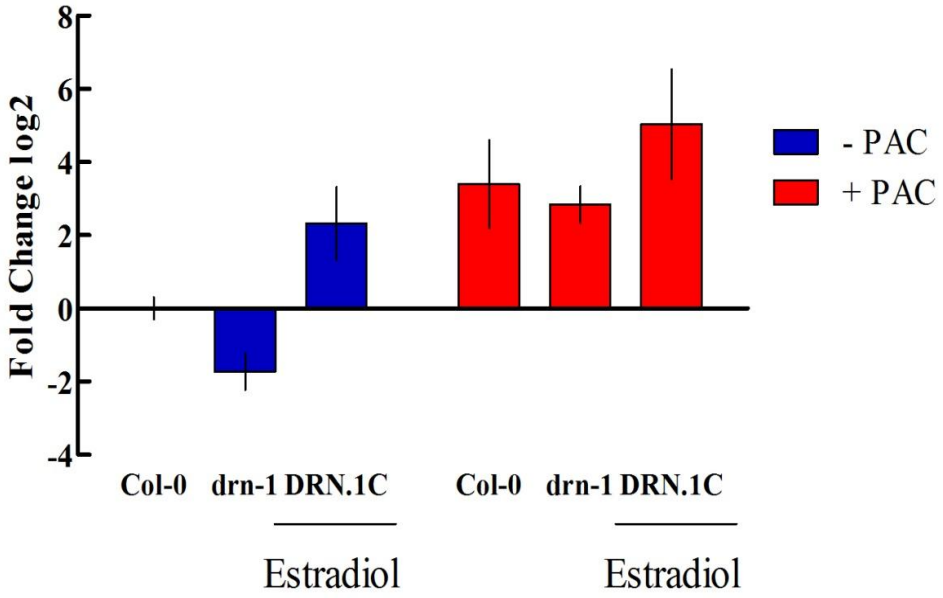


Figure IV.22Transcript levels of *IAMT1* in 3 days old etiolated Col-0, *drn-1* and TPT_DRN seedlings. Seedlings were treated with Mock or 0.4 µM PAC and in the case of TPT_DRN with 10 µM β-Estradiol.
Data represent the mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown.

Interestingly, using the dominant mutant *bes1-D* or treating plants with epibrassinolide (EBR) showed a decrease in the levels of *IAMT1* transcript, this decrease can be reverted by treatment with PAC (Figure IV.23), while the levels of *IAMT1* transcript increased in the *pif4* null mutant and decreased in the PIF4ox (Figure IV.24).

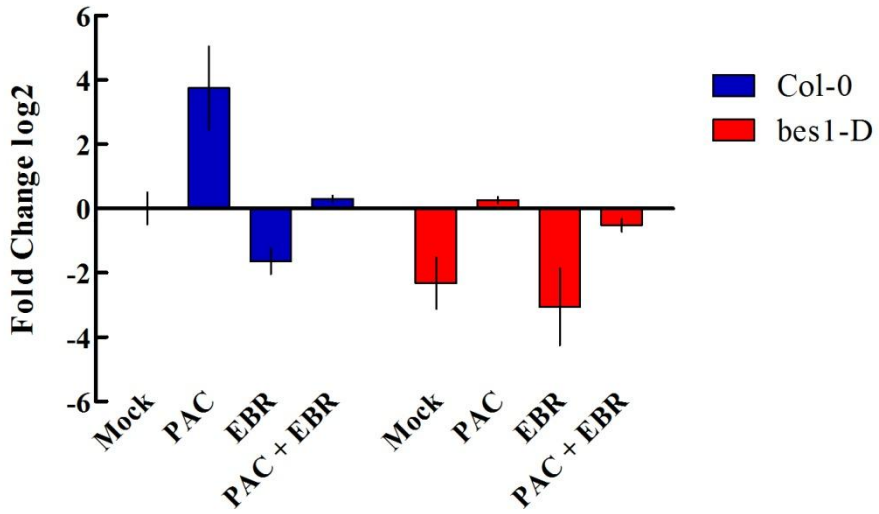


Figure III.23 Transcript levels of *IAMT1* in 3 days old etiolated Col-0 and bes1-D seedlings. Seedlings were treated with Mock, 0.4 µM paclobutrazol, 10 µM epibrassinolide or both.
Data represent the mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown.

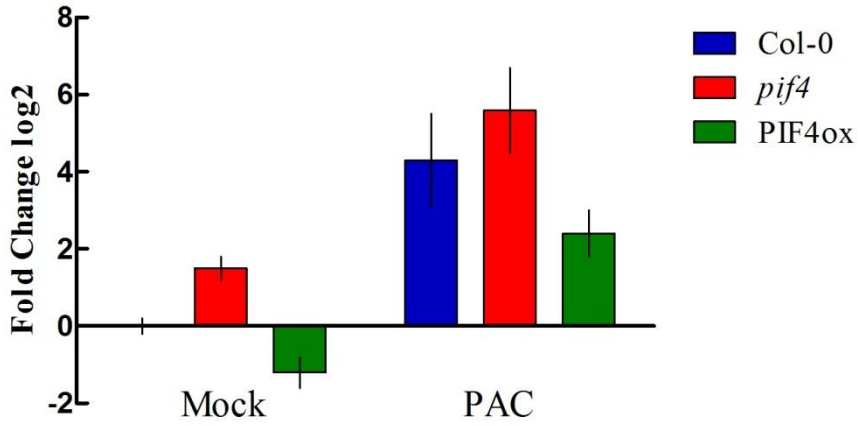


Figure III.24 Transcript levels of *IAMT1* in 3 days old etiolated *pif4* and PIF4ox seedlings. Seedlings were treated with Mock or 0.4 µM PAC.
Data represent the mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown.

Thought transient expression assays were useful to identify the different regulators and GAI partners needed for *IAMT1* regulation we urged ourselves to study the physiological relevance of this regulation. For that end, we studied if the two putative regions discovered are functional in Arabidopsis. We cloned deletion 1, deletion 12 and deletion 14 in front of the GUS gene (β-glucuronidase) and prepared transgenic plants (Figure IV.25). Deletions 1 and 14 and 16 were crossed in the *drn-1* and the estradiol inducible lines TPT_DRN.1C and TPT_DRN.1E.

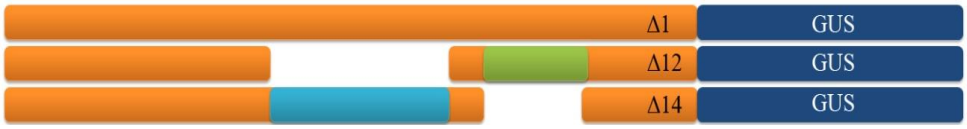


Figure III.25 A scheme depicting the fusion of the different deletions of the promoter of IAMT1 with GUS.

Unlike *ProIAMT1Δ14::GUS* , *ProIAMT1Δ1::GUS* shows decreased levels of GUS in the *drn-1* null mutant background and an increase response upon the induction of DRN in the TPT_DRN.1C by estradiol (Figure IV.26).

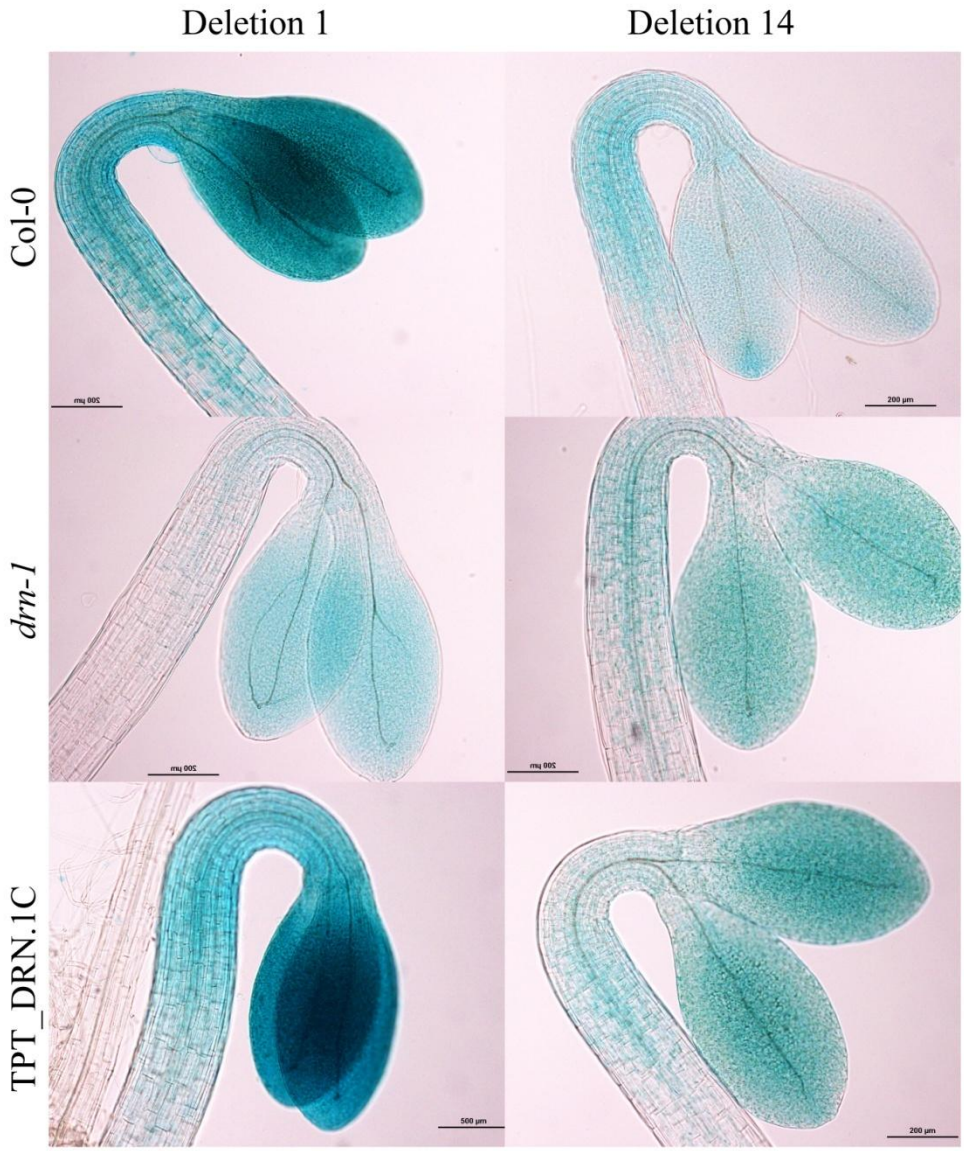


Figure IV.26 The expression pattern of *ProIAMT1Δ1::GUS* and *ProIAMT1Δ14::GUS* in wild type plants, *drn-1* and TPT_DRN.1C.

IV.7 Spatial control of the expression of *IAMT1*

GAI seems to be able to regulate the expression of *IAMT1* through dual routes, one aiding the transcription factor DRN to bind the promoter, and

the other by removing the repression of PIF4 and BES1 from the promoter of *IAMT1*. To see if this regulation is synchronic or independent we checked the transcript levels of *IAMT1* based on our knowledge from the expression pattern in previous works. The expression of IAMT1 using Diurnal tool (http://diurnal.mocklerlab.org/) showed that in long days IAMT1 is highly expressed during the in comparison to night, in agreement to the fact that DELLAs accumulate during the day (Alabadi et al., 2008; Arana et al., 2011), as a consequence we studied the regulation through DRN in light and dark grown seedlings. RT-qPCR showed that the levels of *IAMT1* transcript are indeed lower in etiolated seedlings, and although the *drn-1* exhibit lower levels of *IAMT1* transcript in etiolated seedlings it shows no impact in light grown seedlings (Figure IV.27).

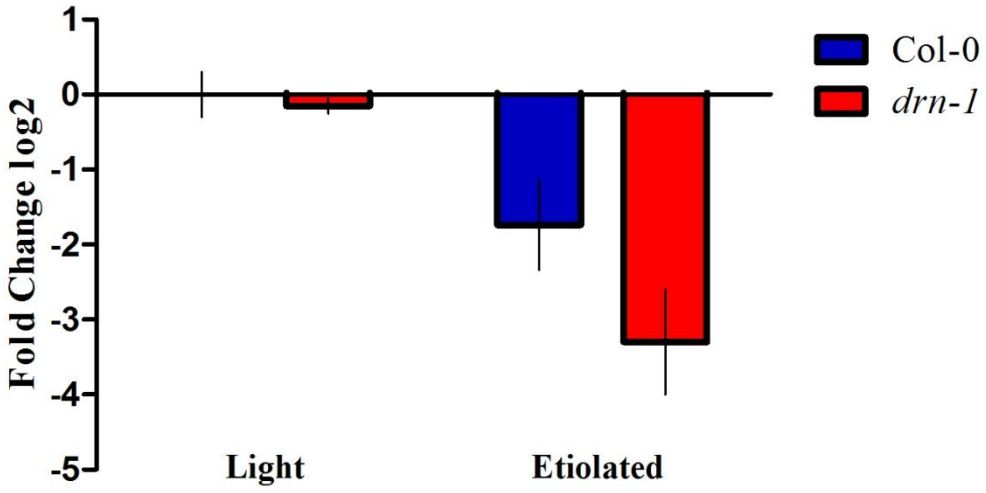


Figure IV.27 Transcript levels of *IAMT1* in Col-0 and *drn-1* in light and etiolated seedlings.
Data represent the mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown.

This specific regulation of *IAMT1* through DRN lead us to believe that DRN might have similar phenotypes in etiolated seedlings to the *iamt1* mutants, for that purpose we studied the response of *drn-1* to gravitropic reorientation. Indeed, *drn-1* exhibit defects reorientation in response to gravity (Figure IV.28) similar to *iamt1-1*.

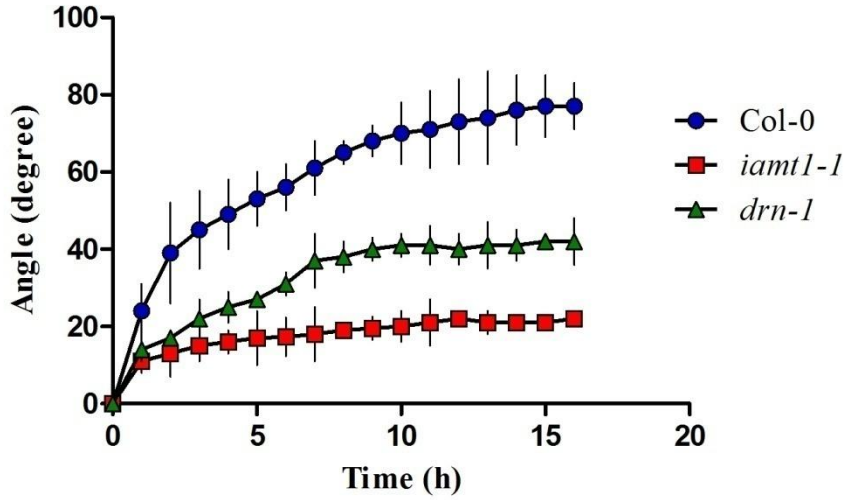


Figure IV.28 Kinetics of gravitropic reorientation of the *iamt1-1*and *drn-1* mutants in correspondence to Col-0.
Data represent the mean and standard deviation of 20 seedlings. Experiments were repeated twice with similar results; results from one representative experiment are shown.

To study the importance of PIF4 regulation on *IAMT1s* expression we looked for physiological phenomena where PIFs' activity is relevant, for instance in the response to changes in ambient temperature (Koini et al., 2009; Stavang et al., 2009). For that purpose we checked if *IAMT1* expression was sensitive to changes to temperature. QRT-PCR revealed that the transcript levels of *IAMT1* strongly decreased after moving seedlings from 22 degrees to 29 degrees (Figure IV.29).

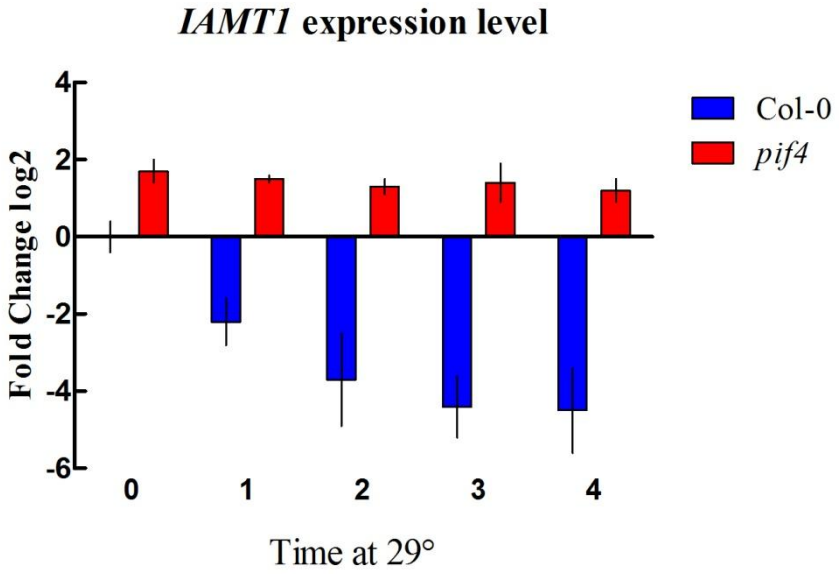


Figure IV.29 *IAMT1* transcript levels *IAMT1* in Col-0 and *pif4* at 22° and 1, 2, 3 and 4 hours at 29°.
Data represent the mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown.

Indeed, the levels of *IAMT1* transcript seems to be stable in *pif4* mutant in response to elevated temperature. To verify that this phenotype is directly related to PIF4 and *IAMT1*, we checked the GUS expression directed by a deleted promoter of *IAMT1* lacking the PIF4 binding site (deletion 12). GUS staining showed that although the levels of GUS decreased in deletion 1 in response to elevated temperature, deletion 12, lacking the PIF4 binding site, is resistant to this decrease and stays elevated even after 3 hours at 29 degrees (Figure IV.27).

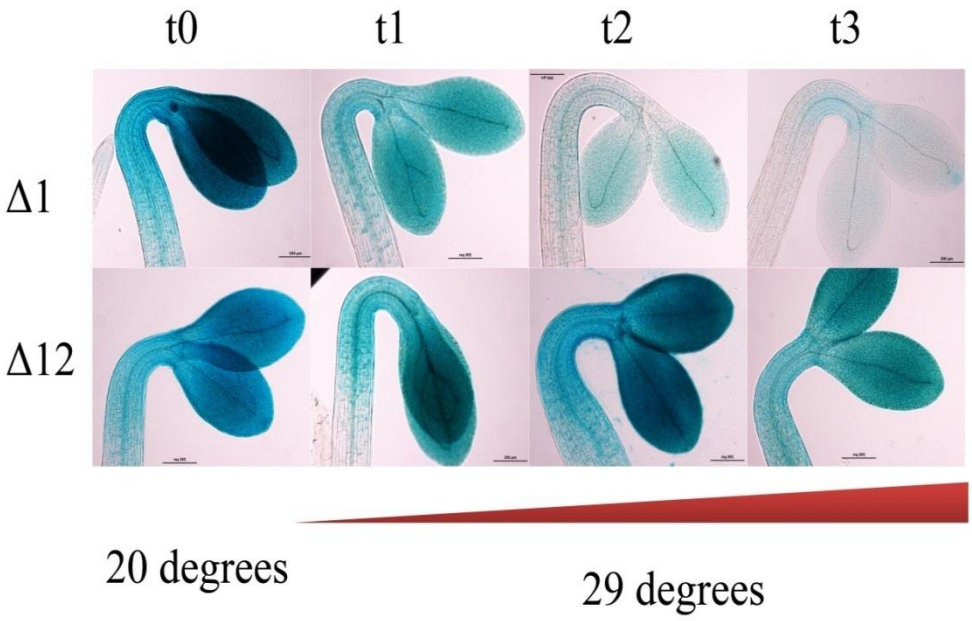


Figure IV.30GUS expression of the ProIAMT1::GUS deletions 1 and 12 at 20 degrees (t0) and after 3 hours at 29 degrees (t1, t2 and t3).

PIF4 is known as well to control cell expansion. The hypocotyl length of *pif4* seedlings grown at 22° and at 29° showed no difference, instead wild type hypocotyl length increased at high temperature (Franklin et al., 2011). In the same sense, 35S::PIF4 over expresser showed increased hypocotyl length at high temperature in comparison to wild type seedlings (Sun et al., 2012). To test whether *iamt1-1* exhibit similar phenotypes such as 35S::PIF4 concerning hypocotyl length in response to elevated temperature, seedlings were grown in continuous light for 7 days either at 20 degrees or 29 degrees. *iamt1-1* seemed to have a longer hypocotyl length at 20 degrees compared to wild type, and this phenotype is exaggerated in plants growing at 29 degrees. It is possible that the phenotypes *iamt1-1* exhibit, similar to the hypocotyl length of 35S::PIF4, might be due to the regulation through PIF4 (Figure IV.31) .

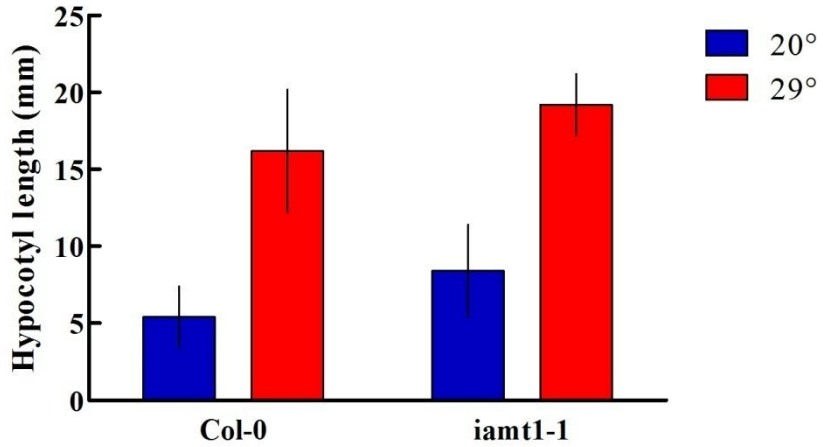
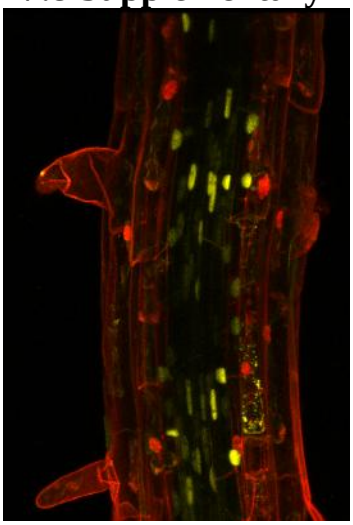


Figure IV.31 Hypocotyl length of continuous light grown seedlings of Col-0 and *iamt1-1* at 20 or 29 degrees.
Data represent the mean and standard deviation of 15 - 20 seedlings measured. Experiments were repeated twice with similar results; results from one representative experiment are shown.

IV.8 Supplementary



Supplementary IV.1 Confocal image of the expression of 35S::DII-VENUS in *iamt1-1* background in the elongation zone of the root.

Supplementary IV.2 List of genes and transcription factors that are co-expressed, co-localized, genetic interactors, physical interactors, and proteins that share domains with IAMT1 using GeneMANIA tool.

Genes positively correlated with IAMT1and their partners	TFs positively correlated with IAMT1 and their partners
ADAP	AT1G71520
AIL5	AT1G49120
AIL7	AT5G21960
ANT	ABR1
AP2	AT1G12890
AT1G01250	AT1G68550
AT1G12630	SHN3
AT1G19210	AT1G50680
AT1G28160	ERF110
AT1G33760	AT4G13040
AT1G36060	AT1G51120
AT1G44830	ERF10
AT1G64380	AT2G40350

AT1G71130	CRF4
AT1G71450	RAP2.9
AT1G75490	ATERF14
AT1G77200	AT1G25470
AT1G77640	DREB2C
AT1G79700	AT4G13620
AT1G80580	CRF5
AT2G20350	AT5G25190
AT2G20880	WRI1
AT2G22200	DRN
AT2G25820	AT5G52020
AT2G44940	ERF1
AT3G16280	AT1G44830
AT3G23220	AT2G25820
AT3G23230	CRF1
AT3G57600	AT1G64380
AT3G60490	ATCBF3
AT4G16750	AT3G16280
AT4G18450	TINY2
AT4G28140	AT3G23230
AT4G31060	ADAP
AT4G32800	ATERF38
AT4G39780	AT1G77200
AT5G07310	AT1G01250
AT5G25190	AT2G44940
AT5G43410	tny
AT5G52020	DRN
AT5G65130	TCP14
AT5G67000	AGl86
AT5G67010	ANAC042
ATCBF3	AT1G33760
ATERF13	HRD
ATERF14	CBF4
ATERF15	AT2G20880
ATERF38	AP2
BBM	TOE3

BIM1	AIL7
CBF4	PLT1
CRF1	DEAR3
CRF3	AT4G16750
CRF5	DREB26
DDF1	AT4G32800
DEAR2	AT5G67000
DEAR3	AT1G71450
DREB26	ddf2
DREB2B	AT1G77640
DREB2C	AT5G67010
DRN	AT3G23220
EDF3	ERF9
ERF1	AT3G57600
ERF2	FTQ4
ERF5	BBM
ERF9	AT1G80580
FTQ4	AT4G18450
HRD	AT3G60490
LEP	AT1G75490
ORA47	AIL5
ORA59	AT2G20350
PHV	RAP2.1
PLT1	AT4G39780
PLT2	AT2G22200
RAP2.1	AT5G65130
SMZ	ORA59
SNZ	AT5G07310
TINY2	DEAR2
ANAC059	ERF5
TOE3	AT4G28140
WRI1	LEP
ddf2	AT1G19210
tny	TOE2
ADAP	SNZ
AIL5	ANT

AIL7	ANAC059
ANAC042	PLT2
AP2	DREB2B
AT1G01250	AT4G31060
AT1G12630	AT1G79700
AT1G19210	ATERF15
AT1G28160	AT5G43410
AT1G33760	bHLH18
AT1G36060	AT1G12630
AT1G44830	ORA47
AT1G64380	ERF2
AT1G71130	AT1G36060
AT1G71450	AT1G28160
AT1G75490	ATERF13
AT1G77200	CRF3
AT1G77640	AT1G71130
AT1G79700	EDF3
AT1G80580	
AT2G20350	
AT2G20880	
AT2G22200	
AT2G25820	
AT2G44940	
AT3G16280	
AT3G23220	
AT3G23230	
AT3G57600	
AT3G60490	
AT4G16750	
AT4G18450	
AT4G28140	
AT4G31060	
AT4G32800	
AT4G39780	
AT5G07310	
AT5G25190	

AT5G43410
AT5G52020
AT5G65130
AT5G67000
AT5G67010
ATCBF3
ATERF13
ATERF15
ATERF38
BBM
BIM1
CBF4
CRF1
CRF3
CRF5
ANAC42
DEAR2
DEAR3
DREB26
DREB2B
DRNL
EDF3
ERF1
TCP14
ERF5
ERF9
FTQ4
HRD
LEP
ORA47
ORA59
PLT1
PLT2
RAP2.1
bHLH18
SNZ

TINY2
TOE2
TOE3
ddf2
tny
AGL86
ERF2
ANT

Supplementary IV.3 List of genes that are negatively correlatedwith IAMT1 using coexpression tool from www.cressexpress.org and angualr expression from www.bar.utorano.ca.

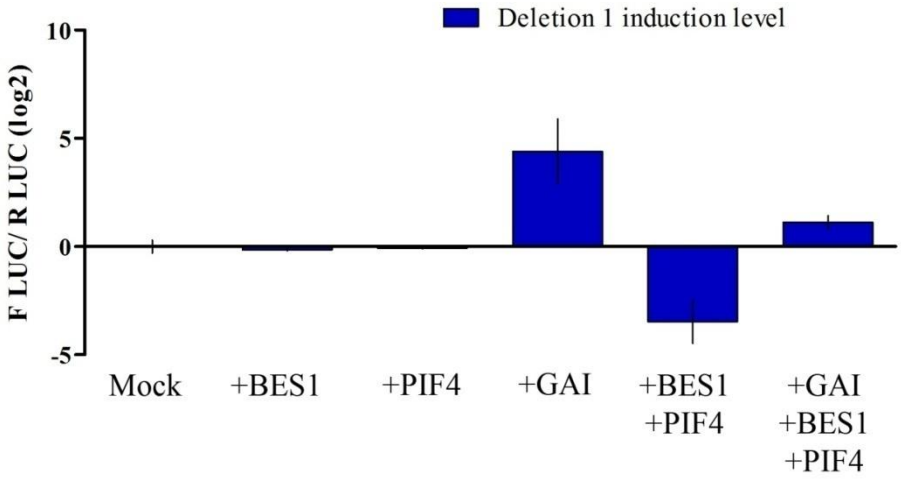
CRESS	BAR.UTORANTO.CA
AT2G26570	AT4G11330
AT2G26520	AT3G25870
AT2G26450	AT3G42120
AT2G26480	AT3G44510
AT2G39730	AT1G07780
AT2G23260	AT3G56150
AT2G23180	AT2G01450
AT2G23160	AT1G13330
AT2G23270	AT1G31270
AT2G39820	AT5G61770
AT2G39830	AT1G33390
AT2G39850	AT5G58810
AT2G40910	AT4G22820
AT2G40830	AT4G00335

AT2G40890	AT2G15960
AT2G41690	AT2G43010
AT2G41520	AT4G19960
AT2G41630	AT3G42550
AT2G41620	AT3G01330
AT2G41560	AT4G27550
AT2G41680	AT2G03090
AT2G47420	AT5G63880
AT2G47450	AT3G02080
AT2G47600	AT5G06370
AT2G45350	AT1G06580
AT2G45340	AT4G26590
AT2G45320	AT5G04760
AT2G45250	AT1G18330
AT2G45420	AT4G16260
AT2G45270	AT2G37730
AT2G45240	AT3G04840
AT5G12450	AT3G01300
AT2G33160	AT1G66480
AT2G33080	AT1G72450
AT2G33230	AT1G23690
AT2G33150	AT4G32750

AT2G47560	AT1G22280
AT2G47540	AT4G15260
AT5G12410	AT3G43240
AT1G67710	AT4G15520
AT1G19350	AT4G20300
AT1G67660	AT4G23570
AT1G67740	AT5G10270
AT5G12270	AT2G43620
AT5G12340	AT5G28820
AT1G44224	AT3G03020
AT4G16143	AT1G19350
AT1G38340	AT5G27820
AT3G29810	AT5G60310
AT4G25610	AT3G56340
AT4G25590	AT1G49830
AT4G25580	AT4G15545
AT1G44510	AT1G22890
AT1G44446	AT1G64790
AT1G44350	AT3G49910
AT1G44318	AT1G02305
AT1G44224	AT3G22650
AT4G17500	AT1G23560

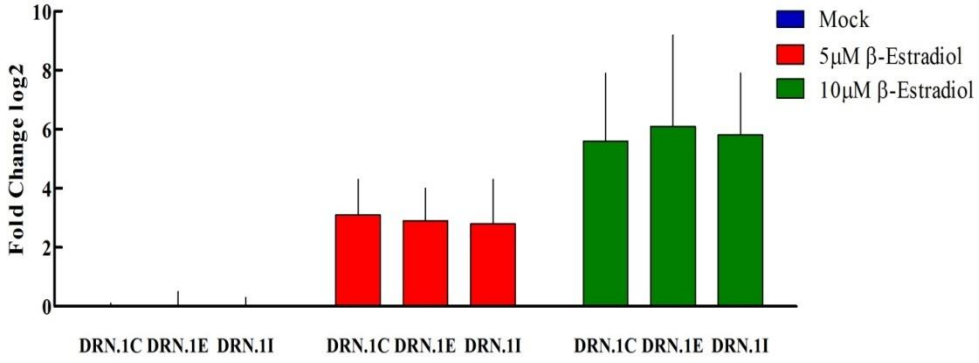
AT4G15440	AT3G26770
AT4G14110	AT1G55280
AT4G16360	AT1G77680
AT4G14385	AT2G17520
AT2G43010	AT1G65390
AT4G15210	AT1G35860
AT4G16780	AT4G00170
AT4G14030	AT5G54430
AT4G15470	AT4G30330
AT4G14340	AT3G27660
AT4G17695	AT3G27550
AT4G16770	AT1G18050
AT4G17486	AT3G10950
AT4G15420	AT3G28470
AT4G16146	AT2G39390
AT4G14130	AT1G23350
AT4G14410	AT3G02190
AT4G16160	AT5G05160
AT4G17090	AT2G06130
AT4G16000	AT2G06310
AT4G14430	AT5G45060
AT4G17460	AT2G02830

AT4G15120	AT5G16505
AT4G17720	AT4G17840
AT4G15975	AT2G21230
AT4G16840	AT2G39200
AT4G17620	AT3G07710
AT4G17650	AT4G09860
AT4G17585	AT4G19620
AT4G17150	AT1G14620
AT4G17160	246974_AT
AT4G17170	AT3G09140
AT4G17180	AT1G59960
AT4G17310	AT3G46390
AT4G17330	AT1G75090
AT4G17360	AT2G36730
AT4G17380	AT2G18250
AT4G17430	AT3G19330
AT4G16670	AT3G58660
AT4G16740	AT1G52520
AT4G16820	AT2G17180
AT4G16860	AT5G12880



Supplementary IV.4 Transient expression assay in *Nicotiana* leaves showing the response of the promoter of *IAMT1*to BES1, PIF4, GAI and a combination of all three.

Error bars represent variation of measurements of three technical replicates of three biological replicates.



Supplementary IV.5 Transcript levels of *IAMT1* in 3 days old etiolated TPT_DRN seedlings. Seedlings were treated with Mock or with 10 µM β-Estradiol.

Data represent the mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown.

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DISCUSSION

The main contributions of this thesis are:

(1) the finding that IAA methylation is an integral component of the generation of lateral auxin gradients that lead to differential growth; and

(2) the elucidation of the mechanism by which DELLAs regulate *IAMT1* expression and, consequently, a new mode of regulation of differential growth by GAs.

V.1 The role of IAA methylation during differential growth

The involvement of IAA methylation in the formation of auxin gradients was initially suspected because of the agravitropic phenotype of iRNA lines targeted to the inactivation of IAMT1 (Qin et al., 2005). However, the high degree of similarity between IAMT1 and other methyl transferases, as well as the extremely severe defects of these lines all along development, raised the doubt as to the specificity of the RNA interference. In fact, the two T-DNA insertion lines characterized in this work display a much milder mutant phenotype, in which only differential growth seems to be affected. The observation that *iamt1-1* mutant seedlings still contain relatively high levels of MeIAA indicates that there exist alternative pathways for IAA methylation, but this does not diminish the relevance of the studies presented here, in which very clear mutant phenotypes are caused by the inactivation of *IAMT1*.

Several lines of evidence point to a role of IAA methylation in the control of polar auxin transport (PAT), necessary for differential growth:

(1) the higher auxin flux through etiolated hypocotyls of the *iamt1* mutant;

(2) the enhanced (and expanded) expression of *PIN* genes in the *iamt1* mutant; and, more importantly,

(3) the alleviation of the agravitropic phenotype of *iamt1* mutants by low doses of NPA.

Contrary to other mutations that impair differential growth by decreasing auxin synthesis, transport or sensitivity (Lehman et al., 1996; Chen et al., 1998; Luschnig et al., 1998; Reed et al., 1998; Stepanova et al., 2008; Chen et al., 2014), the impairment of differential growth in the *iamt1* mutants is associated to excessive auxin transport, and higher local auxin levels in the responding organs, as revealed by DR5 and DII-VENUS examination. This observation clearly indicates that the formation of lateral auxin gradients requires a certain level of basal auxin flux, and any deviation under or over this flux prevents the proper formation of the gradient, and optimal response for instance to tropic stimuli.

How does IAA methylation contribute to control auxin homeostasis for the formation of auxin gradients? It is important to remark that the pool of MeIAA does not account for more than 2% of the total pool of IAA. This may explain why we did not detect statistically significant differences in IAA levels in *iamt1* mutant seedlings. But it also rules out the possibility that IAA methylation is simply a mechanism to inactivate IAA at the source tissues, and thereby decrease the pool of available active auxin.

We envision at least two mechanisms by which IAA methylation could restrict auxin transport. The first one would be the reduction of the auxin pool in the cells of the target tissues. It has been proposed that auxin promotes its own transport as it passes by the cells (Peret et al., 2013).

Thus, local reduction of available IAA in a cell by certain degree of methylation via IAMT1, would maintain the appropriate level of polar auxin transport. On the contrary, loss of IAMT1 function would increase the concentration of free IAA as it passes by the responding cells, and this would enhance the feed-forward loop on auxin transport.

Alternatively, we have to invoke a biological function for MeIAA, rather than constituting an inactive form of auxin. Methylated forms of other hormones have been attributed specific biological roles (Seo et al., 2001; Chen F, 2003), but previous investigations of a possible function of MeIAA have not yielded any positive result (Li et al., 2008). In those assays, MeIAA application caused developmental defects equivalent to those caused by auxin, although at much higher concentrations than IAA. Likely, the presence in plant tissues of methyl esterases (general, or specific for MeIAA), is responsible for the transformation of applied MeIAA to IAA and the subsequent auxin-related effects.

In case MeIAA had a biological function, it would have to be opposite to that of IAA to explain the phenotype of *iamt1* mutants. As depicted in Figure V.1, there are at least three reasonable mechanisms by which MeIAA could act as an IAA antagonist: (1) the inhibition of PIN auxin efflux carriers; (2) the inhibition of AUX/LAX or other auxin influx transporters; and (3) the inhibition of auxin perception by TIR1/AFBs. We have initiated the evaluation of these possibilities, with negative results so far. For instance, we have used the auxin sensor system introduced in human cell cultures (Wend et al., 2013), and MeIAA does not seem to be recognized by TIR1-IAA17 or interfere with the recognition of IAA by this

receptor complex (Matías Zurbriggen and Sophia Samodelov, unpublished).

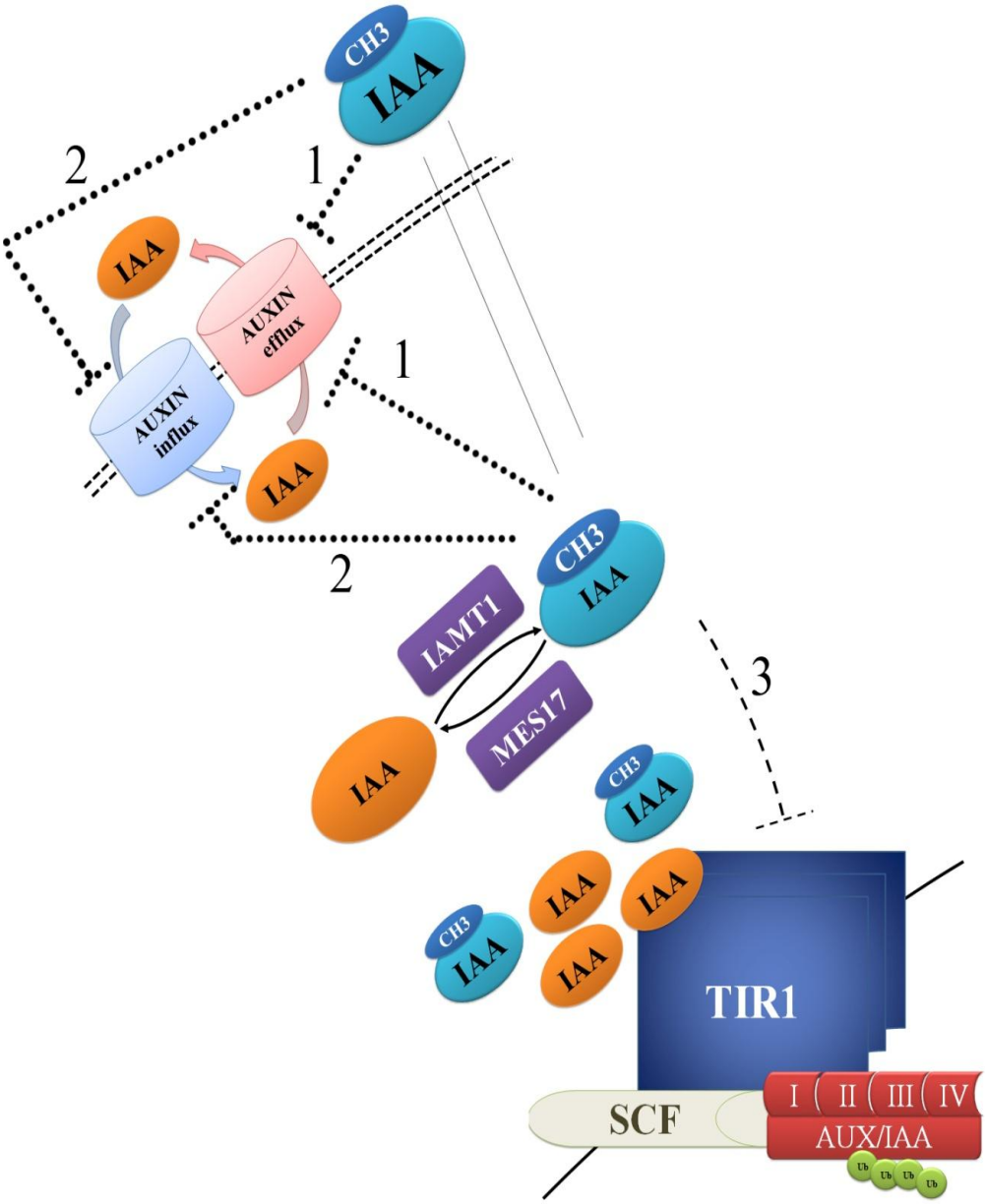

Figure V.1 Schematic model depicting the possible role sand effects of IAMT1 and MeIAA

1: The competition of MeIAA for auxin efflux carriers; 2: The competition of MeIAA for auxin influx carriers; 3: The competition of MeIAA for the auxin receptor TIR1.

V.2 The regulation of *IAMT1* expression by DELLA proteins

The observation that DELLA proteins regulate *IAMT1* expression opened two standing questions: which TFs mediate the regulation by DELLAs? And what is its physiological relevance? Our results have provided some clues on these two issues.

DELLA proteins have been proposed to act as hubs connecting different signaling networks. The mechanism by which transcriptional regulation is achieved by DELLAs is through physical interaction with multiple TFs (Rosa, 2014) and the modification of the activity of these TFs. This modification consists in preventing the binding of the TF to the target promoters, but it has recently been shown that DELLAs can also be recruited by other interacting TFs to the target promoters, where they mostly act as transcriptional coactivators (Lim et al., 2013; Yamaguchi et al., 2014). Here we have found that DELLAs activate *IAMT1* transcription through two alternative TFs, and through the two proposed mechanisms: inhibition of the BZR1/PIF4 repressors, and coactivation via the DRN positive regulator (Figure V.2).

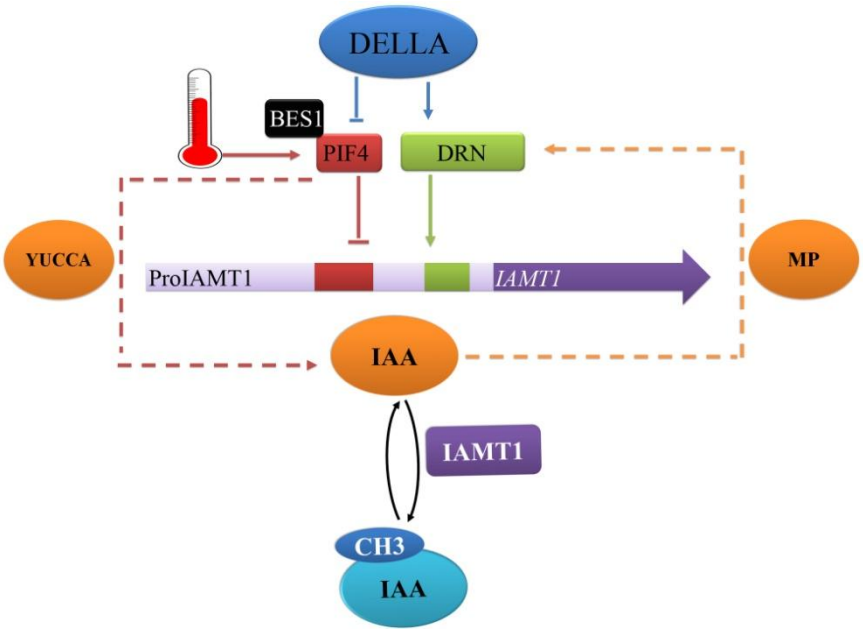

Figure V.2 Schematic model depicting molecular mechanism of regulation of DELLA on *IAMT1*'s expression.
DELLAs are able to repress the repression of the complex PIF4-BES1; BES1, the temperature induced transcription factor, is able to induce IAA biosynthesis through the regulation of the YUCCA genes; IAA in turn is able to induce the expression of DRN through the activity of MP; DRN along side with DELLA is able to induce the expression of *IAMT1* whose activity is important to methylate free IAA.

Interestingly, each mechanism seems to be more relevant in a particular condition; i.e., through BZR1/PIF4 for the regulation in response to temperature, and through DRN in the absence of light. Considering that GAs, auxin, and brassinosteroids are all involved in temperature-dependent regulation of cell expansion (Gallego-Bartolome et al., 2011), the regulation of auxin homeostasis by GAs in this process may represent one of the multiple crosstalk mechanisms in place to provide optimal responses. On the other hand, the relevance of the regulation of *IAMT1* by DELLAs via DRN seems to be major in the regulation of tropic responses, as shown

by the similar agravitropic phenotype caused loss of IAMT1, DRN (this work) or DELLA function, albeit with different severities.

In any case, more work is necessary to solve several issues. Apart from the elucidation of the mechanism that links IAMT1 with the regulation of *PIN* expression, it is important to define the spatial constrains that affect the regulation of IAMT1 in different cell types, not only by DELLAs.

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CONCLUSIONS

- IAMT1 has an essential role in controlling differential growth, especially apical hook development and gravitropic reorientation, through the maintenance of an auxin gradient due to its unique mode of expression in auxin sources and targets thus allowing proper flow of IAA.
- The importance of the regulation of IAA homeostasis through IAMT1 is of a high magnitude that allow proper expression of the auxin efflux carriers PINs, whose proper expression and localization allows proper auxin transport from the cotyledons to downward tissues.
- The molecular regulation of DELLAs on the expression of IAMT1 is dedicated to two regions of the promoter of *IAMT1* used by one side by DELLAs and DRN to co-activate the expression of IAMT1, and on the other side by the repression of DELLA on the repressing complex PIF4-BES1.
- The importance of the dual modes of the regulation of DELLA on *IAMT1*s expression is due to the implication of the DELLA partners in their signaling systems, whereas the regulation through PIF4-BES1 on *IAMT1*s expression is important to allow proper response to light and elevated temperature. While the regulation through DRN is implicated in the proper agravitropic reorientation of etiolated seedlings.

MATERIALS AND METHODS

VII.1 Plant lines and growth conditions

Arabidopsis thaliana mutant plants *iamt1-1 (SALK_072125), mes17* (Yang et al., 2008), *DR5::GUS, DR5rev::GFP* (Sabatini et al., 1999; Friml et al., 2003b), *35S::DII-VENUS* (Brunoud et al., 2011), *PIN2::GUS* (Friml et al., 2003a), *ProPIN3::GFP-PIN3* (Blilou et al., 2005), *drn-1* (Chandler et al., 2007), *pif4-101* (Niwa et al., 2009) *bes1-d* (Yin et al., 2002), *35S::PIF4* (Franklin et al., 2011) *TPT_DRN.1C, TPT_DRN.1E, TPT_DRN.1I* (Coego et al., 2014) were all in the Col-0 ecotype, *iamt1-2 (GT_5_41946)* was in L*er*-0 ecotype. For germination, seeds were surface-sterilized and sown on sterile Whatman filter papers placed in plates of half-strength MS medium (Duchefa) with 0.8% w/v agar and 1% w/v sucrose, and stratified at 4°C for 4 to 7 days in darkness. Germination took place under continuous white fluorescent light (90–100 μmol m−2 sec−1) at 23°C in a Percival growth chamber E-30B then either kept under light or moved to darkness. For differential growth experiments, seedlings were grown on MS without sucrose. Soil-grown plants were kept at 23ºC in cabinets with 16-h photoperiod. For experiments incubated with chemicals, Col-0, *iamt1-1* was germinated on mock MS medium for 3 days in etiolated seedlings then transferred for MS plates containing 0.125, 0.25, 0.5, 1 and 10 μM NPA N-(1-naphthy1)phthalamic acid (Sigma); Col-0 was grown on mock MS plates for 3 days then shifted to MS plates supplied with 0.4 μM paclobutrazol or 10 μM epibrassinolide. For the transplanta lines, upon germination seedlings were transferred to MS plates supplied with 10 μM β-estradiol.

For temperature variation experiments, seedlings were grown at 20°C degrees for 3 days then shifted to 29°C degrees for 3 days. Temperature

growth experiments was conducted as follows, seedlings were germinated on MS then transferred to 20°C or 29°C for 7 days

VII.2 Genotyping *iamt1* mutants

iamt1-1 and *iamt1-2* were obtained from The European Arabidopsis Stock Centre (uNASC). Seedlings were genotyped using primers in table VII.1

Table VII.1 Oligos used to genotype *iamt1-1* and *iamt1-2*.

Oligo Name	Sequence (5'- 3')
SALK_F1	TTAAAGAGAGAAGGAGAGATCCATAGAGA
SALK_F2	GCCACCTTTCATGCTGAGAAG
*IAMT1*GENO_F2	TGCCGGAGAGAAGACAACAAC
*IAMT1*GENO_R2	CATGGCCCCACCTCTCTTAA
FW3	CGCCAACGGCTCATTTG
REV3	AGGCACGACCAACTTCTGATG

VII.3 Growth rate and tropism conditions

For growth rate seedlings and hook analysis were germinated for 8 hours under light then transferred for complete darkness (22 ºC) in a vertical orientation on plates containing half strength MS medium (Duchefa) with 0.8% w/v phytoagar and without sucrose for 7 days.

For reorientation experiments, the conditions were the same as above but after 3 days of growth the plates were reoriented 90º relative to the initial growth angle.

In all experiments, seedlings were recorded every half an hour under infrared light using CCD cameras coupled to Metamorph software. Pictures and angles were measured using ImageJ software.

VII.4 Generation of double mutant lines

iamt1-1 was crossed to *DR5::GUS*, *DR5::GFP*, *35S:DII-VENUS*, *PIN2::GUS* and *PIN3:GFP-PIN3*. F1 and F2 generations were selected based on their resistance to kanamycin and by genotyping for GUS or GFP (Table VII.2). Lines with a 3:1 (resistant: sensitive) segregation ratio were

selected and at least 10 homozygous lines were identified in the F3 generation for each cross.

Table VII.2 Oligos used to sequence GUS and GFP.

Oligo name	Sequence (5' – 3')
GUS-FW	CAACGTCTGCTATCAGCGCGAAGT
GUS-REV	TATCCGGTTCGTTGGCAATACTCC
GFP-FW	ATGAGTAAAGGAGAAGAACTTTTC
GFP-FW	GTATAGTTCATCCATGCCATGTG

VII.5 Real-time quantitative RT-PCR

Total RNA from imbibed seeds was extracted using a modified RNAeasy Mini kit (Qiagen) protocol, where the grinded tissue was previously incubated with 600 µl of RLT-PVP buffer (540RLT buffer from RNAeasy Mini Kit + 60 µl PVP40 10% + 6 µl ß-mercaptoethanol) followed by a 30 s centrifugation, recovery of the supernatant, and application to the lilac column. cDNA synthesis and quantitative PCR, as well as primer sequences for amplification of *PIN1*, *PIN2*, *PIN3*, *PIN7*, *IAMT1* and *EF1-α* genes, have been described previously (Frigerio et al., 2006; Gallego-Bartolome et al., 2011a) (Table VII.3).

Table VII.3 Oligos for qRT for IAMT1, PIN1, PIN2, PIN3 and PIN7.

Oligo name	Sequence (5' - 3')
GT_F3	CTGCCTTCTCCTTGCATTGG
GT_R3	TATCCGTCACACTTTCCGGC
PIN1-FW	TTGCTTCTTATGCCGTTGGC
PIN1-REV	CTCTTTGGCAAACACAAACGGT
PIN2-FW	AAGCCAACGCGAAGAATGC
PIN2-REV	CGCTTTAGTAGCGAGGTTGTCG
PIN3-FW	GAGACGGCTGCTTCCATTGTT
PIN3-REV	CCGTCGTCAATCTCTGCAT
PIN7-FW	CGTCACCGATTTGAGCAG
PIN7-REV	TCCAGAGACTGGTGCGATT

VII.6 Construction of transgenic lines

Different deletions of the promoter of *IAMT1* were cloned using primers present in table VII.4. DNA was amplified from genomic DNA of Col-0. Deletions from 1 to 9 contained *Hin*dIII restriction sites at their 5' end and *Nco*I restriction site at their 3' end. For the construction of ProIAMT1Δ1035SmCaMV and ProIAMT1Δ1135SmCaMV forward primers included *Hin*dIII recognition sites, the reverse primer contained *Bam*HI-*Spe*I and *Not*I recognition sites for deletion 10 and 11 respectively, *Bam*HI and *Not*I were used to fuse the deletions to *Bam*HI and *Not*I present in CAMV35Sm-*Bam*H1-*Not*1-*Nco*I, the CAMV35Sm was annealed to itself to generate a double stranded fragment with *Bam*H1-*Not*1-*Nco*I recognition sites, the fragment was digested with appropriate restriction enzymes and fused to Del 10 and 11. For the construction of ProIAMT1Δ12 and ProIAMT1Δ14, ProIAMT1Δ1-FW-*Hin*dIIIprimer was used with ProIAMT1Δ12-REV and ProIAMT1Δ14-REV to generate the first piece, while ProIAMT1-REV-*Nco*I was used with ProIAMT1Δ12-FW and ProIAMT1Δ14-FW to generate the end piece, the two pieces for each deletion were overlapped using the thermocylcer and a PCR was made using ProIAMT1Δ1-FW-*Hin*dIII and ProIAMT1-REV-*Nco*I to generate the full fragments. For the generation of ProIAMT1Δ15, ProIAMT1Δ1-FW-*Hin*dIII was used along side with ProIAMT1Δ15-Rev to generate the first fragment, ProIAMT1Δ15-MF-*Spe*I and ProIAMT1Δ15-MR-*Not*I were used to generate the second fragment, both fragments were fused by overlapping PCR as above then the recognition site *Not*I was used to fuse it to CAMV35Sm-*Bam*H1-*Not*1-*Nco*I.

To generate ProIAMT1Δ16, ProIAMT1Δ1-FW-*Hin*dIII was used with ProIAMT1Δ12-REV to generate the first piece, the second piece was

generated using ProIAMT1Δ12-FW and ProIAMT1Δ14-REV, the third and last piece was generated using ProIAMT1Δ14-FW and ProIAMT1-REV-*Nco*I, all three pieces were fused using overlapping PCR as stated above. For the generation of ProIAMT1Δ13.ProIAMT1Δ1-FW-*Hin*dIII was used with ProIAMT1Δ12-REV to generate the first piece, ProIAMT1Δ14-FW and ProIAMT1-REV-*Nco*I were used to generate the last pieces, both pieces were fused by overlapping PCR.

All deletions were cloned into PCR8 (invitrogen) then subcloned using gateway technology to the pMDC162 GUS expressing vector (Curtis and Grossniklaus, 2003) to produce *ProIAMT1:GUS*.

The construction of *IAMT1::GFP-IAMT1, SCR::GFP-IAMT1* and *ML1::GFP-IAMT1* was as follows: The *IAMT1* coding sequence was amplified from cDNA of the Col-0 with primers that included the attB1 and attB2 Gateway recombination sites (not shown) (Table VII.4), respectively. The PCR product was cloned into *pDONR221* (Invitrogen) by BP reaction, and then into the binary vector *pSBright:GFP*(Bensmihen et al., 2005) by LR reaction to give rise to *pSBright:GFP-IAMT1* construct. The *ML1* promoter was PCR-amplified using primers described (An et al., 2004) and that included the *Hin*dIII recognition site. The PCR product was cloned into the *pCR8* vector and sequenced. After digestion with *Hin*dIII, the *ML1* promoter was cloned into the *Hin*dIII site of *pSBright:GFP-IAMT1*, to create *ProML1:GFP-IAMT1*. For the generation of *ProSCR::GFP-IAM1*, the promoter of SCARECROW was amplified as described before (Ubeda-Tomás et al., 2008) with primers that included the *Hin*dIII and *Xba*I recognition sites. The PCR product was cloned into the *pCR8* vector and sequenced. After digestion with *Hin*dIII and *Xba*I, the *SCR* promoter was cloned into the *Hin*dIII and *Xba*I sites of *pSBright:GFP-IAMT1*, to create

ProSCR:GFP-IAMT1. For the generation of *ProIAMT1:GFP-IAMT1* the *IAMT1* promoter was cloned using deletion 1 primers that included *Hin*dIII and *Xba*I recognition sites. The PCR product was cloned into the *pCR8* vector and sequenced. After digestion with *Hin*dIII and *Xba*I, the *IAMT1*promoter was cloned into the *Hin*dIII and *Xba*I sites of *pSBright:GFP-IAMT1*, to create *IAMT1::GFP-IAMT1*.

Constructs were introduced in *Agrobacterium* strain C58 and used to transform *Arabidopsis* Col-0 wild type plants and *iamt1-1 DR5::GUS*, *DR5::GFP* and *35S:DII-VENUS*. Transgenic seedlings in the T1 and T2 generations were selected on 50 µM glufosinate ammonium (Sigma). Transgenic lines with a 3:1 (resistant: sensitive) segregation ratio were selected, and 10 homozygous lines were identified in the T3 generation. Data from two representative lines are shown.

Table VII.4 Oligos for the generation of the deletions of *ProIAMT1* and for the generation of IAMT1 CDS.

Oligo Name	Sequence (5'- 3')
ProIAMT1-REV-*Nco*I	GAACCCATGGTCTTTCTCTTTCTC
ProIAMT1Δ1-FW-*Hin*dIII	AAGCTTTAATACTCCTTATG
ProIAMT1Δ2-FW-*Hin*dIII	TTAAGCTTATATATATATCTCAAT
ProIAMT1Δ3-FW-*Hin*dIII	TCAGATATTGAAAAGCTTATA
ProIAMT1Δ4-FW-*Hin*dIII	TATTGATTATTGTGTAAGCTTAT
ProIAMT1Δ5-FW-*Hin*dIII	CTCTTTACTCCAAGCTTAGC
ProIAMT1Δ6-FW-*Hin*dIII	AAGCTTGACAAACCATGTAT
ProIAMT1Δ7-FW-*Hin*dIII	AAGCTTGTATTTTGTTTTTTG
ProIAMT1Δ8-FW-*Hin*dIII	TATATACAAAAGCTTATGTACGC
ProIAMT1Δ9-FW-*Hin*dIII	AAGCTTCATACATTTATCATTTC
ProIAMT1Δ10-FW-*Hin*dIII	TTAAGCTTATATATATATCTCAAT
ProIAMT1Δ10-Rev-*Bam*H1-*Spe*I	ACTAGTGGATCCATTTTCAATATCT GATGTG
ProIAMT1Δ11-FW-*Hin*dIII	AAGCTTGACAAACCATGTAT
ProIAMT1Δ11-Rev-*Not*I	GCGGCCGCAAATGATAAATGTATG AATT
ProIAMT1Δ12-REV-	CTTAAATTTTTCTTCGTGTACATCA TATATTG

ProIAMT1Δ12-Fw-	CAATATATGATGTACACGAAGAAA AATTTAAG
ProIAMT1Δ14-REV-	CAACCTACAGTGCACAAGGAAACA AACATAATG
ProIAMT1Δ14-Fw-	CATTATGTTTGTTTCCTTGTGCACT GTAGGTTG
ProIAMT1Δ15-FW-	TCAGATATTGAAAATTATAAATGA CAAACCATGTAT
ProIAMT1Δ15-MF-*Spe*I	ACTAGTGACAAACCATGTATATAT AC
ProIAMT1Δ15-MR-*Not*I	GCGGCCGCAAATGATAAATGTATG AATTTAG
ProIAMT1Δ15-Rev-	ATACATGGTTTGTCATTTATAATTT TCAATATCTGA
ProIAMT1Δ16-FW-	CTTACTTAAATTTTTCTTTCAGATA TTGAAAATTATACG
ProIAMT1Δ16-Rev	CGTATAATTTTCAATATCTGAAAG AAAAATTTAAGTAAG
CAMV35Sm-FW-*Bam*H1-*Not*1-*Nco*I	GGATCCGCGGCCGCCAAGACCCTT CCTATATAAGGAAGTTCATTTCATT TGGAGAGGCCATGG
CAMV35Sm-REV-*Bam*H1-*Not*1-*Nco*I	CCATGGCCTCTCCAAATGAAATGA ACTTCCTTATATAGAGGAAGGGTC TTGGCGGCCGCGGATCC
IAMT1-CDS-FW	ATGGGTTCTAAGGGAGACAAC
IAMT1-CDS-RV	CTAAGTAAAAGACAAAGAAGCGA C

VII.7 Transient expression assays

For the generation of the luciferase expressing constructs, promoter deletions were cloned using restriction sites *Hin*dIII and *Nco*I to the transient expression vector pGreenII 0800-LUC (Hellens et al., 2005). For the transient expression assay 35S::GAI-TAP was used (Gallego-Bartolome et al., 2012), the CDS for DRN, AGL86, ANAC42, ANAC59, bHLH18, TCP14, BES1 and PIF4 were retrieved from the REGIA (REgulatory Gene Initiative in Arabidopsis) project (Valencia et al., 2002) in pDonor221 and subcloned using gateway technology to pAlligator1 (Gallego-Bartolome et al., 2012) containing a VP16 domain. The CDS for DRN was also cloned

through gateway to the expression vector pEarleyGate201 (Earley et al., 2006).

Constructs were introduced in *Agrobacterium* strain C58 and used to transform 2 week old *Nicotiana benthamiana* leaves. 3 days after transformation 1 cm disc like leaves were collected and the activity of the Firefly luciferase and *Renilla luciferase* was measured according to the Dual Glo Lucifease Assay System (promgea) manual.

VII.8 Confocal microscopy

Seedlings were rinsed for 2 min with 10 μg/ml propidium iodide (PI), then 5 min with water. Fresh stained seedlings were mounted on slides only with water. Images were taken using a Leica TCS SL confocal laser microscope (Leica, http://www.leica.com) and Zeiss 780 Axio Observer (Zeiss, http://www.zeiss.com) with excitation at 488 nm. For GFP and VENUS detection, channel 1 was configured between 500-540 nm; and for PI detection, channel 2 was configured between 590-660 nm.

VII.9 Analysis of reporter lines

β-*glucuronidase* (GUS) staining was performed as described (Zadnikova et al., 2010). Briefly, seedlings were collected and fixed with 90% cold acetone, washed twice with a solution of NaPO4 (pH 7.2) with a final concentration of 50 mM, 0.2 % Triton X (Sigma), 2mM Potassium Ferrocyanide and Potassium Ferricyanide (Sigma), then 1 mM X-GLUC (Sigma) were added. Seedlings were stained overnight (unless mentioned differently) at 37°C then cleared with a series of ethanol washes and with Chloral hydrate (Sigma) for 2 days. Images were taken using Nikon Eclipse E600.

VII.10 *In silico* promoter analysis

Promoter analysis (http://element.cgrb.oregonstate.edu/) was done using the ELEMENT web tool (http://element.cgrb.oregonstate.edu/). Logos were built using the Weblogo web tool (http://weblogo.berkeley.edu/). The cluster lists are formulated by using the highest-count promoter core elements. All longer elements containing the core element are clustered together. PLACE database (http://www.dna.affrc.go.jp/PLACE/) was used to identify any known *cis*-acting element.

VII.11 *In silico* correlation analysis

Co-expression analysis for the identification of DRN was done using GeneMANIA tool (http://www.genemania.org/), all networks were enabled, the network Query-dependent weighting was automatic, options for genes with similar Co-expression, Co-localization, Genetic interactions, Physical interactions, Predicted and Shared protein domains were selected. DRN was identified in the correlation analysis as a physical partner of BIM1 that was identified as a co-expressed protein with IAMT1. Analysis via expression Angular tool in http://bar.utoronto.ca/welcome.htm was set up with the search criteria for genes that are negatively correlated with IAMT1 with an r-value cut off range between -0.01 and -0.1 (approximate lower *r*-values to return the top 25, 50, or 100 best correlated genes are -0.010, -0.011, and -0.012, respectively) and used as a database the AtGenExpress Tissue compendium and checked vegetative rosettes and seedlings. Analysis via CressExpress (http://cressexpress.org/) to look for negatively correlated genes, we used the Version 3.2 (1779 arrays - MAS5 processing) and 0.15 cut-off value for Kolmogorov-Smirnov quality-control statistic (lower r value indicates high quality chips only**)**, the database used was from the ATH1 arrays for young seedlings in entirely

with cotyledons, hypocotyl and primary roots, and looked through all the experiments corresponding to the array type and tissue types selected, the r-squared threshold for pathway-level correlation was 0.9 (higher r-squared threshold signify high correlation).

VII.12 Protein extraction and western blot

Total proteins were extracted by homogenizing seedlings in one volume of cold extraction buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM PMSF, and 1×complete protease inhibitor cocktail (Roche)]. Extracts were centrifuged at 13000 g for 10 min at 4°C. Protein concentration in the supernatants was quantified by the Bradford assay (Bradford, 1976). Aliquots (40 μg) of denatured total proteins were separated in Precise™ 8% Tris–HEPES–SDS gels (Pierce) and transferred onto PVDF membrane (Bio-Rad). GFP-IAMT1 and DII-VENUS fusion was detected using the monoclonal anti-GFP antibody (clone JL-8) from Clontech.

VII.13 Yeast two hybrid assay

A *pENTR* vector carrying the coding sequence (CDS) of *DRN* was from the REGIA project (Valencia et al., 2002). Deleted versions of GAI was amplified by PCR and cloned into *pCR8/GW/TOPO* (Invitrogen) to create *pENTR* vector (Gallego-Bartolome et al., 2012). GAI was transferred into the *pDEST32* to create Gal4 DNA binding domain (Gal4-DBD) fusions (prey vector) by Gateway (Invitrogen). The full length *DRN* CDS was cloned into *pDEST22* as a Gal4 activation domain (Gal4-AD) fusion (bait vector) (invitrogen). Final bait and prey constructs were used to co-transform the yeast strain ATH109 (Clontech). Yeasts were selected in

SD/-Leu/-Trp/-His and with different amounts of 3-aminotriazol (3-AT) (Sigma) to test the interaction.

VII.14 Bimolecular Florescence Complementation

pENTR vectors carrying the CDS of *DRN* and *GAI* were from the REGIA project (Valencia et al., 2002). For bimolecular florescence complementation (BiFC), *DRN* and *GAI* CDSs were transferred into *pMDC43-YFC* and *pMDC43-YFN* (Gallego-Bartolome et al., 2011b), respectively, by Gateway. Vectors for BiFC expressed the fusion proteins from the constitutive *35S* promoter. Each construct was introduced in *Agraobacterium tumefaciens* C58 cells that were used to infiltrate *N. benthamiana* leaves. BiFC analysis was performed as described (Gallego-Bartolome et al., 2012).

VII.15 Auxin transport assays

Seedlings were germinated on control medium and transplanted to treatment plates on the third day after germination in darkness containing 1or 10 µM NPA (sigma) for six hours, the seedlings were allowed to grow on a sheet of parafilm. A droplet containing 6.75 nM [^{3}H]IAA (specific activity 25.0 Ci/mmol, 1µCi/µl, Amersham, Buckinghamshire, UK) and 0.1 % tween-20 (sigma) was applied to the cotyledons for 3 hours after which plants were either allowed to grow straight for 3 more hours or we re-orientated 90° for 3 hours. After which the lowest 5 mm of the hypocotyl was collected and radioactivity was measured using previously described methods (Lewis and Muday, 2009).

VII.16 IAA and MeIAA quantification

Whole or dissected seedlings were immediately frozen in liquid N2. Approx. 100 mg of tissue was pooled per sample and at least 3 biological replicates were harvested for each independent experiment. 1 ml of methanol and 50 pmol of $^{2}H_{2}$-IAA or 100 50 pmol $^{2}H_{5}$-MeIAA was added,

heated for 2 minutes at 60 °C, then further reaction without heating for at least 1 hour. The sample was taken to complete dryness

For purification of IAA and MeIAA, the sediments were dissolved in 2 ml cold sodium phosphate buffer (50 mM, pH 7.0) containing 5% MeOH, followed by a 10 min ultrasonic treatment (Branson B5510DTH, Branson Ultrasonics, Dunbury, USA). Next, the pH was adjusted to 2.5 with 1 M hydrochloric acid, and the sample was purified by solid-phase extraction using 1 ml/30 mg Oasis™ HLB columns (Waters Corporation, Milford, USA) conditioned with 1 ml methanol and 1 ml water, and equilibrated with 0.5 ml sodium phosphate buffer (acidified with 1 M hydrochloric acid to pH 2.5). After sample application, the column washed twice with 1 ml 5% methanol and then eluted with 2 ml 80% methanol. The elute was taken to complete dryness by using a vacuum concentrator (Vacufuge® plus, Eppendorf, Hamburg, Germany). 20 µl of N,O-bis (trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA+TMCS, 99:1 (v/v), Supelco, Bellefonte, USA) were added to each extract. The extracts were transferred into 400 µl GC-MS vials and incubated for 70 min at 60º C.

To analyze IAA and MeIAA contents in the same samples, 1 µL of each sample was injected split less by a CombiPAL autoinjector (CTC Analytics, Zwingen, Switzerland) into a Bruker Scion-455 gas chromatograph (BRUKER Daltonics, Bremen, Germany) equipped with a 30 m x 0.25 mm i.d. fused silica capillary column with a chemical bond 0.25-µm ZB35 stationary phase (Phenomenex, Torrance, USA). Helium at a flow rate of 1 mL/min served as the mobile phase. A pressure pulse of 25 psi over 1 min was used to force the transfer of compounds from the injector into the column. The injector temperature was 250ºC and the

column temperature was held at 50ºC for 1.20 min. Thereafter, the column temperature was increased by 30º C/min to 120ºC. After reaching 120º C, the temperature was further increased by 10º C/min to 325º C and held at that temperature for another 5 min. The column effluent was introduced into the ion source of a Bruker Scion-TQ triple quadruple mass spectrometer. The mass spectrometer was used in EI-MRM mode. The transfer line temperature was set to 250 ºC and the ion source temperature to 200ºC. Ions were generated with -70 eV at a filament emission current of 80 µA. The dwell time was 100 ms, and the reactions m/z 247 to m/z 130 (endogenous IAA), m/z 249 to m/z 132 ([$^2H_2$]-IAA, internal standard), m/z 261 to m/z 202 (endogenous MeIAA),and m/z 266 to m/z 207 ([$^2H_5$]-MeIAA, internal standard) were recorded. Argon set at 1.5mTorr was used as the collision gas. The amount of the endogenous compound was calculated from the signal ratio of the unlabeled over the stable isotope-containing mass fragment observed in the parallel measurements.

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