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Abstract

Most land plant species live in symbiosis with arbuscular mycorrhizal (AM) fungi. This is a very ancient symbiosis dating back to 450 million years. AM fungi are soil fungi that belong to the Glomeromycota. They are present in most terrestrial ecosystems. Thus they can be considered as an integral root component of plants. They form essential functional structures called arbuscules in root cortical cells at which mineral nutrients are released to the plant in exchange of sugars. The phytohormone auxin is involved in many developmental processes in plants, including apical dominance, tropisms, vascular patterning and lateral root formation. The main objective of our work was to investigate further the role of auxin in the mycorrhizal developmental process. We already know that AM symbiosis stimulates the lateral root formation in host plants, which could be due to modification of auxin metabolism, transport or perception. The microRNAs (miRNAs) are ~21-nucleotides noncoding RNAs that target corresponding mRNA transcripts for cleavage and transcriptional repression. Several miRNAs interact with auxin signaling and among them miR393 that targets auxin receptors. We investigated the role of miR393 in AM root colonization. In *Solanum lycopersicum* (Solanaceae), *Medicago truncatula* (Fabaceae) and *Oryza sativa* (Poaceae), expression of the precursors of the miR393 was down-regulated during mycorrhization. In addition DR5-GUS, a reporter for auxin response, was found to be preferentially expressed in root cells containing arbuscules. By over-expressing miR393 in roots and therefore down-regulating auxin receptor genes, arbuscules could not develop normally. As components of auxin receptor complexes, Aux/IAA proteins play a major role in auxin signaling pathway by repressing the activity of ARF type transcription factors. We checked the expression of 25 AUX/IAA genes in AM roots. Among them, we focused on IAA27 that was significantly up-regulated during the early stages of AM symbiosis. IAA27 down-regulation in plants led to a strong decrease of AM colonization and arbuscule abundance. We showed by different approaches that the positive regulation of mycorrhization by IAA27 was linked to strigolactone biosynthesis. Overall these results strongly support the hypothesis that auxin signaling plays an important role both in the early stage of mycorrhization and in the arbuscule formation.

Résumé

La plupart des espèces végétales terrestres vivent en symbiose avec les champignons mycorhiziens à arbuscules (MA). Il s'agit d'une symbiose très ancienne datant de plus de 400 millions d'années. Les champignons MA sont des champignons du sol qui appartiennent aux Gloméromycètes. Ils sont présents dans la plupart des écosystèmes terrestres. Ainsi, ils peuvent être considérés comme une composante intégrale des racines des plantes. Ils forment dans les cellules racinaires corticales des structures fonctionnelles essentielles appelées arbuscules où ils apportent à la plante des minéraux nutritifs en échange de sucres. L'auxine est une phytohormone impliquée dans de nombreux processus de développement des plantes, y compris la dominance apicale, les tropismes, la structuration vasculaire et la formation de racines latérales. Le principal objectif de notre travail était d'étudier de manière approfondie le rôle de l'auxine dans le processus de développement des mycorhizes. On sait déjà que la symbiose MA stimule la formation de racines latérales dans les plantes hôtes, ce qui pourrait être due à une modification du métabolisme de l'auxine, de son transport ou de sa perception. Les microARNs (miARNs) sont des molécules d'ARN non codantes de ~ 21 nucléotides capables de réprimer l'expression de gènes en ciblant et clivant spécifiquement leur ARNm correspondant. Plusieurs miARNs interagissent avec la signalisation de l'auxine et parmi eux miR393 qui cible les récepteurs à l'auxine. Nous avons étudié le rôle de miR393 dans la colonisation mycorhizienne. Nous mettons en évidence que chez *Solanum lycopersicum* (Solanacées), *Medicago truncatul*a (Fabaceae) et *Oryza sativa* (Poaceae), l'expression des précurseurs de miR393 diminue lors de la mycorhization. En outre nous montrons que DR5-GUS, un gène rapporteur de réponse à l'auxine, est préférentiellement exprimé dans les cellules de la racine contenant les arbuscules. En sur-exprimant miR393 dans les racines et donc en régulant négativement l'expression des gènes de récepteurs à l'auxine, nous montrons également que les arbuscules ne se développent pas normalement. En tant que composantes des complexes récepteurs d'auxine, les protéines Aux/IAA jouent un rôle majeur dans la voie de signalisation de l'auxine en réprimant l'activité des facteurs de transcription de type ARF. Nous avons vérifié dans des racines de tomate mycorhizées l'expression de 25 gènes AUX/IAA. Nous nous sommes concentrés sur IAA27 dont l'expression est induite lors des premiers stades de la symbiose MA. Nous observons qu'une répression par ARNi de l'expression de IAA27 dans des plants de tomate conduit à une forte diminution de la colonisation MA et du nombre des arbuscules. Puis nous montrons par des approches différentes que la régulation positive de la mycorhization par IAA27 est liée à la biosynthèse

des strigolactones. Globalement, ces résultats appuient fortement l'hypothèse selon laquelle la signalisation de l'auxine joue un rôle important aussi bien dans le stade précoce de la mycorhization que dans la formation des arbuscules.

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Abreviations

2.4 D: 2.4-dichlorophenoxyacetic acid

4-Cl-IAA: 4-Chloroindole-3-acetic acid

AAO: ACETALDEHYDE OXIDASES

ABA: Abscisic Acid

ABCB: ATP-binding cassette transporters

ABP1: AUXIN BINDING PROTEIN 1

ACC: 1-aminocyclopropane-1-carboxylic acid

AFB: Auxin Receptor F-box

AM: Arbuscular Mycorrhiza

AMI1: AMIDASE 1

ARF: Auxin Response Factor

Asp: Aspartate

Aux/IAA : Auxin/Indole-3-Acetic Acid

AUX/LAX : AUXIN RESISTANT 1/LIKE AUX1

AuxRE: Auxin Responsive cis-Element

CAM: Camalexin

CaMV: Cauliflower Mosaic Virus

CCD: Carotenoid cleavage dioxygenase

CK: Cytokinin

CSSP: Common Symbiotic Signaling Pathway

DBD: DNA binding domain

EAR: Ethylene-responsive element binding factor-associated Amphiphilic Repression

ER: Endoplasmic Reticulum

GA: Gibberellin

GH3: Gretchen Hagen 3

Glu: Glutamate

IAA: Indole-3-Acetic Acid

IAA-glucose: 1-O-indol-3-ylacetyl-beta-D-glucose

IAAId: Indole-3-acetaldehyde

IaaM: TRYPTOPHAN-2-MOOXYGENASE

Iaglu: IAA glucose synthase

IAM: Indole-3-acetamide

IAN: Indole-3-acetonitrile

IAOx: Indole-3-acetaldoxime

IBA: Indole-3-butyric acid

IG: Indoleglucosinolates

IPA: Indole-3-pyruvate

JA: Jasmonate

KPI: Kunitz protease inhibitor

LCOs: Lipochitooligosaccharides

Leu: Leucine

LR: Lateral Root

MFs: Myc factors

NAA: Naphthalene-1-Acetic Acid

NIT: Nitrilase

NPA: N-1-napthylphtalamic acid

NSP1: Nodulation signaling pathway1

NSP2: Nodulation signaling pathway2

OH-IAA-Asp: Oxindole-3-acetic acid

Ox-IAA-Asp: Oxindole-3-acetyl-NAsp

PAM: Plasmalemma-derived periarbuscular membrane

Phe: Phenylalanine

PILS: PIN-LIKEs

PIN: PINFORMED

PPA: Prepenetration Aparatus

qRT–PCR : quantitative Reverse Transcription–PCR

RNAi: RNA interference

SAUR: Small Auxin Up RNA

SCF: SKP1-Cullin-F-box

SCP1: Serine carboxypeptidase

SL: Strigolactone

Sl-IAA: Solanum lycopersicum Auxin/Indole-3-Acetic Acid

TAA: Tryptophan aminotransferase of arabidopsis

TAAR: TIR1 and AFB1-2-3 paralogs

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TAM: Tryptamine

TAR: Tryptophan aminotransferase related

TDC: Tryptophan decarboxylase

TIR1: Transport Inhibitor Resistant1

TPL: Topless

Trp: Tryptophane

VAMPs: Vesicle-associated membrane proteins

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Chapter I – Bibliographic review and objectives

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1. Arbuscular mycorrhizal symbiosis

Arbuscular mycorrhizal (AM) symbiosis represents a vital component in plant ecosystems; it is widely distributed in natural environments and concerns more than 80% of land plant species, from the liverworts to the angiosperms (Smith and Read, 2008). The term mycorrhiza derives from the Greek words “fungus” and “root” which describes a very ancient and mutualistic association between plant roots and soil fungi of the Glomeromycetes phylum (Smith and Read, 2008). Plants can survive without their fungal symbionts, but AM fungi function as obligate biotrophs. AM fungi have a significant role in absorbing soil nutrients, especially phosphate, with their mycelium growing around the roots. These nutrients are then provided to their plant host through specific intraradical, highly branched, structures called arbuscules (Smith and Read, 2008). In return they get their carbon source from their plant host. They also improve plant resistance to various biotic and abiotic stresses (Smith and Read, 2008). For these reasons the AM symbiosis greatly improves plant fitness and plant productivity (Fig. 1), and there is great expectation that AM symbiosis might be exploited in tomorrow’s agriculture for its potentially high environmental and economic value.

The establishment of AM symbiosis involves different steps of fungal development (Fig. 2). Precontact stage (also referred to as presymbiotic stage) is characterized by an intense hyphal-branching triggered by plant-derived exudates including strigolactones (SLs) (Akiyama et al., 2005; Besserer et al., 2006). During this early stage AM fungi produce Myc factors (MFs) such as lipochito-oligosaccharides (LCOs) and COs (Maillet et al., 2011; Genre et al., 2013) that induce in roots the common symbiotic signaling pathway, a pathway also activated in the nodulation process (reviewed by Oldroyd, 2013).

Then AM fungi make hyphopodia on the root surface (Bastmeyer et al., 2002) from which hyphae enter the roots through prepenetration apparatus (PPA; Genre et al. 2008): root cell structures which guides the fungus through epidermal cells.

These hyphae grow intercellularly along the root axis and, via the induction of PPA-like structures in inner cortical cells (Genre et al., 2008); they differentiate in root cortical cells tree shape, highly branched, structures called arbuscules (Parniske, 2008). Arbuscule formation involves significant root cellular re-structuring, including the synthesis of a plasmalemma-derived periarbuscular membrane (PAM). The PAM is formed around the fungal cell wall and creates a periarbuscular space and a large surface area for nutrient exchange (Harrison, 2012). In cortical cells, arbuscules are functional for only few days. Arbuscular branches become fragile and shrink. Then, the phosphate transporters disappear

within few hours. The collapse of arbuscules occurs in the subsequent days (Kobae and Hata, 2010). Kobae and Fujiwara (2014) showed that cells with collapsed arbuscules were rarely recolonized to host new arbuscules. Rather, *de novo* colonization occurs in close tissue contributing to the expansion of root colonization.

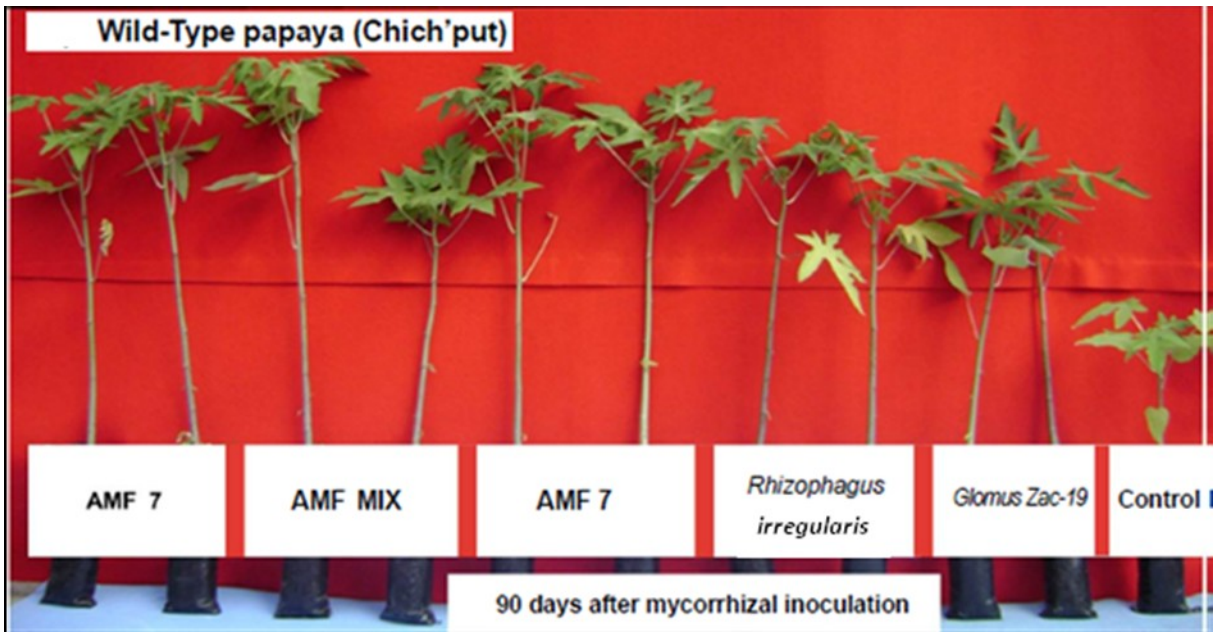

Figure 1. Growth responses of wild type papaya due to the inoculation with arbuscular mycorrhizal fungi (AMF) after 90 days. AMF 7: *Funneliformis constrictum, Claroideoglomus etunicatum, G. aggregatum, Acaulospora* sp. 1 and *Glomus* sp1; AMF Mix: *Glomus* sp. 1, *Glomus* sp. 2, *Glomus* sp. 3, and *Glomus* sp. 4; *Glomus* Zac-19: *Claroideoglomus claroideum, Rhizophagus diaphanus*, and *G. Albidum* (from Alarcón et al., 2012)

Vesicles are storage structures of the fungus that can also be formed between or within the root cortical cells.

Finally, from its mycelium extending out of the root, the fungus produces asexual spores in the soil (Dalpé et al., 2005; Parniske, 2008). These spores are the resting living forms used by the fungus for its propagation.

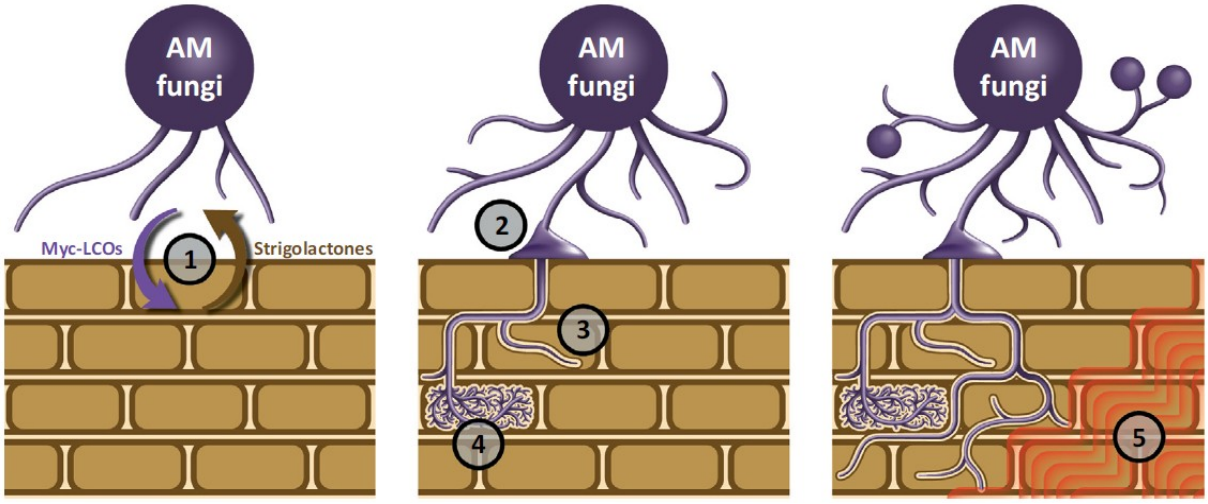

Figure 2. Arbuscular mycorrhizal (AM) symbiosis in flowering plants. (1) The plant root and the AM fungus exchange symbiotic signals that activate the symbiotic program of the other partner. (2) The AM fungus develops a hyphopodium on the surface of the root epidermis. (3) The plant allows inter- and intracellular colonization of the root. (4) In cortical cells, the fungus forms highly branched arbuscules, surrounded by the plant plasma membrane, which are specific interfaces optimized for nutrient exchange. (5) Finally, the host plant controls the spatial colonization of the root. Abbreviation: Myc-LCOs, mycorrhizal lipochito-oligosaccharides (from Delaux et al., 2013).

1.1. Arbuscule development

During arbuscule development that is linked with important changes in plant cellular structure and function, cortical cells are dramatically reprogrammed by the symbiotic stimulation (Harrison, 2012) (Fig. 4). Regarding the combination of plant and AM fungal species, two morphological patterns in arbuscule formation have been reported. *Arum*-type colonization is defined as intercellular hyphal spread, tree-shaped and terminal arbuscules in cortical cells which are the case in most legumes. *Paris*-type colonization requires hyphal passage from cell to cell as well as intracellular hyphal coils that later differentiate into intercalary arbuscules (Bonfante and Genre, 2008; Gutjahr and Parniske, 2013) (Fig. 3).

Upon direct hyphal contact with cortical cells, separated PPA-like endoplasmic reticulums (ER) accumulate in inner cortical cells (Genre et al., 2008). PPA guides the hypha to enter in the middle of the inner cortical cells where arbuscule branching begin (Genre et al., 2008). During arbuscule branching, the ER surrounds the developing arbuscule and both actin filaments and microtubules bundle around the arbuscule branches accumulate

(Blancaflor et al., 2001; Genre et al., 2008; Pumplin and Harrison, 2009). In addition, vacuole deformation and nucleus movement to the center of the cell is observed during arbuscule development (Fester et al., 2001; Lohse et al., 2005; Pumplin and Harrison, 2009). Each branch of the arbuscule is surrounded with the plant-derived periarbuscular membrane (PAM) which prevents the direct contact with plant cytoplasm. Thus, during arbuscule development, the plant-membrane surface increases which makes the nutrient and metabolite exchanges most efficient between symbiotic partners (Pumplin& Harrison 2009).

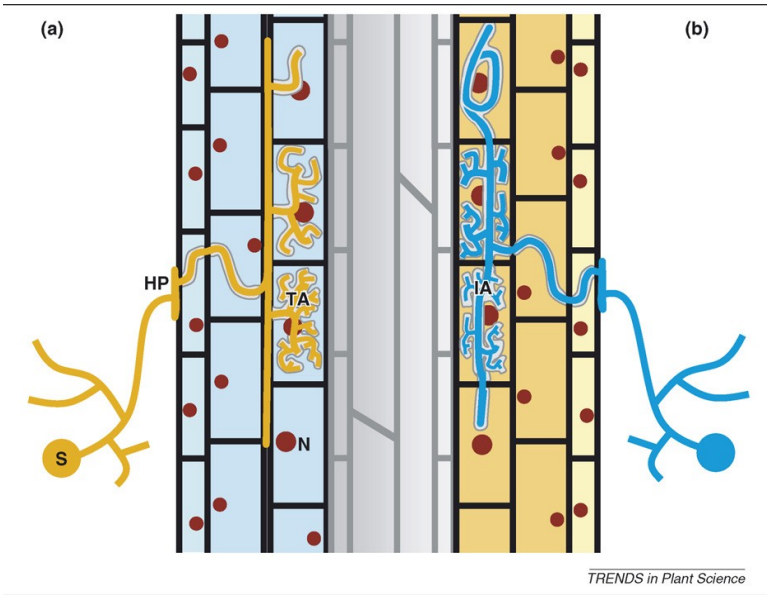

Figure 3. Root colonization by AM fungi. The scheme summarizes the main features of AM fungal development and root colonization patterns in angiosperms. (a) *Arum*-type colonization. (b) *Paris*-type colonization (from Bonfante and Genre, 2008).

1.1.2. Transcriptional reprogramming during arbuscule development

Transcription profiling after laser microdissection of arbuscular mycorrhizal roots shows that the expression of hundreds of genes is modified in arbuscule-containing cells (Gomez et al., 2009; Hogekamp et al., 2011; Gaude et al., 2012). The most induced transcripts belong to transport processes, transcriptional regulation, and lipid metabolism (Gaude et al., 2012). Also, Gaude et al. (2012) by comparing the non-colonized cortical cells of mycorrhizal roots with cortical cells of non-mycorrhizal roots show significant transcriptional differences between them. These results could be interpreted by the presence of intraradical hyphae in non-colonized cortical cells of mycorrhizal roots and/or

transcriptional reprogramming of cortex cells during AM symbiosis (Gutjahr and Parniske, 2013). This would be compatible with the expression patterns of AM-specific marker such as SCP, Bcp1 and SbtM1, which are activated in cells containing intracellular hyphae and during PPA formation (Liu et al., 2003; Hohnjec et al., 2005; Pumplin and Harrison, 2009; Takeda et al., 2009; Takeda et al., 2012). All together it seems that arbuscule formation is characterized by at least two waves of gene expression. The first wave of gene is induced prior to and during arbuscule formation, such as SCP or SbtM1. The second wave is characterised by genes that are induced during arbuscule formation and in arbuscule-containing cells, such as PT4, Cel1, STR, and STR2 (Fig. 4) (Harrison et al., 2002; Liu et al., 2003; Zhang et al., 2010; Gutjahr and Parniske, 2013).

1.1.3. Genes required for arbuscule formation

According to symbiotic plant mutants, arbuscule development can be arrested in different stages. Five distinct stages are defined due to arbuscule morphological analyses of these mutants (reviewed by Gutjahr and Parniske, 2013) (Fig. 4a, b).

PPA structure and fungal cell entry are the first two stages that generate the arbuscule trunk formation. The first stage starts with CSSP genes CYCLOPS/IPD3 and CCaMK/DMI3 which are activated by Myc-LCOs and second and third require VAPYRIN/PAM1. Complete absence of arbuscules have been observed in (*ccamk, cyclops, vapyrin*) mutants or single hyphal bulges that enter cortical cells (pam1) (Demchenko et al., 2004; Kistner et al., 2005; Sekhara Reddy et al., 2007; Yano et al., 2008; Pumplin et al., 2010). Silencing of vesicle-associated membrane proteins (VAMPs), head to dwarf (stage III) arbuscules, showing transition stage IV of arbuscular development (Ivanov et al., 2012; Lota et al., 2013). Importantly, this silencing demonstrates that fungal growth and branching are limited by PAM synthesis and so are regulated by the plant.

The fourth stage describes mature arbuscules that develop from the birdsfoot stage through continuous hyphal branching. Two half-size ABC transporter genes mutation or down-regulation lead to arbuscule developmental arrest at the birdsfoot step (Zhang et al., 2010). RAM2 is encoding a glycerol-3-phosphate acyl transferase (GPAT), needed for colonization of the root by mycorrhizal fungi, as it is both necessary for appropriate hyphopodia and arbuscule branching (Wang et al., 2012). Kunitz protease inhibitor (KPI106) and a serine carboxypeptidase (SCP1) control arbuscular mycorrhiza development in the root

cortex. Both proteins are only induced during mycorrhiza formation and belong to large families whose members are also mycorrhiza-specific (Rech et al. 2013). A default of the transcription factor gene ERF1 generated stunted arbuscules and blocked at the birdsfoot step (Devers et al., 2013), raising the possibility that ERF1 regulates genes that require to be expressed to enable arbuscule branching (Gutjahr and Parniske, 2013).

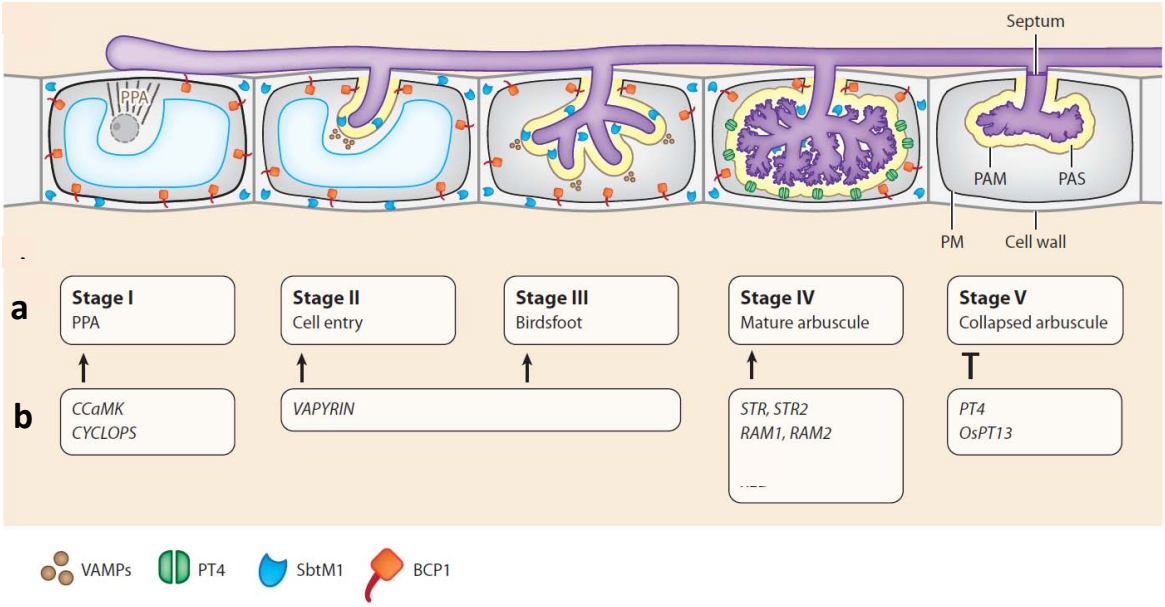

Figure 4. (a) Five different genetically separable stages in arbuscule development. (I) prepenetration apparatus (PPA) structure, displayed here by a conically arranged microtubule array; (II) cell entry and formation of the arbuscule trunk; (III) birds foot, a stage identified by weak and low-order branching; (IV) mature arbuscule, defined by thin, higher-order branches; (V) collapsed arbuscule, characterized by septa, which disconnect this senescence stage from the remaining hyphal network. **(b)** Genes required for the progression to distinct stages of arbuscule development are listed in white boxes. PT4 and OsPT13 are required for arbuscule maintenance, and they delay the progress to senescence stage IV (as signified by the inhibition sign) (from Gutjahr and Parniske, 2013).

1.2. Phytohormones and arbuscular mycorrhizal symbiosis

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The establishment of AM symbiosis is connected with changes in root cells as well as in fungal hyphae development. Environmental and physiological condition of host plants affect on both developmental structures of AM fungi and the percentage of plant root colonization. This indicates that mycorrhizal establishment is, at least partly, under plant control. Phytohormones and hormone signaling pathways have important roles in plant development and response to biotic and abiotic stresses. So explaining how phytohormones manage AM development will provide direction into understanding how plants organize AM symbiosis (Gutjahr, 2014).

1.2.1. Jasmonic acid (JA)

Jasmonic acid is implicated in the plant's systemic response to necrotrophic pathogen attack (see review by Ballaré, 2011). Since AM fungal hyphae penetrate plant cells, this has led to questions regarding its involvement in the regulation of AM development.

Several studies by different groups have been done, but they have not reported completely coherent results. Ludwig-Müller et al. (2002) showed a clear reduction of AM colonization by high levels of JA and Regvar et al. (1996) showed an increase in AM colonization with low levels of JA. These results represent the negative role of JA in AM symbiosis. In the other hand increasing JA level by repeated wounding promoted AM colonization (Landgraf et al., 2012) and using antisense expression of the allene oxide cyclase gene to down-regulate JA level delayed AM colonization (Isayenkov et al., 2005). These observations suggest a positive role for jasmonate in AM development. Tejeda-Sartorius et al. (2008) and Herrera-Medina et al. (2008) showed, respectively, that the JA-deficient *spr2* mutant was defective in AM colonization whereas the JA-insensitive mutant *jai-1*had an increased AM colonization. In general it seems that JA (or derivatives such as methyl jasmonate) can have a range of effects on mycorrhizal colonization, from positive to inhibitory, depending on plant species, dose, timing and nutritional conditions (see review by Gutjahr and Paszkowski, 2009).

1.2.2. Gibberellic acid (GA)

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GA is a phytohormone that controls many aspects of plant growth and development and also influences responses to abiotic and biotic stresses (Hauvermale et al., 2012; Hou et al., 2013). Transcriptome analyses highlight significant changes in the expression of genes encoding proteins involved in gibberellic acid (GA) biosynthesis, degradation and signaling during AM symbiosis (Gomez et al., 2009; Guether et al., 2009; GarcíaGarrido et al., 2010; Hogekamp et al., 2011). It has been reported that GA levels increase significantly in mycorrhizal roots (Shaul-Keinan et al., 2002), while GA treatment repressed the arbuscule number (El Ghachtouli et al., 1996). DELLA proteins are central players in GA signaling. They repress GA responses and restrain the growth (Sasaki et al., 2003; Harberd et al., 2009). Floss et al. (2013) showed that DELLA proteins are required for arbuscule formation (Fig. 5). Arbuscule formation is severely diminished in a *M. truncatula* Mtdella1/Mtdella2 double mutant and GA treatment of wild-type roots phenocopies the della double mutant (Floss et al., 2013).

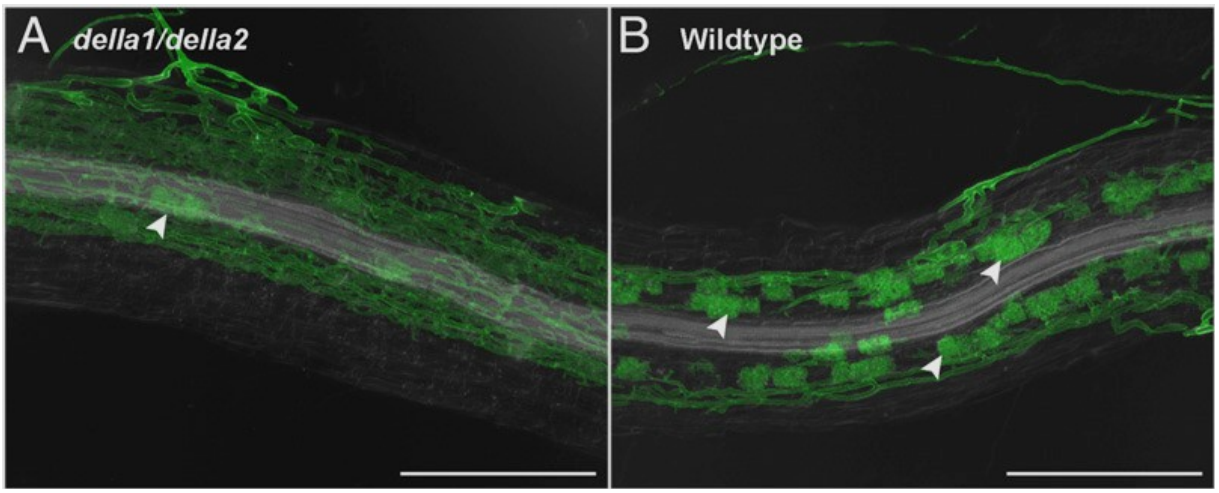

Figure 5. AM phenotype of della1/della2 roots colonized by *Glomus versiforme*. (A and B) Laser-scanning confocal microscope images of *G. versiforme* in *della1*/ *della2* (A) and wild-type (B) roots (from Floss et al., 2013).

1.2.3. Cytokinins (CK)

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Cytokinins play a critical role in regulating the proliferation and differentiation of plant cells. They are known as essential regulators of the plant root system, as they are involved, antagonistically to auxin, in the control of lateral root organogenesis (Sakakibara, 2006; Marhavý et al., 2011). Same as auxin, during the establishment of symbiosis, they repress defense responses of the host (Ludwig-Müller, 2010). Foo et al. (2013) suggested that CKs might not be required in the regulation of AM development. However, several plants which are colonized by AM fungi accumulate more CKs than non-mycorrhizal plants (Allen et al., 1980; Drüge and Schönbeck, 1992; van Rhijn et al., 1997; Torelli et al., 2000; Shaul-Keinan et al., 2002). Cytokinin-like substances have been shown to be produced by axenically grown mycelium of *Glomus mosseae* (Barea and Azcon-Aguilar, 1982). So, participation of AM fungi to regulation of the host CK level is possible but unclear (Barker and Tagu, 2000). A perfect balance between auxin and CK effects are necessary for proper developmental output (Marhavý et al., 2011). However, the inconsistency between high CK content and high branching found in some potential AM-host plants grown under high Pi or colonized by AM fungi is still unclear (Fusconi, 2013).

1.2.4. Abscisic acid (ABA)

ABA-deficient tomato mutants (*sitiens*) which have lower endogenous ABA levels are affected in the *Rhizophagus irregularis* colonization (Herrera-Medina et al., 2007). The results of the authors showed not only a reduction in the frequency and intensity of colonization in the mutant roots, but also less well-developed arbuscular morphology. They showed that frequency and intensity of colonization are completely restored by ABA application. ABA seems to play an important role in the development of the complete arbuscule and its functionality. However, part of this effect of ABA deficiency on fungal infection could be caused by an increased ethylene production in these mutants (Herrera-Medina et al., 2007). Other experiments on *sitiens* and with another ABA-deficient mutant, *notabilis*, as well as the use of transgenic plants and ethylene and ABA biosynthesis inhibitors supported the hypothesis that ABA deficiency enhances ethylene levels, which negatively regulates mycorrhizal intensity/ hyphal colonization (Martín Rodriguez et al., 2010; Martín-Rodríguez et al., 2011).

1.2.5. Ethylene (ET)

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Ethylene plays an important role in adjusting internal and external signals, as well as in some stress responses and interaction of plants with other organisms (López-Ráez et al., 2010; Lei et al., 2011). ET and salicylic acid function as negative regulators of AM symbiosis (Gamalero et al., 2008; Ludwig-Müller, 2010). Ethylene inhibits the induced expression of early symbiotic genes by germinating spore exudates (GSE) (Mukherjee and Ané, 2011). In addition, an active ET inhibitory effect has been observed on fungus entry into roots and on intraradical fungal spread (Martín-Rodríguez et al., 2011; Mukherjee and Ané, 2011). Ethylene inhibits root elongation by reducing cell elongation synergistically with auxin (reviewed by Muday et al., 2012). However, it also acts antagonistically to auxin by inhibiting lateral root (LR) formation in the earliest stages of the LR initiation (reviewed by Fukaki and Tasaka, 2009; Lewis et al., 2011; Muday et al., 2012). The reduced level of ET found in AM plants is, therefore, in agreement with the increased branching of the colonized roots (Fusconi, 2013).

1.2.6. Strigolactone (SL)

Strigolactones (SLs) function as stimulants of the germination of seeds of parasitic plants, such as *Orobanche spp*. and *Striga spp*. (Cook et al., 1966). Also, SLs act as signals that stimulate hyphal branching (Akiyama et al., 2005), spore germination and growth of AM fungi, via the activation of the fungal respiratory metabolism (Besserer et al., 2006; Besserer et al., 2008) (Fig. 6).

Furthermore, SLs function as phytohormones which are synthesized principally in the lower parts of the stem and the roots (Gomez-Roldan et al., 2008; Umehara et al., 2008; Kohlen et al., 2011). They inhibit shoot branching and regulate root development (Ruyter-Spira et al., 2011; Kapulnik et al. 2011; Seto et al., 2012; Brewer et al., 2013). Several genes, isolated from both mono- and dicots, are involved in SL synthesis and signaling (Fig. 7). Strigolactone biosynthetic pathway is beginning with a carotenoid substrate that will be converted by D27, an iron-containing protein involved in the strigolactone biosynthetic pathway in rice, and carotenoid cleavage dioxygenases (CCD7, CCD8) to a compound called carlactone (Fig. 7, 8c, Lin et al., 2009). MAX1, encoding a cytochrome P450 CYP711A1 protein, is involved in the production of SLs and has been proposed to act on a mobile substrate downstream of CCD7 and CCD8 (Stirnberg et al., 2002; Booker et al., 2005, Fig. 7). CCD7/CCD8 are expressed mainly in the root while the highest activity of MAX1 is

found in all vascular-associated tissue throughout the plant (Sorefan et al., 2003; Booker et al., 2004; Booker et al., 2005). SL production in roots is controlled by the GRAS proteins NODULATION SIGNALING PATHWAY1 (NSP1) and NSP2. These two transcription factors are required for the full expression of DWARF27 (D27) (Liu et al., 2011).

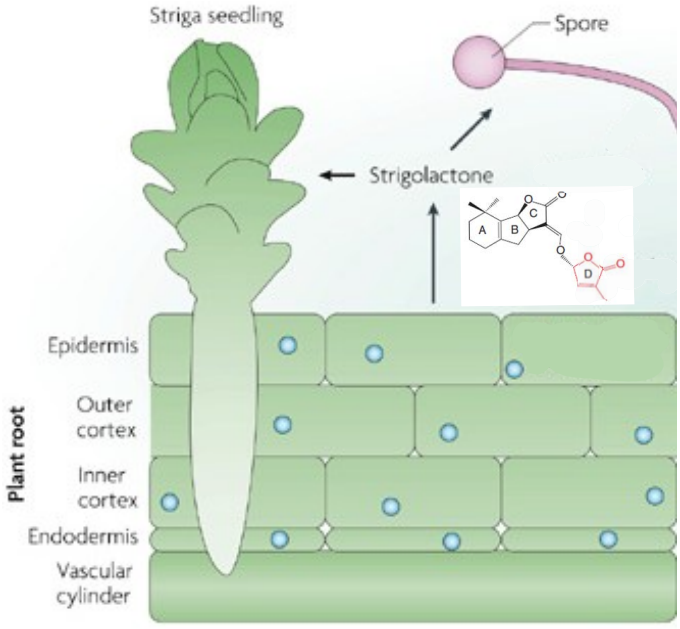

Figure 6. Strigolactones stimulate the germination of parasitic seeds and the hyphal branching arbuscular mycorrhizal fungi. Host plant exudes strigolactones into the soil, which activate dormant parasitic plant seed to germinate. If the germinating seed is close enough to the host root, it will establish a parasitic interaction. Strigolactones are sensed by the hypha of an arbuscular mycorrhizal fungus. In response to strigolactones, the hypha will branch extensively (adapted from Parniske, 2008).

Colonization levels of *L. japonicus* NSP1, and of *M. truncatula* NSP1 and NSP2 mutants are lower than in the wild-types, possibly because of the decreased production of strigolactone, while arbuscule morphology remains normal (Lauressergues et al., 2012; Delaux et al., 2013; Takeda et al., 2013). Colonization of *L. japonicus* NSP1 mutants could not be recovered by exogenous application of GR24 (Takeda et al., 2013). Hence, NSP1 is expected to perform additional roles in AM development (Gutjahr, 2014). In general plant

mutants that produce lower amount of SLs are affected in AM fungal colonization (Gomez-Roldan et al., 2008; Koltai et al., 2010; Liu et al., 2011; Gutjahr et al., 2012; Kretzschmar et al., 2012). SL-insensitive mutants also exhibit mycorrhizal phenotypes. MAX2/D3/RMS4 is a putative component of the SL receptor complex and is required for AM symbiosis (Yoshida et al., 2012; Foo et al., 2013). Colonization blocking at the epidermis, unusual hyphopodia on the root surface and fewer arbuscules have been observed in rice dwarf3 (*d3*) mutant roots. These phenotypes suggest that MAX2/D3/RMS4-mediated signaling is more likely needed in the epidermis rather than in the cortex (Yoshida et al., 2012). In contrast, AM colonization of the rice d14 dwarf mutant, which is another SL-insensitive mutant with a defect in a gene of the α/β-hydrolase family (Arite et al., 2009), was higher than in the wild type (Yoshida et al., 2012).Overall these observations suggest that SLs are playing important roles in the AM symbiosis, and much more work will be necessary to explain the different mechanisms involved.

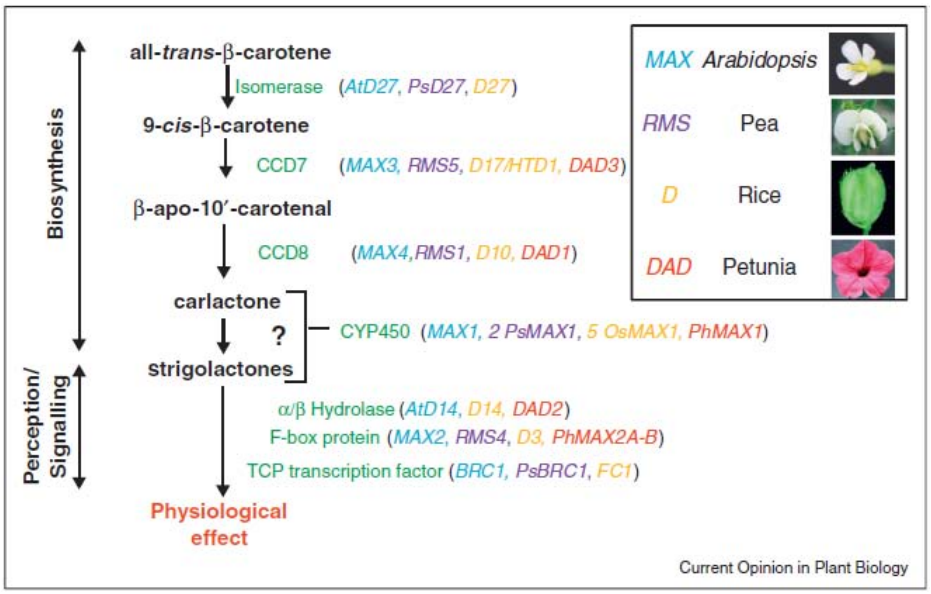

Figure 7. Schematic representation of the SL biosynthesis and signaling pathway for the control of shoot branching with genes so far identified in the four model species (boxed) (from de Saint Germain et al., 2013).

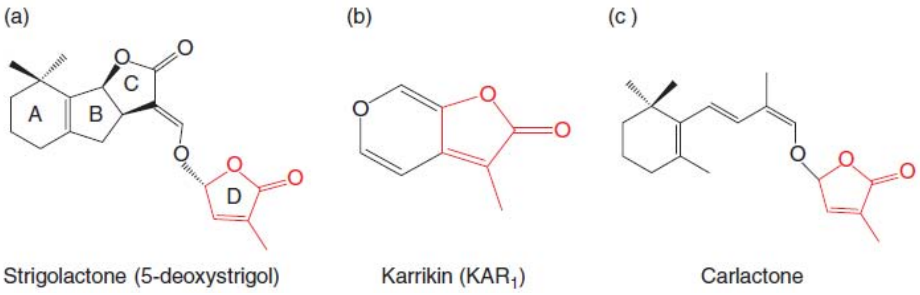

Figure 8. Chemical structures of strigolactones, karrikin and carlactone: (a) structure of the strigolactone 5-deoxystrigol; (b) structure of karrikin; (c) structure of carlactone. Note the butenolide moiety shared by these three compounds (Zhang et al., 2013).

1.2.7 Auxin

Auxin is a principal regulator of plant growth and developmental processes. It promotes plant cell division and elongation and it is the primary regulator of lateral root (LR) formation (Fukaki and Tasaka, 2009; De Smet et al., 2011; Muday et al., 2012; Vanstraelen and Benkova, 2012). The involvement of auxin in mycorrhizal plants has been suggested considering that AM colonization generally increases root branching (Ludwig-Müller, 2010; Hanlon and Coenen, 2011; Sukumar et al., 2013). It has been shown that the application of auxin can increase spore germination and hyphal growth, and influence the rate of colonization (Ludwig-Müller, 2010). Moreover, auxin is suspected to be needed inside the host roots for the early stages of AM formation (Hanlon and Coenen, 2011) and for controlling the SL levels (Foo et al., 2013). Some studies have revealed that auxin level in plant tissues can increase in colonized roots (Ludwig-Müller, 2010), but with no correlation with the amount of colonizing fungus (Jentschel et al., 2007; Ludwig-Müller, 2010). Torelli et al. (2000) have shown that the level of indole-3-acetic acid (IAA), the major endogenous auxin, increases in the mycorrhizal roots of leeks, perhaps explaining the typical modification of the root system architecture with more branched and shorter adventitious roots generally observed during mycorrhization (Berta et al., 1990; Trotta et al., 1991). Given that in soybean roots grown in a split-root system, the accumulation of IAA is only occurring in roots growing in the inoculated side, it seems that AM colonization does not increase IAA systemically (Meixner et al., 2005).

Concentration of Indole-3-butyric acid (IBA) also increased in maize (Kaldorf and Ludwig-Müller, 2000; Fitze et al., 2005) and in *M. truncatula* (Ludwig-Müller and Güther,

2007) when inoculated with AM fungi. IBA acts as a storage form of IAA as it can be converted to IAA that is required for root development (reviewed by Simon and Petrasek, 2011). Moreover, exogenous IBA mimicked the root phenotype of AM plants which raises the possibility that increase IBA concentration during AM symbiosis could be involved in AM root morphogenesis (Kaldorf and Ludwig-Müller, 2000).

The levels of amide conjugates of IAA and IBA are shown to increase in the roots of maize inoculated with *Rhizophagus irregularis* (Fitze et al., 2005). Specifically, accumulation of transcripts for a putative IAA-amidosynthetase and an auxin-responsive GH3-like protein mainly in arbuscule containing cells are reported (Fiorilli et al., 2009). The function of auxin conjugates in AM roots is unclear, but, they are suggested to be implicated in the AM development and in the control of the fungus morphogenesis (Fiorilli et al., 2009). Induction of putative ARFs is found during AM symbiosis in maize, rice and *M. truncatula*, but not in *L. japonicus* (reviewed by Formey et al., 2012). However, comparative transcriptomic analysis among these plant species has not detected any common orthologous auxin-specific genes involved in root development of AM-colonized plants (Formey et al., 2012). Several ectomycorrhizal species are able to synthesize auxin (Niemi et al., 2002, Splivallo et al., 2009). IAA-overproducing mutant of *Hebeloma cylindrosporum* can form greater mycorrhizal associations than the wild-type strain, suggesting a direct role for auxin in establishing mycorrhizal associations (Tranvan et al., 2000). Production of auxin by distinct groups of microorganisms maintains a role of auxin as a potential signal for communication with host plants (Sukumar et al., 2013). All these data point to the probable involvement of auxin in AM root branching. Furthermore, they could also indicate the existence of different regulations of auxin homeostasis and response pathways, possibly on the basis of the plant species as suggested by Formey et al., (2012).

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2. Introduction about auxin

Auxin plays essential roles through the whole life of the plant such as plant cell division, cell elongation and cell differentiation. It has a prominent influence on the final shape and function of cells and tissues in all higher plants. First study of auxin comes from the investigation of coleoptiles bending toward a light source (Darwin, 1880). The signal perceived by coleoptile tip could be transported from the tip to another part of the plant where it induced growth response (Went, 1928). This chemical signal termed auxin has been identified as Indole-3-acetic acid (IAA) (Kögl et al., 1934). Indole-3-acetic acid (IAA), phenylacetic acid (PAA) (Koepfli et al., 1938) and 4-chloroindole-3-acetic acid (4-Cl-IAA) (Porter and Thimann, 1965) are known as endogenous auxins in plant. Except IAA, functions and mechanisms of action of other active auxins have not been well described (reviewed by Simon and Petrasek, 2011). Related auxin functions are regulated at the multiple levels: auxin homeostasis (Biosynthesis, conjugation and degradation), polar transport and signal transduction (Fig. 9).

2.1. Indole-3-acetic acid (IAA)

Among the plant endogenous auxins, the role and mechanism of action of IAA is best studied and understood. There is two major routes for IAA biosynthesis: tryptophan (Trp)-dependent and Trp-independent pathways which are reviewed by Mano and Nemoto, (2012).

2.1.1. The Trp-independent pathway

It has been shown that Trp mutants in *Arabidopsis thaliana* and maize represented no differences in free IAA level compare with control plants (Wright et al., 1991; Normanly et al., 1993), which shows that IAA can be synthesized in the absence of Trp and it might contribute to auxin homeostasis (Chandler, 2009; Normanly, 2010). Indole-3-glycerol phosphate or indole is the likely precursor in Trp-independent IAA biosynthesis, but little is known about the corresponding biochemical pathway and potential intermediates and enzymes (Ouyang et al., 2000; Zhang et al., 2008).

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2.1.2. The Trp-dependent pathway

The Trp-dependent pathway was the most studied. Several Trp-dependent auxin biosynthesis pathways contribute to IAA levels, including the indole-3-acetaldoxime (IAOx), the indole-3-acetamide (IAM), the indole-3-pyruvic acid (IPyA) and the tryptamine (TAM) pathways.

2.1.2.1. The indole-3-acetaldoxime (IAOx) pathway

IAOx that is known to act as a precursor for defense compounds such as camalexin (CAM) and indoleglucosinolates (IGs) (Bak et al., 2001; Zhao et al., 2002; Mikkelsen et al., 2004; Hansen and Halkier, 2005; Normanly, 2010) is also used to produce IAA (Zhao et al., 2002; Sugawara et al., 2009). It has been suggested that IAOx pathway exist only in crucifers (Sugawara et al., 2009); but IAN as a downstream intermediate of this pathway has been detected in maize (Bak et al., 1998). The cytochrome P450 enzymes CYP79B2 and CYP79B3 are responsible for the conversion of Trp to IAOx (Hull et al., 2000; Mikkelsen et al., 2000; Zhao et al., 2002). The over expression of CYP79B2 leads to the increase of IGs (Mikkelsen et al., 2000; Zhao et al., 2002), IAN (Zhao et al., 2002), and free IAA (Zhao et al., 2002) levels. Conversely, the *cyp79b2 cyp79b3* double mutant shows a decrease of IAOx (Zhao et al., 2002; Sugawara et al., 2009), IAN (Zhao et al., 2002; Sugawara et al., 2009) and free IAA (Zhao et al., 2002). Taken together, these results suggest that IAN is a downstream intermediate of the IAOx pathway. However the enzymatic steps between IAOx and IAN have not yet been identified. It has been thought that plant nitrilases convert indole-3-acetonitrile into the plant growth hormone indole-3-acetic acid (IAA) but this view has changed considerably in the last few years (reviewed by Piotrowski, 2008). Recent work on plant nitrilases has shown them to be involved not only in converting IAN to IAA but also in the process of cyanide detoxification. Nitrilase genes (AtNIT1–AtRNIT4) have been isolated from *Arabidopsis thaliana* (Bartling et al., 1992; Bartel and Fink, 1994; Bartling et al., 1994; Hillebrand et al., 1996; Hillebrand et al., 1998). AtNIT1, 2, and 3 gene products were thought to participate in the conversion of IAN to IAA (Bartling et al., 1992; Bartling et al., 1994). AtNIT4 represents an important detoxification mechanism (Howden et al., 2009). All plants possess at least one nitrilase that is homologous to the nitrilase 4 isoform of *Arabidopsis thaliana* (Piotrowski, 2008).

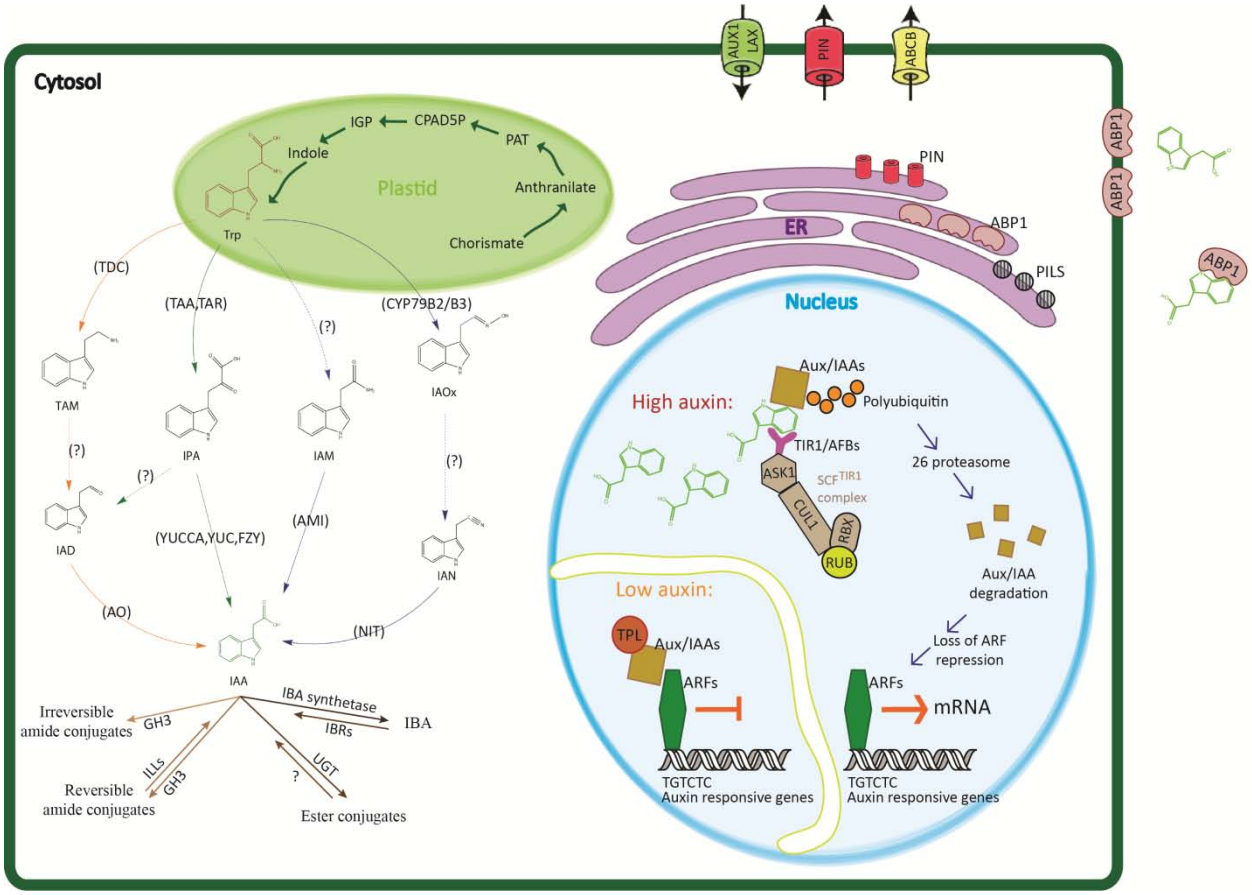

Figure 9. Schematic view of IAA homeostasis, auxin polar transport and auxin signaling. The biosynthesis of IAA precursors, such as IGP for tryptophan independent auxin biosynthesis pathway and Trp for tryptophan dependant IAA biosynthesis pathway, takes place in plastids. Trp, the primary IAA precursor, is generated via the Chorismate pathway. The subsequent Trp-dependent IAA biosynthesis pathways are located in the cytosol. Four putative pathways for Trp-dependent IAA biosynthesis in higher plants is shown: the IAOx, IAM, IPA and TRM pathways. The enzymes known to operate in each pathway are shown in complete arrows with the name, and the unknown enzymes are shown by dashed-arrow with question mark sign. Pathways for IAA degradation and conjugation. IAA can be conjugated to amino acids and sugars. Some IAA conjugates can be regarded as storage forms that can be hydrolysed to form free IAA. Solid arrows indicate pathways in which the enzymes, genes or intermediates are known, and arrows with the question mark indicate pathways that are not well defined. TIR1/AFB auxin receptor is an F-box protein that forms an SCF E3 ubiquitin ligase complex between SKP (ASK1) and cullin1 (CUL1). SCFTIR1/AFB catalyzes the ubiquitination of auxin/IAA proteins (Aux/IAAs) in the presence of auxin. The activity of the auxin response factor (ARF) transcription factors is blocked by Aux/IAA bound to TOPLESS (TPL). Auxin is bound in the small cavity formed between TIR1 and Aux/IAA. The auxin-induced degradation of Aux/IAA repressors recovers the ARF activity and activates the transcription of auxin-responsive genes (auxin

response element, AuxRE: TGTCTC). Auxin-binding protein 1 (ABP1) is an ER-localized protein, but small amounts of functional ABP1 protein act at the plasma membrane as an auxin receptor. Scheme view of ABCBs and PINs as efflux auxin carrier proteins and AUX1/LAX as influx auxin carrier protein. ER marks endoplasmic reticulum which PIN, PIN Like (PILs) and ABP1 seems to be placed on its cell membrane.

2.1.2.2. The indole-3-acetamide (IAM) pathway

IAM, another source of auxin, is present in many plant species such as *Arabidopsis thaliana,* maize, rice and tobacco (Sugawara et al., 2009; Novák et al., 2012). *cyp79b2 cyp79b3* double mutant shows a decrease of IAM level (Sugawara et al., 2009), which is consistent with IAOx contributing to IAM levels. IAOx has been detected in few species such as *Arabidopsis thaliana* and *Brassica campestris* (Ludwig-Müller and Hilgenberg, 1988; Sugawara et al., 2009; Novák et al., 2012) in which IAM has been detected in many species, suggest that IAM also produce independently from IAOx (Sugawara et al., 2009). IAM can be converted to active auxin through IAM hyrolases (AMIDASE) (Pollmann et al., 2003; Nemoto et al., 2009). Indole-3-acetamide hydrolase (AMI) function in conversion of indole-3-acetamide (IAM) into indole-3-acetic acid (IAA), which were thought to exist only in bacteria (reviewed by (Mano et al., 2010). AMI is similar at the amino acid sequence level to translocon (Toc64), a 64 kDa chloroplast outer membrane protein, while these two genes remain evolutionary different from each other (Sohrt and Soll, 2000; Chew et al., 2004; Qbadou et al., 2007; Schlegel et al., 2007). TOC64 contains an inactive amidase domain (Sohrt and Soll, 2000; Kalanon and McFadden, 2008), a C-terminal tetratricopeptide repeat (TPR) motif (Sohrt and Soll, 2000; Lee et al., 2004; Schlegel et al., 2007; Kalanon and McFadden, 2008) and also an N-terminal transmembrane region (TM) that is essential and sufficient for the targeting to the chloroplasts (Sohrt and Soll, 2000; Qbadou et al., 2007; Kalanon and McFadden, 2008). Comparing to TOC64, AMI includes an active amidase domain, but does not contain TM region and TPR domains (Mano et al., 2010).

2.1.2.3. The indole-3-pyruvic acid (IPA) pathway

It seems that the IPA pathway is the main contributor of free IAA. Each step of this pathway from Trp to IAA has been identified (reviewed by Zhao, 2012 and Mano and

Nemoto, 2012). IPA pathway converts IPA to IAA in two-step process. The first process consists in a conversion of Trp to IPA via TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family and its close homologues, TRYPTOPHAN AMINOTRANSFERASE RELATED, TAR1 and TAR2 (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009; Zhou et al., 2011). The second process is the conversion of IPA to IAA via YUCCA (YUC) family of flavin monooxygenases activity (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011). TAA1 encodes an aminotransferase that converts tryptophan to IPA and is closely related to TRYPTOPHAN AMINOTRANSFERASE RELATED 1 to 4 genes (TAR1-4) which make them as a five-member gene family (Stepanova et al., 2008; Tao et al., 2008). TAA1 that is localized in the cytoplasm and the TAR1 proteins do not contain the N-terminal extension, but TAR2–TAR4 proteins contain an N-terminal extension that are predicted to be a signal peptide (Stepanova et al., 2008; Tao et al., 2008). These findings show that TAR1 could be a paralogue of TAA1 and the function of TAR2-4 proteins is different from TAA1 and TAR1 (Mano and Nemoto, 2012). The YUC gene, encodes a flavin monooxygenase-like enzyme, responsible for the conversion of IPA to IAA in *A. thaliana* (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011). Orthologous genes of YUC have been found in other plants as reviewed by Mano and Nemoto, (2012). YUC proteins belong to a multigene family that includes 11 members in *A. thaliana* (YUC1-YU11) and appear to have overlapping functions (Zhao et al., 2001; Cheng et al., 2006, 2007). Genetic, enzymatic, and metabolite-based evidences indicated that TAA and YUC families function in the same auxin biosynthetic pathway in *A. thaliana*. It was proposed that the TAA1–YUC pathway is 'the main auxin biosynthesis pathway in *A. thaliana*' (Mashiguchi et al., 2011). Indole-3-acetaldehyde (IAD) can be an intermediate in this pathway. It has been identified in several plant species (Ernstsen and Sandberg, 1986; Quittenden et al., 2009; Novák et al., 2012) and has previously been hypothesized to be an auxin precursor (reviewed by Mano and Nemoto, 2012).

2.1.2.4. The tryptamine (TAM) pathway

The TAM pathway has recently been proposed as one of the IAA biosynthetic pathways (reviewed by Mano and Nemoto, 2012). Compare with IAA and Trp, TAM is found in very low level (Quittenden et al., 2009; Novák et al., 2012) and is produced by the activity of Tryptophan decarboxylase (TDC) enzyme. It is possible that TAM could function both as a precursor for IAA and in indole alkaloid and serotonin biosynthesis in different

plant species (Quittenden et al., 2009; Mano and Nemoto, 2012). IAD application increased the IAA levels (Larsen, 1949, 1951; Bower et al., 1978; Koshiba et al., 1996; Tsurusaki et al., 1997) which raises the possibility that IAD can be converted to IAA directly *in planta*. It has been suggested that the ALDEHYDE OXIDASE1 (AO1) enzyme converts IAD into IAA (Seo et al., 1998) however the putative role of IAD has recently been questioned (reviewed by Korasick et al., 2013).

2.2. Auxin conjugation and degradation

Free IAA levels can be adjusted via conversion into IBA or conjugation to amides or esters (Rosquete et al., 2012). Auxin conjugates are generally considered as temporary storage of inactive IAA, releasing the free active hormone upon hydrolysis (Fluck et al., 2000). Auxin-conjugation is tissue-specific and strongly regulated. Indeed, genes involved in auxin conjugates synthesis and hydrolysis display tissue specific expression pattern and are regulated by abiotic and biotic stress and hormone treatments (Ludwig-Muller, 2011). Also, auxin can be deactivated by oxidation. The primary oxidation forms of auxin are likely to be 2-oxo-indoleacetic acid (OxIAA), OxIAAaspartate, and O-glucoside.

2.3. Auxin transport

Auxin can be transported by diffusion (passive movement) and by auxin transporter (active movement) (Reviewed by (Zazimalova et al., 2010)). IAA is largely protonated in the apoplasts and can pass through the plasma membrane via diffusion into the cell. Once in the cytosol, it is mainly deprotonated due to the higher pH, and the resulting charged molecule (IAA-) is membrane impermeable. This concept is the basis of the chemiosmotic polar diffusion model or chemiosmotic hypothesis (Rubery and Sheldrake, 1974; Raven, 1975; Goldsmith, 1977). As anionic auxins cannot diffuse across the plasma membrane, thus they require the activity of transporters at the plasma membrane to exit the cells and ameliorate this bottleneck. Up to date some auxin carrier families have been identified, such as AUXIN-RESISTANT 1/LIKE AUX1 (AUX1/LAX) influx carriers, PIN-FORMED (PIN) and ATP-BINDING CASSETTE (ABCB) auxin efflux carriers which mediate auxin distribution (Reviewed by Zazimalova et al., 2010). A major mechanism that regulates auxin distribution

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is polar auxin transport mediated by PIN and AUX/LAX proteins, which control cellular auxin efflux and influx, respectively (Vanneste and Friml, 2009). Their asymmetric distribution across cells and tissues results in directional auxin flow and establishment of auxin gradients (Wisniewska et al., 2006; Bainbridge et al., 2008; Swarup et al., 2008; Petrasek and Friml, 2009). *A. thaliana* contains eight PIN proteins (Paponov et al., 2005), of which several are involved directly in creation of auxin gradients that control diverse developmental processes such as embryogenesis, organ initiation, vascular tissue differentiation and tropisms (Petrasek and Friml, 2009). Similarly, there are four AUX/LAX proteins in *A. thaliana*, with the best characterized, AUX1, mediating high-affinity auxin uptake (Kerr and Bennett, 2007; Bainbridge et al., 2008; Swarup et al., 2008). Auxin signaling regulates cell responses to the different auxin levels that are formed by a combination of auxin metabolism and transport.

2.4. Auxin receptors

Auxin is perceived by two separate classes of receptors:1- transport inhibitor response 1 (TIR1, or auxin-related F-box (AFB)), that controls the transcriptional responses to auxin (reviewed by Mockaitis and Estelle, 2008 and Chapman and Estelle, 2009); 2- The auxin-binding protein 1 (ABP1), which regulates a broad diversity of growth and developmental processes in plants (reviewed by Tromas et al., 2010). The discovery of SCF/TIR1 that promotes the degradation of the Aux/IAA proteins was a significant breakthrough in plant hormone signaling. The *Arabidopsis thaliana* genome encodes five F-box proteins sharing 50–70% sequence identity with TIR1. These proteins have been named auxin signaling F-box protein 1 to 5 (AFB1– AFB5). Genetic and biochemical studies have implicated these proteins in auxin signaling (Dharmasiri et al., 2005; Walsh et al., 2006). Moreover, the diminution of the radio-labeled IAAs level in extracts from mutants lacking TIR1 and AFB1–3, confirmed that these proteins are very likely to function as auxin receptors (Mockaitis and Estelle, 2008).

ABP1 is involved in a wide variety of auxin-dependent responses, including early responses at the plasma membrane, regulation of gene expression, cell division and cell expansion (Shi and Yang, 2011). ABP1 is important throughout the life of plants, including embryogenesis, and differentially affects the cells depending on the developmental context.

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Comparative analysis of the *A. thaliana* ABP1 sequence against available genome sequence databases of land plants revealed that ABP1 is present in all species from bryophytes to flowering plants thus highlighting the significance of its function. All experimental data support that ABP1 is a crucial component of auxin signaling and can be considered as the auxin binding subunit of a plasma membrane auxin receptor (Tromas et al., 2010). Tromas et al. (2013) recently reported that ABP1 is a negative regulator of the SCF (TIR1/AFB) pathway and is genetically upstream of TIR1/AFBs.

2.5. Auxin Signaling

2.5.1. Transcriptional auxin signaling

One important pathway involving auxin receptor TIR1 and linking auxin perception to gene expression is now well established. In the corresponding model, high concentration of IAA in the cells leads to the ubiquitination of AUXIN RESISTANT/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) proteins by the TIR1/AFB subunit of the $SCF^{TIR1/AFB}$ ubiquitin ligase and the degradation of Aux/IAA proteins by the 26S proteasome, which then releases the Aux/IAA-mediated inhibition of AUXIN RESPONSE FACTORs (ARFs) and allows these transcription factors to modulate the expression of their target genes (Fig. 10 reviewed by Chapman and Estelle, 2009).

2.5.1.1. Aux/IAA and TPL co-repressor gene families

Aux/IAA genes have been found in many plant species but not in bacteria, animal or fungi and are probably unique to plants. All Aux/IAA members have been identified in *Arabidopsis thaliana*, maize, rice, poplar, sorghum and tomato (Audran-Delalande et al., 2012). Their identification was performed thanks to sequence similarity with already known Aux/IAA genes. According to the diversity of Aux/IAA-related phenotypes and the size of the family, it has been suggested that Aux/IAAs are excellent candidates for providing specificity in auxin responses (Lokerse and Weijers, 2009). Most Aux/IAAs have four domains: auxin-induced degradation through interaction with TIR1/AFBs (DII) and dimerization with the ARFs and other IAAs (DIII and DIV). Aux/IAAs inhibit the activity of ARFs by recruiting the co-repressor TOPLESS (TPL) and related proteins (TPRs) through an

ethylene response factor-associated amphiphilic repression (EAR) motif in domain I (Szemenyei et al., 2008). The interactions at each of these domains may act as tuning knobs to specify the output properties for a given auxin signal (Pierre-Jerome et al., 2013). In tomato, six full-length SlTPL genes were identified, as well as additional three pseudo genes with incomplete coding sequences (Hao et al., 2014). Protein–protein interaction between TOPLESS and auxin/indole-3-acetic acid (Aux/IAA) proteins shows the two classes of TOPLESS. One group such as SlTPL1, SlTPL3, SlTPL4, which are interacting with the most of Aux/IAA, and another group e.g. SLTPL6, with limited capacity for interaction with these protein partners (Hao et al., 2014).

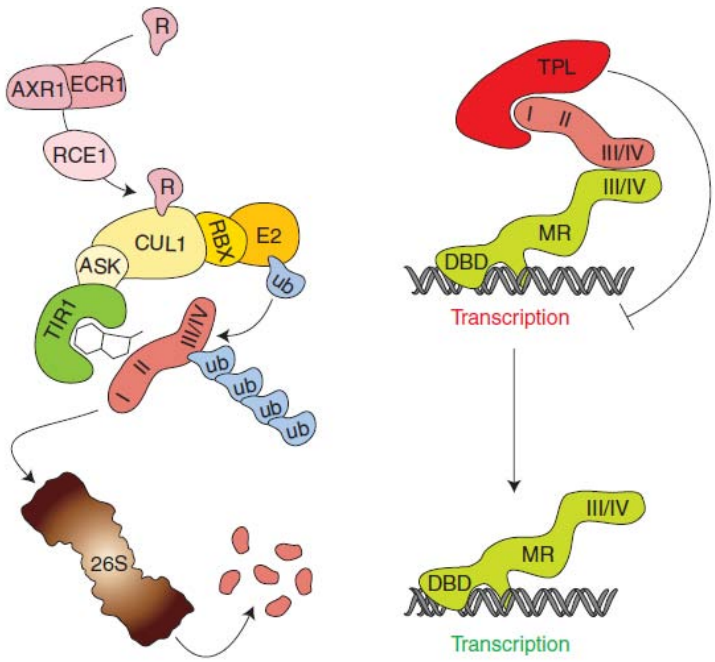

Figure 10. Transcriptional auxin signaling. When auxin is perceived by its receptor TIR1, the affinity for the Aux/IAA proteins increases. The Aux/IAAs are subsequently ubiquitinated and degraded by the 26S proteasome. The ubiquitination of Aux/IAAs in *A. thaliana* involves a ubiquitin activating enzyme (E1, not shown), ubiquitin conjugating enzyme (E2), and the ubiquitin ligase (E3) SCFTIR1 that consists of a cullin protein, an RBX protein, one of the Arabidopsis SKP1(ASK) proteins, and the F-Box protein TIR1. Under low auxin concentrations, the Aux/IAA proteins bind and inhibit the ARF proteins by recruiting the TPL corepressor. When auxin levels rise, the Aux/IAA proteins are ubiquitinated and degraded, thereby releasing the ARF proteins to exert their function as transcriptional activators or repressors (from Moller and Weijers, 2009).

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2.5.1.2. ARF gene family

Interaction of ARF transcription factors with the AUX/IAA repressors and TPL co-repressors mediates the auxin sensitivity (Guilfoyle et al., 1998; Tiwari et al., 2003; Wong and Struhl, 2011). Most of the ARF members display three conserved domains, an amino-terminal DNA binding domain (DBD) and domains III and IV at their carboxy-terminal part (Guilfoyle et al., 1998; Tiwari et al., 2003). The ARF DBD is a plant-specific B3-type domain, which is found in lots of plant transcription factors (Guilfoyle et al., 1998). The binding of ARF to DNA is facilitated by the presence of carboxy-terminal parts in ARF proteins (Ulmasov et al., 1999; Tiwari et al., 2003). The ARF binding to DNA occurs by the recognition of specific DNA elements called AuxRE (TGTCTC or TGTCCC) which allow activation or repression of auxin-responsive genes (Ulmasov et al., 1999). Among the 23 ARFs in *A. thaliana*, only five are classified as transcriptional activators and the rest as repressors.

ARFs were classified as repressors based on the shared absence of glutamine enrichment in their middle regions (Guilfoyle et al., 1998; Ulmasov et al., 1999; Tiwari et al., 2003). The amino acid sequences of domains III and IV present at their carboxy-terminal part are related to Aux/IAA proteins. ARFs can either form ARF homodimers or Aux/IAA-ARF heterodimer. Nevertheless domains III and IV are absent in four AtARFs: ARF3, ARF13, ARF17 and ARF23, suggesting that these proteins cannot interact with Aux/IAAs (Guilfoyle et al., 1998; Liscum and Reed, 2002) (Fig. 11). 22 different ARFs are identified regarding tomato (*Solanum lycopersicum*) reference genome (Zouine et al., 2014). The ARFs can be regulated by ethylene and auxin which suggests their potential contribution to signaling pathways of these two hormones (Zouine et al., 2014). The complexity of the expression of ARF genes at the transcriptional and post-transcriptional levels enables auxin to manage a wide range of physiological processes in a highly precise and coordinated manner (Zouine et al., 2014).

2.5.2. Non-transcriptional auxin signaling

Auxin signaling machinery that is involved in non-transcriptional regulation is not well characterized. Knowledge of the signaling components and the physiological events involved

in this rapid auxin response is limited. Auxin-binding protein 1 (ABP1) is one of the receptor candidates implicated in non-transcriptional auxin signaling (Hayashi, 2012).

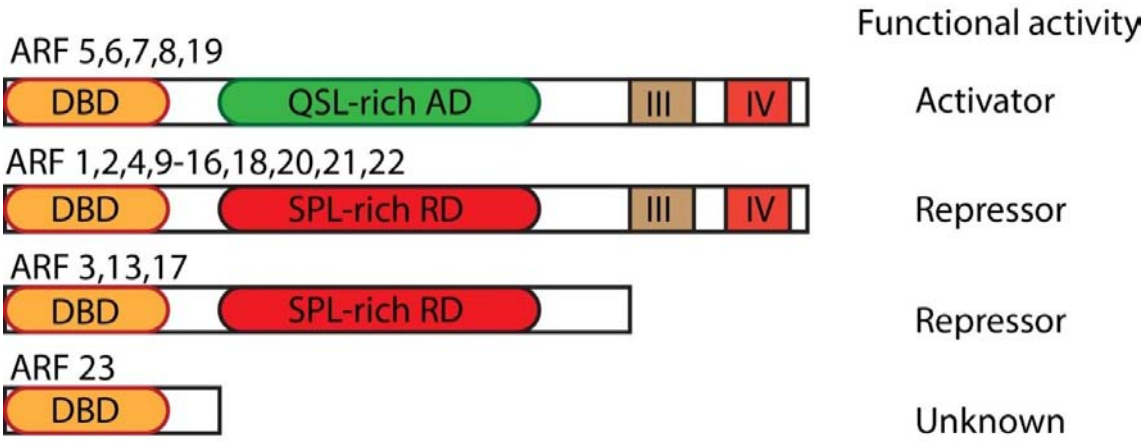

Figure 11. Structure of ARF family in *Arabidopsis thaliana*. ARFs 5, 6, 7, 8, 29 are activators, and the rest of ARFs in *A. thaliana* are repressors except ARF 23 whose function is unknown. Among the repressors, some doesn't have the III and IV domain: ARF 3, 13, 17.

2.6. Auxin signaling is regulated by miRNAs

MicroRNAs (miRNAs) are ~21 nucleotide noncoding RNAs and have important roles in nutrient homeostasis, development, abiotic and biotic stress responses via interactions with specific target mRNAs (Meyers et al., 2008). miRNAs negatively regulate expression of target genes by mRNA cleavage or inhibition of translation (Lanet et al., 2009). Moreover, miRNAs regulate the auxin signalling, and it seems to be conserved among many plant species (Sanan-Mishra et al., 2013). A complicated interaction between auxin levels and miRNAs has been indicated during plant development (Fig. 12). Auxin and miRNAs independently regulate the ARF levels to direct normal growth and development of aerial organs as well as lateral root production.

miR393 targets the auxin receptors consisting of the four closely related F-box genes, including the auxin receptor TIR1, AFB1, AFB2, and AFB3 by guiding the cleavage of their

mRNAs (Ruegger et al., 1998; Gray et al., 2001; Sunkar and Zhu, 2004). miR393 is also known to target At3g23690, a basic helix-loop helix transcription factor that is homologous to GBOF-1 from tulip, which is annotated as an auxin-inducible gene. The role of miR393 in plant susceptibility to microbes was first described in *Arabidopsis thaliana* when miR393-mediated down-regulation of auxin receptors (TIR1, AFB1-3) led to the suppression of auxin signaling and to a higher resistance of the plant (Navarro et al., 2006). Later, Navarro et al. (2008) found that the *Pseudomonas syringae* effector proteins that promote the bacterial infection, targeted the miR393 pathway. It has been demonstrated that ARF10, ARF16 and ARF17 are regulated by miR160 (Rhoades et al., 2002; Bartel and Bartel, 2003). miR160 regulated ARF17 acts directly to repress GH3.5 and DFL1/GH3.6 transcripts providing an important mechanism for controlling levels of free IAA. ARF10 and ARF16 dictate the root cap cell formation to control the direction of root tip growth. In the absence of miR160-mediated regulation of ARF10 and ARF16, severe developmental abnormalities occur (Mallory et al., 2005; Wang et al., 2005). These include abnormal embryos, defects in root growth, leaf curling, serrated laminas, early flowering with altered floral morphology and reduced fertility (Mallory et al., 2005). miR167 negatively regulates the expression of ARF6 and ARF8 (Mallory et al., 2005; Sorin et al., 2005; Williams et al., 2005). The miR167 guided cleavage of ARF8 transcript apparently negatively regulates free IAA levels by controlling GH3-like early auxin responsive gene expression (Tian et al., 2004). miR164 is known to target five members of the NAM/ATAF/CUC (NAC) domain transcription factor family (Rhoades et al., 2002; Guo et al., 2005). Of these, NAC1 is involved in modulating lateral root development (Mallory et al., 2004; Mallory et al., 2004; Guo et al., 2005; Hibara et al., 2006). NAC1 acts downstream of TIR1 to transmit auxin signals promoting lateral root emergence. NAC1 is a transcriptional activator of the auxin-responsive genes DBP (DNA Binding Protein of unknown function) (Xie et al., 2000). In addition miR390 indirectly controls the ARF2, ARF3 and ARF4 regulated leaf patterning, developmental timing and lateral root initiation (Fahlgren et al., 2006; Marin et al., 2010; Yoon et al., 2010).

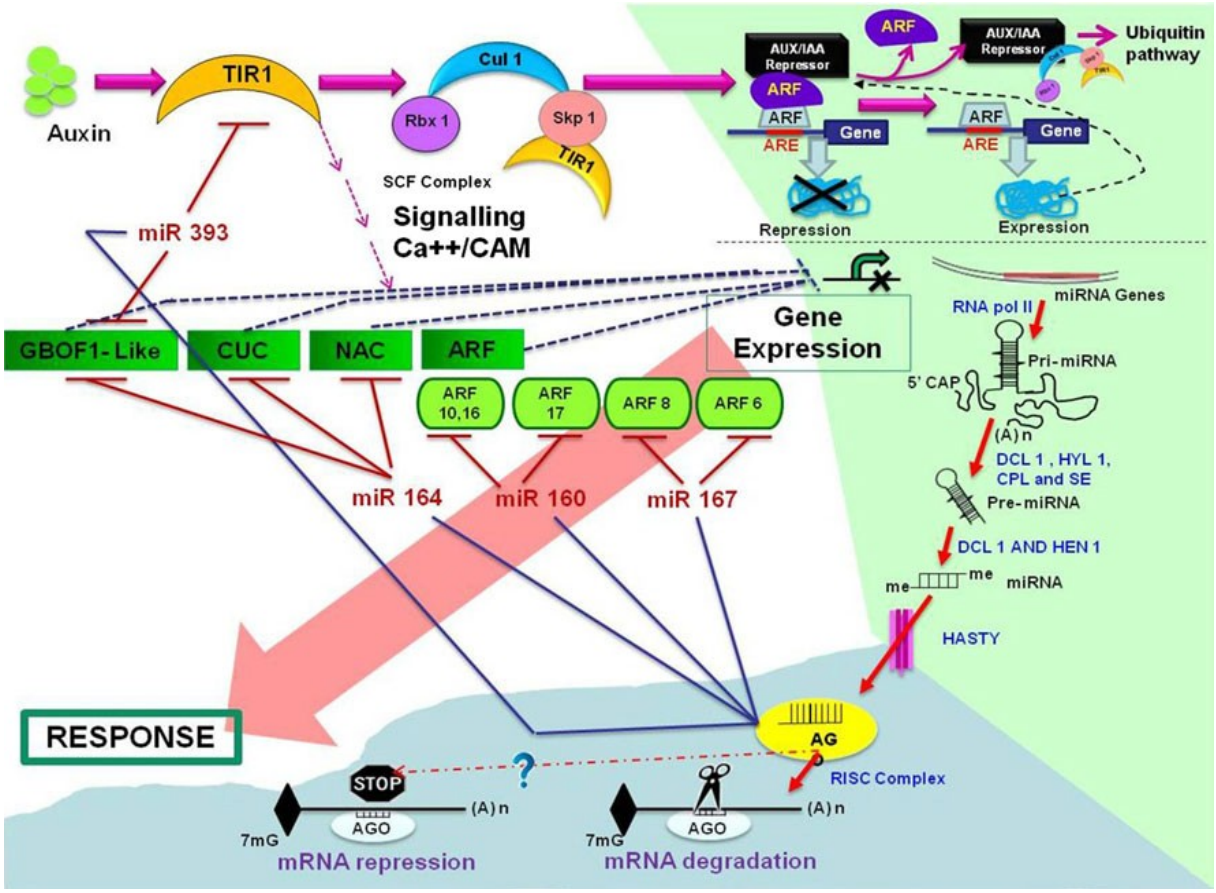

Figure 12. A schematic representation showing the mutual constrain exerted by miRNA and auxin cascade on each other. The bluntly ended lines depict the repression activity, while arrows represent activational processes. ARF auxin response factor; TIR1 transport inhibitor response 1; SCF Complex Skp1, Cullin, and F-box protein-type complex; ARE auxin response element; CUC cup-shaped cotyledon; Aux/IAA auxin/indole-3-acetic acid; TPL topless (from Sanan-Mishra et al., 2013).

2.7. Auxin-responsive genes

Small auxin-up RNAs (SAURs), Aux/IAAs and a group of GH3 proteins are three known gene families that are rapidly induced by auxin (Hagen and Guilfoyle 2002).

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2.7.1. Gretchen Hagen3 (GH3) gene family

Gretchen Hagen3 (GH3) was first identified in *Glycine max* (soybean) as an early auxin-responsive gene (Hagen and Guilfoyle, 1985). Based on GH3 genes sequence similarities and the substrate specificities of their products, they are classified into three groups (I, II, and III) in *Arabidopsis thaliana* (Staswick et al., 2002; Staswick and Tiryaki, 2004). Among these three groups, Group II were demonstrated to be active on IAA and functions in a negative feedback regulation of IAA concentration (Staswick et al., 2002; Staswick et al., 2005). Group II GH3s conjugate extra IAA to amino acids, either for storage or degradation (Staswick et al., 2005). Recent research has shown a reduction of auxin content by over-expressing GH3, (Zhang et al., 2009). According to the *A. thalina* microarray data, Yuan et al. (2013) reported that in Group II enzymes AtGH3.1, AtGH3.2, AtGH3.3, and AtGH3.4 were raised over 10-fold after IAA treatment. AtGH3.5 and AtGH3.6 were induced just 2.5-fold to 8-fold; however, AtGH3.9 and AtGH3.17 expression displayed no significant changes in response to auxin (Okrent and Wildermuth, 2011; Yuan et al., 2013). Kumar et al. (2012) identify fifteen genes encoding GH3 members in tomato and among them only 11 SlGH3s were up-regulated by exogenous auxin treatment (Kumar et al., 2012).

2.7.2. Small Auxin-Up RNA (SAUR)

SAUR genes are originally characterized in soybean (McClure and Guilfoyle, 1987) and currently count 82 members together with SAUR-like genes on the TAIR website (www.arabidopsis.org) in *Arabidopsis thaliana* (Markakis et al., 2013). Although many SAUR genes have been identified in different plant species, only some of them have been functionally characterized. SAUR members have been reported to be localized in membrane and/or cytoplasm (Chae et al., 2012; Spartz et al., 2012). Their expression could be induced within 2 to 5 min by active auxin, indicating that auxin plays an important role in transcriptional regulation of SAUR genes (McClure and Guilfoyle, 1987; Kong et al., 2013). In addition, many SAUR genes are also regulated posttranscriptional due to a highly conserved downstream (DST) element in their 3'-untranslated region (UTR) that contributes to mRNA instability in an auxin-independent manner (Newman et al., 1993; Park et al., 2012). Therefore, regulation of SAURs may occur at the transcriptional, posttranscriptional and protein levels (Esmon et al., 2006; Spartz et al., 2012). SAURs negatively influence synthesis of auxin and proteins for polar auxin transport (Kant et al., 2009; Kant and

Rothstein, 2009). 99 members of SAUR gene family are characterized in tomato which some of them response to exogenous IAA treatment (Wu et al., 2012). It has been shown that abiotic (cold, salt and drought) stresses significantly modified transcript levels of Sl-SAURs genes (Wu et al., 2012).

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Aim of the study

The objective of my research was to understand the relation between auxin and AM symbiosis (Fig. 13).

More defined objectives of this project were to investigate the following questions:

1. Is the auxin signaling pathway involved in the mycorrhization process?
2. Does the perception of auxin affect the mycorrhization process?
3. If the host's auxin signaling pathway is involved in the mycorrhization process:
    a. What step(s) of this process is (are) particularly concerned
    b. Is there any link between Aux/IAAs and strigolactone biosynthesis during mycorrhization?

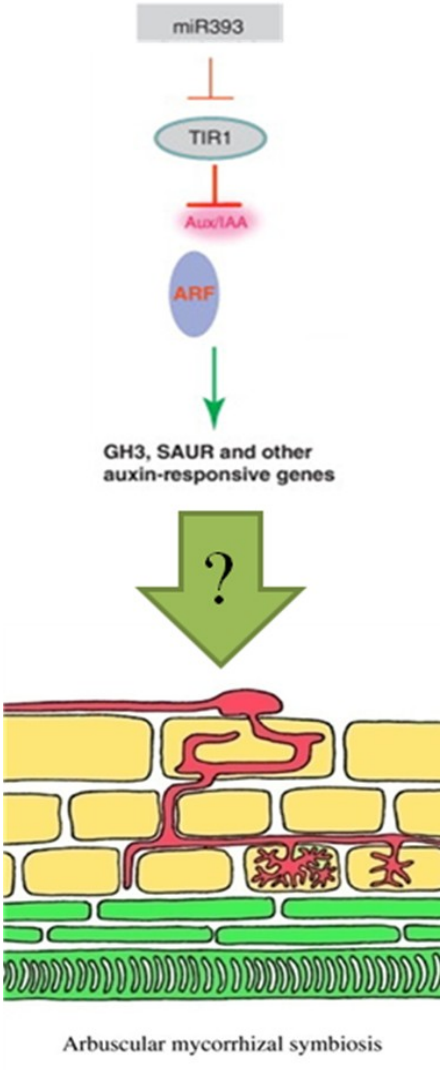

Figure 13. The schematic view of our objectives.

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Chapter II – Involvement of Auxin and miR393 in arbuscular mycorrhizal symbiosis

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1. Introduction

Devers et al. (2013) via high throughput (Illumina) sequencing of small RNAs and degradome tags of *Medicago truncatula* roots, disclosed the influence of miRNAs and miRNA-mediated mRNA cleavage on root cell during AM symbiosis. However, up to now, only a few miRNAs have been functionally characterized during mycorrhizal symbiosis. NSP2, a GRAS transcription factor already known for its role during nodulation, has also been shown to be required for proper AM fungal colonization (Maillet et al., 2011). miR171h is a miRNA that targets NSP2 (Devers et al., 2013), and when it is overexpressed fungal colonization is reduced, mimicking the phenotype of the *nsp2* mutant (Lauressergues et al., 2012). In contrast, fungal colonization increases and extends into the root elongation zone in transformed roots where the nsp2 gene is modified to be resistant to miR171h cleavage. These data have raised the hypothesis that the plant might limit the colonization of AM fungi via a miRNA171h-mediated negative regulation of NSP2.

Another miRNA which affects mycorrhizal colonization is miR396 (Bazin et al., 2013). This miRNA is one of the most conserved miRNAs in angiosperms and gymnosperms and targets members of the family of growth-regulating factor genes (Debernardi et al., 2012). Over-expression of miR396b in roots reduces root growth and mycorrhizal colonization (Bazin et al., 2013).

We have seen in our general introduction that several phytohormones, including auxin, are involved in the regulation of mycorrhizal symbiosis (Foo et al., 2013). Because auxin is a major regulator of root development and because some miRNAs are involved in auxin perception or signaling, my first aim was to investigate whether auxin and auxin-related miRNAs could play a role during AM symbiosis.

2. Publication miR393

Auxin Perception Is Required for Arbuscule Development in Arbuscular Mycorrhizal Symbiosis[1][W]

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Most land plant species live in symbiosis with arbuscular mycorrhizal fungi. These fungi differentiate essential functional structures called arbuscules in root cortical cells from which mineral nutrients are released to the plant. We investigated the role of microRNA393 (miR393), an miRNA that targets several auxin receptors, in arbuscular mycorrhizal root colonization. Expression of the precursors of the miR393 was down-regulated during mycorrhization in three different plant species: *Solanum lycopersicum*, *Medicago truncatula*, and *Oryza sativa*. Treatment of *S. lycopersicum*, *M. truncatula*, and *O. sativa* roots with concentrations of synthetic auxin analogs that did not affect root development stimulated mycorrhization, particularly arbuscule formation. DR5-GUS, a reporter for auxin response, was preferentially expressed in root cells containing arbuscules. Finally, overexpression of miR393 in root tissues resulted in down-regulation of auxin receptor genes (*transport inhibitor response1* and *auxin-related F box*) and underdeveloped arbuscules in all three plant species. These results support the conclusion that miR393 is a negative regulator of arbuscule formation by hampering auxin perception in arbuscule-containing cells.

Arbuscular mycorrhiza (AM) is a widespread symbiosis between soil fungi (*Glomeromycota* spp.) and most land plant species. The fungus colonizes the roots of its host plant, where it obtains carbohydrates (Bago et al., 2003). In exchange, it provides mineral nutrients to the plant, especially phosphate, that are taken up from the soil by its extraradical mycelium, thus considerably improving plant nutrition in soils of low fertility (Bago et al., 2003; Smith and Smith, 2011). After spore germination, the fungus forms a hyphopodium on the root surface, penetrates the rhizodermis through a prepenetration apparatus, colonizes the root tissue intercellularly, and eventually, forms highly ramified structures called arbuscules in cortical cells, where mineral nutrients are released to the host (Parniske, 2008; Harrison, 2012). Each branch of the arbuscule is surrounded by a plant-derived periarbuscular membrane that prevents fungal penetration of the root cell cytosol and controls nutrient and signal exchange between the symbionts. Arbuscule formation relies on drastic reorganization of the plant cell and the implementation of a plant genetic program that remains poorly known, because only few genes associated with this process have been identified so far (for review, see Delaux et al., 2013; Gutjahr and Parniske, 2013).

It is well established that plant hormones are key regulators of plant physiologic and developmental processes (Santner et al., 2009), and it has recently become apparent that some of them are also important for arbuscule development. For example, the abscisic acid-deficient mutant *sitiens* was impaired in arbuscule formation and viability, indicating an important function

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jean-Philippe Combier (combier@lrsv.ups-tlse.fr).

[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.114.246595

of abscisic acid in arbuscule maintenance (Herrera-Medina et al., 2007). Conversely, gibberellic acid (GA_3) treatment or constitutive GA_3 signaling in DELLA-deficient mutants suppresses arbuscule formation, and a reduction of GA_3 signaling by stabilized DELLA proteins promotes arbuscule development (Floss et al., 2013; Foo et al., 2013). Auxin has also been suggested to play a role in AM symbiosis, although its exact role in this type of plant-microbe interaction remains elusive (Hause et al., 2007; Hanlon and Coenen, 2011). Although an increase in auxin content in mycorrhizal roots has been previously reported for *Medicago truncatula*, maize (*Zea mays*), and soybean (*Glycine max*; Ludwig-Müller et al., 1997; Kaldorf and Ludwig-Müller, 2000; Fitze et al., 2005; Ludwig-Müller and Güther, 2007), no change in auxin content in mycorrhizal roots of tobacco (*Nicotiana tabacum*) and leek (*Allium porrum*) has been observed (Torelli et al., 2000; Shaul-Keinan et al., 2002). Meixner et al. (2005) found that indole-3-acetic acid (IAA) levels were higher in mycorrhizal soybean roots. This increase of IAA content in mycorrhizal roots was lower in the mutant *nark*, which is deficient in autoregulation of nodulation, suggesting that IAA might play a role in the autoregulation of mycorrhization. Congruently, a strong decrease of AM colonization but with normal fungal structures was observed in both the auxin-resistant tomato (*Solanum lycopersicum*) mutant *diageotropica* and the auxin hypertransporting tomato mutant *polycotyledon*, indicating that, indeed, auxin could play a role in AM colonization (Hanlon and Coenen, 2011). It is, however, unknown whether reduced colonization was a direct consequence of the defective auxin signaling or transport or whether it was a consequence of cross talk with other hormone signaling or biosynthesis pathways (Hanlon and Coenen, 2011). Auxin signaling has also been implicated in legume root symbiosis with nitrogen-fixing rhizobia (Suzaki et al., 2012). However, disturbance of auxin signaling only had an impact on nodule development but not infection, which shares cell biological features with AM colonization (Mao et al., 2013; Turner et al., 2013).

Auxin was the first plant hormone to be described, possibly because of its paramount importance for most plant developmental processes. Among its many hormonal functions (Overvoorde et al., 2010), it is crucial to regulate cell elongation and cell and organ polarity (Lau et al., 2008; Kramer, 2009) and could potentially also exert such a role in plant cell developmental changes during symbiont accommodation. Auxin is perceived by nuclear-localized F-box domain-containing proteins (Dharmasiri et al., 2005; Kepinski and Leyser, 2005), and one important pathway linking auxin perception to gene expression is now well established. It involves the ubiquitination of Auxin (Aux)/IAA proteins by the transport inhibitor response1 (TIR1)/auxin-related F-box (AFB) proteins subunit of the Skp, Cullin, F-box containing $complex^{TIR1/AFB}$ ubiquitin ligase and the degradation of Aux/IAA proteins by the 26S proteasome. This degradation then releases the Aux/IAA-mediated inhibition of auxin response factors and allows these transcription factors to modulate the expression of their target genes (Hayashi, 2012). *TIR1* and several *AFB* genes encoding auxin receptors are regulated posttranscriptionally by microRNA393 (miR393) during root development and response to pathogens (Navarro et al., 2006, 2008; Parry et al., 2009; Vidal et al., 2010).

miRNAs are small noncoding RNAs that regulate the expression of target genes having complementary sequences by cleaving their mRNAs or inhibiting their translation (Voinnet, 2009). miRNAs are involved in most biological processes, such as development, response to stresses, and interactions with microorganisms. To date, only a few miRNAs have been characterized with respect to their role in AM symbiosis. miR171h quantitatively regulates mycorrhizal root colonization by targeting the *NODULATION SIGNALING PATHWAY2* (*NSP2*) transcription factor gene (Lauressergues et al., 2012), and miR396 regulates lateral root formation during fungal colonization (Bazin et al., 2013).

The suppression of auxin signaling by miR393 plays an important role in plant resistance to bacteria (Navarro et al., 2006). Extrapolating from it, we were interested to explore whether miR393 could also be involved in regulating AM interactions. We found that expression of miR393 was down-regulated in mycorrhizal roots, indicating that an active auxin perception might be needed during AM symbiosis. In parallel, treatment of plants with auxin analogs increased the quantity of arbuscules. Accordingly, the Direct repeat5 (DR5)-GUS promoter, which serves as an indicator of auxin response, was activated in mycorrhizal roots, specifically in arbuscule-containing cells. Overexpression of miR393 led to a down-regulation of auxin receptor expression and a concomitant strong defect in arbuscule formation. We showed this for three different plant species (tomato, *M. truncatula*, and rice [*Oryza sativa*]), indicating that auxin perception and/or auxin signaling are important for arbuscule development.

RESULTS

The Expression of miR393 Is Down-Regulated during AM Symbiosis

To examine whether miR393 might be involved in the regulation of AM colonization, we assessed the expression of miR393 in tomato roots colonized by the AM fungus *Rhizophagus irregularis*. The precursors of miR393 in tomato were identified using the MIReNA software (Mathelier and Carbone, 2010) and the miR393 of Arabidopsis (*Arabidopsis thaliana*) as query. The tomato genome contains only one miR393 copy, for which the mature sequence is identical to the Arabidopsis miRNA (Supplemental Fig. S1; Lin et al., 2013). Tomato roots inoculated with *R. irregularis* were harvested and assessed for root colonization and the induction of a mycorrhiza-specific plant marker gene, phosphate transporter4 (*PT4*; Harrison et al., 2002; Paszkowski et al.,

2002; Nagy et al., 2005; Supplemental Fig. S2). Root colonization (approximately 60%) was accompanied by a strong *PT4* induction (Supplemental Fig. S2). Furthermore, we observed a decrease in microRNA precursor miR393 transcript level and mature miR393 accumulation (Fig. 1A; Supplemental Fig. S3). To know if the down-regulation of miR393 is a general feature of mycorrhization, we measured the expression of precursors of miR393 during mycorrhizal colonization in two phylogenetically distant species: the model plants *M. truncatula* and rice. The genome of both plants contains two precursors of miR393 according to miRbase (www.mirbase.org; Supplemental Fig. S1). We first monitored root length colonization and the expression of mycorrhiza-specific plant *PT4* and *PT11* in *M. truncatula* and rice, respectively (Supplemental Fig. S2; Harrison et al., 2002; Paszkowski et al., 2002; Nagy et al., 2005). As for tomato, the miR393 precursors of *M. truncatula* and rice accumulated to lower levels in colonized roots compared with noncolonized roots (Fig. 1, B and C). In a time course experiment, the down-regulation of miR393 in *M. truncatula* correlated with the onset of arbuscule formation, which was revealed by *PT4* expression, at 3 weeks after inoculation and continued until 9 weeks postinoculation when the symbiosis was well established (Supplemental Fig. S4). This down-regulation of miR393 was not detected in roots treated with exogenous applications of Myc-LCOs and COs (Supplemental Fig. S5). These molecules are symbiotic molecular signals released by the fungus before colonization (Maillet et al., 2011; Genre et al., 2013). This suggests that down-regulation of miR393 specifically occurs later during root colonization. To support this hypothesis, the down-regulation of miR393 during mycorrhization was not observed in a *doesn't make infection3* (*dmi3*) mutant, which is impaired in the formation of symbiotic structures (Supplemental Fig. S6).

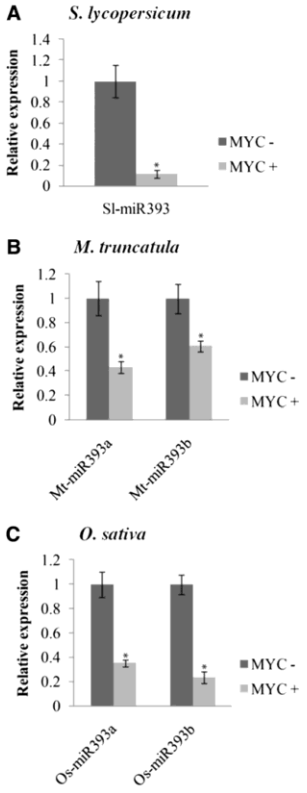

Figure 1. Down-regulation of premiR393 in AM symbiosis. Quantification by quantitative reverse transcription (qRT) -PCR of the expression of premiR393 in nonmycorrhizal (MYC−) and mycorrhizal (MYC+) roots of tomato (A), *M. truncatula* (B), and rice (C) colonized by *R. irregularis*. The measured transcripts were normalized to the relative expression value in nonmycorrhizal roots. Error bars represent SEM. *, Significant difference between the two treatments according to the Kruskal-Wallis test ($n = 6$, $P < 0.05$).

Auxin Treatment Increases Arbuscule Abundance

The previous experiments showing that miR393 was down-regulated in AM-colonized roots suggested that auxin might positively affect mycorrhizal colonization. To test this hypothesis, we treated tomato and *M. truncatula* plants with the synthetic auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D; Song, 2013). Because high concentrations of 2,4-D are lethal to plants or can strongly influence root development, we first monitored the effect of 2,4-D concentration on root development. Because the tomato root system produced no lateral roots under our in vitro conditions, we tested the effect of several concentrations of 2,4-D on primary root length. For *M. truncatula*, we measured both root length and root branching. Concentrations less than 10^{-8} M influenced neither the root length of tomato plants nor the root length and root branching of *M. truncatula* (Supplemental Fig. S7). Therefore, we treated tomato and *M. truncatula* plants three times per week with 10^{-10} M 2,4-D during mycorrhiza development. Whereas the root development was not affected by prolonged watering with low concentrations of 2,4-D, treatment with 2,4-D resulted in a significant increase of tomato root length colonization (+16%) compared with treatment with water (Fig. 2A), and particularly, the proportion of arbuscules was significantly higher in 2,4-D-treated roots (+32%) compared with control roots (Fig. 2A). Treatment of *M. truncatula* roots led to comparable results (i.e. root length colonization [+57%] and arbuscule [+119%] abundance were increased by the 2,4-D treatment; Fig. 2B). Because monocotyledonous plants are hardly sensitive to 2,4-D, we used 10^{-10} M naphtyl acetic acid (NAA) to examine the effect of auxin

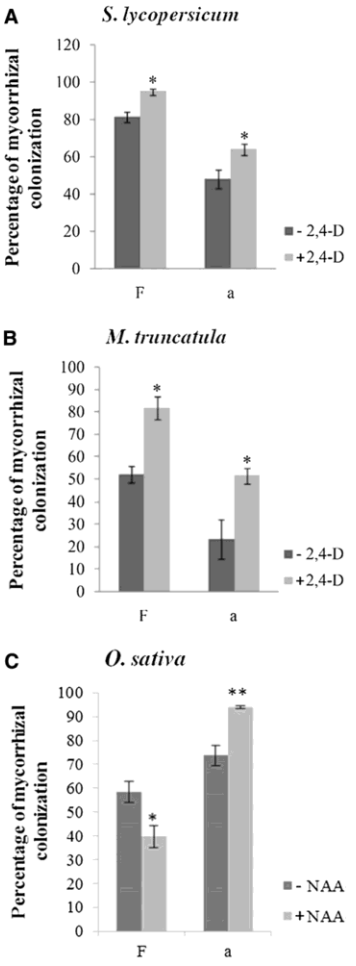

Figure 2. Frequency of colonization and arbuscule abundance increased in response to auxin treatment. Frequency (F) of mycorrhization and arbuscule abundance (a) in roots of tomato (A) and *M. truncatula* (B) in response to solvent control (−2,4-D) or 10^{-10} M 2,4-D (+2,4-D). C, F of mycorrhization and a in roots of rice in response to solvent control (−NAA) or 10^{-10} M NAA (+NAA). Error bars represent SEM. Asterisks indicate significant difference between the two treatments according to the Kruskal-Wallis test ($n = 6$). *, $P < 0.05$. **, $P < 0.01$.

treatment on colonization of rice. At this NAA concentration, the frequency of colonization was slightly reduced (−19%). Nevertheless, arbuscule abundance in the colonized areas was strongly increased (+ 20%; Fig. 2C). Taken together, these data showing a lower miR393 expression in mycorrhizal roots and a higher arbuscule formation in response to exogenous auxin treatments suggest that auxin signaling may be involved in arbuscule development.

Auxin Response Is Activated in Arbuscule-Containing Cells

Auxin regulates the formation of lateral roots, and it has been shown that overexpression of miR393 leads to a decreased number of lateral roots (Vidal et al., 2010). It is also known that mycorrhizal root systems are generally more ramified (Oláh et al., 2005; Gutjahr et al., 2009; Mukherjee and Ané, 2011). Thus, the decreased expression of miR393 that we observed in mycorrhizal roots could be related to a stimulatory effect of the fungus on lateral root formation. However, auxin and auxin signaling could also play a more specific and direct role in the establishment of the symbiosis. To reveal the stage of mycorrhizal colonization at which auxin signaling intervenes, we attempted to identify cells within colonized roots that would display a higher auxin response. We visualized the activity of *DR5*, a synthetic auxin-inducible promoter, fused to the *GUS* reporter gene (Ulmasov et al., 1997; Chaabouni et al., 2009). We first characterized the *DR5-GUS* expression pattern in nonmycorrhizal tomato roots. GUS staining was detected in the root tips and lateral root primordia (Supplemental Fig. S8), which is a common pattern of *DR5* activity in roots (Chaabouni et al., 2009). In mycorrhizal roots, strong additional GUS staining was present in larger patches localized in the cortex and apparently not linked to meristems (Fig. 3, A and B; Supplemental Fig. S9). To assess whether this staining corresponded to colonization units, we used specific fluorescent dyes: ImaGene Green, which is a fluorescent substrate of GUS (see "Materials and Methods"), and Uvitex2B or fluorescein-conjugated wheat germ agglutinin (WGA-FITC) to visualize the fungus (Diagne et al., 2011); each label was confirmed to be specific to cells expressing GUS or the fungus, respectively, except an unspecific labeling of lignified cell walls by ImaGene Green (Supplemental Fig. S10). Interestingly, dual labeling revealed that the nonmeristematic regions displaying GUS activity corresponded to root cortical cells containing arbuscules (Fig. 3, D–I). To investigate whether this specific localization of GUS activity could be generalized to other plant species, similar experiments were performed on stable transgenic *M. truncatula* and rice DR5-GUS lines (Scarpella et al., 2003; Herrbach et al., 2014). For both species, GUS staining was observed in root meristems (data not shown) and arbuscule-containing cells. However, whereas the GUS labeling in *M. truncatula* roots was highly specifically correlated to the presence of arbuscules (Fig. 4, A–F), the GUS staining in rice was more diffuse across all tissue layers including root hairs. However (apart from root meristems), its highest intensity was restricted to arbuscule-containing root portions (Fig. 4, G and H).

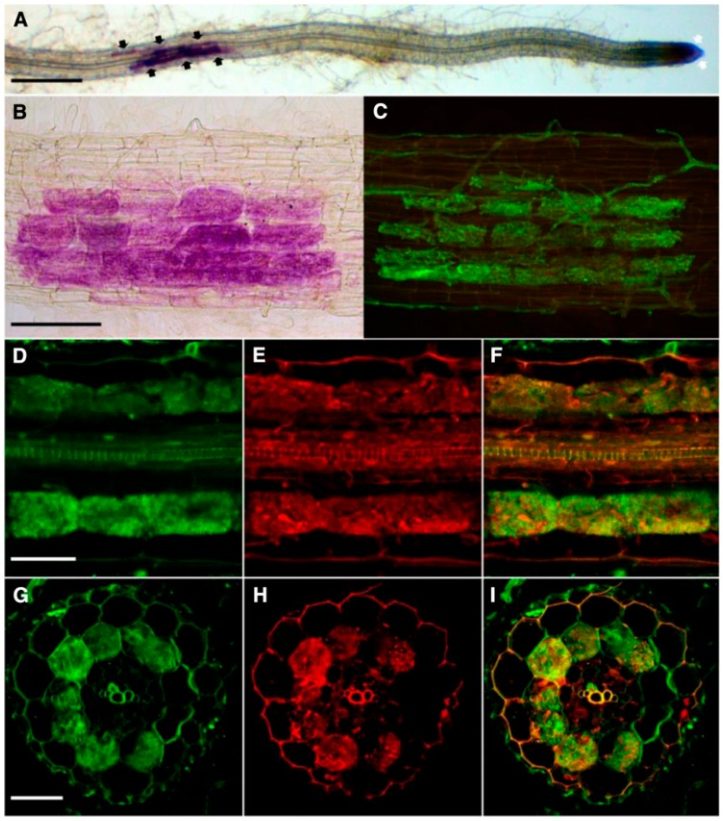

Figure 3. DR5:GUS expression in arbuscule-containing cells in roots of tomato colonized by *R. irregularis*. A, DR5:GUS staining (5-bromo-6-chloro-3-indolyl-β-D-glucuronic acid) of root tips (white arrows) and colonized root tissue (black arrows) by *R. irregularis*. Bar = 500 μm. B, Higher magnification of DR5:GUS staining of a colonized root. Bar (for B and C) = 100 μm. C, Fungal staining of the same root segment using WGA-FITC. D to F, Longitudinal root confocal section containing arbuscules. Bar = 50 μm. G to I, Confocal root cross section containing arbuscules. Bar = 50 μm. D and G, Fungal staining using Uvitex2B. E and H, DR5:GUS staining using ImaGene Green. F and I, Overlaps of images D and E and images G and H, respectively.

These data suggest that arbuscule development or functioning is accompanied by an auxin response.

Overexpression of miR393 Causes Inhibition of Arbuscule Development

To specifically investigate the impact of altered auxin signaling on mycorrhiza development, we transformed tomato roots using *Agrobacterium rhizogenes* with a vector to overexpress the precursor of miR393 under the control of the 35S promoter. As expected, these transgenic roots significantly overexpressed the precursor of miR393 and the mature miR393 (Supplemental Fig. S11). Accordingly, transcripts of miR393 target genes were detected at lower levels than in control roots (Supplemental Fig. S12). The three potential target genes of miR393 in tomato had been identified by using psrnatarget (Dai and Zhao, 2011) and according to their homology, with the TIR1-AFB gene family members of Arabidopsis (Supplemental Figs. S13 and S14). We analyzed the transformed roots of 15 chimeric plants and repeated the experiment three times. Although the overall colonization of miR393-overexpressing roots was slightly decreased (Fig. 5A), arbuscule formation was strongly reduced (Fig. 5, A and D). Reduction in arbuscule formation caused by miR393 overexpression was confirmed by the expression level of the gene *PT4*, which is exclusively expressed in arbuscule-containing cells and therefore, an unequivocal marker for arbuscule abundance (Fig. 5B). Observation of arbuscule morphology revealed that miR393-overexpressing roots only allowed the formation of stunted arbuscules with coarse, lower-order branches and no fine branches (Fig. 5, D and H). Moreover, we observed finger-like hyphal protrusions into cortical cells, indicating that, in some cases, arbuscule formation was already blocked at the stage of cell penetration (Fig. 5, F and G).

To investigate whether miR393 overexpression would perturb arbuscule development in other plant species, we transformed *M. truncatula* roots with a vector containing

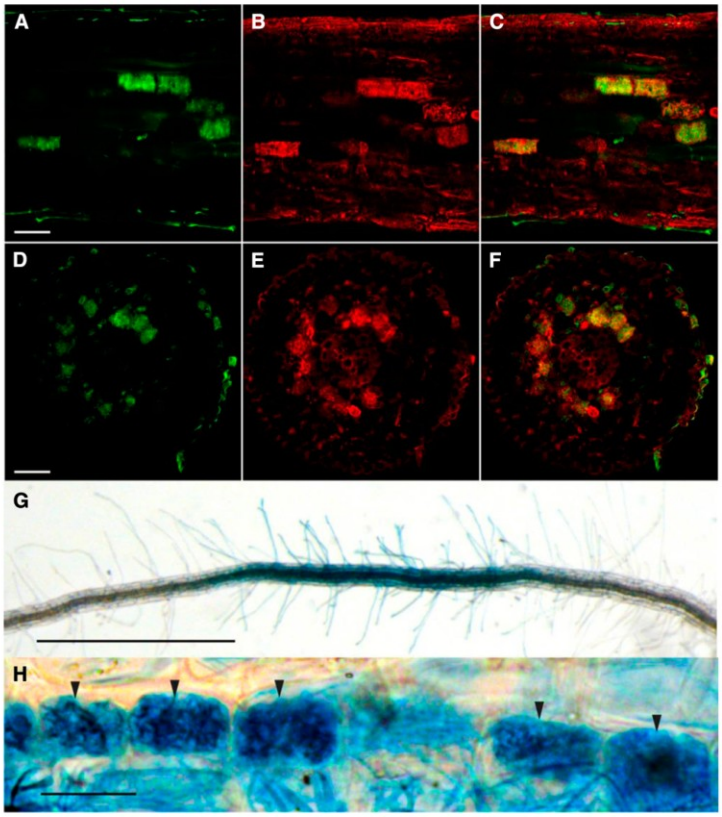

Figure 4. DR5:GUS expression in arbuscule-containing cells of *M. truncatula* and rice roots colonized by *R. irregularis*. A to C, Longitudinal *M. truncatula* root confocal section containing arbuscules. Bar = 50 μm. D to F, Confocal *M. truncatula* root cross section containing arbuscules. Bar = 50 μm. A and D, Fungal staining using Uvitex2B. B and E, DR5-GUS staining using ImaGene Green. C and F, Overlaps of images A and B and images D and E, respectively. G, DR5:GUS staining (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt) shows a colonized root of rice by *R. irregularis*. Bar = 1 mm. H, Higher magnification of DR5-GUS staining of a colonized root of rice. Black arrows show the arbuscule-containing cells. Bar = 5 μm.

the p35S-miR393 cassette. *M. truncatula* hairy roots transformed with the p35S-miR393 construct showed higher transcript levels of miR393 and lower transcript levels of the miR393 target genes *TIR1*, *AFB2*, and *AFB4* (Supplemental Figs. S12 and S13). They were also less sensitive to auxin treatment, which was determined by root elongation assays and DR5-GUS expression (Supplemental Fig. S15). As for tomato, the three potential target genes of miR393 in *M. truncatula* were identified by BLAST homology with the TIR1-AFB protein family members of Arabidopsis (Supplemental Figs. S13 and S14). Similar to tomato, the expression of the arbuscule marker *PT4* was decreased compared with control roots (Fig. 6A), and arbuscule formation was defective: roots overexpressing miR393 contained many hyphal protrusions into cortical cells that did not develop into arbuscules, and arbuscules had a lower magnitude of branching compared with control arbuscules (Fig. 6, B–E). Two independent stable transgenic lines of the monocot rice, overexpressing the miR393 (Xia et al., 2012), revealed a similar phenotype. The roots or 35S-miR393-transformed plants did not contain any mature arbuscules like in control roots (Fig. 6G) but instead, numerous abortive or poorly branched arbuscules (Fig. 6, H–J). The arbuscule phenotype was confirmed by decreased transcript accumulation of the arbuscule marker gene *PT11* in miR393-overexpressing roots compared with control roots (Fig. 6F). In summary, miR393 overexpression hampers arbuscule development in three distantly related plant species.

DISCUSSION

Previous studies in several plant species had shown a different auxin level in mycorrhizal roots compared with nonmycorrhizal roots but without any consensual role (Jentschel et al., 2006; Campanella et al., 2007). Some tomato mutants with pleiotropic phenotypes related to impaired auxin signaling or transport exhibited a defect in mycorrhizal colonization but without any arbuscule defect (Hanlon and Coenen,

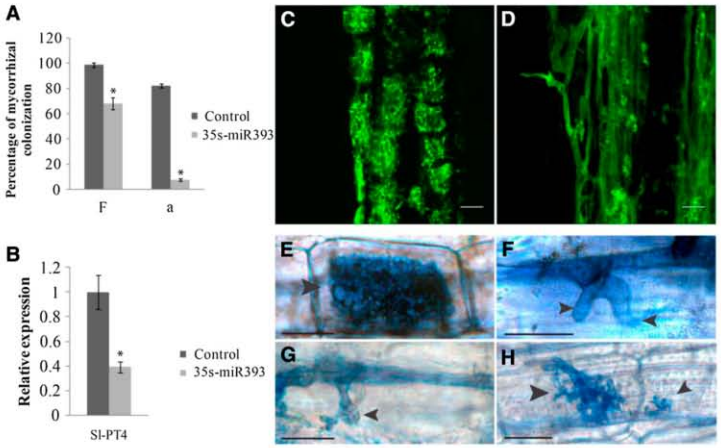

Figure 5. Overexpression of miR393 in tomato roots inhibits formation of arbuscules. A, Frequency (F) of mycorrhization and arbuscule abundance (a) in control and miR393-overexpressing roots of tomato. B, Expression measured by qRT-PCR of mycorrhiza-specific plant *PT4* in control and miR393-overexpressing roots. The measured transcripts were normalized to the relative expression value in control (empty vector-transformed) roots. Error bars represent SEM. *, Significant differences between the genotypes according to the Kruskal-Wallis test ($n = 6$, $P < 0.05$). C and D, Confocal microscopy images showing mycorrhizal colonization stained with WGA-FITC of control (C) and miR393-overexpressing (D) roots. Bars = 25 μm. E to H, Arbuscules (arrows) images in control (E) and miR393-overexpressing roots (F–H) stained with ink. Bars = 25 μm.

2011). Here, we collected evidence that auxin perception is required for arbuscule development, because (1) the miR393, which is known to target auxin receptor transcripts, was down-regulated in mycorrhizal roots, (2) treatments of roots with low concentrations of 2,4-D or NAA increased arbuscule abundance, (3) the expression of the DR5-GUS reporter for auxin response was mainly restricted to arbuscule-containing cells, and (4) overexpression of miR393 strongly impaired arbuscule development. These phenomena were observed in three plant species, including monotyledons and dicotyledons, indicating that the requirement of auxin perception for arbuscule development is conserved, at least across the angiosperms. To date, only two miRNAs have been reported to be regulated and play a role during AM symbiosis (Lauressergues et al., 2012; Bazin et al., 2013). We show here that miR393 is another miRNA regulated in AM symbiosis with a potential negative impact on arbuscule formation.

Several fungi, such as plant pathogens (Reineke et al., 2008) or ectomycorrhizal fungi (Tranvan et al., 2000; Felten et al., 2009), are able to synthesize auxin. Our data on DR5-GUS activity show that the auxin response increased in roots colonized by *R. irregularis*, mainly in cells containing arbuscules. This increase in auxin response could be caused by increased auxin accumulation in arbuscule-containing cells. Although a previous study has shown that AM fungi alone do not produce auxin (Jentschel et al., 2006), we cannot exclude that they are capable of producing this hormone in planta to improve their colonization success.

Fu and Harberd (2003) have shown that, in Arabidopsis, auxin stimulates GA_3-mediated DELLA protein destabilization. In this context, the promoting effect of auxin signaling on mycorrhiza formation seems not to be in agreement with the study by Floss et al. (2013) showing that DELLA proteins, by repressing GA_3 signaling, are positive regulators of arbuscule formation. However, DELLA expression in the vasculature was sufficient to drive arbuscule formation in the cortex (Floss et al., 2013), although we have observed auxin responses in arbuscule-containing cortical cells. It is, therefore, possible that DELLA and auxin act in different cell types. Furthermore, we have seen that DELLA gene expression is decreased in *M. truncatula* roots with reduced sensitivity to auxin (Supplemental Fig. S16).

Arbuscule development is preceded in the cortical cell by the formation of a prepenetration apparatus corresponding to cytoplasmic aggregations that organize the apoplastic space in which the arbuscule will develop (Genre et al., 2008). The fungus can then penetrate the cell by producing an arbuscular trunk from which coarse and later, fine hyphal branches will emerge to form the mature arbuscule (Gutjahr and Parniske, 2013). This process is severely hampered in cortical cells of miR393-overexpressing roots, which only display arbuscular trunks or stunted arbuscules with highly reduced and disorganized ramifications. This is reminiscent of the phenotype of *vapyrin* mutants, which are defective

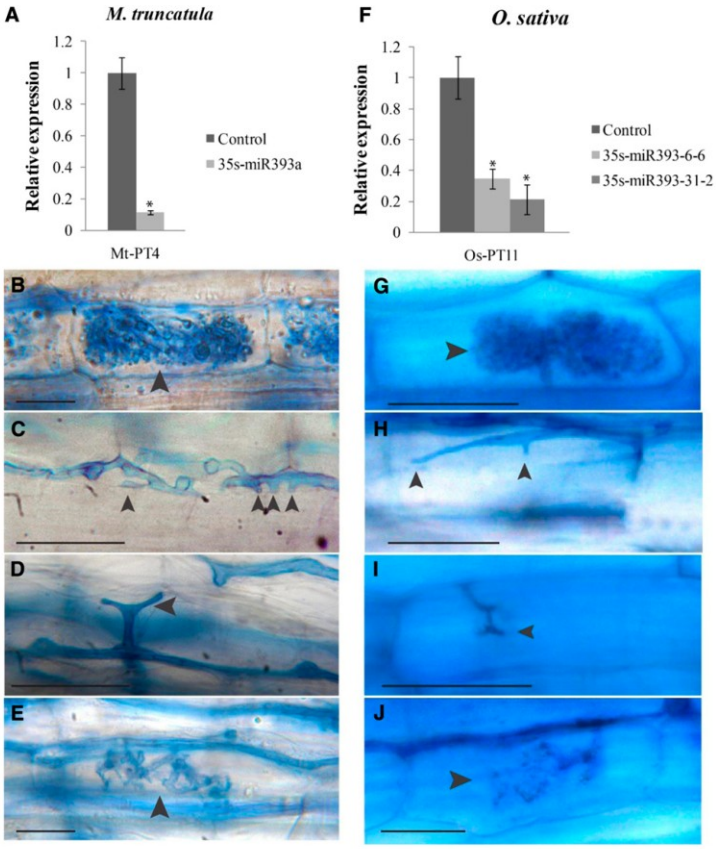

Figure 6. Overexpression of miR393 in *M. truncatula* and rice roots inhibits formation of arbuscules. A and F, Expression, measured by qRT-PCR, of mycorrhiza-specific plant PT4 (A) and PT11 (F) in control roots (transformed with an empty vector) compared with miR393-overexpressing roots of *M. truncatula* (A) and rice (F). The measured transcripts were normalized to the relative expression value in control roots. Error bars represent SEM. *, Significant differences between the genotypes according to the Kruskal-Wallis test ($n = 6$, $P < 0.05$). Fungal structures (arrows) observed in control (B and G) and miR393-overexpressing (C–E and H–J) roots of *M. truncatula* (B–E) and rice (G–J) inoculated with *R. irregularis*. Fungal structures are stained with ink (B–E) or trypan blue (G–J). Bars in B to E = 25 μm. Bars in G to J = 5 μm.

in a protein of unknown function but proposed to be an executor of intracellular accommodation (Feddermann et al., 2010; Pumplin et al., 2010; Gutjahr and Parniske, 2013). Continuous arbuscule branching is accompanied by the formation in cortical cells of a periarbuscular membrane (PAM), which corresponds to an exocytosis-mediated massive extension of the plasma membrane surrounding each fine arbuscular branch. Interestingly, mutants altered in the exocytotic machinery also show arbuscule branching defects (Ivanov et al., 2012; Lota et al., 2013), indicating that exocytosis is required for PAM extension and arbuscule formation. The PAM contains a distinct set of proteins and can be considered as a unique membrane domain that differs from the peripheral plasma membrane (Pumplin and Harrison, 2009; Kobae and Hata, 2010; Zhang et al., 2010). Arbuscule development requires the polarization of individual cortical cells within the tissue context, and vesicle trafficking for PAM construction is likely to be cytoskeleton dependent (Brandizzi and Wasteneys, 2013). Polarization during arbuscule development is accompanied by rearrangement of the actin and microtubule cytoskeleton, such that, finally, actin filaments run along arbuscule branches and microtubules form a basket-like structure around the arbuscule (Genre and Bonfante, 1998). Auxin is regarded to be a crucial signaling substance for development and maintenance of cell polarity in plants (Yang, 2008), and artificial elevation of auxin concentration in epidermal cells leads to a reorganization of actin filaments and microtubules (Holweg et al., 2004; Nick et al., 2009). Furthermore, local auxin maxima lead to a cell-specific repolarization of membrane-localized pinoid proteins, which are auxin efflux carriers, in a TIR1-dependent manner (Sauer et al., 2006). Taking together these published data, it is tempting to speculate that the malformation of arbuscules in roots overexpressing miR393 (i.e. with altered expression of auxin receptors and thus, auxin signaling) could result from a defect in cytoskeletal rearrangement and cell polarization. AUXIN BINDING PROTEIN1 (ABP1) is another plasmamembrane and

endoplasmic reticulum-localized type of auxin receptor (Sauer and Kleine-Vehn, 2011) that, in Arabidopsis, has been implicated in regulating leaf pavement cell polarity through activation of ρ-like guanosine triphosphatases and cytoskeletal changes (Xu et al., 2011). It will be interesting to investigate whether ABP1 also plays a role in AM development.

The mechanisms underlying the establishment of AM symbiosis are far from being fully understood. Nevertheless, showing additional evidence that they are likely conserved across plant kingdom, at least with regard to the requirement for an auxin signaling, provides new leads toward deciphering this highly complex developmental process.

MATERIALS AND METHODS

Biological Materials

Medicago truncatula 'Jemalong' genotype A17 and tomato (*Solanum lycopersicum* 'MicroTom') seeds were surface sterilized by bleach and water for 2 to 5 min and then rinsed five times in sterile water. *M. truncatula* and tomato plants were cultivated in 250-mL pots filled with Oil-Dri US special substrate (Damolin) for 8 and 12 weeks, respectively, in a growth chamber (*M. truncatula*: 16-h/8-h at 22°C/20°C day-night cycle, 400 μmol m^{-2} s^{-1}; tomato: 16-h/8-h at 25°C/25°C day-night cycle, 600 μmol m^{-2} s^{-1}) and watered every 2 d with modified Long Ashton medium containing a low concentration (7.5 μM) of phosphate (Balzergue et al., 2011). *Rhizophagus irregularis* (formerly named *Glomus intraradices*) DAOM197198 sterile spores were purchased from Agronutrition. Tomato and *M. truncatula* roots were inoculated with 400 spores of *R. irregularis* per plant. *M. truncatula* DR5-GUS plants were provided by Sandra Bensmihen (Laboratoire des Interactions Plantes-Microorganismes; Herrbach et al., 2014). For auxin treatment, plants were watered three times a week with Long Ashton medium supplemented or not with 10^{-10} M 2,4-D during mycorrhiza development. Fifteen plants (for mycorrhization) and six plants (for quantitative PCR) per experiment were used for all experiments with three different biological replicates. One representative of three independent experiments is shown.

Control and miR393 overexpressing seeds of *Oryza sativa* ssp. 'Japonica' ZH11 were provided by Mingyong Zhang (Chinese Academy of Science; Xia et al., 2012). The *O. sativa* ssp. 'Japonica' Taichung 65 DR5-GUS line (Scarpella et al., 2003) was provided by Pieter B.F. Ouwerkerk (University of Leiden, Leiden, The Netherlands). Rice seedlings pregerminated for 4 d in the dark were inoculated with 500 spores of *R. irregularis* (SYMPLANTA) in 128-mL pots filled with quartz sand (16-h/8-h at 26°C/26°C day-night cycle, 223 μmol m^{-2} s^{-1}). They were fertilized two times a week with 10 mL of one-half-strength Hoagland solution containing 25 μM phosphate and 0.001% (w/v) Sequestrene rapid (Syngenta). For auxin treatment, 10^{-10} M NAA was added to the fertilizer or water and supplied three times a week. Rice roots were harvested 6 weeks postinoculation. Six plants were used in each experiment. The root system of each plant ($n = 6$) was divided into two equal parts: one-half was used for AM quantification ($n = 3$), and one-half was used for RNA extraction ($n = 3$, quantitative PCR analysis).

For root length measurement, composite plants were transferred to a modified Fahraeus medium containing 2,4-D (0.2 μM) or solvent control. Root apices were directly marked on the petri dishes at time point zero to monitor the root elongation. At 14 d, root elongation was scored from digital images of petri dishes using ImageJ software.

Plasmid Construction

Precursor of miR393 was amplified using Pfu polymerase (Promega), and the primers are shown in Supplemental Table S1. They were cloned using *Xho*I and *Not*I restriction enzymes into the pPEX-discosoma RED (DsRED) plasmid (Combier et al., 2008) for overexpression under the control of the strong constitutive Cauliflower mosaic virus 35S promoter.

Plant Transformation

Root transformations of tomato and *M. truncatula* were performed with *Agrobacterium rhizogenes* as described by Boisson-Dernier et al. (2001). Transformed roots were selected by observation of DsRED fluorescence using a fluorescence binocular (Leica). Control roots corresponded to roots transformed with *A. rhizogenes* carrying the empty vector pPEX-DsRED.

Expression Analyses

Total RNA was extracted using a Plant RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated by DNase I (Promega) to remove any genomic DNA contamination. Reverse transcription was performed using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) on 500 ng of total plant RNA. For each experiment, six independent plants or transformants were analyzed. Quantitative PCR amplifications were conducted on a Roche LightCycler 480 System (Roche Diagnostics) under the following conditions: 95°C for 5 min and then 45 cycles of 95°C for 15 s and 60°C for 1 min. The various primer sets used are described in Supplemental Table S1. The measured transcripts were normalized to the relative expression value in nonmycorrhizal roots. For the miR393 overexpressing lines inoculated with *R. irregularis*, expression of genes of interest was normalized to the relative expression value of mycorrhizal control roots. Actin, ubiquitin, and cyclophilin (Supplemental Table S1) were used as reference genes for normalization of gene expression of tomato, *M. truncatula*, and rice, respectively.

Northern-blot analyses were performed as described by Lauressergues et al. (2012).

Identification of Target Genes and Phylogenic Tree

Putative miR393 target genes in *M. truncatula* and tomato were found by using psRNAtarget (Dai and Zhao, 2011). The protein sequences of putative targets were extracted, and we performed a phylogenetic analysis. Amino acid alignments were made using Tcoffee, and phylogenetic trees were performed using Mega5 (maximum likelihood, bootstrap = 100; Tamura et al., 2011; Supplemental Figs. S13 and S14).

Histochemical Staining and Microscopy Studies

5-Bromo-6-chloro-3-indolyl-β-D-GlcA cyclohexyl ammonium salt GUS staining was performed as described by Combier et al. (2008). GUS expression at the cellular/tissue level was detected by treating the transgenic tissue in 50 μM ImaGene Green C12FDGlcU substrate (ImaGene Green GUS Gene Expression Kit; Invitrogen) in phosphate buffer (pH 7) at 37°C for 2 h in the dark. GUS activity was detected by fluorescence microscopy using the Leica SP2 Confocal Microscope.

Fungal structures were visualized by staining with 0.01% (w/v) Uvitex2B in phosphate buffer (pH 7) for 30 min at room temperature (Diagne et al., 2011). Root sections (50 μm) were made using the vibratome VT1000S from samples embedded in 4% (w/v) agarose. For root mycorrhizal phenotyping, roots were cleared in 10% (w/v) KOH, rinsed in sterile water, treated for 30 min with WGA-FITC (Invitrogen), which binds fungal chitin, washed three times for 10 min in PBS, and observed using an inverted light microscope or a confocal microscope (Leica). Alternatively, they were stained with Schaeffer black ink as described by Vierheilig et al. (1998). Quantification of mycorrhizal colonization was performed as described by Trouvelot et al. (1986): the frequency of mycorrhiza in the root system and the arbuscule abundance (percentage) were calculated in the colonized root sections using Mycocalc software (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). Fifteen root systems of tomato and *M. truncatula* and two mutant lines (6-6 and 31-2) of rice were analyzed, and each experiment was repeated three times. Trypan blue staining of rice roots was performed as described (Gutjahr et al., 2008)

Statistical Analyses

The mean values for relative gene expression ($n = 6$) or mycorrhization rates ($n = 15$) were compared using the Kruskal-Wallis test, and when significant, a pairwise comparison was made using the nonparametric Mann-Whitney test. In the figures, asterisks indicate significant differences compared with the control ($P < 0.05$ or $P < 0.01$), and error bars represent the SEM.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Conservation of mature miR393.

Supplemental Figure S2. Expression of mycorrhiza-induced plant phosphate transporter and root length colonization.

Supplemental Figure S3. Expression of tomato mature miR393.

Supplemental Figure S4. Expression of *M. truncatula* precursor of miR393 during mycorrhization.

Supplemental Figure S5. Relative expression of *M. truncatula* precursor of miR393 in response to mycorrhizal-lipochito-oligosaccharides and chito-oligosaccharides.

Supplemental Figure S6. Expression of *M. truncatula* precursor of miR393 during mycorrhization in wild-type and *dmi3* mutant.

Supplemental Figure S7. Effect of different concentration of 2,4-D on root development.

Supplemental Figure S8. Expression of the *DR5-GUS* auxin response marker in tomato roots.

Supplemental Figure S9. Expression of the *DR5-GUS* auxin response marker in mycorrhizal tomato and *M. truncatula* roots.

Supplemental Figure S10. Specificity of Uvitex2B and ImaGene Green root staining.

Supplemental Figure S11. miR393 is overexpressed in 35S miR393 roots.

Supplemental Figure S12. Expression of miR393 targets in miR393-overexpressing roots.

Supplemental Figure S13. Phylogenetic analysis of putative target genes of miR393.

Supplemental Figure S14. Identity of mature miR393 and targets.

Supplemental Figure S15. Overexpression of miR393 reduces the sensitivity to exogenous application of 2,4-D in *M. truncatula*.

Supplemental Figure S16. Expression of a DELLA gene in tomato mycorrhized roots.

Supplemental Table S1. List of primers used in this study.

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Supplementary Data

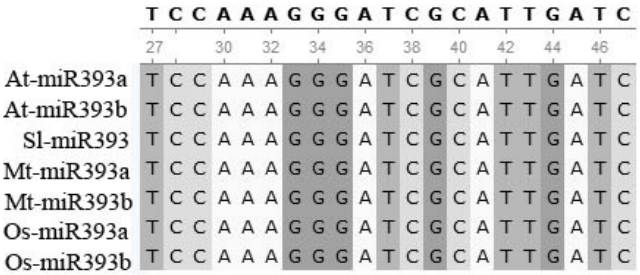


Fig. S1. Conservation of mature miR393. Sequence alignment of mature miR393 of *A. thaliana* (At-miR393a, b), tomato (Sl-miR393), *M. truncatula* (Mt-miR393a, b) and rice (Os-miR393a, b).

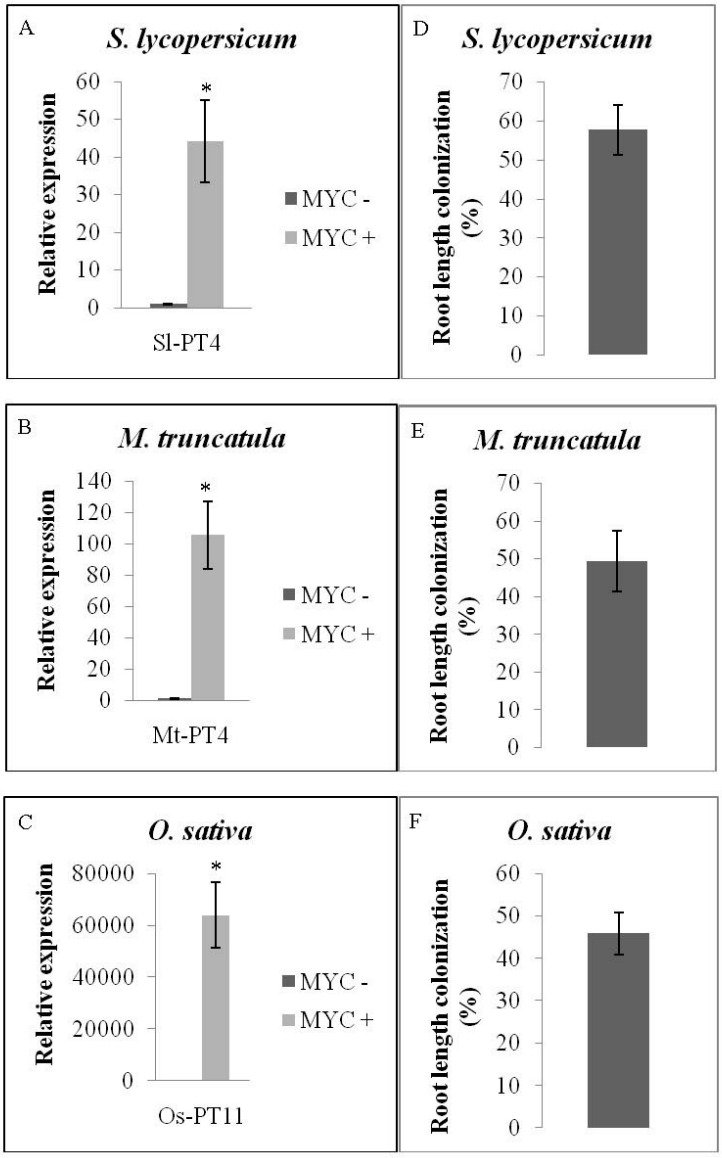


Fig. S2. Increased expression of mycorrhiza-induced plant phosphate transporter (PT) and root length colonization. A, B, C, Quantification by qRT-PCR of the expression of mycorrhiza-

induced plant phosphate transporter genes in non-mycorrhizal (MYC -) and mycorrhizal (MYC +) roots of tomato (Sl-PT4), *M. truncatula* (Mt-PT4) and rice (Os-PT11). The measured transcripts were normalized to the relative expression value in non-mycorrhizal roots. D, E, F, Mycorrhization rate (%) in roots of *S. lycopersicum*, *M. truncatula* and *O. sativa* colonized by *R. irregularis*, as calculated with the intergrid method (Trouvelot et al., 1986). Error bars represent standard error of the mean (SEM). The asterisks indicate a significant difference between the two treatments according to the Kruskal–Wallis test ($n = 6$, $P < 0.05$).

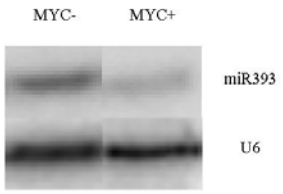
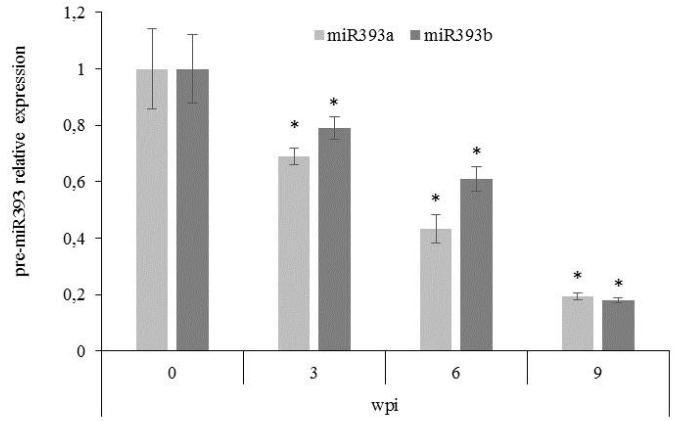


Fig. S3. Decreased expression of tomato mature miR393 in roots inoculated with *R. irregularis* (MYC+) compared to uninoculated (MYC-) roots, analysed by Northern blot.

A



B

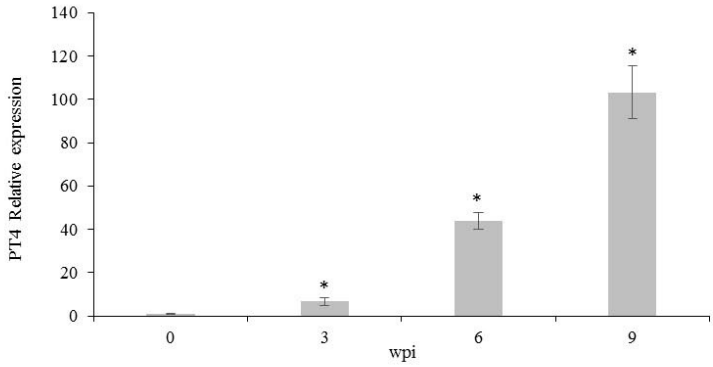


Fig. S4. Decreased expression of *M. truncatula* pre-miR393 (A) during mycorrhization correlates with increased expression of *PT4* (B). Expression was measured by qRT-PCR. The measured transcripts were normalized to the relative expression value in non-mycorrhizal roots. Error bars represent standard error of the mean (SEM). The asterisks indicate a significant difference between the control and the inoculated plants according to the Kruskal–Wallis test ($n = 6$, $P < 0.05$). wpi: weeks post inoculation.

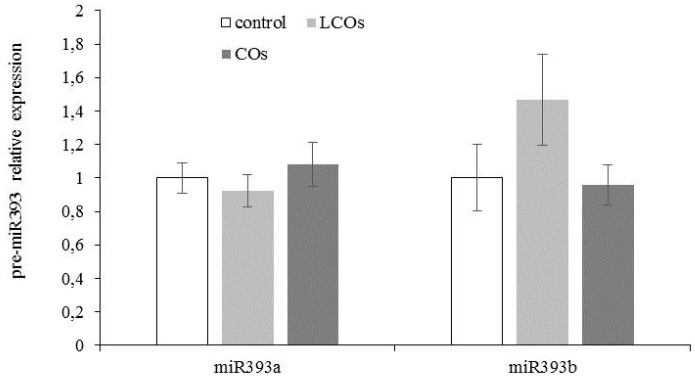


Fig. S5. Relative expression of *M. truncatula* pre-miR393 is not affected in response to Myc-LCOs (LCOs) and COs. Expression was measured by qRT-PCR. The measured transcripts were normalized to the relative expression value in control roots. Error bars represent standard error of the mean (SEM). The asterisks indicate a significant difference between the control and the treated plants according to the Kruskal–Wallis test ($n = 6$, $P < 0.05$).

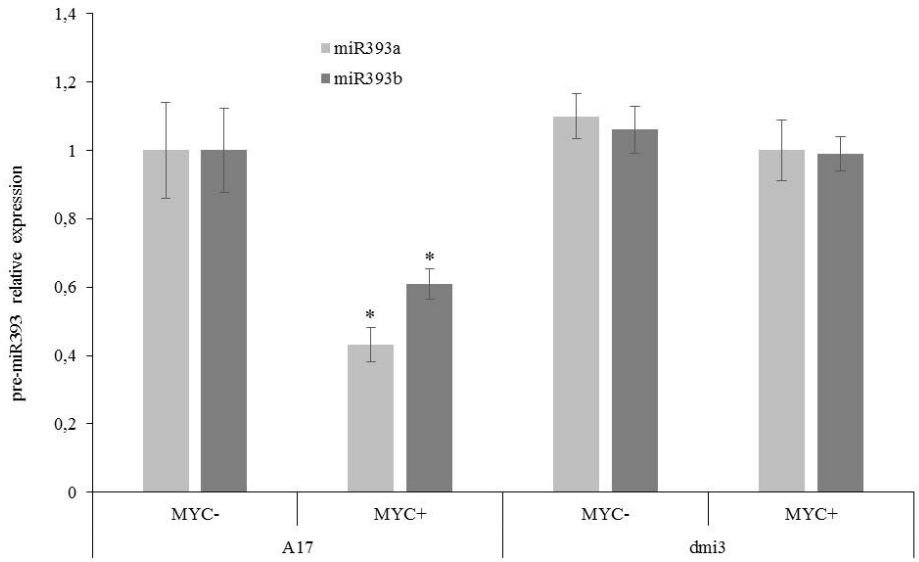


Fig. S6. Expression of *M. truncatula* pre-miR393 is decreased during mycorrhization (MYC+) compared to uninoculated roots (MYC-) in wild type (A17) but not in *dmi3* mutant. Expression was measured by qRT-PCR. The measured transcripts were normalized to the relative expression value in A17 non-mycorrhizal roots. Error bars represent standard error of the mean (SEM). The asterisks indicate a significant difference between the control and the inoculated plants according to the Kruskal–Wallis test ($n = 6$, $P < 0.05$).

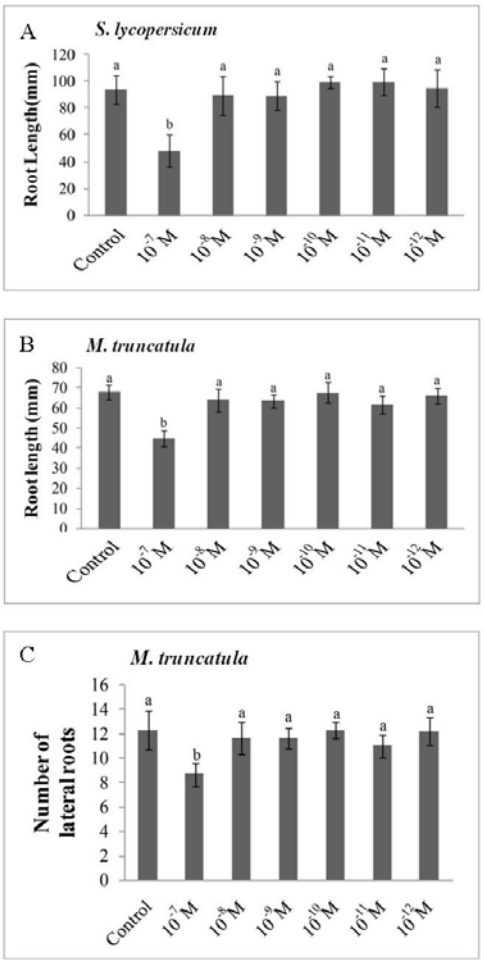


Fig. S7. Effect of different concentration of 2,4-D on root development. Effect of different concentrations of 2,4-D (10^{-7} to 10^{-12} M) on tomato (*S. lycopersicum*) (A) and *M. truncatula* (B) root length. (C) Effect of different concentrations of 2,4-D (10^{-7} to 10^{-12} M) on *M. truncatula* lateral root number. Student's t-test was performed between non-treated root with 2,4-D as control and 2,4-D treated samples. Different letter indicates significant difference when compared to the control (n = 32 for each concentration, $P < 0.05$).

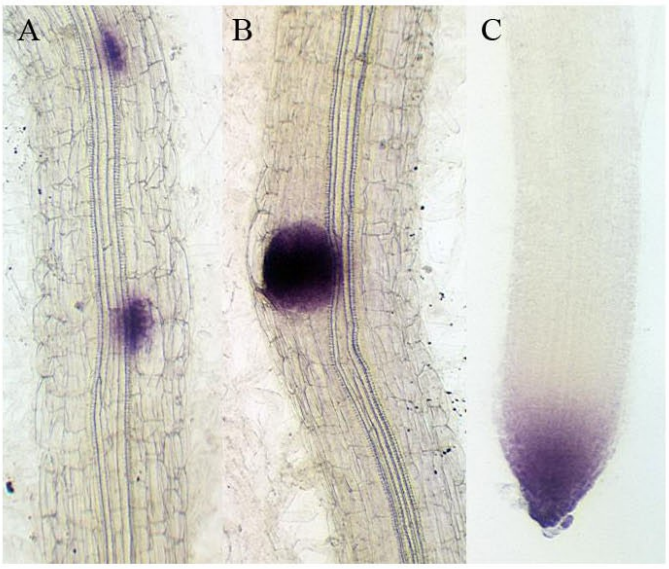


Fig. S8. Expression of the *DR5-GUS* auxin response marker in tomato roots. *DR5-GUS* expression stained by Magenta GLUC in: A, lateral root primordia, B, lateral root emerging and, C, root tip.

`

A

B

Fig. S9. Expression of the *DR5-GUS* auxin response marker in mycorrhizal tomato (A) and *M. truncatula* (B) roots is localized in lateral root emergences and zones colonized by the fungus. *DR5-GUS* expression is stained by Magenta/X-GLUC.

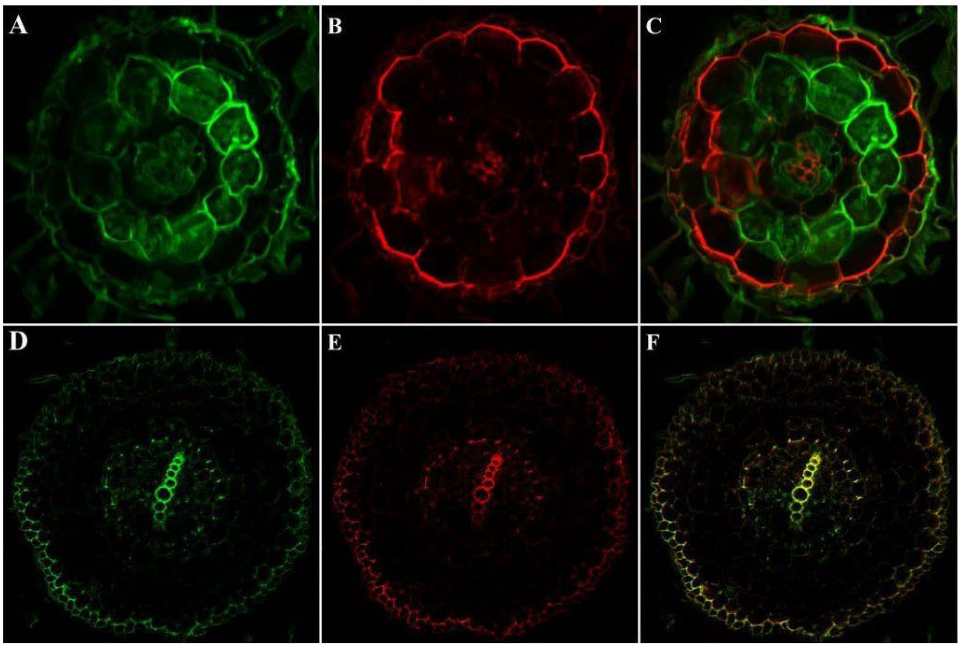


Fig. S10. Specifity of Uvitex2B and ImaGene Green root staining. A, B, C, confocal cross section of a colonized root of tomato inoculated with *R. irregularis*. D, E, F, confocal cross section of a non-colonized root of tomato. A, D, Uvitex2B staining shows arbuscule staining and cell wall staining in (A) and cell wall staining in (D). B, E, autofluorescence after excitation with the wavelength (λ excitation= 490 μm, λ emission= 525 μm) appropriate for fluorescence visualization of ImaGene Green. C, F, merge of Uvitex2B with autofluorescence images (gamma = 490 μm).

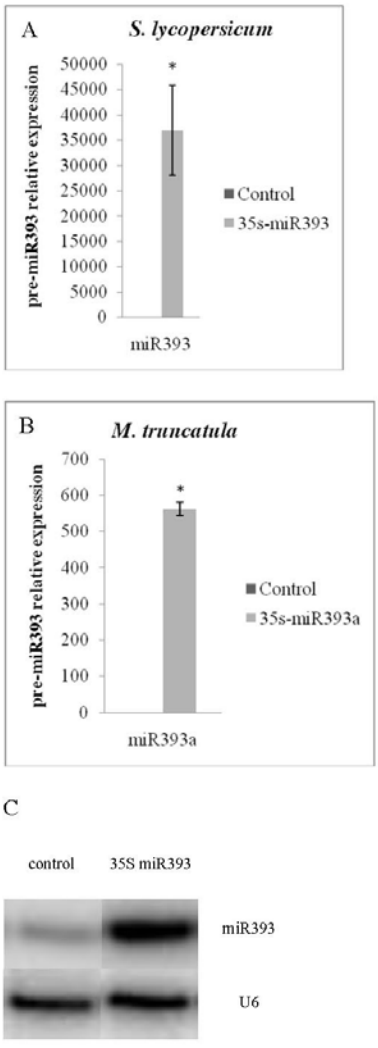
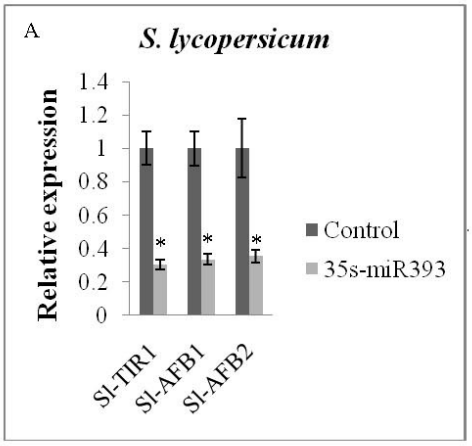
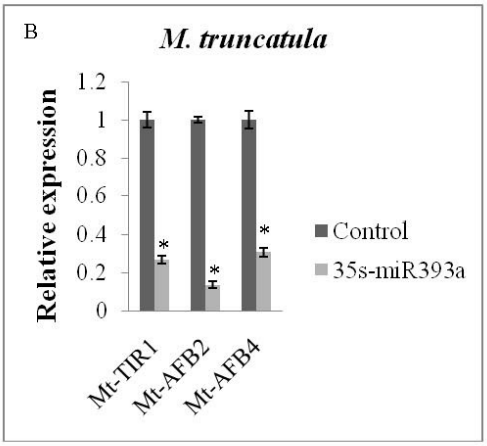


Fig. S11. miR393 is overexpressed in 35S pre-miR393 roots. A, B, Expression, measured by qRT-PCR, of pre-miR393 in control roots (transformed with an empty vector) and in pre-miR393-over-expressing roots of tomato (*S. lycopersicum*) and *M. truncatula*, respectively. The measured transcripts were normalized to the relative expression value in control. Errors bars represent standard error of the mean (SEM). The asterisk indicates a significant difference between the two genotypes according to the Kruskal–Wallis test ($n = 6$, $P < 0.05$). C, Expression of tomato mature miR393 in control roots or roots overexpressing the pre-miR393, analysed by Northern blot.


A
S. lycopersicum
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sl-TIR1
Sl-AFB1
Sl-AFB2
Control
35s-miR393


B
M. truncatula
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Mt-TIR1
Mt-AFB2
Mt-AFB4
Control
35s-miR393a

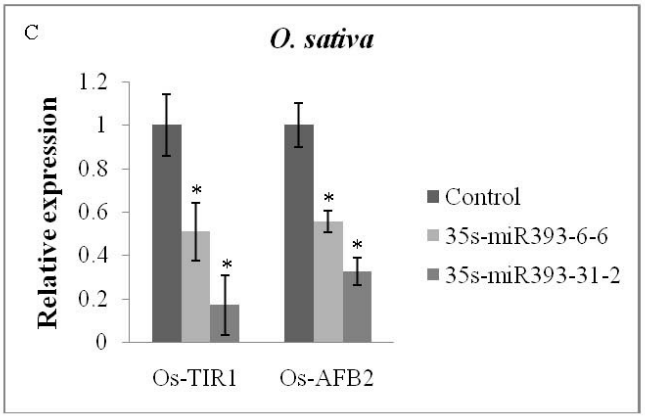

C
O. sativa
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Os-TIR1
Os-AFB2
Control
35s-miR393-6-6
35s-miR393-31-2

Fig. S12. Expression of miR393 targets is downregualted in pre-miR393-overexpressing roots. A, B, expression, measured by qRT-PCR, of miR393 target genes in control roots (transformed with an empty vector) and in miR393 over-expressing roots of tomato (*S. lycopersicum*) and *M. truncatula*, respectively. C, Expression of miR393 target genes in two lines of stable rice (*O. sativa*) plants overexpressing miR393 which are indicated as 35s-miR393-6-6 and 35s-miR393-31-2. The measured transcripts were normalized to the relative expression value in control. Errors bars represent standard error of mean (SEM). The asterisks indicate a significant difference between the two treatments according to the Kruskal–Wallis test (tomato, *M. truncatula* $n = 6$, $P < 0.05$ and rice $n = 9$, $P < 0.05$).

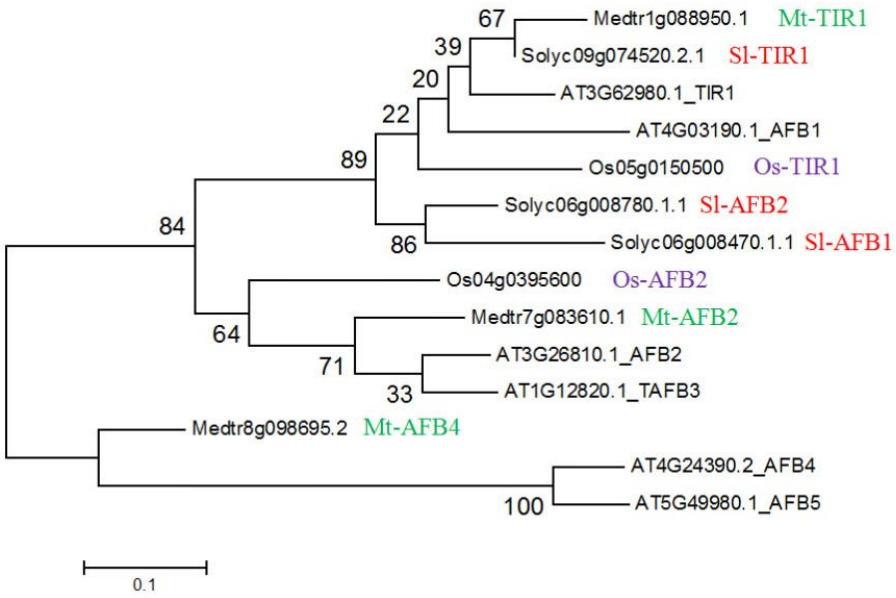


Fig. S13. Phylogenetic analysis of putative target genes of miR393 (TIR1 and AFBs) based on amino acid sequence alignments. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. AT: *Arabidopsis thaliana*, Solyc: *Solanum lycopersicum*, Medtr: *Medicago truncatula*, Os: *Oryza sativa*.

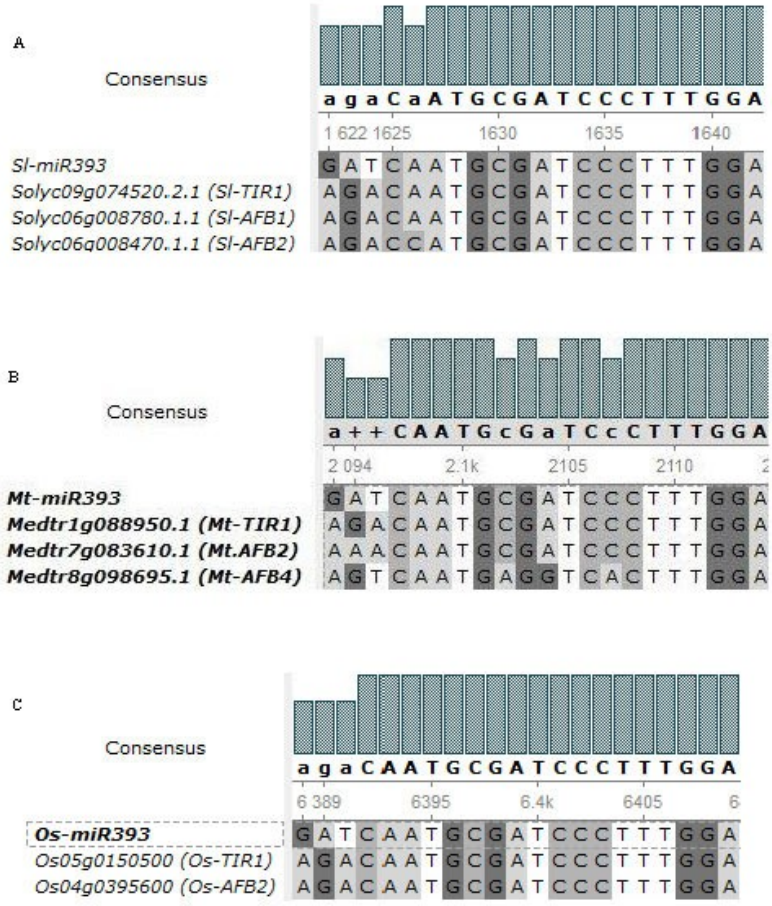
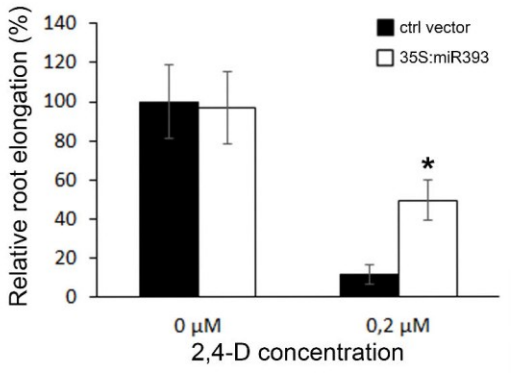


Fig. S14. Identity of mature miR393 and targets. Sequence alignment of mature miR393 and targets of tomato (Sl-miR393) (A), *M. truncatula* (Mt-miR393a, b) (B) and rice (Os-miR393a, b) (C).

A


Relative root elongation (%)
140
120
100
80
60
40
20
0
ctrl vector
35S:miR393
*
0 µM
0,2 µM
2,4-D concentration

B

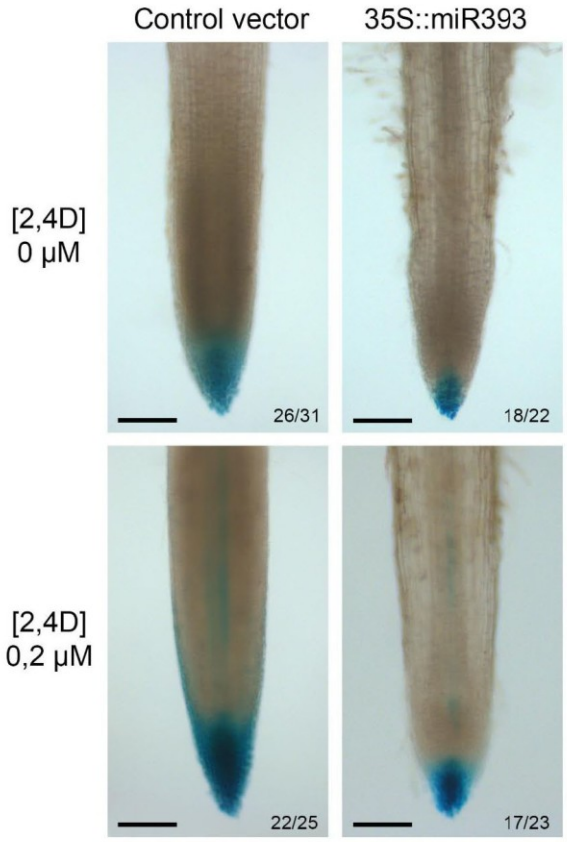

Control vector
35S::miR393
[2,4D]
0 µM
26/31
18/22
[2,4D]
0,2 µM
22/25
17/23

Fig. S15: Overexpression of miR393 reduces the sensitivity to exogenous application of 2,4-D in *M. truncatula*. A. Loss of root elongation inhibition by 2,4-D in *Medicago* roots overexpressing miR393. The error bars represent the standard error at the mean (n>30 per construct and condition). Asterisks indicate a statistically significant difference between control and 35S miR393 plants using a Mann and Whitney U test (P<0.001). B. Reduced DR5::GUS response of the miR393 overexpressing roots. The number of independent roots showing the presented pattern out of the total root number is indicated on the right lower corner of each panel. Scale bars = 50 µM.

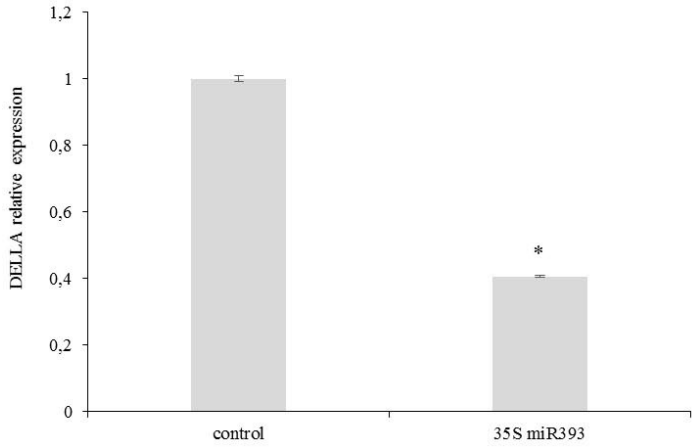


Fig. S16. Expression of a DELLA gene in tomato control roots compared to roots overexpressing miR393. The measured transcripts were normalized to the relative expression value in control. Errors bars represent standard error of mean (SEM). The asterisks indicate a significant difference between the two treatments according to the Kruskal–Wallis test ($n = 6$, $P < 0.05$).

Table S1. List of primers used in this study

Gene/reference	Name	Annotation	Sequence	Note
(Nagy et al., 2005)	Sl-PT4	Pi transporter 4	GATTCTGGCTTGGATTTGGA	q-PCR Fw
(Nagy et al., 2005)	Sl-PT4	Pi transporter 4	GCAAAAACAGCAGCAATGAA	q-PCR Rev
Solyc09g074520.2	Sl-TIR1	Auxin F-box protein	CCGGAAGGTTGGGGAGCT	q-PCR Fw
Solyc09g074520.2	Sl-TIR1	Auxin F-box protein	CAAGCCATGGATAAGACCTAGAC	q-PCR Rev
Solyc06g008470.1	Sl-AFB1	Auxin F-box protein	GGGATCGCATGGTCTCCAC	q-PCR Fw
Solyc06g008470.1	Sl-AFB1	Auxin F-box protein	GTAAGTTGGAGATTAGAGACTGC	q-PCR Rev
Solyc06g008780.1	Sl-AFB2	Auxin F-box protein	GGACAGTGTCAGGAAGGAG	q-PCR Fw
Solyc06g008780.1	Sl-AFB2	Auxin F-box protein	GAAGAAGCCAAAGAGAAATCCCA	q-PCR Rev
Sly-miR393	Sl-miR393		CGCATTGATCCTGTGTTCCA	q-PCR Fw
Sly-miR393	Sl-miR393		CGCATGATCCCATAGTCCCA	q-PCR Rev
	Sl-Actin		TGTCCCTATCTACGAGGGTTATGC	q-PCR Fw
	Sl-Actin		AGTTAAATCACGACCAGCAAGAT	q-PCR Rev
	SlDELLA		TGGGTCTTCGTCTTCAGCTT	q-PCR Fw
	SlDELLA		ATTGTTCACCGGAGGTTGAG	q-PCR Rev
	Mt-PT4		ACGTTCTTGGTGACGGAAAC	q-PCR Fw
	Mt-PT4		GAGCCCTGTCATTTGGTGTT	q-PCR Rev
	Mt-TIR1		ATTGAGCCTCGAACTCCTGA	q-PCR Fw
	Mt-TIR1		CTGTGAGAAGCCCTGAAAGG	q-PCR Rev
	Mt-AFB2		AAGGCCACCATTCTCAAGTG	q-PCR Fw
	Mt-AFB2		CCACAGTGGCGGATAAGTTT	q-PCR Rev
	Mt-AFB4		TTTATGGGTGGCTTCAGCAT	q-PCR Fw
	Mt-AFB4		TGCATGCAGAGTATAGAACTTGG	q-PCR Rev
	Mt-miR393a		CAACTTGAGGAGGCATCCAA	q-PCR Fw
	Mt-miR393a		GGATGGCATGATCCCAATTA	q-PCR Rev
	Mt-miR393b		TCGCATTGATCCCAAATCTA	q-PCR Fw
	Mt-miR393b		CCCAAATATTATGAGATATTAAGGAAA	q-PCR Rev

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	MtmiR393 5'Xho		TCAGTCCGCTCGAGAACTGCAACTTGAGGAGGCA	cloning
	MtmiR393 3'Not		AAGGAAAAAAGCGGCCGCGTAGCTACTAAAGGAGAAATC	cloning
	Mt-Ubiquitin		GCAGATAGACACGCTGGGA	q-PCR Fw
	Mt-Ubiquitin		AACTCTTGGGCAGGCAATAA	q-PCR Rev
(Gutjahr et al., 2008)	OsPT11		GAGAAGTTCCCTGCTTCAAGCA	q-PCR Fw
(Gutjahr et al., 2008)	OsPT11		CATATCCCAGATGAGCGTATCATG	q-PCR Rev
(Bian et al., 2012)	Os-MIR393a		ATGGCGGTCGTCGTCTACA	q-PCR Fw
(Bian et al., 2012)	Os-MIR393a		CGTCTGAACCCAACAATGAAG	q-PCR Rev
(Bian et al., 2012)	Os-MIR393b		CGGCCTGAGGAAACTAGTGGA	q-PCR Fw
(Bian et al., 2012)	Os-MIR393b		GGAAGATGAGGAGGCGGAAG	q-PCR Rev
(Bian et al., 2012)	Os-TIR1		GATGCTTTCAATCGCCTTCG	q-PCR Fw
(Bian et al., 2012)	Os-TIR1		ACAAGCACGACGACATCCAAAG	q-PCR Rev
(Bian et al., 2012)	Os-AFB2		CGTTTGTCAATATCTGGTC	q-PCR Fw
(Bian et al., 2012)	Os-AFB2		GACATCCAAAGGGATCGCA	q-PCR Rev
(Bian et al., 2012)	Os-Actin		CAACACCCCTGCTATGTACG	q-PCR Fw
(Bian et al., 2012)	Os-Actin		CATCACCAGAGTCCAACACAA	q-PCR Rev
(Gutjahr et al., 2008)	Os-CYC2		GTGGTGTTAGTCTTTTTATGAGTTCGT	q-PCR Fw
(Gutjahr et al., 2008)	Os-CYC2		ACCAAACCATGGGCGATCT	q-PCR Rev

Bian H, Xie Y, Guo F, Han N, Ma S, Zeng Z, Wang J, Yang Y, Zhu M (2012) Distinctive expression patterns and roles of the miRNA393/TIR1 homolog module in regulating flag leaf inclination and primary and crown root growth in rice (Oryza sativa). New Phytol **196:** 149-161

Gutjahr C, Banba M, Croset V, An K, Miyao A, An G, Hirochika H, Imaizumi-Anraku H, Paszkowski U (2008) Arbuscular mycorrhiza-specific signaling in rice transcends the common symbiosis signaling pathway. Plant Cell **20:** 2989-3005

Nagy R, Karandashov V, Chague V, Kalinkevich K, Tamasloukht M, Xu G, Jakobsen I, Levy AA, Amrhein N, Bucher M (2005) The characterization of novel mycorrhiza-specific phosphate transporters from Lycopersicon esculentum and Solanum tuberosum uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. Plant J **42:** 236-250

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Chapter III – Aux/IAA27 is required for fungal colonization of the root

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1. Introduction

Auxin signaling pathway has been shown to be needed for the early steps of arbuscular mycorrhiza (AM) formation, including during presymbiotic signal exchange (Hanlon and Coenen, 2011). Studies on *A. thaliana* have identified various components of auxin signaling, among which the Aux/IAA family of transcriptional regulators (Abel et al., 1995). Aux/IAA genes constitute one of the three major groups of primary auxin-responsive genes which also include Small Auxin Up RNA (SAUR) and Gretchen Hagen 3 (GH3) (Theologis et al., 1985; Oeller et al., 1993). Aux/IAAs are short-lived proteins that contain four conserved domains (Reed, 2001). They represent the capacity to function as transcriptional repressors because of a conserved leucine motif (LxLxLx) located in the domain I (Tiwari et al., 2004). This repression domain is similar to the ethylene-responsive element binding factor-associated amphiphilic repression (EAR) (Kagale et al., 2010; Kagale and Rozwadowski, 2010, 2011). It is well known that Aux/IAA genes have a double activity, one as a transcriptional regulator, and another in the auxin signaling complex. In low level of auxin, Aux/IAAs bind ARFs (auxin response factors) through domains III and IV present in both proteins and recruit the TOPLESS (TPL) co-repressors, thus blocking ARFs from activating the transcription of their target genes (Guilfoyle and Hagen, 2007; Szemenyei et al., 2008).

Sl-IAA27 protein has a unique motif of unknown function that is present in Sl-IAA9 and conserved in monocot and dicot species (Bassa et al., 2012). Sl-IAA27 silencing leads to higher auxin sensitivity, altered root development and reduced chlorophyll content in leaves (Bassa et al., 2012). Due to the root phenotype of Sl-IAA27, we investigated whether this gene could play a role in the mycorrhization.

2. Publication

SHORT COMMUNICATION

Plant Signaling & Behavior 8:10, e25637; October 2013; © 2013 Landes Bioscience

Sl-IAA27 gene expression is induced during arbuscular mycorrhizal symbiosis in tomato and in *Medicago truncatula*

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Keywords: auxin, Aux/IAA genes, root, tomato, arbuscular mycorrhizal fungus

Aux/IAA genes play a pivotal role in auxin transcriptional regulation. Their functions were mainly studied in *Arabidopsis* through analysis of gain-of-function mutants. In the tomato, the Solanaceae reference species, different studies on *Sl-IAA* down-regulated lines showed specific role for *Sl-IAA* genes. Our recent work revealed that the Sl-IAA27 gene displays a distinct behavior compared with most *Aux/IAA* genes, being down-regulated by auxin. Interestingly, the silencing of *Sl-IAA27* leads to altered chlorophyll accumulation in leaves, reduced fertilization, altered fruit development and altered root formation. Here we report that *IAA27* could be a key auxin signaling gene involved in AM in tomato and also in *Medicago* model plant. Indeed both *Sl-IAA27* and its closest homolog in *Medicago truncatula, Mt-IAA27,* are overexpressed in mycorrhized roots. These data are in line with the putative role of auxin in arbuscular mycorrhization.

The plant hormone auxin has an essential role in plant growth and development processes. Auxin is involved in the regulation of cell growth affecting both cell division and cell elongation and also in specific differentiation events such as embryogenesis, root development, vascular differentiation, apical dominance, gravitropic and phototropic response, fruit set, and fruit development.1,2 The auxin signaling pathway is modulated by the interaction of 3 main multigenes family coding for the TIR1/AFB receptors, the Aux/IAA repressors and the auxin response factor (ARF) transcription factors. The binding of auxin to the TIR1/AFB nuclear receptors leads to the degradation of the Aux/IAA proteins via the ubiquitin-proteasome pathway and allows the release of the ARF activators that could activate or repress their target auxin responsive genes.3-6

In the tomato, a reference species for Solanaceae plants, 25 Aux/IAA genes were identified and the understanding of their function in planta was achieved for 4 of them through the characterization of down-regulated plants.7-14 Our recent publication aims with the functional characterization of a specific tomato Aux/IAA gene, the Sl-IAA27 gene.8 Indeed, the Sl-IAA27 protein harbors the 4 typical Aux/IAA domains but we also identified another motif in the protein that is conserved among putative orthologous IAA27 proteins in monocot and dicot species. Moreover, the Sl-IAA27 auxin expression is atypical being downregulated by auxin treatment whereas the expression of Aux/IAA genes is generally overexpressed by auxin. Interestingly, our functional analysis of Sl-IAA27 by reverse genetic revealed an implication of this gene in various plant developmental processes. First, the level of chlorophyll content was reduced in leaves of Sl-IAA27 downregulated lines and was correlated with downregulation of many genes involved in chlorophyll synthesis. Second, the reproductive development of the Sl-IAA27 RNAi lines was altered at different levels. Indeed, the size of the Sl-IAA27 RNAi fruits was smaller with an enlarged placenta and the fertility of both ovule and pollen were dramatically reduced compared with wild-type plants. Moreover, the transgenic lines displayed an alteration of root development with an increased primary root length and more lateral roots. Interestingly, Sl-IAA27 overexpressing plants displayed a reduced primary root growth and no lateral root formation at the opposite of Sl-IAA27 RNAi lines.8

Previous studies have shown that AM symbiosis positively affects tomato plant productivity and tomato phenology. Indeed, mycorrhization accelerates flowering and fruit development, increases fruit yield and enhances the nutritional and

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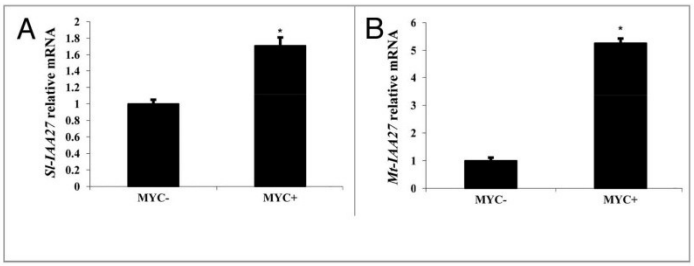

Figure 1. Accumulation of *Sl-IAA27* (**A**) and *Mt-IAA27* (**B**) transcripts in uninoculated roots (MYC–) and in mycorrhized roots (MYC+) of 12-week-old tomato plants and 8-week-old *Medicago truncatula* plants, respectively. qRT-PCR analyses were performed according to Lauressergues et al.,[23] with n = 6, error bars represent SEM. Stars indicate significant differences when compared with corresponding control, according to the Kruskal–Wallis test ($P < 0.05$).

nutraceutical value of the fruit.15-17 Following inoculation with AM fungi, a significant increase in the level of free auxin was observed in different species.18 However, even though such an increase in auxin levels has not been reported yet in tomato, analysis of mycorrhization in 2 auxin tomato mutants revealed that auxin signaling is required for normal fungal infection.19 Partial transcriptomic analysis using the TOM2 microarray showed that several genes involved in auxin metabolism, such as GH3-like protein, are differentially expressed in mycorrhized tomato plants compared with the controls.20 All these data suggest a role for auxin during the process of tomato mycorrhization. Since our previous work indicated that modulation of Sl-IAA27 expression impacts root development, and considering that AM symbiosis greatly impacts root development, we investigated the potential effect of mycorrhization on Sl-IAA27 expression. Transcript accumulation of Sl-IAA27 assessed by qRT-PCR revealed a slight but significant upregulation of Sl-IAA27 in tomato mycorrhized roots with a level of expression 1.7 fold higher than in uninoculated roots (**Fig. 1A**). To uncover whether this induction of Sl-IAA27 expression is a common feature during AM symbiosis, we also assessed the expression of the putative ortholog of Sl-IAA27 in Medicago truncatula, a plant species widely used as a model for the study of the mycorrhization process. We performed TBLASTN analysis on the M. truncatula genome using Sl-IAA27 protein sequence (http://www.plantgdb.org/MtGDB/). This in silico search identified Medtr2g122570.1, here named Mt-IAA27, as the closest homolog Sl-IAA27 in M. truncatula. The Mt-IAA27 protein displays 67% of amino acid identity with Sl-IAA27 and presents the specific domain (YxGLS) of tomato Aux/IAA clade B comprising Sl-IAA27 (**Fig. 2**).8 Interestingly, Mt-IAA27 transcript accumulation is significantly induced in mycorrhized roots with a level more than 5-fold higher than in uninoculated roots (**Fig. 1B**). The upregulation of IAA27 expression in mycorrhized roots uncovered in this study in 2 different plant species, strongly suggests a role of this gene during AM symbiosis. These data add putative new functions to Aux/IAA members and open novel opportunities to study auxin signaling during AM symbiosis. Future work will focus on the response of the IAA27 gene to the Myc-LCOs and COs and its role during AM symbiosis.21,22

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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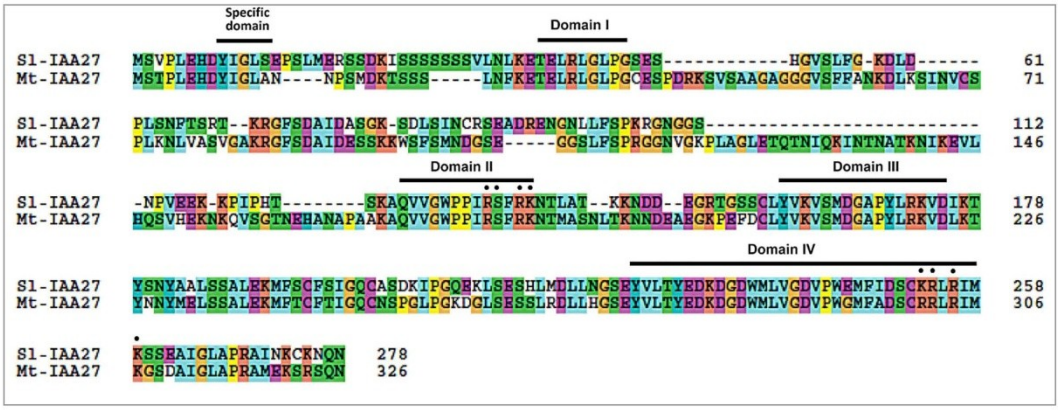

Figure 2. Sequence alignment of Sl-IAA27 protein and its closest homolog in *Medicago truncatula*, Mt-IAA27 obtained with ClustalX and manual correction. Amino acid residues, part of nuclear localization signal, are indicated by stars. The amino acid position is given on the right of each sequence.

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3. Complementary results regarding the role of Sl-IAA27 in the AM symbiosis

Materials and Methods

Biological materials

Tomato (*Solanum lycopersicum* cv. MicroTom) seeds were germinated directly in pots. Tomato plants were cultivated in 250 mL pots filled with Oil-Dri US special substrate (Damolin) for 12 weeks, in a growth chamber (16h day/8h night, 25°C, 60 µmoles/m^2/s), and watered every 2 days with modified Long Ashton medium containing a low concentration (7.5 µM) of phosphate (Balzergue et al., 2011). *Rhizophagus irregularis* DAOM197198 sterile spores were purchased from Agronutrition (Carbone, France). Tomato roots were inoculated with 400 spores of *R. irregularis* per plant. Three different lines of IAA27 RNAi and IAA27:: GUS seeds of tomato were provided by Corinne Audran-Delalande (Bassa et al., 2012).

Quantitative RT-PCR analyses

Total RNA was extracted using a Plant RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated by DNase I (Promega) to remove any genomic DNA contamination. Reverse transcription was performed using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) on 500 ng of total plant RNA. For each experiment, six independent plants or transformants were analyzed. Quantitative PCR amplifications were conducted on a Roche LightCycler 480 System (Roche Diagnostics) under the following conditions: 95°C for 5 min, then 45 cycles of 95°C for 15 sec and 60°C for 1 min. The measured transcripts were normalized to the relative expression value in non-mycorrhizal roots. Actin was used as reference genes for normalization of gene expression of tomato.

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Histochemical staining and microscopy studies

X-GLUC (5-Bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt) GUS staining was performed as described by Combier et al. (2008). Root sections (50 µm) were made using a vibratome VT1000S from samples embedded in 4% agarose. For root mycorrhizal phenotyping, roots were cleared in 10% w/v KOH and rinsed in sterile water, treated for 30 min with fluorescein-conjugated wheat germ agglutinin (WGA-FITC) (Invitrogen), which binds fungal chitin, then washed three times for 10 min in PBS and observed using an inverted light microscope or a confocal microscope (Leica). Alternatively, they were stained with Schaeffer black ink as described by Vierheilig et al. (1998). Quantification of mycorrhizal colonization was performed as described by Trouvelot et al. (1986): the frequency (F) of mycorrhiza in the root system and the arbuscule abundance (a) (percentage) were calculated in the colonized root sections using Mycocalc software (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

Biochemical

The Myc-LCOs used in this study are an equimolar mix of the four Myc-LCOs LCO-IV(C16:0), LCO-IV(C16:0,S), LCO-IV(C18:1ΔZ) and LCO-IV(C18:1Δ9Z,S described by Maillet et al. (2011), at a final concentration of 10^{-7} M. And COs treatment is used with final concentration of 10^{-8}.

Statistical analyses

The mean values for relative gene expression (n=6) or mycorrhization rates (n=15) were compared using the Kruskal–Wallis test, and, when significant, a pairwise comparison was made using the non-parametric Mann–Whitney test. Asterisks indicate significant differences compared to the control ($P < 0.05$) and error bars represent the standard error of the mean (SEM).

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Results

IAA27 expression is induced by the fungus

We have already shown, with a qRT-PCR analysis that Sl-IAA27 expression is up-regulated during mycorrhization (Bassa et al., 2013). In order to confirm this result, and to have a visualization of IAA27 expression during mycorrhization, we used a transcriptional fusion of the promoter of IAA27 with GUS coding sequence (Bassa et al., 2012). Interestingly, whereas the expression of IAA27 was restricted to the primary root and the proximal area of secondary roots when non-inoculated (Fig. 1A), the inoculation of roots with *Rhizophagus irregularis* increased expression of IAA27 in extended areas from this basal expression, even before penetration of the fungus in the root, early after inoculation (Fig. 1B). Later, when the symbiosis was well-established, the expression pattern was the same, i.e. IAA27 expressing in older parts of the roots, but interestingly, not in arbuscule containing zones (Fig. 1C, D, E, F). These observations suggest that IAA27 expression is induced by diffusible signals released by the fungus. Up to now, two kinds of such fungal molecules have been identified, Myc-LCOs and COs (Maillet et al., 2011, Genre et al., 2013). To know if IAA27 expression could be modulated by these molecules, we treated pIAA27-GUS plants with 10^{-7} and 10^{-8} M of Myc-LCOs or COs respectively. However, we detected no induction of the expression of IAA27 by these molecules (fig. S1), suggesting that other, or additional, fungal signaling molecules are necessary to trigger IAA27 expression. We have previously shown that auxin signaling is increased during mycorrhization, and especially in arbuscule-containing cells (Etemadi et al., 2014). Because IAA27 expression is down-regulated by treatment with auxin (Bassa et al., 2012), the lack of expression of IAA27 in arbuscule-containing cells might be due to the activation of auxin signaling of these cells.

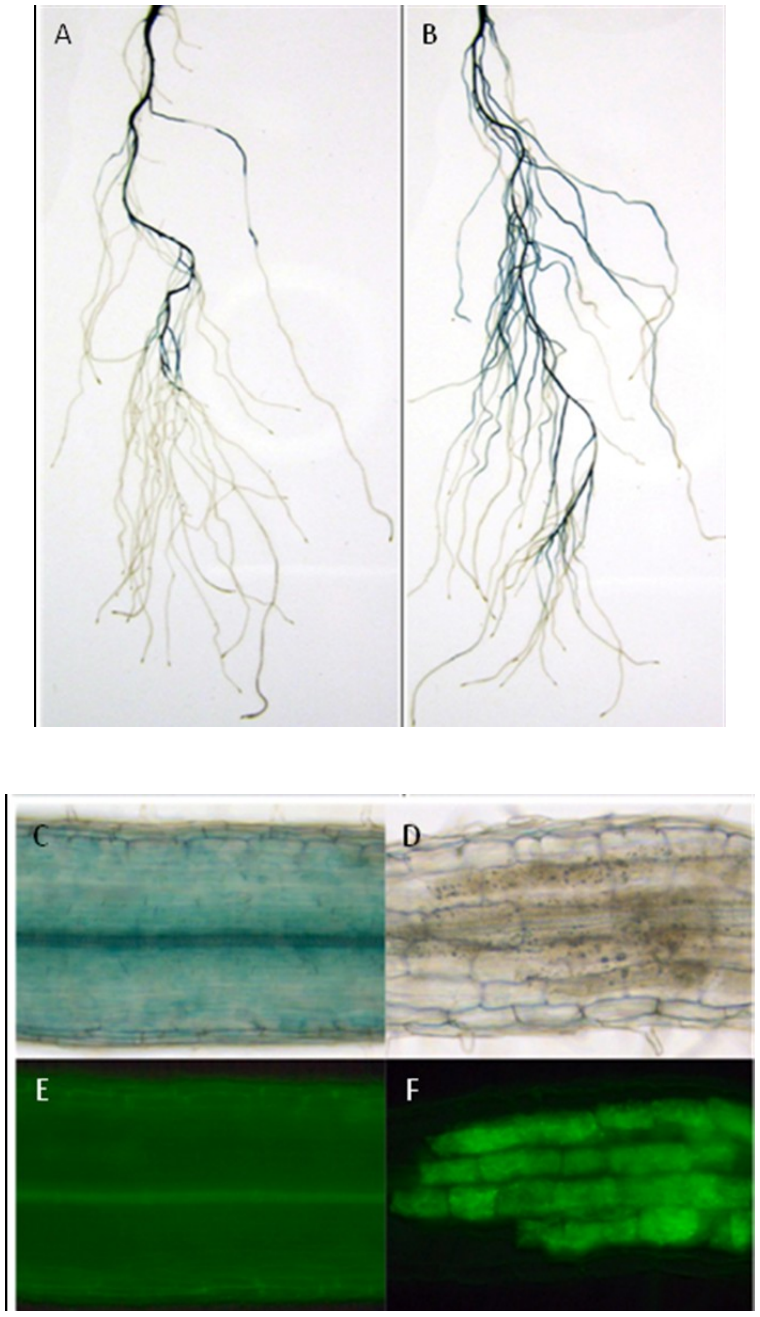

Figure 1. IAA expression with GUS. IAA27 GUS expression in non-inoculated condition (A). IAA27 GUS expression in inoculated condition with *R. irregularis* (B). IAA27:GUS staining (X-GLUC) (C,D) and fungal staining of the same root segment using WGA-FITC (E,F).

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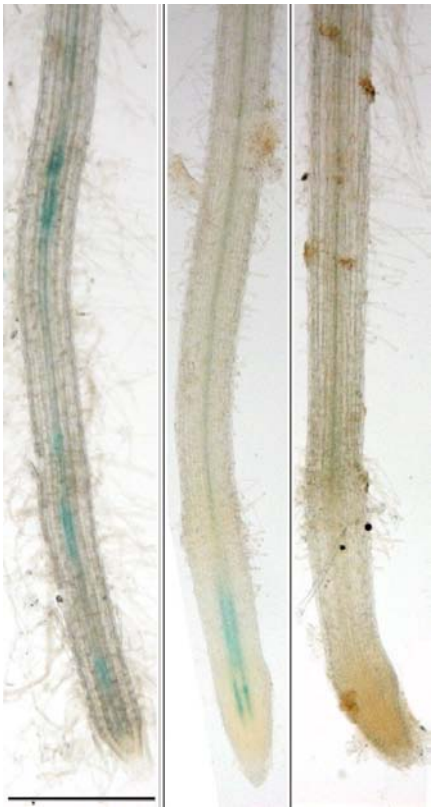

Figure S1. Expression in response to water (left), Myc-LCOs (middle), and COs (right).

IAA27 is required for mycorrhization

We next investigated whether IAA27 could play a role during mycorrhization. For this, we used tomato lines silenced for the expression of IAA27 (Bassa et al, 2012), inoculated them with *R. irregularis*, and harvested them 10 weeks after inoculation. We analyzed them as indicated in Trouvelot et al. (1986) and we observed a strong defect in the presence of the fungus in the root. As shown in Figure 2, both the colonization rate (F) by the fungus and the abundance of arbuscules (a) in colonized sections of the RNAi IAA27 roots, were severely decreased when compared to control roots. However, the shape and the size of arbuscules in these lines appeared normal (data not shown), suggesting that IAA27 controls the root colonization by the fungus but not the arbuscule development.

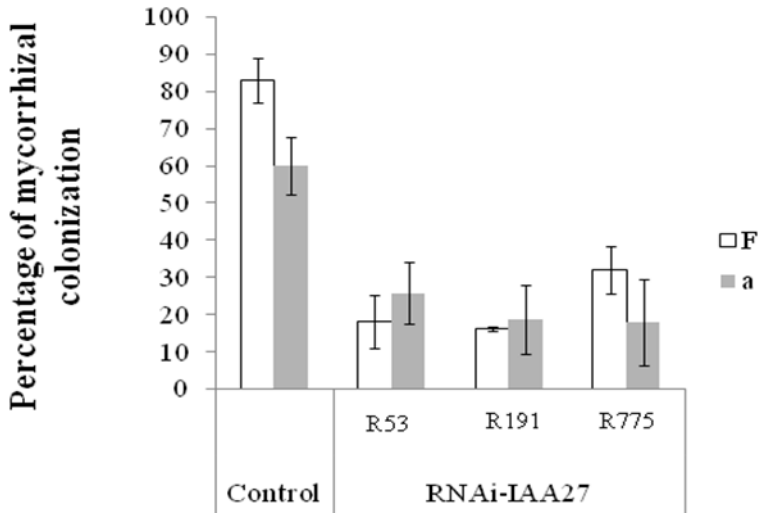

Figure 2. Frequency of colonization and arbuscule abundance decreased in IAA27-RNAi lines. frequency (F) of mycorrhization and arbuscule abundance (a) in root system of *S. lycopersicum* in control IAA27-RNAi lines. Error bars represent standard error of the mean (SEM). The asterisks indicate a significant difference between the two treatments according to the Kruskal–Wallis test (n = 6, $P < 0.05$)

IAA27 controls NSP1 expression

We have previously shown that the GRAS transcription factors NSP1 and NSP2 are involved in the control of root colonization by the fungus (Maillet et al., 2011; Lauressergues et al., 2012; Delaux et al., 2013). In order to identify if there is a link between IAA27 and the expression of these genes, we performed expression analysis in mycorrhizal control and RNAi IAA27 roots by qRT-PCR. NSP1 expression was lower in mycorrhizal roots of all RNAi lines (Fig. S2), whereas NSP2 and miR171h expression were not affected (data not shown). Liu et al (2011) have shown that NSP1 regulates the expression of strigolactone biosynthesis genes, MAX1 and D27. In the agreement with this, the expression of these two genes was also down regulated in roots of the RNAi IAA27 lines (Fig. S2). To determine if the lower expression of MAX1 and D27 was simply a consequence of a decrease of root colonization, or if this down regulation was more directly linked to the silencing of IAA27, we performed the same expression analysis but with non mycorrhizal plants. Even when the fungus was absent, NSP1, D27 and MAX1 expression was down regulated in the RNAi IAA27 roots compared to control ones (Fig. 3). These data suggest that IAA27 controls the biosynthesis of strigolactones by regulating the expression of NSP1.

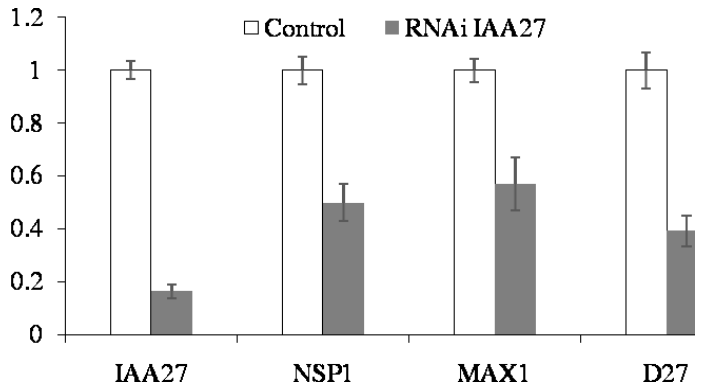

Figure 3. Expression of SL biosynthesis genes on non mycorrhized roots of IAA27RNAi. Quantification of the expression of AUX/IAA27 gene in non-mycorrhizal roots of IAA27RNAi tomato (*S. lycopersicum*). The measured transcripts were normalized to the relative expression value in control roots. Error bars represent standard error of the mean (SEM). The asterisks indicate significant differences between the genotypes according to the Kruskal–Wallis test (n = 6, P < 0.05).

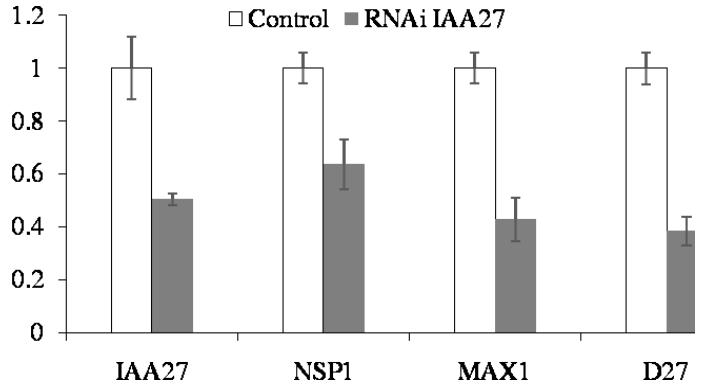

Figure S2. Expression of SL biosynthesis genes on mycorrhized roots of IAA27RNAi. Quantification of the expression of AUX/IAA27 gene in mycorrhizal roots of IAA27RNAi tomato (*S. lycopersicum*). The measured transcripts were normalized to the relative expression value in control roots. Error bars represent standard error of the mean (SEM). The asterisks indicate significant differences between the genotypes according to the Kruskal–Wallis test (n = 6, P < 0.05).

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Silencing of IAA27 decreases strigolactone content of the roots

To confirm the role of IAA27 in the regulation of strigolactone biosynthesis, we used an in vivo assay to quantify root strigolactones based on the stimulation of seed germination of the parasitic plant *Phelipanche ramosa*, a close species of the Orobanche species (Pouvreau et al., 2013). Indeed these biological tests using seeds of parasitic plants are a hundred time more sensitive than mass spectrometry analyses (Puech-Pages, personal communication). Therefore they are more appropriate for strigolactone quantification. We analyzed the germination rate of *P. ramosa* seeds and found a good correlation between the quantity of GR24, a synthetic strigolactone, and the germination rate of *P. ramosa* seeds (Fig. S3). Then, we performed a strigolactone extraction of roots according to Gomez-Roldan et al. (2008) and treated *P. ramosa* seeds with the obtained extracts. Whereas the extracts of control roots were able to stimulate the germination of P. ramosa seeds as efficiently as 10^{-11} M GR24, the extract of RNAi roots were nearly 10 fold less active, strongly suggesting than these plants were affected in their capacity to produce strigolactones (Fig. 4).

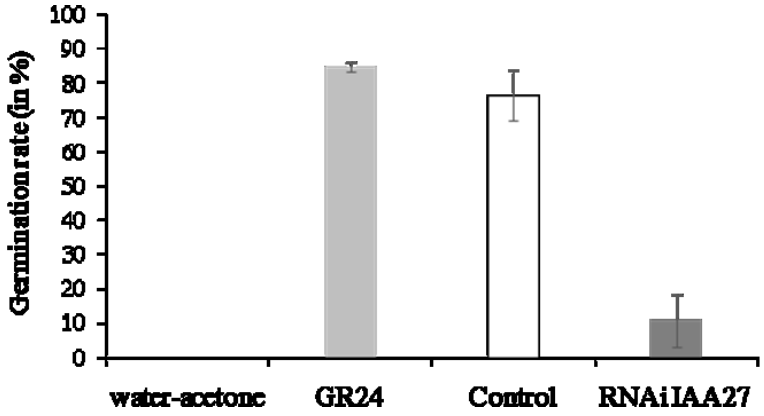

Figure 4. Decrease orobanche germination in SL extraction of IAA27RNAi. Error bars represent standard error of the mean (SEM). The asterisks indicate significant differences between the genotypes according to the Kruskal–Wallis test.

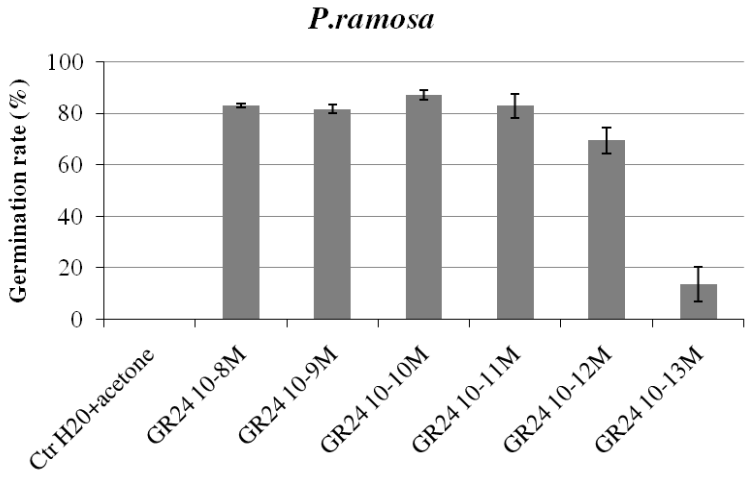

Figure S3. **Phelipanche ramos*a* seeds germination in response to different concentration of GR24**. Error bars represent standard error of the mean (SEM).

Finally, in order to further confirm that the lower mycorrhization observed in the IAA27 RNAi lines was a direct consequence of lower strigolactone contents in roots, we performed a mycorrhization assay in the presence or not of 10^{-7}M GR24. GR24 addition was able to complement the mycorrhizal defect of RNAi IAA27 plants (Fig. 5), showing that the lower mycorrhization of these plants is indeed due to lower strigolactone content.

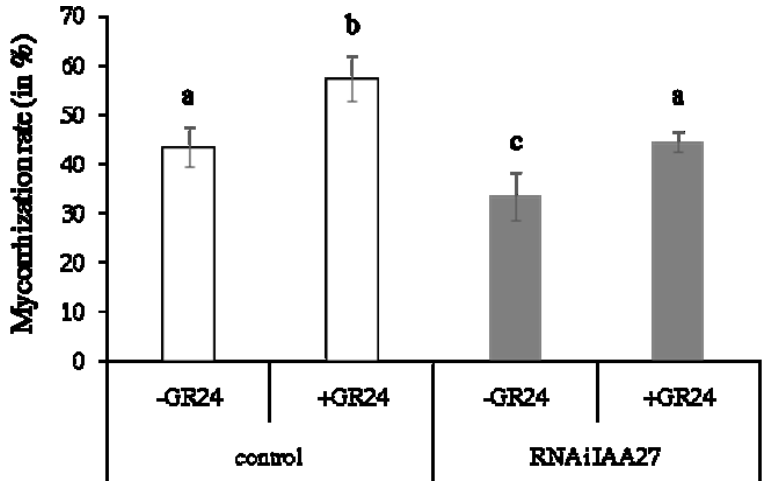

Figure 5. AM colonization in root of non-treated (-GR24) and treated with GR24 (+GR24). Error bars represent standard error of the mean (SEM). Student's *t*-tests were used to compare different samples (n = 5, P < 0.05).

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Discussion

The strong reduction of AM fungal colonization of the AUX/IAA27 RNAi lines suggests that AUX/IAA27 is a positive regulator of the AM symbiosis. Given that *NSP1*, *MAX2* and *D27*, three genes involved in the biosynthesis of strigolactones (Liu et al. 2011), were also down regulated in the RNAi lines, we propose that AUX/IAA27 positively controls AM fungal colonization by activating the synthesis of strigolactones. This hypothesis was supported by the observation that a GR24 treatment of the RNAi lines could restore normal mycorrhization. The fact that the expression of IAA27 was strongly induced in roots growing in the presence of the fungus, even in the absence of root colonization, indicates that AUX/IAA27 positively regulates the early stage of AM symbiosis. Our study suggests that COs and Myc-LCOs are not the fungal signals activating AUX/IAA27 gene expression during the early symbiotic stage. Before concluding that other fungal metabolites are involved, additional experiments will have to be performed with a mix of COs and LCOs (in case of synergistic action), or with specific LCOs (rather than with a mix), or with different molecular LCO/CO concentrations.

In agreement with the fact that AUX/IAA27 could be a positive regulator of the early stage of AM symbiosis, we detected no expression of AUX/IAA27 in later stages of the symbiosis such as in arbuscule-containing cells. We know that, in contrast with most AUX/IAAs, AUX/IAA27 is down-regulated by auxin (Audran-Delalande et al., 2012). Given that, in three plant species, including tomato, Etemadi et al. (2014) found that arbuscule formation is under a positive control of auxin, we could hypothesize that in arbuscule-containing cells, while stimulating arbuscule formation, auxin would also repressed AUX/IAA27. This would participate to eventually lower the strigolactone content in roots, as it has been shown in well established mycorrhizal plants (Lopez-Raez et al., 2011). Several authors have shown in different plant species that auxin is a positive regulator of strigolactone biosynthesis (Hayward et al., 2009; Foo et al., 2013). If our hypothesis above is correct, both positive and negative regulations of strigolactone synthesis would therefore coexist in the later stages of the symbiotic interaction. A more careful spatio-temporal investigation will be necessary to decipher where and when these opposite regulations are taking place, and if the negative regulation of strigolactone synthesis participates in a local autoregulation of AM root colonization.

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General discussion and perspectives

The role of plant hormones in the development and maintenance of the AM symbiosis has been an emerging area of research in recent years. Several studies have shown that most plant hormones are more or less directly involved in AM development (Foo et al., 20013; Bucher et al., 2014). A great challenge when studying plant hormones is that they regulate a great deal of overlapping physiological and developmental plant responses, and some of these plant responses can impact indirectly mycorrhizal development and functioning. The aim of my Ph.D. was to establish whether auxin and auxin signaling pathways are specifically (directly) involved in the arbuscular mycorrhizal development.

Previous studies have shown in several plant species an elevated auxin level in mycorrhizal roots. Some tomato mutants with pleiotropic phenotypes related to impaired auxin signaling or transport exhibited a defect in mycorrhizal colonization, but without any arbuscule defect (Hanlon and Coenen, 2011). Hanlon and Coenen (2011) proposed that auxin signaling is required for presymbiotic plant–fungus interactions in root and that host auxin responses guide the exchange of diffusible signals between plant and fungus. This proposition is in line with our results suggesting that Aux/IAA27 is a positive regulator of the early stage of the AM interaction. Given that ARF proteins interacting with Aux/IAA27 are gene activators (recent results in GBF laboratory) we must hypothesize that the activated genes are repressors of the early steps of mycorrhization. How IAA27 does regulate positively *NSP1* transcription is another question. Aux/IAA proteins, including IAA27, have no DNA binding domains, suggesting the requirement of intermediate proteic partners to activate *NSP1* expression. Another hypothesis is that Aux/IAA27 activates indirectly this gene by repressing a hypothetical *NSP1* repressor. We see that much more work will be necessary to elucidate the mechanism by which SlAux/IAA27 positively regulates the early steps of AM interaction.

Our results showing an increase of auxin concentration in arbuscule-containing cells (Etemadi et al., 2014) and a substantial defect in arbuscule formation resulting from the down-regulation of auxin receptors are also in favor of an active role of auxin signaling in later stages of the symbiosis and more specifically for arbuscule development. Moreover, contrary to several studies showing that, depending on the plant species, auxin levels increase or decrease in mycorrhizal roots, our study shows that this new auxin regulation appears to be shared in di- and monocotyledonous plants, supporting evolutionary conservation of this

regulation in plants. The promoting effect of auxin-signaling on mycorrhiza formation is in line with a study by Navarro et al. (2006) who reported a negative role of auxin signaling in plant immunity. Thus, it could be also proposed that root cells that are to be colonized by a symbiotic fungus rely on a local reduction of plant immunity. Auxin signalling can repress SA levels and SA signaling (Robert-Seilaniantz et al., 2011), so it is possible that AM fungi, as biotrophic organisms, developed ways to use auxin-mediated suppression of SA to improve the susceptibility of the host (Chen et al., 2007).

Thus it appears that auxin regulation of the mycorrhization process is a complex, multi component, regulation. First, during the early stage of the root-fungus interaction the presence of the fungus induces *IAA27* expression. We propose that this induction leads to an increase of strigolactone synthesis and a promotion of root colonization. An interesting challenge is to determine if fungal diffusible signals are involved here and if so, to determine what their chemical structures are. Then, the plant would accumulate auxin in arbuscule-containing cells to promote arbuscule formation. This auxin increase would down regulate *IAA27* expression as it has been shown in previous studies (Bassa et al., 2012) and as it is revealed in our study by the absence of pIAA27-GUS staining in area of root tissues containing arbuscules. We hypothesize that this local down-regulation of *IAA27*, probably associated with a lower strigolactone synthesis, is one reason for the short life time of arbuscules and perhaps participates to autoregulate fungal proliferation in neighboring cells. How this complex regulation system, occurring in different root tissues and combining positive and negative regulation of fungal development, is spatiotemporally controlled, is still far from being understood. For further investigation, a more careful cartography of the expression pattern of *NSP1*, *MAX2* and *D27*, and of the strigolactone concentration in different tissues, will represent a first step to confirm some of our hypotheses.

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