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Research article

Knock-down of a member of the isoflavone reductase gene family impairs plant growth and nodulation in *Phaseolus vulgaris*

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ABSTRACT

Flavonoids and isoflavonoids participate in the signaling exchange between roots of legumes and nitrogen-fixing rhizobia and can promote division of cortical cells during nodule formation by inhibiting auxin transport. Here, we report the characterization of a member of the common bean isoflavone reductase (EC 1.3.1.45, PvIFR1) gene family, an enzyme that participates in the last steps of the biosynthetic pathway of isoflavonoids. Transcript levels of PvIFR1 were detected preferentially in the susceptible zone of roots, augmented upon nitrogen starvation and in response to *Rhizobium etli* inoculation at very early stages of the interaction. Knockdown of PvIFR1 mediated by RNA interference (RNAi) in common bean composite plants resulted in a reduction of shoot and root length. Furthermore, reduction of PvIFR1 mRNAs also affected growth of lateral roots after emergence, a stage in which auxins are required to establish a persistent meristem. Upon inoculation, the number of nodules formed by different strains of *R. etli* was significantly lower in IFR RNAi than in control roots. Transcript levels of two auxin-regulated genes are consistent with lower levels of auxin in PvIFR1 silenced roots. These results suggest a complex role of PvIFR1 during plant growth, root development and symbiosis, all processes in which auxin transport is involved.

1. Introduction

Legume plants are able to establish a symbiotic interaction with nitrogen-fixing bacteria collectively known as rhizobia. This mutually beneficial association accounts for most of the nitrogen incorporated biologically into natural and agronomic ecosystems. Reduction of nitrogen takes place inside the nodule, a new organ developed predominantly in the root system to allocate bacteria and provide the microanaerobic environment required for nitrogenase activity. The complex interaction between these prokaryotic and eukaryotic organisms involves several signaling molecules that are secreted and recognized by the symbiotic partners as a previous step for bacterial penetration and nodule development, two separated but synchronized processes that occur on the root [1].

Flavonoids and isoflavonoids are secondary metabolites produced as part of the phenylpropanoid metabolism that play multiple roles in plant biology, acting as antimicrobial compounds, attractants of pollinators, UV protection molecules, pigments, etc [2]. Previous studies revealed that flavonoids play essential roles at different stages of the symbiotic interaction between legumes and rhizobia. The first role is played in the rhizosphere, where plants secrete (iso) flavonoids when nitrogen in the soil is scarce [3]. Perception of these compounds by rhizobia activates the transcription of bacterial nodulation genes, leading to the synthesis of Nod factors [4]. This exchange of signals between both symbionts constitutes a first level of host specificity. After attachment of bacteria to the surface of the root hair, a microcolony is formed and infection proceeds through a tubular structure formed by invagination of the trichoblast plasma membrane, called the infection thread (IT) [5]. Continuous secretion of (iso) flavonoids is necessary to maintain Nod factor production during the progression of bacteria inside the IT, providing a second layer of host specific recognition [6,7]. The third proposed role of (iso) flavonoids during nodulation also takes place inside the root and is related to the well-known capacity of (iso) flavonoids to inhibit auxin transport [8]. Re-activation of cortical cell divisions during nodule

Abbreviations: bp, base pair; CHS, chalcone synthase; DPI, days post infection; ESTs, expressed sequence tags; GFP, green fluorescence protein; IFR, isoflavone reductase; IFS, isoflavone synthase; IT, infection thread; LR, lateral root; ORF, open reading frame; qRT-PCR, reverse transcription followed by quantitative PCR; RNAi, RNA interference.

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organogenesis is preceded by an inhibition of auxin transport produced by flavonoids [9]. Also, flavonoid-deficient roots generated by post-transcriptional gene silencing of the enzyme chalcone synthase (CHS) in *Medicago truncatula* displayed enhanced auxin transport, resulting in plants unable to form nodules [10]. On the other hand, soybean roots silenced in the isoflavone synthase (IFS) gene showed reduced nodulation, but inhibition of auxin transport was not essential for nodule organogenesis, since strains of *Bradyrhizobium japonicum* hypersensitive to flavonoids formed nodules normally in IFS silenced roots [6]. It has been proposed that this apparent contradiction is caused by the difference between indeterminate and determinate types of nodules [7,11]. Indeterminate nodules, produced by temperate legumes as alfalfa and pea, are cylindrical and grow by cell division localized in a persistent meristem, whereas determinate nodules, produced by tropical legumes as soybean and common bean, are spherical organs originated by a transient cell division followed by cell enlargement. Indeterminate nodules arise from the inner cortex and the pericycle, whereas determinate nodules are formed by cell divisions in the outer cortex.

In a previous work we screened for genes involved in the strain-specific response in the common bean (*Phaseolus vulgaris*)–*Rhizobium etli* interaction [12]. We used a suppressive subtractive approach to identify differential cDNA clones from a Mesoamerican cultivar of common bean inoculated with an efficient strain of *R. etli*, SC15 (tester), compared with plants inoculated with a less efficient strain, 55N1 (driver). One of these differential cDNA clones, PvRHS24-07, showed high sequence similarity with isoflavone reductases (IFR, EC 1.3.1.45) from other species and was highly expressed in roots grown in the absence of nitrogen, as compared with aerial tissue and nodules. Moreover, transcripts of this gene accumulated early after infection with the efficient strain SC15, but not in response to 55N1. The IFR enzyme is part of the isoflavonoid biosynthetic pathway, acting in the reduction of the 2,3 bond of the central ring of isoflavonoids. Subsequent reactions give rise to more complex compounds, as prenylated isoflavones, glycosides, rotenoids, pterocarpans, 3-arylcoumarins and coumestans [13,14]. In the present study we used a reverse genetic approach in order to understand the role of the IFR in the symbiotic interaction between common bean and its symbiotic partner *R. etli*. Post-transcriptional gene silencing of this IFR, named PvIFR1, by RNA interference (RNAi) in composite plants showed a complex phenotype, affecting plant growth, lateral root elongation and the number of nodules developed upon inoculation with high or low efficient strains of *R. etli*.

2. Results

2.1. PvRHS24-07 encodes for a putative isoflavone reductase

As previously reported, PvRHS24-07 was a partial cDNA clone that showed a high percent of sequence identity with IFRs from other plant species [12]. In order to obtain the full length sequence of this clone, we performed a BLAST search against the DFCI bean gene index database. The ESTs with high sequence similarity were assembled with PvRHS24-07, resulting in a consensus sequence with an ORF of 924 base pairs (bp), encoding a putative protein of 307 amino acids, with an estimated molecular weight of 33.9 kDa and an isoelectric point of 5.7. A BLAST search against the recently released version 1.0 of the *P. vulgaris* genome (http://www.phytozome.org/) identified the transcript Phvul.002G033300 as the region that displayed the highest sequence identity (98.49%). The predicted transcript includes 5′ and 3′ untranslated regions of 167 bp and 196, respectively. Other sequences encoding putative reductases were identified in the common bean genome, displaying amino acid sequence identities that ranged from 86.4% to 37.3%. Two clusters of 4 genes each were identified in chromosomes 2 and 11, suggesting recent amplification events at these locations. A phylogenetic analysis of the common bean sequences is shown in Fig. 1A. The protein encoded by Phvul.002G033300 was aligned with IFRs from other species, including legume and non-legume plants (Fig. 1B), revealing a high sequence conservation along the entire protein. Percentages of sequence identity ranged from 88% to 84% for the legumes soybean and medic barrel (*M. truncatula*) to 69% and 64% for non-legumes, as *Vitis vinifera* and Arabidopsis, respectively. Based on these analyses, PvRHS24-07 was renamed as PvIFR1 for *P. vulgaris* IsoFlavone Reductase 1.

2.2. PvIFR1 is highly expressed in the susceptible zone of the root

We previously reported that PvIFR1 (PvRHS24-07) was highly expressed in roots and, at a lesser extent, in 7-day old nodules as compared with shoot tissues [12]. Reverse transcription followed by quantitative PCR analysis (qRT-PCR) showed that PvIFR1 is repressed about 70% when plants were grown in Fahraeus medium supplemented with nitrogen as compared with plants grown in the absence of nitrogen (Fig. 2A). Considering that this gene was identified in a screening designed to identify root transcripts that accumulate in response to infection with high efficient rhizobial strains, we aimed to explore the expression pattern in the different regions of the root. Zone I has been defined as the hairless region that extends from the tip to the emerging root hairs, zone II corresponds to the area were root hairs are actively growing and zone III contains only fully elongated root hairs. Since nodulation requires redirectioning of the root hair polar growth, infection occurs mainly in the zone II, also called the susceptible zone. For this reason, it is expected that genes involved in symbiosis will show increased expression in this part of the root. qRT-PCR analysis showed that PvIFR1 mRNAs accumulate in the zone II of uninoculated roots at higher levels than in zones I and III (Fig. 2B). The expression of PvIFR1 was compared with putative orthologs from other legumes using publicly available expression data (see Materials and methods). Transcript accumulation was higher in roots than in aerial tissues of *M. truncatula* (Fig. 2C) and soybean (Fig. 2E), resembling the expression pattern observed in common bean. More interestingly, mRNA levels of IFR were higher in zone II than in the zone I in *M. truncatula* and *Lotus japonicus* (Fig. 2C and D), whereas in soybean the expression of the best homolog genes was higher in the whole root than in the tip, indicating that the region of the root that contains zones II and III accumulated high levels of IFR transcripts (Fig. 2E). On the other hand, the best homolog from Arabidopsis showed high levels of mRNA accumulation in flowers, seeds and lateral roots (Fig. 2F). This comparative analysis reveals that the expression pattern of IFR is similar in legume plants, supporting a role for this enzyme in the symbiotic association with rhizobia.

2.3. Shoot, root and lateral root elongation is affected in IFR RNAi composite plants

To gain insight into the function of PvIFR1 in common bean, we used a reverse genetic approach. Post-transcriptional gene silencing was generated in roots expressing a hairpin-structured RNA that acts as a precursor of small RNAs complementary to PvIFR1 (IFR RNAi). *Agrobacterium rhizogenes* inoculation in the cotyledon insertion region produced the emergence of transgenic hairy roots in the infection area. After these roots were developed, the wild type root system was cut, resulting in a composite plant with transgenic roots and an untransformed aerial part. Reduction of PvIFR1 mRNA levels ranged from 60 to 85% in different roots as compared with roots transformed with a GUS RNAi construct used

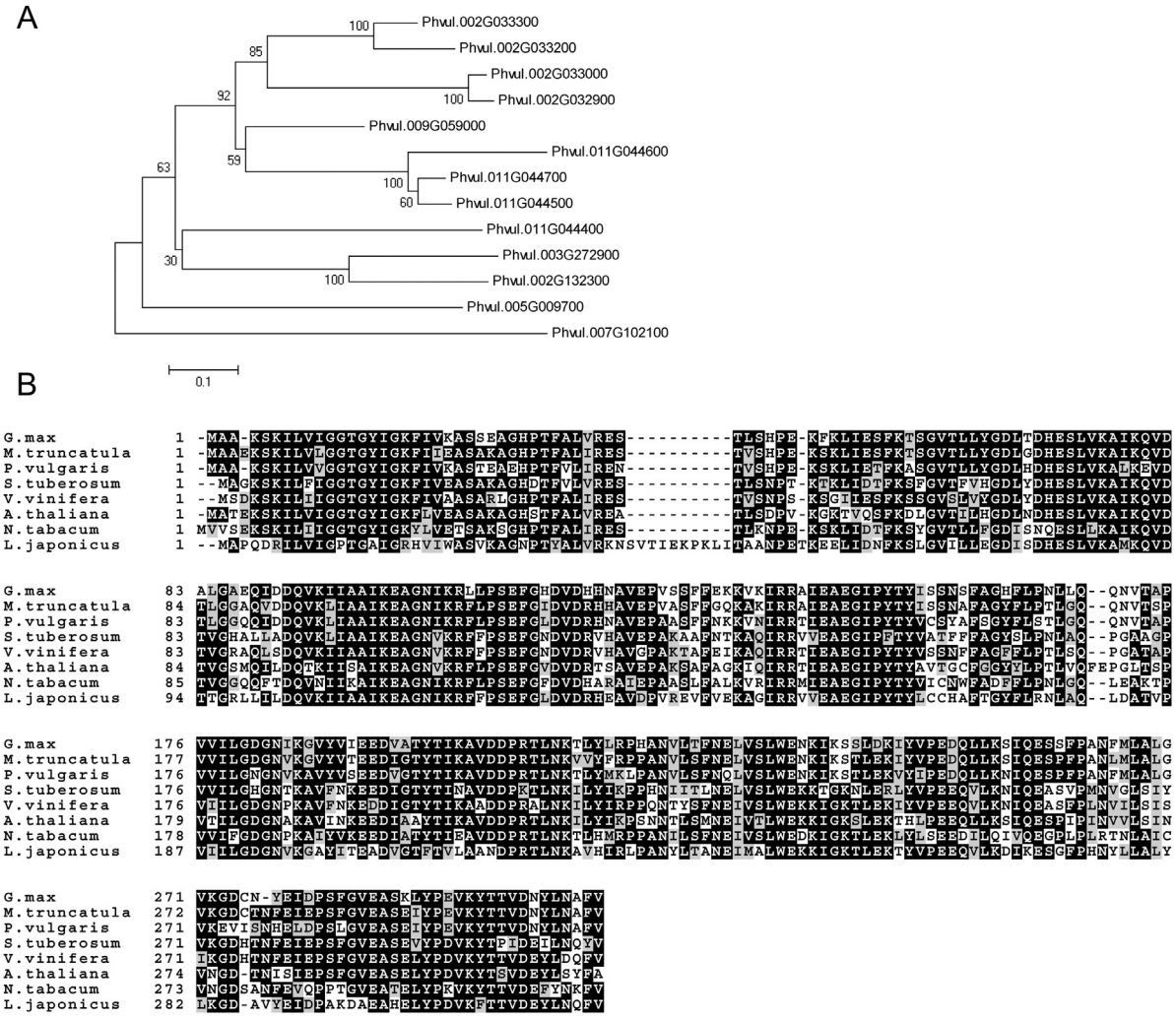

Fig. 1. A. Phylogenetic analysis of PvIFR1 (Phvul.002G033300) and homolog sequences identified in the common bean genome. The relationship between sequences was inferred using the Neighbor-joining method. Numbers indicate the percentage of trees in which the associated taxa cluster together (1000 replicates in a bootstrap analysis). Evolutionary distances are expressed as substitution of aminoacids per site. B. The multiple alignment of PvIFR1 with homologous proteins from other plant species was made with the *Clustal W* program. Black residues represent identical amino acid, whereas gray marks indicate conservative substitutions. Numbers on the left indicate the amino acid position on the protein. Accession numbers were P52578 for *Solanum tuberosum*, NP_565107.1 for *Arabidopsis thaliana*, AAF17577.1 for *Glycine max*, BAF34847.1 for *Lotus japonicus*, ACJ84329.1 for *Medicago truncatula*, P52579.1 for *Nicotiana tabacum*, and CAI56335.1 for *Vitis vinifera*.

as control (Fig. 3A). In order to assess the specificity of the RNAi-mediated silencing, we quantified the mRNA levels of the closest homolog genes (Fig. 3A). Phvul.002G033200 showed a reduction that was not statistically significant, whereas Phvul.002G032900 and Phvul.002G033000, the following closest homologs of PvIFR1, did not show a reduction in their mRNA levels in IFR RNAi as compared with control roots. We also selected two other genes that belong to other clades (see Fig. 1) to verify that silencing was restricted to PvIFR1. As seen in Fig. 3A, Phvul.009G059000 and Phvul.002G132300 did not show differences between IFR and GUS RNAi, suggesting that the designed RNAi produced post-transcriptional gene silencing of PvIFR1, with a partial effect on the closest homolog gene, Phvul.002G033200.

Visual examination of the plants revealed substantial differences between IFR and GUS RNAi plants (Fig. 3B). Both hairy root and shoot lengths were reduced by 30–40% in IFR RNAi plants as compared with controls (Fig. 3C and D). The reduction in shoot length was unexpected since composite plants have transgenic roots, whereas the aerial part of the plant is wild type. We consider unlikely that poor shoot development is a consequence of the shortness of the hairy roots because the phenotype was evident very early, when growth of the plant was still supported by the wild type root (see Material and methods). No differences in mRNA levels of PvIFR1 were observed between shoots of IFR and GUS RNAi composite plants, indicating that the shoot phenotype was not caused by systemic silencing of PvIFR1 (Fig. 3E).

To further investigate whether silencing of PvIFR1 had an impact on root architecture, we examined and quantified the number of emerged lateral roots (LRs) per cm of root (LR density) in IFR and GUS RNAi plants. In *A. rhizogenes* transformation, each hairy root represents an independent transformation event; hence, we took advantage of the *gfp* gene present as a selectable marker in the IFR RNAi construct to discriminate between transgenic and adventitious roots. Fluorescence microscopy revealed a high percentage of transformed roots, which ranged from 80 to 90% of all the roots emerged at the site of *A. rhizogenes* inoculation. As illustrated in Fig. 4A–C, approximately 64% of the transgenic roots expressing the IFR RNAi formed very short LRs (less than 1 mm, indicated by arrows in Fig. 4A), whereas the untransformed non-fluorescent roots showed LRs similar in length to those formed in

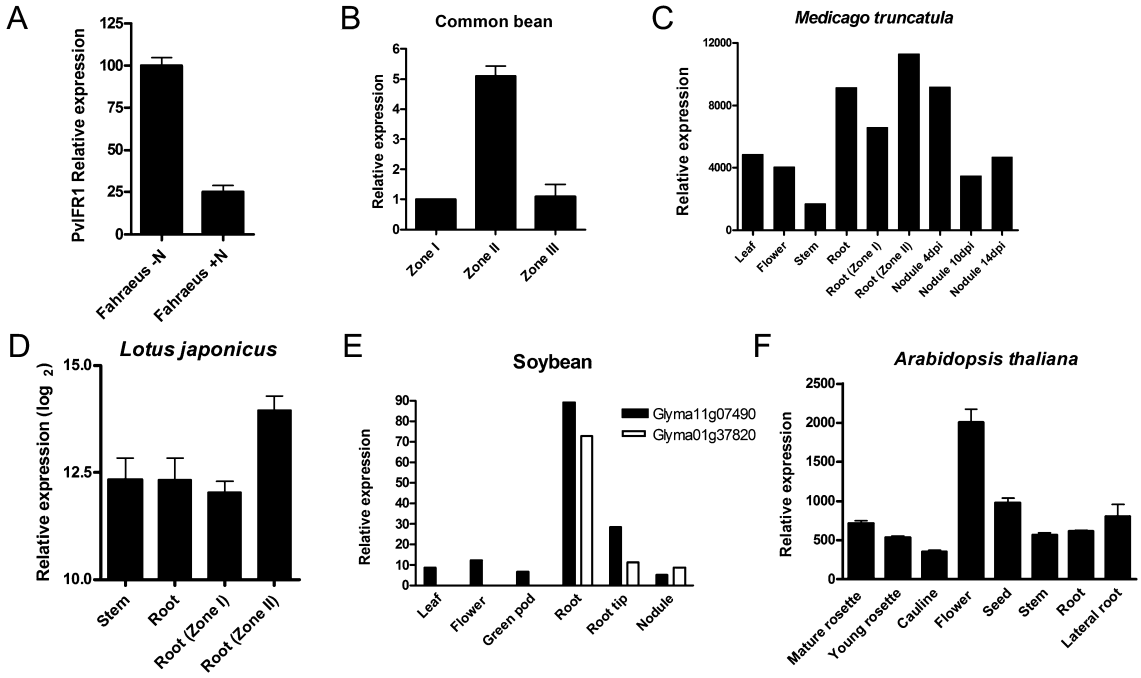

Fig. 2. Relative expression of IFR in different organs of leguminous plants and Arabidopsis. A and B. qRT-PCR analysis of PvIFR1. Steady-state levels of the PvIFR1 transcript were estimated in roots of plants grown in the absence (Fahraeus − N) or presence (Fahraeus + N) of nitrogen (A) or from zones I (hairless), II (containing elongating root hairs) and III (mature root hairs) of the root (B). Transcript levels were normalized to EF1α and presented relative to the value measured in zone I. Error bars represent SD of three technical replicates. C. Relative expression levels of *M. truncatula* IFR best homolog (Mtr8604.1.S1_at) in leaf, flower, stem, zone I, II and whole root and nodules of 4, 10 and 14 DAI. D. Expression analysis of PvIFR1 best homolog in *L. japonicus* (chr2.CM0249) in stem, zone I, II and the whole root. Data is shown in arbitrary units derived from the normalization of microarray data. E. Relative expression levels of soybean IFR homologs (Glyma11g07490 and Glyma01g37820) in leaf, flowers, green pods, whole root, root tip and nodules. Data is shown in arbitrary units derived from normalization of mRNA sequencing data. F. Relative expression value of an Arabidopsis IFR like gene (At1g75280) in mature and young rosette leaf, cauline leaf, flower, seed, stem, root and lateral root tissue. Data is shown in arbitrary units derived from the normalization of microarray data. Error bars represent SD.

GUS RNAi transformed roots (Fig. 4D). The cell organization of these LR primordia did not revealed obvious differences between IFR RNAi and LRs formed by GUS RNAi roots at a comparable stage (Fig. 4E and F). Reduction of PvIFR1 levels did not affect significantly the density of LRs, but provoked a reduction of the length of LRs after emergence (Fig. 4G and H). Many of the LRs in IFR RNAi transgenic roots seem to cease growing after emergence (see Fig. 4A). A comparison of IFR and GUS RNAi roots revealed that silencing of PvIFR1 produced an increase of 4 fold of LRs that stop growing after emergence (Fig. 4I), which is reflected in the average length of the emerged LRs (Fig. 4H). All these results suggest that PvIFR1 may play a role in the growth of post-emerged LRs.

2.4. IFR RNAi affects the number of nodules

PvIFR1 was identified in a screening of genes that are differentially expressed in a highly efficient symbiotic interaction between *R. etli* and *P. vulgaris*; thus, the possible role of this gene in nodule formation was also investigated. No obvious differences were observed between nodule primordia formed in GUS and IFR RNAi roots (Fig. 5A and B). However, the number of nodules formed was significantly reduced in IFR RNAi roots as compared with that of control roots (Fig. 5C). Differences were observed as early as 5 days post inoculation (DPI), when nodules became clearly visible in common bean, and persisted at 10 DPI. A similar reduction in the number of nodules was observed in IFR RNAi as compared with control plants upon inoculation either with the high efficient strain SC15 (reduction of 53.3%) or the low efficient strain 55N1 (63.5%; Fig. 5C). This result suggests that products of the PvIFR1 enzymatic activity are required for nodule organogenesis in common bean, and furthermore, that this requirement is independent of the efficiency of the interaction.

Nitrogen availability is directly associated to the production of aboveground biomass. Therefore, shoot dry weight of the plants was determined in uninoculated and *R. etli* inoculated plants. Comparison of uninoculated IFR RNAi plants respect to GUS RNAi controls showed a reduction of approximately 40% in the aerial biomass when these plants were grown in the absence of nitrogen (Fig. 5D), consistently with the reduced shoot development of IRF RNAi plants shown in Fig. 3B. Upon inoculation with either SC15 or 55N1 strain, IFR RNAi displayed a reduction in dry weight of approximately 24% compared with GUS RNAi plants; however, this difference was smaller that the 40% observed between uninoculated plants. Despite the reduction in the numbers of nodules observed in IFR RNAi plants, nodules of these plants were able to fix nitrogen, since dry weight was much higher in inoculated than non-inoculated plants. In addition, shoot dry weight was lower in plants inoculated with the low efficient strain 55N1 than in those inoculated with strain SC15, either in IFR or GUS RNAi plants. This result supports the hypothesis that the requirement of PvIFR1 is independent on the efficiency of the *R. etli* strain.

2.5. Expression of auxin-regulated genes in IRF and GUS RNAi roots in response to rhizobia

The phenotype observed in IFR RNAi composite plants, i.e. poor shoot and root development, as well as reduced lateral root growth and nodule formation, could be related to defects in auxin distribution. To further explore the involvement of auxin in the pleiotropic phenotype observed, we measured the level of two transcripts that respond positively and negatively to this hormone in both uninoculated and rhizobia inoculated GUS and IFR RNAi roots. First, we confirmed silencing of IFR RNAi (composite plants showed a reduction of PvIFR1 mRNA compared with GUS RNAi

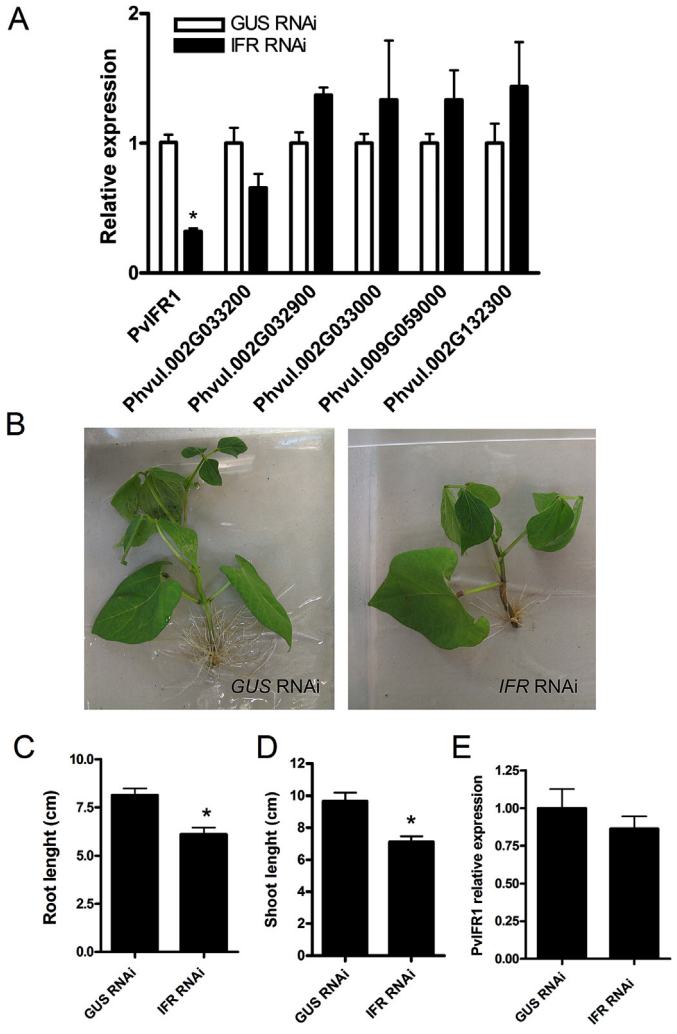

Fig. 3. Effect of IFR RNAi expression on plant growth. A. qRT-PCR analysis of PvIFR1 (Phvul.002G033300) and homolog genes in hairy roots from NAG12 composite plants, expressing GUS or IFR RNAi. Transcript levels were normalized to EF1α. Error bars represent SD of at least three technical replicates. The asterisk indicates a value that is significantly different than its control in an unpaired two-tailed *t*-test with $P < 0.05$. B. Compared phenotype of GUS RNAi (left) and IFR RNAi (right) uninoculated composite plants. C and D. Root and shoot length were measured in GUS or IFR RNAi composite plants, respectively. Error bars represent SEM of at least 25 independent measurements and is representative of three independent biological experiments. Asterisks indicate values significantly different than those of GUS RNAi roots in an unpaired two-tailed *t*-test with $P < 0.05$. E. PvIFR1 transcript levels determined by qRT-PCR in leaves from GUS or IFR RNAi composite plants. Transcripts levels were normalized to EF1α. Error bars represent SD of three technical replicates.

plants) and verified that control plants responded to SC15 and 55N1 inoculation in terms of IFR transcript levels as previously reported for wild type roots (Fig. 6A) [12]. Then, we selected GH3 as a gene that responds positively to auxin and whose promoter from soybean has been extensively used as a marker of auxin accumulation [16,17]. The best homolog of the soybean GH3 gene was identified in the common bean EST database and used to design primers for qPCR. As expected, PvGH3 mRNA levels dramatically increased by auxin treatment in wild type plants (Fig. 6B). Transcripts of PvGH3 were reduced by 42% in uninoculated IFR RNAi plants as compared with controls (Fig. 6B), indirectly suggesting that auxin levels are reduced in IFR RNAi roots. On the other hand, PvRHS24-02 was identified in the same screening than PvIFR1 and is homologous to ARG10, a gene down-regulated by auxins [18]. Transcript levels of PvRHS24-02 were reduced approximately 75% by auxin treatment (Fig. 6C). Comparison of IFR and GUS RNAi roots showed that silencing of PvIFR1 produced a 50% increase of PvRHS24-02 mRNAs (Fig. 6C). The transcriptional response of PvGH3 and PvRHS24-02 is consistent with reduced levels of intracellular auxin in IFR as compared with GUS RNAi roots. Interestingly, both genes were up-regulated by strains of *R. etli* in IFR and GUS RNAi roots. In the case of PvRHS24-02, transcripts accumulated at higher levels with the more efficient strain SC15 than with 55N1 as previously reported [12]. Moreover, the effect of PvIFR1 silencing and rhizobia inoculation on PvRHS24-02 transcript accumulation seems to be additive, whereas they have an opposite effect in the case of GH3. These results suggest that expression of GH3 and PvRHS24-02 during the symbiotic interaction is positively regulated by signals from the bacteria, independently of the levels of PvIFR1 transcripts and/or auxin concentration in the whole root.

3. Discussion

In this work, we post-transcriptionally silenced PvIFR1, a gene that encodes for a NAD(P)H dependant oxidoreductase that is part of the biosynthetic pathway of isoflavonoids. Our results showed that reduction of PvIFR1 levels affects the development of aerial and root organs in *P. vulgaris*. Shoots of composite plants transformed with IFR RNAi were reduced in size as compared to control plants, indicating that silencing of PvIFR1 in the emerging hairy roots could affect growing of the aerial tissue. We did not detect systemic silencing of PvIFR1 in non-transformed tissue, suggesting that signals other than small RNAs produced by IFR RNAi hairy roots can move to the shoot and affect the development program of leaves and stems. Transcript accumulation of two genes that respond to auxin suggests that auxin present in the whole root can be negatively affected in IFR RNAi plants, probably as a consequence of direct inhibition of the auxin transport or by an indirect effect on the hormone distribution. This evidence is an indication of the possible participation of auxin in the observed pleiotropic phenotype detected in IFR RNAi silenced plants.

During the symbiotic association, formation of nodules was reduced in PvIFR1 silenced plants with either of the *R. etli* strains used in this work. Considering that plant growth is also altered, these results should be interpreted with caution, since the reduction of shoot development might limit the availability of photosynthates, indirectly affecting the number of nodules formed by IFR RNAi plants. The fact that reduction of both, nodule number and dry weight, was similar for SC15 and 55N1, argues against a role of this enzyme in the affinity between the host and particular rhizobial genotypes, as it could be expected from the screening that led to the identification of this cDNA clone. Nodules formed by IFR RNAi roots were normal in size and color, and early events of the legume rhizobia interaction, such as root hair curling and IT formation and progression, were not affected in these nodules (data not shown). This is in accordance with previous studies in *M. truncatula* and soybean, where depletion of flavonoids did not affect the initial steps of bacterial infection [6,10].

The steady-state levels of mRNA observed in common bean and other legumes support a role of the IFR in roots, particularly in the differentiation zone, where nodules and lateral roots are formed. In the non-leguminous plant *Arabidopsis thaliana*, the pattern of mRNA accumulation is different, suggesting that IFR might be involved in other function of the plant, such as the defense response or pollinator attraction. Genes with high sequence similarity to IFRs have been identified in non-legume plants and are called isoflavone reductases like (IRL) [19]. In most cases, the actual substrates and products of the enzyme are not known, since IFRs from different plants can render different products in each species. The presence of several genes with high sequence identity to IFRs in

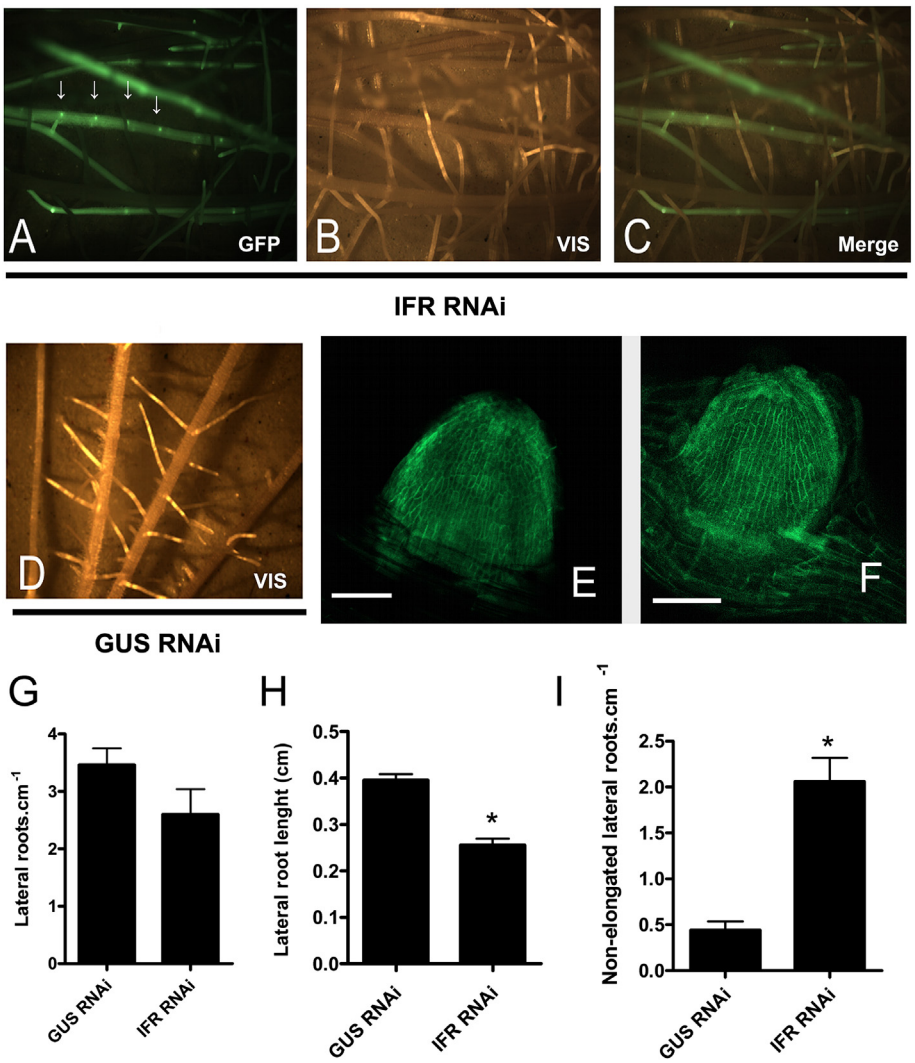

Fig. 4. The number and length of LRs are affected in IFR RNAi composite plants. Roots from IFR RNAi composite plants were visualized under UV (A) or visible light (B), and images were merged (C). Arrows in A indicate LRs that have not elongated after emergence on fluorescent transgenic roots. Normal LRs can be observed in non-transgenic roots (non-fluorescent) (C) and GUS RNAi composite control plants (D). Cell-organization of emerged lateral roots was visualized by confocal fluorescence microscopy. Images were obtained by z integration of 5 μm sections obtained from GUS (E) or IFR RNAi plants (F). Bars, 100 μm. LR density (G), length of LR (H) and density of LRs that do not elongate after emergence (I) were determined in GUS and IFR RNAi composite plants. Error bars represent SEM of at least 25 independent measurements and are representative of three independent biological experiments. Asterisks indicate values different than those of GUS RNAi roots in an unpaired two-tailed *t*-test with $P < 0.05$.

the genome of common bean suggest that the resulting reductases can function on different substrates, or, alternatively, they can be expressed in different tissues or in response to other environmental stimuli in which the response of the plant involves changes in the profile of isoflavonoids.

Plants expressing IFR RNAi produced lateral roots that form properly, but are unable to grow normally after emergence. A similar phenotype was observed in *rml1* [20], *alf3* [21] and *mdr1* [22] mutants of *A. thaliana*. This stage of elongation of lateral roots seems to depend on the acropetal transport of auxin to the emerged lateral root, whereas reduction of the basipetal transport has no effect on this developmental stage [22]. Here, we found that two auxin regulated transcripts have an altered expression pattern in IFR RNAi as compared to control GUS RNAi roots, suggesting that either auxin concentration or distribution is affected by silencing of PvIFR1 in common bean. When plants were completely depleted of isoflavonoids by silencing of IFS, contradicting results were observed in soybean [6] and Medicago [23] in terms of auxin transport, suggesting that the mechanisms can differ among species. Also, levels of GH3 increased in IFS RNAi soybean plants, but decreased when the chalcone reductase was silenced [6]. Considering that we observed a reduction of GH3 levels, it is possible to speculate that silencing of distinct enzymes of the phenylpropanoid pathways can lead to accumulation or reduction of particular isoflavonoids with different effects on auxin transport.

As previously mentioned, flavonoids can modulate the establishment of auxin gradient blocking auxin efflux from the cell [24,25]. Localization of PIN proteins, which act as auxin transporters, is altered in mutants that are deficient or overproduce (iso) flavonoids [26]. This intracellular relocalization of PIN transporters is regulated by phosphorylation of vesicular trafficking proteins [27]. Interestingly, in the same screening that PvIFR1, we identified RabA2, a small GTPase of the Rab family involved in vesicle transport [12], which proved to be required at early stages of the interaction between common bean and *R. etli* [28]. Since it has been reported that monomeric GTPases participate on PIN localization and the flux of basipetal auxin transport [29,30], it will be of interest to explore a possible connection between isoflavonoids and the function of RabA2 during symbiosis.

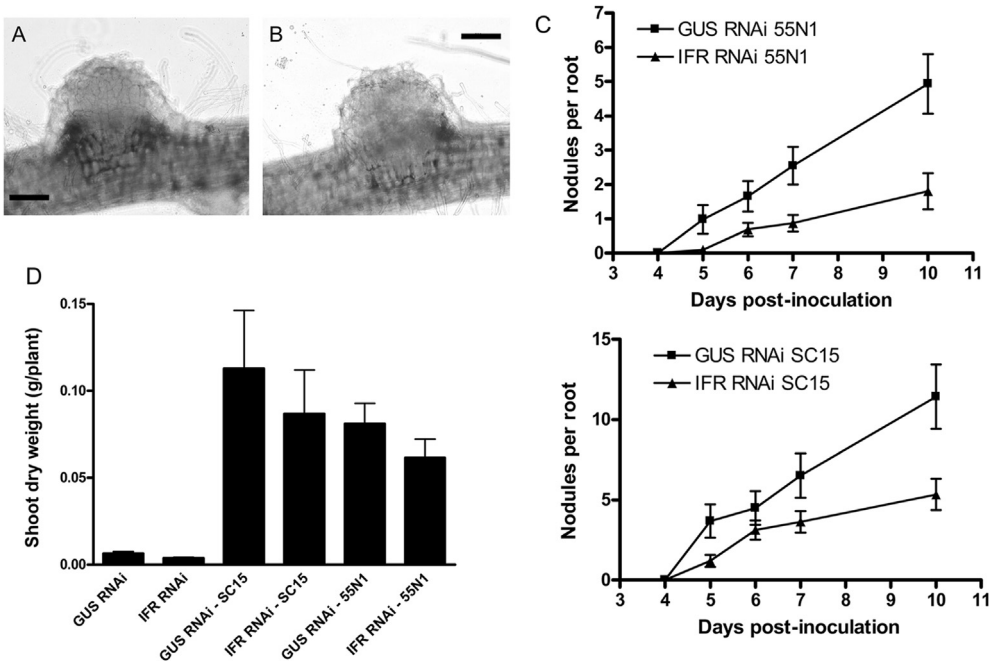

Fig. 5. IFR RNAi plants present a reduced number of nodules. Nodule primordia of 4 DPI with *R. etli* SC15 were similar between GUS (A) and IFR RNAi plants (B). Bars, 500 μm. C. Time-course of nodule formation comparing IFR and GUS RNAi composite plants inoculated with the *R. etli* strains 55N1 or SC15. Values represent the number of nodules per root ($n > 30$) and error bars represent SEM. The experiment is representative of three independent biological replicates. D. Shoot dry weight (g per plant) of GUS and IFR RNAi plants, uninoculated and inoculated with *R. etli* strains SC15 or 55N1. The measurement was performed at 28 DPI. Data represent the average value of 6 plants per treatment and error bars SEM data.

Taking together, our results suggest that PvIFR1 plays a critical role of in the growth of plant organs. It is possible to speculate that the complex phenotype displayed by composite IFR RNAi plants can be a consequence of changes in the isoflavonoid composition or concentration produced in the root, which might affect the establishment of auxin polar gradients, influencing the activity of root and lateral root meristems, nodule formation and even the shoot apical meristem acting through a diffusible signal. Experiments that examine auxin localization and transport in IFR RNAi roots would certainly help to contrast this hypothesis and increase our understanding of the role of isoflavonoids in plant growth and development, including the organogenesis of root nodules.

4. Materials and methods

4.1. Biological material and plant transformation

Seeds of *P. vulgaris* cultivar NAG12 (Mesoamerican) were provided by Susana García Medina (INTA, EEA Salta, Cerrillos, Argentina). *R. etli* strains SC15 and 55N1 were previously reported [31]. Plant growth and transformation were performed essentially as previously described [15,28].

4.2. Plasmid construction

To create a construct for RNAi mediated silencing of PvIFR1, a fragment corresponding to the coding region of this gene was amplified by PCR using the primers 5′-*CACC*GAATTCGGGCTT-GATGTGGAC-3′ and 5′-GCTTCGACGTTTCCATCTCCTAG-3′ and cDNA from common bean roots as template. The four first bases of the forward primer, indicated in italics, were added for cloning into the pENTR/D-TOPO entry vector following manufacture's instructions (Invitrogen, Carlsbad, CA, USA) and recombined into the Gateway compatible destination vector pK7GWIWG2D(II) [32] to produce the IFR RNAi construct. The selected vector carries a screenable marker composed by the *rol*D promoter fused to the coding sequences of the green-fluorescent protein (GFP) linked to the endoplasmic reticulum-targeting signal (EgfpER) and the 35S terminator for early visualization and selection of transgenic roots. The GUS RNAi construct was previously generated [28].

4.3. Phenotypic analysis

Composite plants were generated as described [28]. The main root was cut 1 cm below the inoculation site and then transferred to acrylic boxes containing slanted agar-Fahraeus. Transgenic roots were identified using fluorescence of the GFP encoded in the vector. Phenotypic analysis was performed using composite plants 10 days after transplantation to boxes. Root length was measured from the tip to the site of hairy root emergence on the stem. For shoot length, the distance between the cotyledon insertion zone (where the inoculation with *A. rhizogenes* was performed) to the apex was taken. LR length was measured as the distance between the primary root and the tip of the LR. Lateral and emerging roots were counted and normalized by lineal cm of root. Five roots per plant were selected from 10 independent plants for each construct (GUS or IFR RNAi). Statistical significance was calculated from unpaired two tailed *t*-tests.

Wild type or transgenic roots were inoculated with *R. etli* strains as described [12,28]. The number of nodules per composite plant and per independent transgenic root was counted after 4, 5, 6, 7 and 10 DPI, on 30 independent transgenic roots. Dry weight was recorded 21 DPI as previously described [15].

4.4. Sequence analysis

Sequences with high sequence identity to *P. vulgaris* IFR were obtained from the DFCI gene index (http://compbio.dfci.harvard.

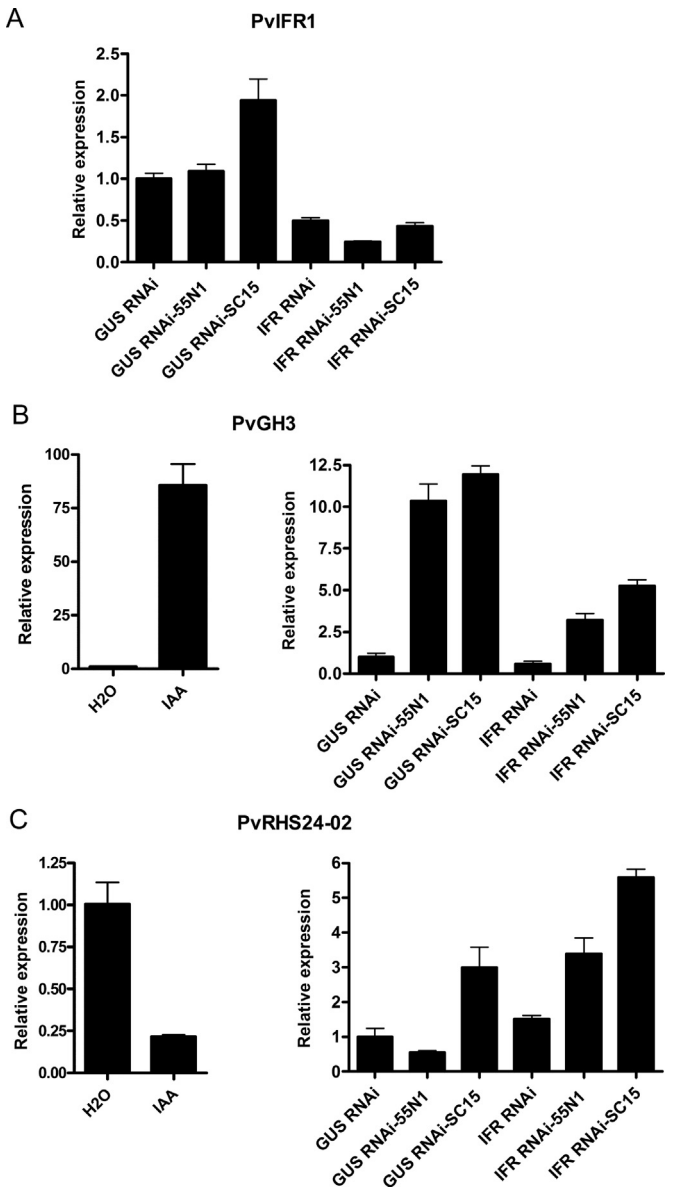

Fig. 6. Expression analysis of PvIFR1 and auxin-regulated genes in IFR RNAi roots. Relative expression levels of (A) PvIFR1, (B) PvGH3 and (C) PvRHS24-02 in roots of composite plants 24 h post-inoculation with *R. etli* strains SC15 or 55N1, or with YEM (control). Auxin treatment (IAA) in wild type plants was included as a control of PvGH3 and PvRHS24-02 regulation in response to the hormone. Roots were treated with a 2 μg/ml solution of indol acetic acid (IAA) during 24 h, whereas controls were treated with water. Transcripts levels were normalized to EF1α and presented relative to the values of control GUS RNAi roots. Error bars represent SD of three technical replicates.

edu/tgi/plant.html) and the version 1.0 of the common bean genome produced by the US Department of Energy Joint Genome Institute, available at Phytozome (http://www.phytozome.org/). We performed a BLASTN search [33] using a partial cDNA sequence named PvRHS24-07 obtained from the subtractive hybridization library [12].

Sequences of IFR from other species were retrieved from genomic databases: *A. thaliana* (http://www.arabidopsis.org/Blast/), *M. truncatula* (http://www.medicago.org/genome/), *G. max* (http://www.phytozome.net/soybean), and *L. japonicus* (http://www.kazusa.or.jp/lotus/). *N. tabacum*, *V. vinifera* and *S. tuberosum* sequences were retrieved from the GenBank non-redundant database in a BLASTX search using the *P. vulgaris* IFR complete nucleotide sequence as the query.

Alignment of the common bean amino acid sequence with IFR sequences from other species was generated with Clustal W and shaded with the BOXSHADE 3.21 software (K. Hofmann and M.D. Baron, pretty printing and shading of multiple-alignment files, 1996; http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic tree was made with MEGA5 using the Neighbor joining method, with a boostrap of 1000 replicates [34].

4.5. Quantitative RT-PCR assays

RNA extraction, cDNA synthesis and qRT-PCR assays were performed essentially as previously described [12]. For each primer pair, the presence of a unique product of the expected size was verified on ethidium-bromide-stained agarose gels after PCR reactions. Absence of contaminant genomic DNA was confirmed in reactions with DNAse-treated RNA as template. Amplification of *P. vulgaris* elongation factor 1α (EF1α) was used to normalize the amount of template cDNA. At least three biological replicates were performed per condition. Conditions and primers used to quantify PvIFR1 (PvRHS24-07), PvRHS24-02 and EF1α were previously described by Peltzer Meschini et al. [12]. Primers were designed from the version 1.0 of the common bean genome available at Phytozome; Phvul.002G033200: 5′-ATCCTGAGGTCAAATACACTAC-3′ and 5′- TAACTAACCAAACGAAAGACAATA-3′; Phvul.002G032900: 5′-TTACCTACAAAACAAGAAAACCA-3′ and 5′-GCTTGTTAACAGTGGG TGGAGTG-3′; Phvul.002G033300: 5′-ACTACGGTGGACGACTATT-3′ and 5′-GCAGCAAGCTAAAGGAA3'; Phvul.009G059000: 5′-GAGCCTTCTTTTGGGGTTGA-3′ and 5′-AAGTAGCAGAAGGCAGAGA-TAAAA-3′; Phvul.002G132300: 5′-AAAAATTGGGAGTTGTTA-3′ and 5′-ATTATTTTCTTCTATGTTTG-3′. Primers for GH3 were designed from the EST contig PUT-181a-Phaseolus_vulgaris-5214 (Plant Genome Database assembling based on the GenBank release 181): 5′-AATTGGCCCAGTTTTTGACAGAA-3′ and 5′-AGAGGGCTTGCA-CATTGGATTTAG-3′.

4.6. Expression analysis in other species

The expression pattern of IFR genes was analyzed in *M. truncatula* (Mtr.8604.1) and *L. japonicus* (chr2.CM0249) using the data available at the *Gene Expression Atlas* (http://mtgea.noble.org/v2) [35] and The Lotus Transcript Profiling Resource (http://cgi-www.cs.au.dk/cgi-compbio/Niels/index.cgi) [36], respectively. Expression data of soybean Glyma11g07490 and Glyma01g37820 were obtained from The Bio-Array resource for Plant Biology using Soybean eFP browser (http://bar.utoronto.ca/efpsoybean/cgi-bin/efpWeb.cgi) and the Arabidopsis expression data for At1g75280 was obtained from GENEVESTIGATOR Plant Biology (https://www.genevestigator.com/gv/plant.jsp).

4.7. Microscopy and imaging

The bright-field and epifluorescence images of transgenic roots and nodule primordia expressing green fluorescent protein GFP were performed using a Leica MZ8 stereo microscope (Leica, Wetzla, Germany). Microscopic observations were performed with a Nikon Eclipse Ti inverted microscope using white and UV light with appropriated filters (Nikon Instruments Inc., Melville, NY, USA). Confocal images of lateral roots were obtained with a Leica SP5 using transformed roots that express GFP from the pK7GWIWG2D(II) vector. Images were captured with the Leica Application Suite software and processed with Image J (W.S. Rasband, Image J, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

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