Sl-IAA27 regulates strigolactone biosynthesis and mycorrhization in tomato (var. *MicroTom*)

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Summary

- Root colonization by arbuscular mycorrhizal (AM) fungi is a complex and finely tuned process. Previous studies have shown that, among other plant hormones, auxin plays a role in this process but the specific involvement of Aux/IAAs, the key regulators of auxin responses, is still unknown.
- In this study, we addressed the role of the tomato *Sl-IAA27* during AM symbiosis by using Sl-IAA27-RNAi and *pSL-IAA27*::*GUS* stable tomato lines.
- The data show that *Sl-IAA27* expression is up-regulated by the AM fungus and that silencing of *Sl-IAA27* has a negative impact on AM colonization. *Sl-IAA27*-silencing resulted in down-regulation of three genes involved in strigolactone synthesis, *NSP1, D27* and *MAX1*, and treatment of *Sl-IAA27*-silenced plants with the strigolactone analog GR24 complemented their mycorrhizal defect phenotype.
- Overall, the study identified an Aux/IAA gene as a new component of the signaling pathway controlling AM fungal colonization in tomato. This gene is proposed to control strigolactone biosynthesis via the regulation of *NSP1*.

Introduction

The arbuscular mycorrhiza (AM), a symbiosis between soil fungi of the *Glomeromycota* phylum and nearly 80% of terrestrial plant species, is characterized by a two-way trade in which the fungus provides mineral nutrients to the plant in exchange for carbohydrates. The initiation of this symbiosis is known to require a molecular communication between the two partners. The plant secretes several signal molecules in its root exudates, including strigolactones (SLs), a class of plant hormones playing an important role in the rhizosphere for the establishment of AM symbiosis (Gomez-Roldan *et al.*, 2008). SLs stimulate AM fungal metabolism and hyphal proliferation (Akiyama *et al.*, 2005; Besserer *et al.*, 2006, 2008) and from its side, the AM fungus produces trace amount of chitinic signals (Maillet *et al.*, 2011; Genre *et al.*, 2012, 2013). Upon this successful mutual recognition, the fungus penetrates the roots through the epidermis, grows between root cells and forms highly branched structures called arbuscules inside cortical root cells, where most nutrient exchanges occur between the two partners.

The control of the mycorrhizal symbiosis is a finely tuned process at multiple levels. An increasing number of reports point to the important role of several plant hormones, besides that of SLs, in the regulation of early recognition/colonization steps up to the final arbuscular formation (reviewed in Hause *et al.*, 2007; de Los Santos *et al.*, 2011; Foo *et al.*, 2013; Gutjahr, 2014). For instance, auxin is involved in both the general development of the fungus *in planta* and the formation of arbuscules, whereas SLs are involved in presymbiotic growth of the fungus but not in arbuscule differentiation.

With regard to auxin, several studies have shown an increase in auxin content in AM roots and a stimulation of fungal growth and mycorrhization by exogenous auxin treatment (reviewed in Gutjahr, 2014). This was recently confirmed in tomato by the observation that the synthetic auxin-responsive gene DR5-GUS promoter undergoes a net activation in mycorrhized roots and, more precisely, in arbuscule-containing cells (Etemadi *et al.*, 2014). Further supporting the role of auxin in the AM symbiosis, the mycorrhization rate was strongly decreased, although showing normal fungal structures and arbuscules, in the pea *bushy* mutant that produces three times less auxin in its roots, and also in the auxin-resistant tomato mutant *diageotropica* as well as in the auxin hypertransporting tomato mutant *polycotyledon* (Hanlon & Coenen, 2011; Foo, 2013). Interestingly, the low mycorrhization rate of *bushy* was attributed to a decreased SL biosynthesis, suggesting a possible crosstalk between auxin and SL in the regulation of AM (Foo, 2013).

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Auxin perception and/or signaling appear(s) to be critical for arbuscule development, as the inhibition of auxin receptors by the overexpression of the microRNA393 leads to a defect in arbuscule formation in addition to a reduced mycorrhization (Etemadi *et al.*, 2014). Upon auxin recognition, the auxin receptors TIR/AFBs (Transport Inhibitor Response/Auxin signaling F-Box) become associated with the SKP1-Cullin-F-box (SCF) complex, leading to a rapid proteasome-mediated degradation of Aux/IAAs (Dharmasiri *et al.*, 2005; Kepinski *et al.*, 2005; Leyser, 2006; Tan *et al.*, 2007; Chapman & Estelle, 2009), a release of auxin response factors (ARFs) that can then activate the transcription of auxin-regulated genes through binding to auxin-responsive elements present in their promoter region (Hagen *et al.*, 1991; Ulmasov *et al.*, 1997; Hagen & Guilfoyle, 2002). Therefore, one can hypothesize that Aux/IAAs and/or ARFs play a role in the regulation of mycorrhization.

In tomato, 25 *Aux/IAA* genes were identified (Imaizumi-Anraku *et al.*, 2005; Herrera-Medina *et al.*, 2007; Chaabouni *et al.*, 2009a,b; Wang *et al.*, 2009; Audran-Delalande *et al.*, 2012; Bassa *et al.*, 2012; Deng *et al.*, 2012a,b; Su *et al.*, 2014). Among these, *Sl-IAA27* was shown to display an intriguing expression pattern: a down-regulation upon exogenous auxin treatment and an up-regulation during mycorrhization (Bassa *et al.*, 2012, 2013).

To gain further insight into the role of auxin, and more specifically that of Aux/IAAs, in the mycorrhization process, we analyzed in *MicroTom* plants the expression pattern of *Sl-IAA27* in mycorrhized and nonmycorrhized roots, the mycorrhizal phenotype of *Sl-IAA27*-silenced plants, and we compared the ability to produce SLs of wild-type and *Sl-IAA27*-silenced plants. The data suggest that *Sl-IAA27* positively regulates mycorrhization via the induction of *NSP1* transcription and SL biosynthesis.

Materials and Methods

Plant and fungal materials, growth and conditions

Seeds of tomato (*Solanum lycopersicum* cv *MicroTom*) wild-type, *pSl-IAA27*: GUS lines (three independent lines, 14/6/1, 10/10/1 and 37/4/1) and RNAi *Sl-IAA27* (three independent lines, 5/3, 19/1 and 77/7, named Rline1, Rline2 and Rline3, respectively) were obtained as already described (Bassa *et al.*, 2012). Seeds of the parasitic plant *Phelipanche ramosa* L. Pomel (genetic type 1; Voisin *et al.*, 2011) were provided by P. Simier (LBPV, University of Nantes, France).

Tomato seeds were surface-sterilized for 1 min in 2.3% sodium hypochlorite and washed eight times with sterile deionized water. They germinated on a solid water agar plate in the dark at 23°C for 6 d.

For mycorrhization assays and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses, seedlings were grown in 250 ml pots (one seedling per pot) filled with Oil-Dri US special substrate (Damolin, Etrechy, France) for 12 wk, in a growth chamber (16 : 8 h, 24 : 22°C, day : night, 120–150 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). They were inoculated with *Rhizophagus irregularis* DAOM 197198 sterile spores (400 spores per liter of substrate) purchased from Agronutrition (Carbone, France). For β-glucuronidase (GUS) staining experiments, seedlings were inoculated with a higher inoculum (2000 spores l^{-1} of substrate) and harvested 4 wk after inoculation.

For *in vitro* culture, germinated seedlings were grown on modified Long Ashton medium (7.5 μM of phosphate), gelled with 0.8% agar (Agar HP 696; Kalys Biotech, http://biotech.kalys.com) in 12-cm-square plates (five seedlings per plate) in a growth chamber (16 : 8 h, 24 : 22°C, day: night, 60 μmol m^{-2} s^{-1}). After 6 d, a cellophane membrane (Couvre confiture, Hutchinson, Chalette/Loing, France) covered with 500 *R. irregularis* germinating spores was laid on seedling roots for another 3 days, so that the membrane prevented physical contact, but not the chemical communications, between the two partners. Before this step, the spores had been incubated on the cellophane membrane laid on the same solid modified Long Ashton medium for 6 d at 30°C and 2% CO_2.

Homologous gene identification

BlastN analyses were conducted on Phytozome11 https://phytozome.jgi.doe.gov/pz/portal.html, with the latest version of *Solanum lycopersicum* iTAG2.3 genome.

Strigolactone treatment

The SL analog GR24 was purchased from Chiralix BV (Nijmegen, the Netherlands). For *P. ramosa* seed germination tests (see later) 10^{-8}–10^{-13} M water solutions of SLs were prepared from a 10^{-3} M stock solution in acetone. For treatment of tomato plants grown in pots, 10^{-7} M GR24 was dissolved in the low-phosphate Long Ashton medium and watered (10 ml per pot) three times a week. Control plants were watered with 0.0001% (v/v) acetone. To minimize the amount of GR24 used, 12 plants for control and four plants per RNAi *Sl-IAA27* line were used. For the RNAi *Sl-IAA27* lines, the mean values of Fig. 4(b) (see later) represent the average obtained with the three lines.

Gene expression analyses

For qRT-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 genomic DNA contamination. Reverse transcription was performed using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) on 1 μg of total plant RNA. For each experiment, six to 12 independent plants were analyzed. Quantitative PCR amplifications were conducted on a Roche LightCycler 480 System (Roche Diagnostics, Meylan, France) under the following conditions: 95°C for 5 min, then 45 cycles of 95°C for 15 s and 60°C for 1 min. The various primer sets used are described in Supporting Information Table S1. The measured transcripts were normalized by using the Sl-Actin gene.

For histochemical GUS analysis, root tissues of *pSL-IAA27::GUS* tomato lines were soaked in GUS staining solution

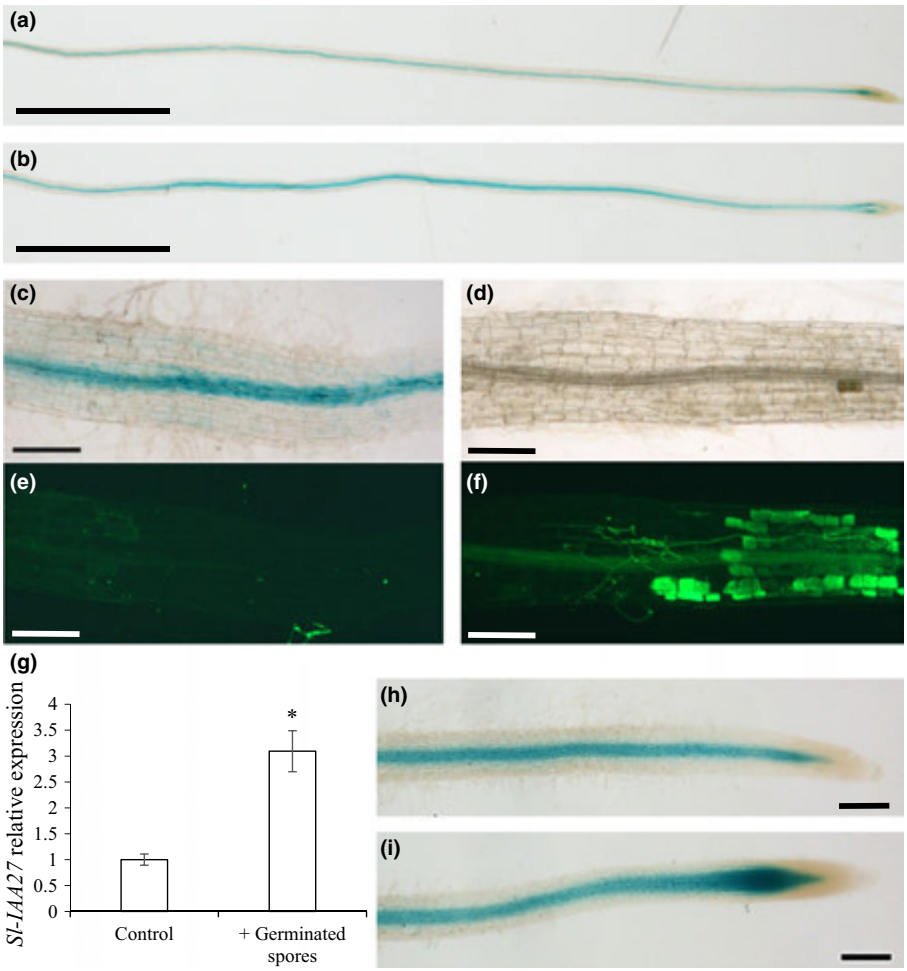

Fig. 1 Localization of *Sl-IAA27* expression in roots of *pSl-IAA27*:: *GUS* plants inoculated or not with *Rhizophagus irregularis*. (a, b) β-glucuronidase (GUS) expression mainly found in the inner cortex and the central cylinder of young roots: (a) in a root of an uninoculated plant; (b) in an uncolonized root of an inoculated plant. (c, d) GUS expression in uncolonized (c) and colonized (d) root sections of an inoculated plant. (e, f) Corresponding fluorescent images confirming the absence (e) and presence (f) of the fungus stained with fluorescein-conjugated wheat germ agglutinin. (g) Quantification by quantitative reverse transcription polymerase chain reaction (qRT-PCR) of *Sl-IAA27* gene expression in roots of plants cultivated *in vitro* in the presence or absence (control) of germinating spores separated with a cellophane membrane. Error bars represent ± SEM. Asterisks indicate a significant difference when compared with controls according to the Kruskal–Wallis test: $n = 5$, $P < 0.05$. (h, i) GUS expression in a control root (h) and in a root cultivated in the presence of germinating spores (i) separated by a cellophane membrane. The GUS pictures shown in this figure were obtained with transformed line 10/10/1. Bars: (a, b), 2 cm; (c–f, h, i), 200 μm.

(100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Triton X-100, 0.3 mg ml^{-1} X-Gluc) under vacuum for 15 min. Root tissues were then incubated for 6–12 h in GUS staining solution at 37 °C. GUS pictures shown in Fig. 1(a–d,h, i) and Fig. S1 represent a staining pattern found in all *pSl-IAA27*: GUS lines. For fluorescent images shown Fig. 1(e,f), roots were GUS-stained as already described, then cleared with KOH and stained with fluorescein-conjugated wheat germ agglutinin (WGA-FITC) as described later for mycorrhizal phenotyping.

For transversal root sections, root tissues after GUS staining were included in low melting 5% agar and cut into 50 μm sections using a vibratome, before observation under an Axio Zoom V16 Zeiss stereomicroscope.

Mycorrhizal phenotyping and fungus staining

Roots were cleared in 10% w/v KOH for 8 min at 95°C and rinsed in sterile water. Then they were treated for 30 min with WGA-FITC (Invitrogen), which binds fungal chitin, washed three times for 10 min in PBS and observed using an Axio Zoom V16 Zeiss stereomicroscope. Alternatively, roots were stained with Schaeffer black ink as described by Vierheilig *et al.* (1998). The percentage of mycorrhization was established using the grid intersect method described by Giovannetti & Mosse (1980) and with two additional mycorrhization indices: F, mycorrhization frequency and a, arbuscule abundance in colonized root sections, according to Trouvelot *et al.* (1986). Arbuscule size and shape were analyzed using an Axio Zoom V16 Zeiss and an AxioPlan Zeiss.

Statistical analyses

Means were calculated with values of six to 15 replicates ($n < 25$) depending on the experiments (indicated in figure legends) and were therefore compared using the Kruskal–Wallis test. Each experiment was repeated two to three times.

P. ramosa germination assay

Root extracts One gram of powdered N_2-frozen roots of tomato (12 wk old) grown in pots as described earlier was suspended in 2 ml of 100% ethyl acetate and sonicated for 10 min in 4°C water. After centrifugation at 450 ***g*** for 10 min at 4°C, the upper organic phase was transferred into a new tube and the extraction of the pellet was repeated with 2 ml of fresh ethyl acetate. The two organic phases were pooled before being washed with 0.2 M K_2HPO_4 buffer and then dried under nitrogen flow. Root extracts used to stimulate germination of *P. ramosa* seeds were resuspended in 25% acetone and diluted 1000–100 000 times in sterile deionized water before use.

Phelipanche ramosa seeds were surface-sterilized by vigorous agitation in a 2.3% sodium hypochlorite solution for 5 min. They were then washed with sterilized deionized water three times for 30 s and three times for 5 min, and then they were transferred for 10 d in the dark at 24°C in 12-well plates (*c.* 300 seeds per well) containing 0.5 ml of sterilized deionized water per well. After this preconditioning period, water was removed and replaced by 0.5 ml of diluted root extract. After 7 d, seeds were stained with 0.5% (w/v) neutral red and germination rate was assessed under a Leica MZ75 stereomicroscope.

Results

Sl-IAA27 expression is induced by the AM fungus *R. irregularis*

We have previously shown by qRT-PCR that *Sl-IAA27* expression is globally up-regulated in mycorrhized roots of tomato (Bassa *et al.*, 2013). To gain additional information on *Sl-IAA27* expression in roots of mycorrhized plants, we used transgenic *pSL-IAA27::GUS* tomato lines (Bassa *et al.*, 2012). We observed that in uncolonized roots of mycorrhized plants, *Sl-IAA27* expression was higher than in roots of nonmycorrhized plants (Fig. 1a,b). In both types of roots, GUS expression was higher in young tissues and was mainly localized in the central cylinder and the inner cortex (Figs 1a,b, S1a–c). Interestingly, GUS expression was completely absent in the colonized root sections of mycorrhized plants (Fig. 1d,f). These observations suggest the occurrence of a subtle regulation of *IAA27* expression in mycorrhized roots. While this expression seems to be positively regulated by the general presence of the fungus, it is negatively regulated in the immediate vicinity of intraradical fungal structures. To test if diffusible signal compounds released by the fungus are responsible for the positive regulation of *IAA27* transcription, we cultivated *pSl-IAA27::GUS* tomato seedlings (7 d old) *in vitro* for 3 d in the presence of germinating fungal spores. Roots and spores were separated by a membrane allowing chemical exchanges but preventing physical contact. The presence of the fungus significantly increased *Sl-IAA27* expression as shown by qRT-PCR (Fig. 1g) and GUS expression analyses (Fig. 1h,i), indicating that *Sl-IAA27* up-regulation in mycorrhized roots could be caused by diffusible fungal compound(s).

Sl-IAA27 is a positive regulator of mycorrhization

We next investigated whether *Sl-IAA27* plays a role during mycorrhization. We used three independent tomato lines silenced for the expression of *Sl-IAA27* (named RNAi *Sl-IAA27* lines, Rline1, Rline2 and Rline3; Bassa *et al.*, 2012). Silencing of *Sl-IAA27* results in higher auxin sensitivity and reduced Chl content in leaves. Both ovule and pollen display a dramatic loss of fertility and the internal anatomy of the flower and the fruit are modified (Bassa *et al.*, 2012). As *Sl-IAA27*-RNAi lines were also described to have longer primary roots and higher number of lateral roots when grown *in vitro* on rich medium (MS/2) (Bassa *et al.*, 2012), we assessed their root architecture in our growth conditions using low-phosphate Long Ashton medium. After 2 wk *in vitro* or 4 wk in pots, we observed no differences between the RNAi lines and the wild-type, for both the primary root length and the number of lateral roots (Fig. S2). We then inoculated the plants with *R. irregularis* spores and analyzed the root colonization rate at 12 wk after inoculation. Total root colonization was strongly reduced in the RNAi *Sl-IAA27* lines (average of the three lines) compared with the wild-type (Fig. 2a). In agreement with this, the expression of the phosphate transporter gene *Sl-PT4*, the tomato homolog of *Medicago truncatula PT4* (Nagy *et al.*, 2005), which is specifically induced during mycorrhization (Harrison *et al.*, 2002; Nagy *et al.*, 2005), was also lower in the RNAi *Sl-IAA27* lines (Fig. 3a). A closer look at the mycorrhization pattern showed that this lower colonization was a result of a strong decrease of the infection frequency and arbuscule abundance (Fig. 2b). On the other hand, the shape and size of arbuscules looked identical in control and *Sl-IAA27*-silenced roots (Fig. 2c–f). Altogether, these data suggest that *Sl-IAA27* is not involved in the process of arbuscule differentiation but rather in the control of fungal root penetration and intraradical colonization.

Sl-IAA27 influences *NSP1* expression

We have previously reported that, in *M. truncatula*, one important GRAS transcription factor of the nodulation process, NSP1, is involved in the control of mycorrhizal root colonization (Delaux *et al.*, 2013). To assess the potential link between *Sl-IAA27* and *NSP1*, we compared the expression of its closer homologous gene in *S. lycopersicum*, in mycorrhized roots of control and RNAi *Sl-IAA27* tomato plants. Only one homologous gene was found by direct BLAST on *S. lycopersicum* genome (Solyc03g123400.1.1). *Sl-NSP1* expression was down-regulated in *Sl-IAA27*-silenced roots compared with control roots (Fig. 3a). As Liu *et al.* (2011) showed in *M. truncatula* and rice that NSP1 regulates the expression of *D27* and *MAX1*, two genes involved in the SL biosynthetic pathway, we also measured the expression of the closest homolog of these two genes, *Sl-D27* and *Sl-MAX1*, in mycorrhized tomato (Challis *et al.*, 2013). Our assumption that *Sl-D27* and *Sl-MAX1* were the best possible homologs of *Mt-D27* and *Mt-MAX1* was based on a previous study (Challis *et al.*, 2013), a BLASTN analysis that we performed using the very last updated genome of *S. lycopersicum* (*Solanum lycopersicum* iTAG2.3 genome), confirming that only one homologous gene of *Mt-D27* and *Mt-MAX1* could be found with a good score and E-value, and on the fact that the *S. lycopersicum D27* and *MAX1* homologs had 3 and 5 exons like *Mt-D27* and *Mt-MAX1*, respectively. Moreover, as expected, the four genes *Sl-D27, Sl-MAX1*, *Sl-CCD7* and *Sl-CCD8* were regulated by phosphate (Fig. S3a), as has been shown for *Mt-MAX1*, *Mt-D27*, *Mt-CCD7* and *Mt-CCD8* in *M. truncatula* (Liu *et al.*, 2011; Bonneau *et al.*, 2013; Van Zeijl *et al.*, 2015). We found that *Sl-D27* and *Sl-MAX1* expressions were also down-regulated in the three RNAi *Sl-IAA27* tomato lines compared with control plants (Fig. 3a). The same results were obtained in nonmycorrhized plants (Fig. 3b), indicating that the observed down-regulation of *NSP1*, *MAX1*

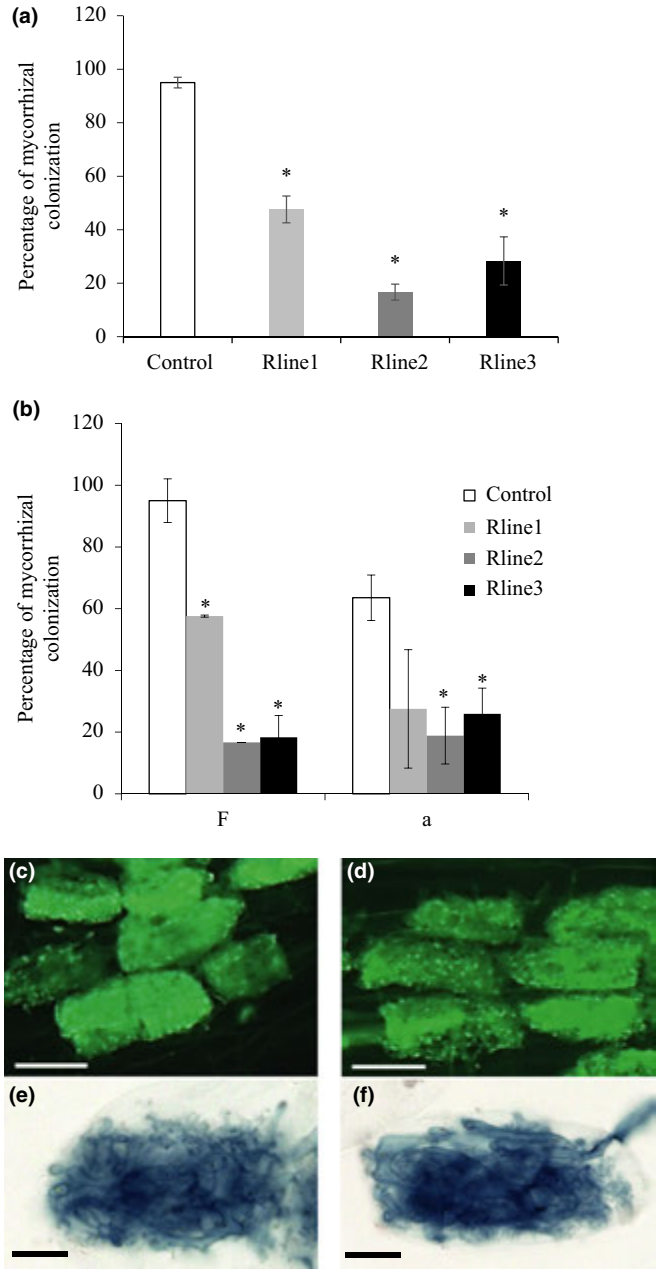

Fig. 2 Mycorrhizal phenotype of control and RNAi *Sl-IAA27* lines 12 wk after inoculation with *Rhizophagus irregularis*. (a) Mycorrhizal rate in control and three RNAi *Sl-IAA27* lines as measured by the grid-intersect method (Giovannetti & Mosse, 1980). (b) Quantification of mycorrhization in control and in the three RNAi *Sl-IAA27* lines separately, according to Trouvelot's method (Trouvelot *et al.*, 1986). 'F', frequency of colonization in the root system; 'a', arbuscule abundance (percentage) in the colonized root sections. (c–f) Root sections showing arbuscules of control (c, e) and Rline2 roots (d, f) stained with fluorescein-conjugated wheat germ agglutinin (c, d) and ink (e, f). Error bars represent ± SEM. Asterisks indicate a significant difference when compared with controls according to the Kruskal–Wallis test: $n = 10$, $P < 0.05$. Bars: (c, d), 50 μm; (e, f), 10 μm.

and *D27* in mycorrhizal *Sl-IAA27*-silenced roots was not a result of the lower mycorrhization rate.

We also examined if the silencing of *Sl-IAA27* could affect *Sl-CCD7* and *Sl-CCD8*, SL biosynthesis genes and presumably

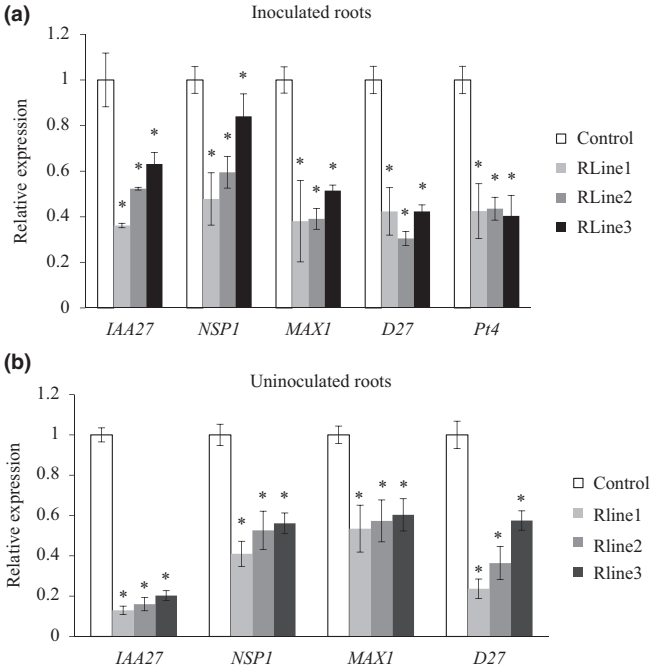

Fig. 3 Expression of *Sl-IAA27*, *Sl-NSP1*, *Sl-MAX1* and *Sl-D27* in *Sl-IAA27*-silenced lines, with or without mycorrhization. Quantification of *Sl-IAA27*, *Sl-NSP1*, *Sl-MAX1* and *Sl-D27* gene expression by quantitative reverse transcription polymerase chain reaction in inoculated (a) and uninoculated (b) control and RNAi *Sl-IAA27* plants. Error bars represent ± SEM. Asterisks indicate a significant difference when compared with control according to the Kruskal–Wallis test: (a) $n = 10$, (b) $n = 9$, $P < 0.05$.

not linked to NSP1 (Vogel *et al.*, 2010; Kohlen *et al.*, 2012). In the three RNAi lines, we found no significant down-regulation of these three genes, suggesting that *Sl-D27* and *Sl-MAX1* expressions are specifically affected via Sl-NSP1 (Fig. S3b).

It has been reported that a close *IAA27*-related gene, *AUX/IAA9*, was up-regulated in young fruits of the *Sl-IAA27* RNAi lines (Bassa *et al.*, 2013). Therefore we analyzed the expression of *AUX/IAA9* in nonmycorrhized roots to check whether this up-regulation in fruit was also effective in roots. We detected no significant changes of *Sl-IAA9* expression in roots of silenced *Sl-IAA27* lines. In addition, we assessed in the *Sl-IAA27* RNAi lines the expression of the closest homologous genes of *Sl-IAA27* and *Sl-IAA9*, *Sl-IAA8* (Fig. S3c; Audran-Delalande *et al.*, 2012), and we detected no significant changes.

These data suggest that the down-regulations of *NSP1*, *MAX1* and *D27* in *Sl-IAA27* lines were not the result of some indirect *IAA9* and/or *IAA8* regulation. They support the hypothesis that Sl-IAA27, among other as yet unknown regulatory roles, could be an Aux/IAA specifically involved in the regulation of *NSP1* expression and therefore indirectly involved in the regulation of SL biosynthesis.

Mycorrhizal defect of RNAi *Sl-IAA27* lines can be complemented by GR24 addition

To investigate further the possible role of Sl-IAA27 in the regulation of SL biosynthesis, we performed mass spectrometry analyses of root extracts of wild-type and *Sl-IAA27*-silenced plants to

compare their SL content. We could not detect the presence of SL in any of those extracts, probably because they are in trace amounts in *S. lycopersicum* cv *MicroTom*. Therefore we compared the ability of the extracts to stimulate seed germination of the parasitic plant *Phelipanche ramosa*. This *in vivo* assay has long been used to detect the presence of SL in plant extracts (Dörr *et al.*, 1994; Bouwmeester *et al.*, 2003; Echevarría-Zomeño *et al.*, 2006; Yoneyama *et al.*, 2010; Dor *et al.*, 2011). It can detect SLs with a much higher sensitivity (down to 10^{-13} M; Fig. S4) than that of a mass spectrometry analysis (10^{-9} M; V. Puech-Pagès, pers. comm.), and it provides a better dynamic range for their quantification. As expected, when treated with the control solvent, the germination rate of *P. ramosa* seeds was null, while 73% germination was obtained in the presence of 10^{-11} M GR24 (Fig. 4a). A similarly high rate of germination (55%) was obtained when seeds were treated with exudates of control roots, whereas zero or very few seeds germinated when treated with root extracts of RNAi *Sl-IAA27* plants. Moreover, the addition of GR24 (10^{-11} M) to the RNAi *Sl-IAA27* root extract stimulated *P. ramosa* seed germination as efficiently as when added to the solvent or to the control root extract, showing the absence of germination inhibitors in the RNAi *Sl-IAA27* root extracts (Fig. 4a). These results indicate that root extracts of RNAi *Sl-IAA27* plants were at least 10 times less active than extracts of control roots (Figs 4, S4), therefore suggesting that SL synthesis of RNAi *Sl-IAA27* roots could be strongly down-regulated.

To ask whether the mycorrhizal deficiency of the RNAi *Sl-IAA27* plants could result from this SL down-regulation, we performed a mycorrhization assay with control and *Sl-IAA27*-silenced plants in the presence or not of 10^{-7} M GR24. The addition of the synthetic SLs complemented the mycorrhizal defect of RNAi *Sl-IAA27* plants, especially by increasing the infection frequency as well as arbuscule abundance (Fig. 4b), strongly suggesting that the mycorrhizal defect of these plants was a result of a lower SL biosynthesis.

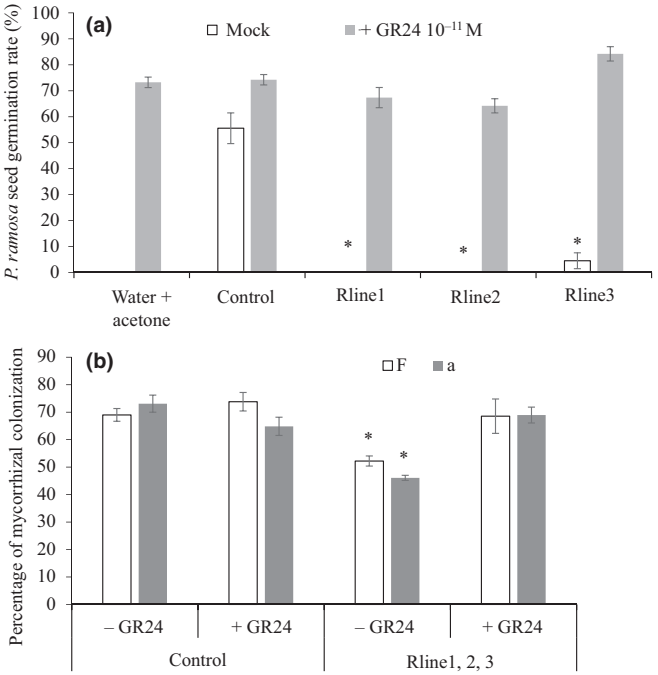

Fig. 4 Quantification of strigolactones in roots of control and RNAi *Sl-IAA27* plants, and effect of GR24 on mycorrhization of control and RNAi *Sl-IAA27* plants. (a) Germination rate of seeds of *Phelipanche ramosa* in response to solvent and to root exudates of control and RNAi *Sl-IAA27* plants, with or without addition of 10^{-11} M of synthetic strigolactone (GR24). (b) Percentage of mycorrhizal colonization in roots of control and RNAi *Sl-IAA27* plants with or without 10^{-7} M of GR24, 12 wk after inoculation, according to Trouvelot's method (Trouvelot *et al.*, 1986). 'F', frequency of colonization in the root system; 'a', arbuscule abundance (percentage) in the colonized root sections. Four replicates per RNAi *Sl-IAA27* line were used here and mean values represent the average obtained with the three lines. Error bars represent ± SEM. Asterisks indicate a significant difference between control and RNAi *Sl-IAA27* plants according to Kruskal–Wallis test: (a) $n = 15$, $P < 0.01$; (b) $n = 12$, $P < 0.05$.

Discussion

Here we collected several pieces of experimental evidence suggesting that the auxin-related gene *Sl-IAA27* positively regulates the mycorrhization process of tomato by controlling the SL synthesis via direct or indirect regulation of *NSP1*, a transcription factor that activates the SL biosynthesis genes *D27* and *MAX1* (Liu *et al.*, 2011). Indeed, we showed that the mycorrhizal defect of *Sl-IAA27*-silenced plants was correlated with a down-regulation of *NSP1*, *D27* and *MAX1* expression and arguably with a lower SL content in roots, which could be complemented by exogenous GR24 treatments.

We demonstrate for the first time the importance of an Aux/IAA in the regulation of SLs biosynthesis, showing an additional crosstalk link between auxin and SL (Foo, 2013; Koltai, 2015). Given that Aux/IAAs are known to interact with ARF partner proteins, preventing them from binding to target promoters, we can speculate that *Sl*-IAA27 represses an ARF that acts as a repressor of *NSP1* expression. This repressor ARF remains to be identified, and it would be interesting to check the occurrence of this regulation in nonmycotrophic species, such as *Arabidopsis thaliana*.

Interestingly, we found that *Sl-IAA27* expression is induced by the fungus very early in the mycorrhizal interaction, even before any root–fungus physical contact. We hypothesize that this induction is caused by some diffusible or volatile fungal signals and may result in the up-regulation of *Sl-NSP1*, thereby promoting *Sl-D27* and *Sl-MAX1* expression and SL synthesis in the roots. This would lead to an increase in SL content in root exudates and to the activation of the fungus metabolism in the rhizosphere (Besserer *et al.*, 2006, 2008).

We have previously shown in *M. truncatula* the importance of *NSP1* in the mycorrhization process (Delaux *et al.*, 2013). The frequency of the fungal entries and the abundance of the arbuscules (with no change in their morphology) were reduced in the *nsp1* mutant, similar to the mycorrhizal phenotype of *Sl-IAA27*-RNAi lines. Given that NSP1 positively regulates *D27* and MAX1 expression (Liu *et al.*, 2011), Delaux *et al.* (2013) speculated that the *nsp1* phenotype could result from a strong decrease in SL biosynthesis affecting fungal stimulation. Here, in tomato, we add some evidence to support this hypothesis: the

perturbation of *NSP1* expression, via the silencing of *IAA27*, has a negative impact on AM colonization that can be partially reversed by the addition of exogenous GR24. It would be interesting to determine whether a mutation of the ortholog of *Sl-IAA27* in *M. truncatula* also down-regulates *Mt-NSP1* expression and perturbs the mycorrhization and the nodulation processes.

Furthermore, the fact that the silencing of *Sl-IAA27* only affects the expression of *Sl-NSP1* and of the NSP1 target genes *Sl-D27* and *Sl-MAX1*, but not of *Sl-CCD7* and *Sl-CCD8*, points to the specificity of the *Sl*-IAA27 action. Intriguingly, the expression of *Sl-CCD7* (but not of *Sl-CCD8*) was found to be up-regulated during mycorrhization, suggesting the occurrence of another IAA27-independent signaling pathway for the activation of *Sl-CCD7* expression in mycorrhizal tomato (López-Ráez *et al.*, 2014).

Our results indicate that in the root the presence of the fungus would switch off the transcription of *Sl-IAA27* locally while up-regulating this expression systemically in as yet uncolonized root tissue. Further investigation will be needed to determine if this *IAA27* transcriptional activation is a result of intra- and/or extraradical diffusible fungal signals and what role this activation could have in the mycorrhization process. The local down-regulation of *Sl-IAA27* transcription in colonized root tissue is reminiscent of previous observation of a strong localized activation of DR5-GUS, an auxin-reporter construct, in arbuscule-containing cells (Etemadi *et al.*, 2014). Given that *Sl-IAA27* expression of tomato can be down-regulated by treatment with exogenous auxin, at least in 12-d-old seedlings (Bassa *et al.*, 2012), the lack of *Sl-IAA27* expression in the inner cortex and the vascular tissue of colonized root sections might be the result of an activation of auxin signaling in neighboring tissues.

We assume that a clear difference has to be made between the early colonization stages, when fungal growth has to be stimulated, and the later colonization stages, when mycorrhization and trophic exchanges have to be tightly controlled and balanced (notably to minimize the carbon cost for the host plant; Peng *et al.*, 1993). During these late colonization stages, when the plant is well colonized, it is commonly known that SL content in roots decreases (Lendzemo *et al.*, 2009; López-Ráez *et al.*, 2011, 2014), while auxin content increases (reviewed in Fusconi, 2014). Here we speculate that the auxin-mediated down-regulation of *Sl-IAA27* transcription in colonized root sections, by negatively regulating *NSP1* expression and SL synthesis, participates in the complex process of autoregulation of mycorrhization and perhaps also in the process of arbuscule degeneration.

The present study illustrates the importance of careful spatiotemporal analyses in understanding the regulation mechanisms underlying the complex developmental process of mycorrhization. Further studies are necessary to fully understand why and how *Sl-IAA27* expression is regulated at different stages of the mycorrhization process.

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Author contributions

J-P.C., C.A., M.B. and G.B. planned and designed the research; B.G. and M.E. performed experiments; J-P.C., B.G. and G.B. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Localization of *Sl-IAA27* expression in roots of 4-wk-old nonmycorrhized tomato plants.

Fig. S2 Root architecture of control and RNAI *Sl-IAA27* tomato plants.

Fig. S3 Control expression analyses.

Fig. S4 Germination rate of seeds of *Phelipanche ramosa* in response to solvent and to a range of concentrations of synthetic strigolactones (GR24).

Table S1 List of primers used, with the corresponding gene annotation