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Enhancing arbuscular mycorrhiza symbiosis effectiveness through the involvement of the tomato GRAS transcription factor SCL3/SlGRAS18

Jonathan D. Avilés-Cárdenas [a], Nuria Molinero-Rosales [a], Jacob Pérez-Tienda [a], Tábata Rosas-Díaz [b], Araceli G. Castillo [b], José M. García-Garrido [a,*]

[a] Department of Soil and Plant Microbiology, Estación Experimental del Zaidín (EEZ), CSIC, Calle Profesor Albareda n◦1, 18008, Granada, Spain
[b] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora" (IHSM "La Mayora"), Universidad de Málaga-Consejo Superior de Investigaciones Cientificas (UMA-CSIC), Campus Teatinos, 29010, Málaga, Spain

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi improve plant growth, nutrition, fitness and stress tolerance while AM fungi obtain carbohydrates and lipids from the host. This whole process of mutual benefit requires substantial alterations in the structural and functional aspects of the host root cells. These modifications ultimately culminate in the formation of arbuscules, which are specialized intraradical and highly branched fungal structures. Arbuscule-containing cells undergo massive reprogramming to hosting arbuscule and members of the GRAS transcription factor family have been characterized as AM inducible genes which play a pivotal role in these process. Here, we show a functional analysis for the GRAS transcription factor SCL3/SlGRAS18 in tomato. SlGRAS18 interacts with SlDELLA, a central regulator of AM formation. Silencing of *SlGRAS18* positively impacts arbuscule development and the improvement in symbiotic status, favouring flowering and therefore progress in the formation and development of fruits in *SlGRAS18* silenced plants which parallel to a discernible pattern of mineral nutrient redistribution in leaves. Our results advance the knowledge of GRAS transcription factors involved in the formation and establishment of AM symbiosis and provide experimental evidence for how specific genetic alterations can lead to more effective AM symbiosis.

1. Introduction

The reciprocal relationship between plants and microorganisms significantly influences the phenology, ecology and productivity of plants. Notably, in forestry and agriculture, the beneficial symbiotic relationships between plants and microorganisms, such as rhizobacteria and rhizospheric fungi, play a crucial role in augmenting plant growth, improving tolerance to various stresses, and combating both biotic and abiotic challenges. In this context, the mutualistic symbiotic association Arbuscular Mycorrhiza (AM), formed between plant roots and arbuscular mycorrhiza fungi (AMF), are of particular relevance to biofertilization processes and agrobiotechnological techniques to increase plant productivity (Smith and Read, 2008).

AM fungi are soil microorganisms that establish mutual symbiosis with the majority of higher plants. This mutually beneficial interaction is tightly controlled by both organisms, operating at the cellular, molecular, and genetic levels, while the high degree of compatibility between the plant and AMF is a consequence of their long common evolutionary history. In fact, AMF-plant associations played an important role in the initial colonization of land by the ancestors of terrestrial plants (Wang et al., 2010).

Plants benefit from AM formation, which increases their mineral nutrition, growth, fitness and stress tolerance (Clark and Zeto, 2000), while AM fungi obtain carbohydrates and lipids from the host plant (Bago et al., 2003; Luginbuehl et al., 2017). This whole process of mutual benefit, intricately tied to environmental and biological factors (Smith and Read, 2008), requires substantial structural and functional alterations in host root cells. This transformation culminates in the development of arbuscules, specialized intraradical fungal structures characterized by extensive branching (Gutjar and Parniske, 2013).

Functional arbuscule development requires genetic changes affecting cell morphology in host cell (Berta et al., 1995; Balestrini et al., 2005) that affect cell size/proportions and root morphometric parameters (Russo et al., 2019; Heck et al., 2016; Seemann et al., 2022). Transcriptional reprogramming in cells hosting arbuscules, as well as hormone signalling have been proposed as molecular mechanisms

* Corresponding author.
E-mail address: josemanuel.garcia@eez.csic.es (J.M. García-Garrido).

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underlying these adaptive changes in roots (Floss et al., 2013; Pimprikar and Gutjahr, 2018). Thus, complex transcriptional regulation is required for AM development and functioning. Genome-wide studies, which have revealed that transcription factor (TF) genes are activated during AM symbiosis in Petunia (Rich et al., 2017), Lotus (Xue et al., 2015), Medicago (Hartmann et al., 2019) and tomato plants (Ho-Plágaro et al., 2019), have also reported that the GRAS gene family is the main class of AM-inducible TF genes. Furthermore, strong evidence shows that plant hormones are pivotal regulators in the establishment and functioning of arbuscular mycorrhizal (AM) symbiosis (Ho-Plágaro and García--Garrido, 2022a), while several studies of pea, Medicago and rice suggest that the giberellin-DELLA complex is required for correct mycorrhizal colonization and arbuscule formation (Floss et al., 2013; Foo et al., 2013; Yu et al., 2014).

In addition to the DELLA protein, other members of the GRAS TF family play a crucial regulatory role in symbiotic processes. Thus, AM-induced GRAS genes belonging to the Required for Arbuscule Development 1 (RAD1), Required for Arbuscular Mycorrhization 1 (RAM1), Mycorrhiza Induced GRAS1 (MIG1), Nodulation Signalling Pathway 1 (NSP1) and NSP2 subfamilies have been found to be involved in mycorrhization (reviewed by Ho-Plágaro and García-Garrido, 2022b). A model has emerged from these discoveries, in which DELLA protein constitute a central hub regulating the formation/functioning of arbuscules, forming transcriptional regulation complexes with multiple additional transcription factors also being required to regulate morphological and functional cell modifications leading to the formation of arbuscules (Floss et al., 2013, 2016, 2017; Heck et al., 2016; Pimprikar et al., 2016). Recently, GRAS TF members of the SCARECROW (SCR), SHORT-ROOT (SHR), and SCARECROW-like 3 (SCL3) subfamilies, which are involved in radial root organization and mediate GA-promoted cell elongation during root development (Heo et al., 2011; Zhang et al., 2011), have also integrated into the intricate system governing the development of arbuscular mycorrhizal (AM) symbiosis in roots (reviewed by Ho-Plágaro and García-Garrido, 2022b).

Members of the GRAS subfamilies SHR, SCL3, SCR, and SCL32 have been characterized as AM inducible genes in tomato and show specific expression in cells containing arbuscules (Ho-Plágaro et al., 2019). These genes include *SlGRAS18*, an arbuscular mycorrhiza (AM)-inducible SCL3 gene, which, together with *SLGRAS11*, has been characterized as orthologs of Arabidopsis *AtSCL3* (Yang et al., 2021). *SlGRAS11* is not inducible by mycorrhization (Ho-Plágaro et al., 2019) and has been implicated in the regulation of volatile terpene biosynthesis and glandular trichome development in tomato (Yang et al., 2021). While *SlGRAS11* showed significantly higher expression in trichomes than in leaves and was detected throughout the plant, with abundance in hypocotyl, stem, leaf, and flower tissues rich in glandular trichomes, the transcript abundance of *SlGRAS18* was significantly lower in trichomes than in leaves (Yang et al., 2021). Instead, it was most abundant in roots and developing fruit, consistent with previous results (Huang et al., 2015).

A *SlGRAS18* orthologue in Medicago have recently been characterized as negative regulators of cortical cell size and arbuscule formation (Seemann et al., 2022). Seeman et al. proposed a hypothetical model of cortical cell size regulation during AM formation in *Medicago truncatula* which is mediated by the interplay of MIGs, DELLA and SCL3 GRAS. According to this model, AM symbiosis triggers the expression of MIG3, which forms a transcriptional complex with SCL3 to regulate the activity of the central regulator DELLA. This interaction counteracts the positive effects of MIG1 and DELLA on cortical cell size (Seemann et al., 2022). In this study, we carried out a functional analysis for the SCL3 GRAS transcription factor *SlGRAS18* during arbuscular mycorrhizal formation in tomato, which confirmed its negative regulatory effect on arbuscule formation. We show that *GRAS18* gene silencing leads to an improvement in symbiotic efficiency measured as enhanced physiological traits associated with AM plant response. This study advances our knowledge of regulatory AM-inducible genes and of how specific genetic alterations can lead to more effective AM symbiosis.

2. Material and methods

2.1. Biological material, transformation and plant growth conditions

Solanum lycopersicum (Moneymaker cultivar) was used in this study. Seeds underwent surface sterilization through a 5-min immersion in 2.35% w/v sodium hypochlorite, followed by shaking at room temperature for 1 day in darkness. Subsequently, they were germinated on sterilized moistened filter paper for 4 days at 25 °C in darkness. *S. lycopersicum* plants were transformed as described by Ho-Plágaro et al. (2018), utilizing *Agrobacterium rhizogenes* strain MSU440 containing respective overexpression, RNA interference (RNAi), and promoter-GUS constructs. The screening and selection of DsRed (transformed) hairy roots were carried out through observation under a fluorescent stereomicroscope Leica M165F.

After selection, each seedling was transferred to a 500-ml pot containing an autoclave-sterilized (20 min at 120 °C) mixture of expanded clay, washed vermiculite and coconut fibre (2: 2:1 v/v/v) and growth took place in a growth chamber (day: night cycle, 16 h: 8 h, 24 °C: 20 °C; relative humidity 50%). At this moment, in the AM inoculated treatments, the plants were inoculated with around 200 spores of *Rhizophagus irregularis* (DAOM, 197198 or DAOM 240403) and infected carrot roots coming from monoxenic culture in Gel-Gro medium produced according to Chabot et al. (1992). Non-inoculated pots were supplemented with a piece of Gel-Gro medium containing only uninfected carrot roots.

Distinct plant growing conditions were used according with the nature of the experiment. Functional analysis of the mycorrhizal phenotype of *SlGRAS18* RNAi and *SlGRAS18* overexpressing and promoter reporter studies were conducted with plants growing in chamber under the above described conditions. In studies focussed on mycorrhizal efficiency and physiological traits determination, plants were grown in a greenhouse under controlled climatic conditions (18–24 °C, 50–60 % relative humidity, 16 h:8 h light (600 μmol m^{-2} s^{-1}): dark). In this late case, seedlings were individually sown in 500 ml pots, inoculated with AM fungi and pre-grown in chamber conditions during 50 days. After this period, plants were transplanted to 3 L pots and transferred to greenhouse conditions. Plants were harvested at different times after inoculation. Usually, the root system rinsed with tap water, excised from the shoot and used for the different measurements. At least eight plants were analysed at each harvest. The number of analysed biological replicates used to obtaining results are shown in the legend of each figure.

AM inoculated plants were watered every 2 d with half strength Long Ashton nutrient solution containing 25% (325 μM) of the standard phosphorous concentration to avoid mycorrhizal inhibition. In the case of non-mycorrhizal plants, the same modified Long Ashton solution was applied amended with the standard concentration of Pi (1.3 mM).

2.2. Plasmids and constructs

RNAi construct was previously obtained (Ho-Plágaro et al., 2019). The RNAi fragments of *SlGRAS18* (Solyc01g008910.2.1) were amplified from *S. lycopersicum* cDNA mycorrhizal roots, introduced in pENTR/D-TOPO (Invitrogen) vector and subsequently recombined into pK7GWIWG2_II-RedRoot2. For overexpression analyses, the coding sequence of *SlGRAS18* was amplified from *S. lycopersicum* cDNA of roots infected by the AM fungus *R. irregularis* and cloned into the destination vector pUBIcGFP-DR (Kryvoruchko et al., 2016). Amplifications were carried out by PCR using the iProof High Fidelity DNA-polymerase (BioRad) and specific primers (Table S1).

2.3. Gene expression analysis

RNA extraction from roots, cDNA synthesis and qPCR was performed

as described in Ho-Plágaro et al. (2021). Experiments were carried out on four biological replicates, and the threshold cycle (Ct) value for each biological replicate was determined from three technical replicates. The relative expression was calculated by using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) with the gene-specific primers described in Table S1. Expression values were normalized using the housekeeping gene SlEF-1α (accession no. X14449) encoding the tomato translation elongation factor-1α. The RT-qPCR data for each gene were shown as relative expression with respect to the reference treatment in which it was assigned an expression value of 1.

2.4. Yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays

For protein–protein interaction assays, the *SlGRAS18* and *SlGAI1* (DELLA) gene ORFs from *S. lycopersicum* were previously cloned into the pENTR-D-TOPO vector. These genes of interest contained in the pENTR/D-TOPO® entry vector was recombined into the corresponding destination vectors through a Gateway LR reaction (Thermo Scientific). The Gal4-based yeast two-hybrid system (Clontech Laboratories Inc.) was used for testing the interaction between SlGRAS18 and SlGAI1 and both genes were cloned using the Gateway technology in pGBKT7 and pGADT7 destination vectors. pGBKT7-p53 (a fragment from murine $p53_{(72\text{-}390)}$), pGBKT7-LamC (a fragment of human lamin $C_{(66\text{-}230)}$) and pGADT7-$AgT_{(84\text{-}708)}$ (a fragment of SV40 large T-antigen) from Clontech Laboratories Inc., and pGADT7-TTL3 (TETRATRICOPEPTIDE THIOREDOXIN-LIKE 3 (Amorim-Silva et al., 2019) were used as controls for the interactions. The bait and prey plasmids were transformed into *Saccharomyces cerevisiae* strain AH109 as described in Gietz and Schiestl (1995) and transformants were grown on plasmid-selective media (SD/-Trp-Leu). Plates were incubated at 28 °C for 4 days and independent colonies for each bait-prey combination were resuspended in 200 μl of sterile water. 10-fold serial dilutions were made and 5 μl of each dilution were spotted onto three alternative interaction-selective medium (SD/-Trp-Leu-His+3-AT (3-amino-1, 2, 4-triazole, 2 mM), SD/-Trp-Leu-Ade, and SD/-Trp-Leu-Ade+3-AT). Plates were incubated at 28 °C and photographed 3 or 7 days later.

For BiFC we used the pDEST-^{GW}SCYCE (VYCE, N-terminal half of Venus) and pDEST-SCYNE^{GW} (VYNE, C-terminal half of Venus) vectors. All inserts were verified with DNA sequencing. BiFC constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Single colonies of transformants were grown overnight in low-salt LB medium. Agrobacterium cells were pelleted, washed and resuspended in the infiltration medium (1 × Murashige-Skoog salts, 10 mM morpholineethanesulfonic acid [pH 5.6], 2% [wt/vol] sucrose, 200 μM acetosyringone) to an OD_{600} = 0.5–1. Next, bacterial suspensions were incubated at room temperature in the dark for 2–4 h before infiltration into the abaxial side of 4-week-old Nicotiana benthamiana leaves with a 1 mL needleless syringe. A. tumefaciens suspensions carrying BiFC constructs were mixed at 1:1 ratio before infiltration. AtBIN2^{VYCE} and AtBIN2^{VYNE} plasmids constructs were used as negative controls for BiFC interactions (AtBIN2, BRASSINOSTEROID-INSENSITIVE 2 from *Arabidopsis thaliana*) (Amorim-Silva et al., 2019). Two days later, samples fluorescence was detected in epidermal cells 2–3 days after infiltration, using a confocal microscope (Zeiss LSM 880) using the preset settings for YFP (Ex: 514 nm, Em: 525–575 nm).

2.5. Physiological parameters

The chlorophyll content in leaves was assessed using a Chlorophyll Content Measurement System CL-01 device (SPAD, Hansatech Instruments Ltd., Norfolk, UK), which calculates relative chlorophyll levels in leaf samples by detecting dual optical absorbance at wavelengths of 620 and 940 nm. These measurements were conducted on the second fully expanded leaf of each plant.

Shoots were separated from root systems at each harvest time and fresh weights were determined. When it proceeded, fruit weights were also determined and traits such as plant height, leaves, flowers and fruit numbers were also quantified and annotated. To measure nutrient content, frozen leaves were ground to a fine powder and lyophilized. Measurement was performed by the Analytical Service of the EEZ, CSIC (Granada, Spain). C and N concentration (% d. wt) were determined by mass spectrometry (ELEMENTAL LECO TruSpec CN) and others mineral determinations were performed by inductively coupled plasma-optical emission spectrometry technique (ICP-OES; Varina ICP 720-ES).

2.6. Quantification of mycorrhizal parameters

Non vital trypan blue histochemical staining procedure and quantification or arbuscular mycorrhizal colonization was carried out as described by Ho-Plágaro et al. (2020). The abundance of arbuscules (% a) along the whole root length were analysed by the magnified intersections method according to McGonigle et al. (1990) in root pieces. For each biological replicate, a minimum of five microscope slides were examined, each containing 30 root segments measuring 1 cm. Additionally, 120 root intersections from these slides were analysed to determine the prevalence of arbuscules across three distinct developmental stages and to identify the presence or absence of vesicles. Following the methodology outlined by Ho-Plágaro et al. (2021), three arbuscule typologies were established: class I, characterized by small arbuscules without fine branches; class II, featuring arbuscules with moderate trypan blue stain intensity occupying most of the plant cell; and class III, exhibiting arbuscules with high trypan blue stain intensity filling the entire plant cell.

2.7. Data processing and statistical analysis

Data were processed by two-way analysis of variance (ANOVA) with hairy root genotype (G) and time post inoculation (T) (Fig. 1) or with hairy root genotype (G) and inoculum (I) (Fig. 6) as sources of variation. In the case of significant interaction between factors, all treatments were compared with each other. In the case of a non-significant interaction between factors, data were analysed by one-way ANOVA considering as independent variable the combination of the variables genotype and time, followed by LSD test. Both, two and one-way ANOVA analysis and box-plots figures generation were performed using R (www.r-project.org).

Principal component analysis (PCA) was also used to compare nutrient and physiological traits with those obtained under different experimental conditions using MetaboAnalyst 5.0 (Metabohttps://www.metaboanalyst.ca/MetaboAnalyst/upload/StatUploadView.xhtml).

3. Results

3.1. Silencing of SlGRAS18 positively impacts arbuscule development

Previous studies have shown that SCARECROW-LIKE3 (SCL3; SlGRAS18) transcripts are induced upon mycorrhization. A time-course expression of SlGRAS18 gene during mycorrhization in tomato roots showed that it expression increased at the late stages of the interaction when the roots reached a colonization ratio ranking 50% when arbuscules are abundant in the whole root (Ho-Plágaro et al., 2019). Additionally, the analysis of the *SlGRAS18* promoter activity in transgenic *S. lycopersicum* roots showed that colonization by *R. irregularis* clearly activated SlGRAS18 gene expression in arbusculated cells (Ho-Plágaro et al., 2019). This is also true in Medicago, where *SCL3* expression is induced in arbuscule-containing cells during symbiosis (Seemann et al., 2022). Since hairy-root transformed composite tomato plants have been used and there is known variation in RNAi efficiency between transgenic roots, we conducted several independent experiments with RNAi plants. We observed certain variable phenotypes concerning the mycorrhizal colonization ratio; sometimes, we detected lower mycorrhizal capacity

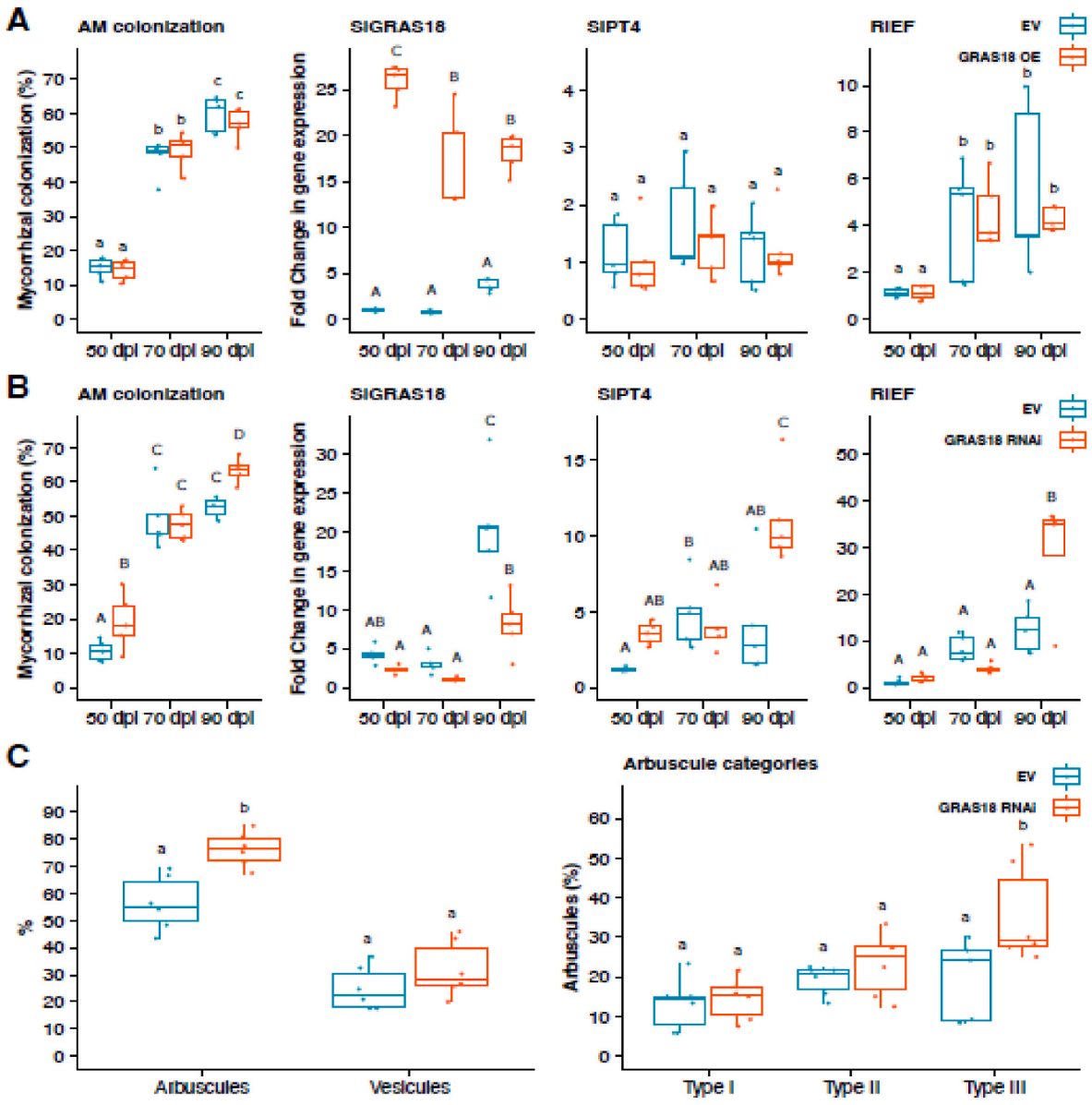

Fig. 1. - Mycorrhizal phenotype characterization of *SlGRAS18* RNAi and *SlGRAS18* overexpressing composite tomato plants. Time course experiments comparing sets of control (EV), overexpressed (*GRAS18* OE) and silenced (*GRAS18* RNAi) composite plants in presence of *R. irregularis* inoculation. Colonization and gene expression of *SlGRAS18*, *SlPT4* and *RiEF* (*R. irregularis* Elongation factor) were analysed in overexpressed (A) and silenced (B) composite plants. Data were analysed by two-way ANOVA with hairy roots genotype (G) and time post inoculation (T) as sources of variation followed by LSD test. In the case of significant interaction between factors, all treatments were compared with each other. Different upper case letters indicate significant differences among treatments ($P \leq 0.05$). In the case of a non-significant interaction between factors, data were analysed by one-way ANOVA considering as independent variable the combination of the variables genotype and time, followed by LSD test. Different lower case letters show differences between particular treatment combinations ($P \leq 0.05$). RT-qPCR data represent the relative expression of the gene (fold change) with respect to its expression in EV plants at 50 dpi, in which its expression was designated as 1, except in the case of the SlGRAS18 gene in (B), where the treatment with the lowest expression (GRAS18 RNAi 70 dpi) was taken as a reference treatment. (C) Percentage of root length containing arbuscules and vesicles in mycorrhizal roots and the percentage of root length of each arbuscule typology were analysed after 90 dpi (days post inoculation) in *SlGRAS18* RNAi and control (EV) hairy roots genotypes. Data were analysed by one-way ANOVA followed by LSD test ($n = 6$). Different letters indicate significant differences between control and silenced composite plants ($P \leq 0.05$).

in silenced plants (Ho-Plágaro et al., 2019), while other times we did not. Thus, using material from the experiment published by Ho-Plágaro et al. (2019), we studied the evolution of the SlPT4 gene, observing a clear induction of the transcriptional activity of this gene at 84 dpi (Fig. S1), a time at which we had not detected any changes in the quantification of the different mycorrhization parameters (Ho-Plágaro et al., 2019). To resolve this inconsistence and confirm the role of *SlGRAS18* during AM formation, we carried out here an in-depth functional analysis of the mycorrhizal phenotypes of *SlGRAS18* RNAi and *SlGRAS18* overexpressing (OE) composite tomato plants during time course experiments of mycorrhizal colonization.

We tested the expression levels in both experiments and obtained successful overexpression and repression of the *SlGRAS18* gene (Fig. 1A and B, respectively). Compared to the control, *SlGRAS18* OE did not show any significant changes in the colonization process, and the expression patterns of fungal *RiEF* and the tomato arbuscule-specific marker gene *SlPT4* were similar in both control and *SlGRAS18* OE plants (Fig. 1A).

A significant interaction between factors (plant genotype and time post-inoculation) was observed in the RNAi experiment. A weak but

significant increase in the percentage of root length colonized by the AM fungus was observed at late harvest time (90 dpi) in *SlGRAS18* RNAi plants compared to control plants (Fig. 1B), paralleling a significant increase in the transcription levels of the RiEF and *SlPT4* genes at that harvest time (Fig. 1B). In EV roots, the stabilization phase of mycorrhization began to become evident between days 70 and 90 post-inoculation (dpi). However, this steady phase in colonization was not observed in *GRAS18*-silenced hairy roots, aligning with the heightened activity of plant and fungal genes associated with symbiotic activity (SlPt4 and *RiEF)* at 90 dpi.

We then investigated how the RNA silencing impacted on arbuscule development measuring the percentage of arbuscules and vesicles in mycorrhizal roots after 90 dpi (Fig. 1C). *SlGRAS18* gene silencing positively affects arbuscule abundance at a late stage in the mycorrhizal colonization dynamic. A slight increase in the number of vesicles was also observed in *SlGRAS18* RNAi plants, although it was not statistically significant. When comparing different types of arbuscules at this late stage of colonization, large and well developed arbuscules (type III) were found to be more abundant in *SlGRAS18* RNAi mycorrhizal roots with respect to control (EV) roots. Values of 13.06%, 19.17% and 19.67% for type I, II and III arbuscules, respectively, were obtained following EV treatment as compared to values of 14.44%, 23.06% and 35.56% obtained with the hairy roots genotype *RNAi18* (Fig. 1C). The relative distribution of the different categories of arbuscules shows that in *SlGRAS18* RNAi plants, the most abundant arbuscules (50% of the total) are mature arbuscules, while in control plants, a more equitable distribution among the different categories was observed (Fig. S2). The analysis of arbuscule phenotype in *SlGRAS18* OE hairy roots did not show significant differences compared to composite control plants (Fig. S3).

On the whole, *SlGRAS18* ectopic overexpression did not cause any significant alteration in overall mycorrhizal colonization, either due to

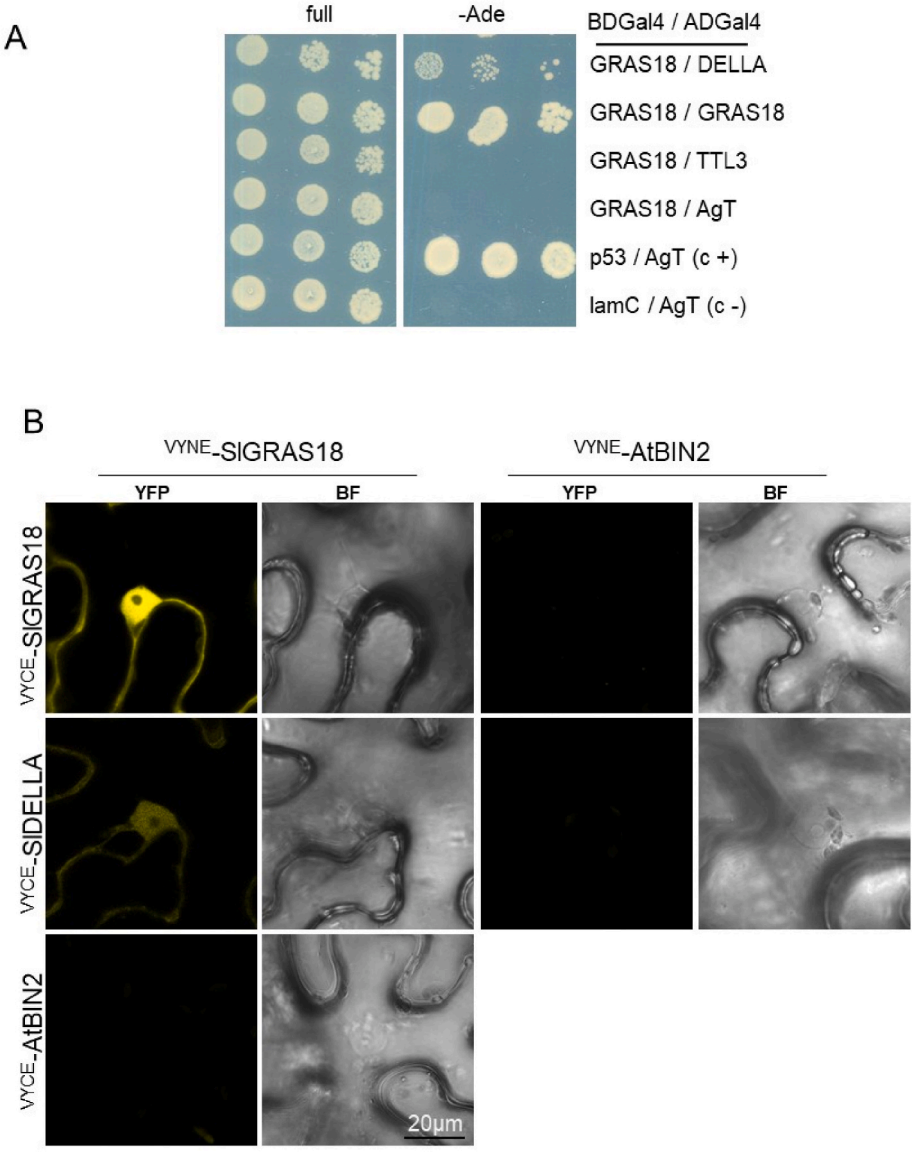

Fig. 2. SlGRAS18 physically interacts with SlDELLA and with itself. (A) Protein-protein interaction by yeast two-hybrid (Y2H). Growth on plasmid-selective media (full) and interaction-selective media (lacking adenine) are shown. Interaction of BD-SlGRAS18 with AD-AgT and AD-TTL3 was also included to discard the self-activation capacity of BD-SlGRAS18. Interaction of BD-p53 and AD-AgT and BD-lamC and AD-AgT, were included as positive and negative controls for protein-protein interaction, respectively. AD: Gal4 activation domain; BD: Gal4 binding domain. (B) SlGRAS18 and SlDELLA interaction detected by bimolecular fluorescence complementation (BiFC) in *N. benthamiana* leaves. VYNE-AtBIN2 and VYNE-AtBIN2 construct were included as negative controls for the SlGRAS18 and SlDELLA interactions. VYNE: N-terminal half of Venus; VYCE: C-terminal half of the Venus. Images were taken at two days post-infiltration (dpi). Scale bar = 20 µm. This experiment was repeated at least three times with similar results.

insufficient transcript accumulation under our experimental conditions or due to a lack of some necessary interacting factors acting in concert with GRAS18 protein. Similarly, the ectopic overexpression of the *SCL3* orthologue to the tomato *SlGRAS18* in Medicago It didn't affect either the frequency or intensity of root colonization by *R. irregularis*, and *MtPT4* gene expression, even though the number of arbuscules decreased (Seemann et al., 2022).The silencing of *SlGRAS18* consistently increased the number of mature arbuscules concomitant with an increase in *SlPT4* expression, as has also been showed for *SCL3* in Medicago (Seemann et al., 2022); This suggest that, like its orthologue in Medicago, the SlGRAS18 protein in tomato contributes to restraining arbuscule development.

3.2. SlGRAS18 interacts with SlDELLA

We then investigated whether SlGRAS18 interacts directly with the SlDELLA protein in a similar manner to how AtSCL3 interacts with DELLA in Arabidopsis plants (Zhang et al., 2011) or how SCL3 in Medicago interacts with DELLA proteins (Seemann et al., 2022). The results obtained by Yeast two-hybrid (Y2H) and BiFC assays highlighted the existence of SlGRAS18 and SlDELLA interaction, and SlGRAS18 self-interaction (Fig. 2). BD-SlGRAS18 interacts with AD-SlDELLA as it activates GAL2-ADE2 expression (co-transformed yeast grown on minimal media lacking Adenine) suggesting that the interaction was direct. Moreover, BD-SlGRAS18 interacts with AD-SlGRAS18 but not with other two unrelated proteins such us AD-AgT (SV40-T antigen, Clontech) or AD-TTL3 (TETRATRICOPEPTIDE THIO-REDOXIN-LIKE protein, a member of the cellulose synthase complex; Kesten et al., 2022) (Fig. 2A). Therefore, we can conclude that SlGRAS18 interacts also with itself and that BD-SlGRAS18 does not auto-activate the Y2H reporter genes (Fig. 2A).

To confirm the presence of SlGRAS18-SlDELLA interaction *in planta*, we performed a BiFC assay on *Nicotiana benthamiana* leaves through *Agrobacterium*-mediated transitory expression. A weak but positive interaction was detected in the nucleus and cytoplasm when VYCE-SlDELLA and VYNESlGRAS18 were co-expressed (Fig. 2B). Interestingly, SlGRAS18 was also found to strongly interact with itself (Fig. 2B), in the nucleus and cytoplasm, suggesting that the formation of homodimers may take an important contribution to its functionality. To determine the specificity of SlGRAS18 and SlDELLA interaction, we co-expressed each protein with an unrelated protein, AtBIN2 (Arabidopsis thaliana BRASSINOSTEROID INSENSITIVE2), fused either to VYCE- or VYNE-, and no fluorescent signal was found in any of the combinations assayed (Fig. 2B).

Previous studies have proposed a model in which DELLA, SCL3, and indeterminate domain (IDD) family proteins cooperate to control GA signalling in the endodermis by regulating downstream gene expression. Based on this model, the Arabidopsis DELLA protein RGA positively regulates *SCL3* expression through interactions with IDD, binding to specific DNA sequences containing AGACAA as a core motif (Yoshida et al., 2014). To pinpoint a similar *SCL3* regulation mechanism during mycorrhiza formation mediated by the DELLA/IDD complex, we searched for sequences homologous to the consensus target motifs of the DELLA/IDD complex and found one candidate carrying AGACAA from −790 to −784 (from the ATG) in the SlGRAS18 promoter (Fig. S4). This 1548 bp *SlGRAS18* promoter sequence was previously shown to direct β−glucuronidase (GUS) reporter gene expression to root cells containing arbuscules (Ho-Plágaro et al., 2019).

3.3. Positive impact of SlGRAS18 silencing on mycorrhizal efficiency

To address the question of how changes in the AM phenotype imposed by *SlGRAS18* silencing affect the development and effectiveness of the AM fungus in the cortex, we evaluated its effects on certain physiological traits, yields and nutrient contents of tomato plants grown under greenhouse conditions with the aid of two complementary experiments. Firstly, plants were inoculated with *R. irregularis,* whose physiological responses related to plant growth, photosynthetic performance, flowering, fruits development and nutrients content in leaves were evaluated. In a second experiment, mycorrhizal parameters and physiological responses related to the plant growth, flowering and fruit performance of composite control and *SlGRAS18* RNAi tomato plants inoculated with different AM fungi were measured in order to validate and reinforce results with different AM fungi.

In the first analysis, to eliminate any inherent effects due to *SlGRAS18* gene silencing, we designed a time course experiment comparing sets of control and silenced plants both in the presence and absence of *R. irregularis* inoculation. The experimental design established was not intended to show the already known differences between inoculated and non-inoculated treatments, but to determine variations in the Mycorrhizal responsiveness due to the silencing of *SlGRAS18*. Thus, un-inoculated plants were supplemented with Pi in order to simulate Pi acquisition conditions in mycorrhized plants.

Uninoculated and inoculated seedlings were individually pre-grown under chamber conditions for 50 days. Following this period, the plants were transplanted into 3 L pots and transferred to greenhouse conditions. At the time of transplant, the average mycorrhizal colonization was 15.7 ± 3.58 for control plants and 17.22 ± 3.56 for RNAi plants. Tomato plant height, flower and fruit numbers and leaf chlorophyll content were measured in a time course experiment, and shoot fresh weight (excluding fruit weight), leaves number and fruit yield were determined at the last harvest time (12 weeks after transplanting) (Fig. 3; Fig. 4). Similar measurements of these traits being carried out for the un-inoculated plants (EV NM and *GRAS18* RNAi NM). AM colonization in *SlGRAS18* RNAi plants clearly showed an improvement in these parameters with respect to control AM plants (EV M treatment). In *R. irregularis* inoculated treatments, plant height of the *SlGRAS18* RNAi plants was higher (around 20%) than that of the control plants with respect to all the measurements carried out over time (Fig. 3). Leaf chlorophyll content, which was measured by SPAD six and ten weeks after transplanting, was clearly reduced (30%) in control plants as compared to *SlGRAS18 RNAi* following mycorrhizal treatment. No differences were detected in the fresh weight of the shoot, and there was only a slight increase (10%) in the number of leaves in *SlGRAS18* RNAi plants compared to control plants following mycorrhizal treatment. For these traits, un-inoculated plants showed values similar to those for RNAi mycorrhizal plants regardless of genotype (Fig. 3). Differences in the time to initiation of the first inflorescence and consequent fruits emergence were also clearly observed, with a delay of two weeks between control and *SlGRAS18* RNAi-inoculated plants (Fig. 4). No change in these traits was observed when comparing un-inoculated plants. As a result of the progress in flowering and fruit appearance, the harvest yield, measured as fruit weight per plant at the end of the trial, was significantly higher in *SlGRAS18* RNAi plants (Fig. 4). The data presented are from an experiment where pots in the greenhouse were randomly placed to prevent spatial arrangement interference. To ensure reliability, the experiment was repeated under comparable conditions. Fig. S5 displays results from plants inoculated with *R. irregularis* in the repeated experiment, showing consistent trends across both experiments.

The principal impact observed in our results was the shortening in the initiation time for the first inflorescence (and fruit emergence) in AM *SlGRAS18* RNAi plants. Given the increase in nutrient uptake rates at flowering through fruiting as well as this demand becomes increasingly competitive with vegetative tissues, we then analysed nutrient content in order to determine the redistribution of nutrients from leaves. A time course analysis was carried out by measuring nutrient content in leaves at three points times: prior to flowering, at the appearance of the first inflorescences and at the end of harvest time which correspond to three, six and ten weeks after transplantation respectively (Table S2). Changes in nutrient concentrations at each harvest time observed among genotypes were confirmed by principal component analysis (PCA) and

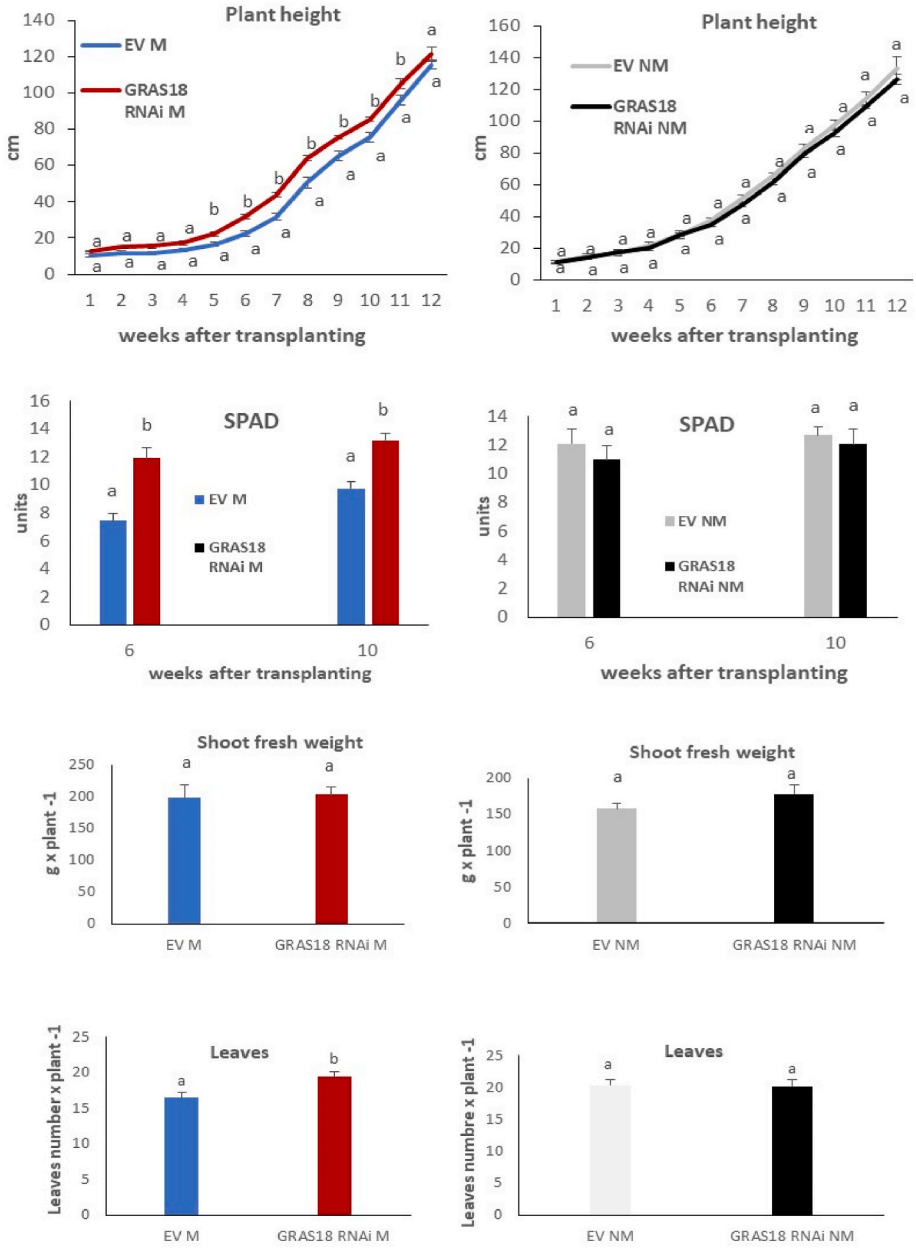

Fig. 3. - Plant height, leaf chlorophyll content, plant weight and leaves number in control and *SlGRAS18* RNAi silenced plants. Time course experiment comparing sets of control (EV) and silenced composite plants (*GRAS18* RNAi) both in the absence (NM) and presence of *R. irregularis* inoculation (M). Plant fresh weight and leaves number were recovered at twelve weeks after transplanting. Data represents means ± SE (n = 8). Significant differences ($P < 0.05$) observed by the LSD multiple comparison test are labelled with different letters.

heatmap plots representation comparing un-inoculated and inoculated treatments with respect to both hairy roots genotypes (Fig. 5).

An in-depth analysis of the evolution of concentrations of certain mineral elements in leaves showed a discernible pattern of nutrient redistribution associated with the genotype of mycorrhizal plants. In general, nutritional levels in the first harvest (3 wat) were lower than in the second and third harvests (6 and 10 wat), with a slight decrease in mineral content observed in the third harvest (10 wat), possibly due to crop depletion (Table S2). PCA analysis of nutrient concentrations in leaves at each harvest times showed that nutrient composition in tomato plants varied in response to AMF and hairy roots genotype (Fig. 5A). Axes PC1 and PC2 explained 74.7%, 97.4% and 96.2% of data variability at 3, 6 and 10 weeks after transplantation respectively. The scores plots indicate a clear division between mycorrhizal *SlGRAS18* RNAi and mycorrhizal control treatments, while the non-mycorrhizal treatments overlapped (Fig. 5A).

Heatmap analysis (Fig. 5B) confirmed these clearly separated profiles, while the non-mycorrhizal treatments (EV NM; RNAi NM) were clustered together next to mycorrhizal *SlGRAS18* RNAi (RNAi M) and separately from the mycorrhizal control treatment (EV M). These analyses of nutrient accumulation showed a set of nutrients with an accumulation profile in leaves associated with AM formation (highlighted by yellow squares in Fig. 5B). Colonization by *R. irregularis* positively correlated with increases in the concentrations of Fe and Cu at the first harvest; with concentration of P, Na, Fe and Li at the second harvest and with concentrations of K, P and N at the late harvest time. Colonization correlated with a decrease in concentrations of Si, S and Al in the first harvest and in concentrations of Na and Al at last harvest time. All these changes were more marked in AM control plants than in *SlGRAS18* RNAi mycorrhizal plants (Fig. 5B).

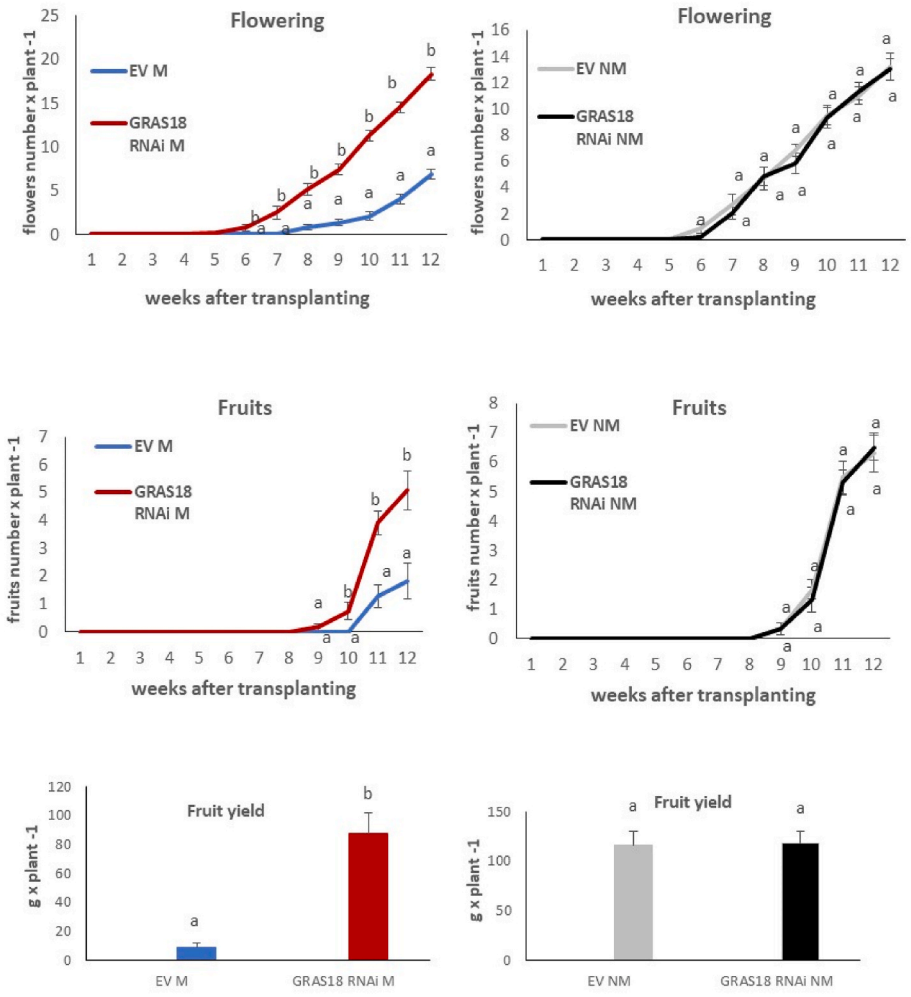

Fig. 4. - Physiological traits in control and *SlGRAS18* RNAi silenced plants. Time course experiment comparing sets of control (EV) and silenced composite plants (GRAS18 RNAi) both in the absence (NM) and presence of *R. irregularis* inoculation (M). Fruit yield was recovered at twelve weeks after transplanting. Data represents means ± SE (n = 8). Significant differences ($P < 0.05$) observed by the LSD multiple comparison test are labelled with different letters.

However, what is most noteworthy is the pattern of nutrient accumulation associated with the genotype of inoculated plants. Increases in the levels of P and Zn (denoted by black square, Fig. 5B) and decreases in the levels of in K, Na, and Mn (denoted by white square, Fig. 5B) closely correlate with the AM control treatment (EV M) at the first harvest time. At the second harvest time, minerals such as Ca, Sr, Mg, Si, S, Al, and N showed an accumulation pattern in leaves characterized by an increase in their content in AM control plants with respect to the other treatments (highlighted by a black square, Fig. 5B), while a reduction in nutrients such as Cu, Zn, Mn and C was observed (highlighted by a white square, Fig. 5B). In addition, a similar pattern of accumulation was observed for Fe, Mn, Si, Ca, Mg, and Sr, whose levels decreased significantly in AM control plants at the third harvest (highlighted by a white square, Fig. 5B). Some minor correlations appear to be associated with the hairy roots genotype regardless of mycorrhizal status. Thus, an increase in Mg at the first harvest, in K at the second harvest time and in Li, Cu and Zn at the third harvest time, as well as a reduction in C at the third and second harvest times, respectively, were observed in *SlGRAS18* RNAi plants (highlighted by pink squares, Fig. 5B).

It is also noteworthy that the un-inoculated plants and *SlGRAS18* RNAi inoculated plants showed quite similar nutrient with respect to most of the nutrients analysed, which closely correlates with previous results regarding physiological traits such as flowering, plant height, fruit number and chlorophyll levels (Fig. 3; Fig. 4).

As mentioned above, in the second experiment, we compared plants inoculated with 2 a.m. fungi isolates (*Rhizophagus irregularis* DAOM, 197198 and DAOM 240403) and also measured physiological parameters of growth and performance. The results obtained show that some traits were significantly and positively affected by *SlGRAS18* silencing. Plant height, as well as the number of leaves, flowers and fruits were determined twelve weeks after transplantation (Table 1). Only slight differences (not significant) in plant height, numbers of leaves and fresh weights between treatments were observed (Table 1). However, flower numbers per plant and fruit yield increased significantly in *SlGRAS18* RNAi plants inoculated with DAOM 197198 compared to control plants. Although less significant, plants inoculated with DAOM 240403 exhibited the same trend.

Plants were collected 12 weeks after transplantation to pots and AM fungal structures were stained within the roots. Mycorrhizal symbiosis was observe to be well established all treatments. AMF colonization was more abundant in Ri DAOM 240403 roots than in Ri DAOM 197198 roots, although no significant differences between control and RNAi silenced genotypes were observed (Fig. 6A). *SlGRAS18 RNAi* silenced plants and Ri DAOM 240403 control plants showed similar higher levels of arbuscules in roots (around 80%), while those in control Ri DAOM 197198 roots reached only the 60% (Fig. 6A). Functional symbiosis was evaluated by analysing the type of arbuscules present in the root system (Fig. 6B). The same pattern of arbuscule distribution was observed regardless on the AM fungus used for inoculation, as the percentage of large, well developed arbuscules (type III) was higher in RNAi plants

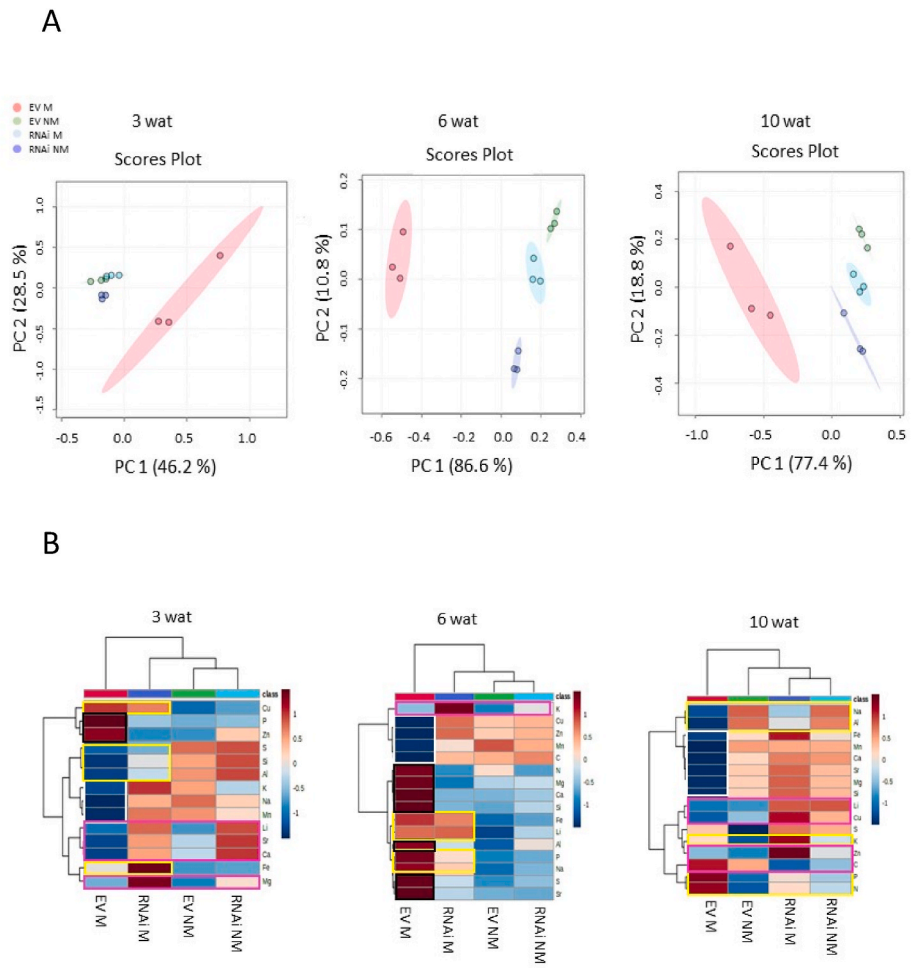

Fig. 5. Analysis of nutrient content redistribution in leaves of control and *SlGRAS18* RNAi silenced composite tomato plants. Time course experiment comparing sets of control (EV) and silenced plants (*GRAS18* RNAi) both in the absence (NM) and presence of *R. irregularis* inoculation (M). Nutrients were measured at three (3wat), six (6wat) and ten weeks (10wat) after transplantation respectively and changes in nutrient concentrations at each harvest time (n = 3) were analysed. Principal Component Analysis (PCA) representation of the major sources of variability (A) and heatmap plots (B) representation comparing un-inoculated and inoculated treatments with respect to both hairy roots genotypes. Clusters of nutrients showing a similar pattern of accumulation are highlighted in the same colour. In the PCA score plots each point represents one plant.

(over 40% for any AM fungus) than in inoculated control plants (22% and 32%, respectively, in Ri DAOM, 197198 and DAOM 240403).

Principal component analysis of all the parameters of the different treatments showed that two main components accounted for 68.9% of the variability observed in the data, with PC1 accounting for 55.3% and PC2 for 13.6% (Fig. 7). Inoculation with AMF had similar effects regardless of the AM fungus used, although the hairy roots genotype has a greater impact on data variability. The scores plot indicates a clear division between mycorrhizal *SlGRAS18* RNAi and mycorrhizal control treatments (EV). The first Principal component (PCA 1) had moderate positive loadings for almost all the parameters determined and strong loadings for fruit yield and arbuscule type III, meaning that the *GRAS18* RNAi phenotype correlates positively with these parameters. Thus, based on the results obtained, it is possible to assume that, in tomato, SlGRAS18 performs similar functions in arbuscule-containing cells regardless of the AM fungus.

4. Discussion

Continuous advances in the discovery and identification of new regulatory molecular mechanisms involved in the formation and establishment of AM symbiosis will provide us with a better understanding of how plants can be prepared for the establishment of AM symbiosis. Therefore, uncovering crucial target genes, regulatory modules, and subsequent processes involved in arbuscular mycorrhizal (AM) formation and function is imperative to enhance the effectiveness of AM symbiosis. Knowledge of the fundamental processes regulating the development of AM formation mediated by the action of specific genes will enable us to genetically alter the way in which plants perceive and regulate their own responses to AM fungi in order to achieve more effective AM symbiosis.

In this study, a further contribution is made to our knowledge of how the transcription factor SlGRAS18 in tomato impacts AM efficiency. *SlGRAS18* was identified as a member of the SCL3 group of GRAS transcription factors, which, in Arabidopsis (AtSCL3) has been shown to be a positive regulator of GA signalling (Heo et al., 2011; Zhang et al., 2011) and whose expression is associated with arbuscule-containing cells in tomato (Ho-Plágaro et al., 2019). Furthermore, SCL3 is involved in a novel negative regulatory module in which MIG3 induces and interacts with SCL3, both of which modulate the activity of the central regulator DELLA, thus restraining cortical cell growth and expansion required for arbuscule development in *M. truncatula* (Seemann et al., 2022).

As in Arabidopsis and *M. truncatula*, we demonstrated that SCL3/GRAS18 interacts with DELLA in tomato. Interestingly, SlGRAS18 also interacts strongly with itself suggesting that the formation of

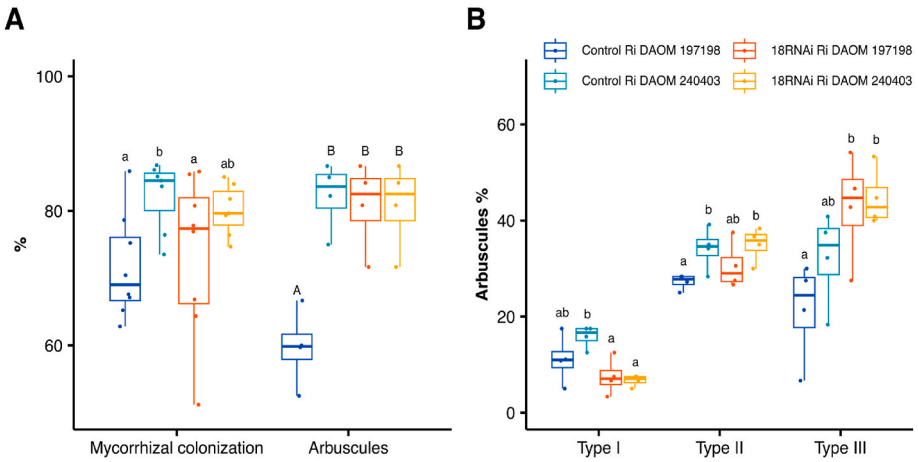

Fig. 6. - Mycorrhizal colonization parameters in roots of *SlGRAS18* RNAi AM-inoculated composite tomato plants. Percentage of root length containing fungal colonization and arbuscules (A) and percentage of root length of each arbuscule arbuscule typology (B) in hairy roots inoculated with *R. irregularis* strains (DAOM, 197198 and DAOM 240403) and harvested at 12 weeks after transplantation. Data were analysed by two-way ANOVA with hairy roots genotype (G) and inoculum (I) as sources of variation ($n \geq 4$). The significance of sources of variation as well as their interaction (G x I) was evaluated by P-value. In the case of significant interaction between factors, all treatments were compared with each other. Different upper case letters are significantly different ($P \leq 0.05$) according to LSD test. In the case of a non-significant interaction between factors, data were analysed by one-way ANOVA considering as independent variable the combination of the variables genotype and inoculum, followed by LSD test. Different lower case letters show differences between particular treatment combinations ($P \leq 0.05$).

Table 1
Physiological traits of tomato plants inoculated with 2 a.m. fungi isolates (*Rhizophagus irregularis* DAOM, 197198 and DAOM 240403) and harvested twelve weeks after transplantation.

Treatments	Plant Height (cm)	Number/plant			Weight (g)		
	Plant	Flowers	Leaves	Fruits	Fruit yield (g plant^{-1})	Fresh root	Fresh shoot
Control Ri DAOM 197198	97.94 ± 4.25^{a}	5.10 ± 0.80^{a}	25.70 ± 1.03^{a}	1.10 ± 0.18^{a}	16.07 ± 2.84^{a}	7.22 ± 0.83^{a}	51.79 ± 3.42^{a}
Control Ri DAOM 240403	103.50 ± 2.54^{a}	5.80 ± 0.74^{a}	24.40 ± 0.72^{a}	1.80 ± 0.29^{a}	22.66 ± 4.83 ^{ab}	8.67 ± 1.11^{a}	61.72 ± 3.95^{ab}
18RNAi Ri DAOM 197198	101.60 ± 4.01^{a}	8.10 ± 0.77^{b}	27.90 ± 1.31^{a}	1.50 ± 0.27^{a}	38.46 ± 9.91^{b}	7.83 ± 1.11^{a}	59.24 ± 4.44^{a}
18RNAi Ri DAOM 240403	109.30 ± 4.34^{a}	6.90 ± 0.48^{ab}	26.80 ± 1.33^{a}	2.00 ± 0.21^{a}	36.33 ± 4.58^{b}	9.45 ± 1.45^{a}	72.29 ± 5.47^{b}

Data represents means ± SE (n = 10). Data were analysed by one-way ANOVA considering as independent variable the combination of the variables genotype and inoculum, followed by LSD test. Different lower case letters show differences between particular treatment combinations ($P \leq 0.05$). Control = EV transformed plants; *18RNAi* = *SlGRAS18* RNAi plants.

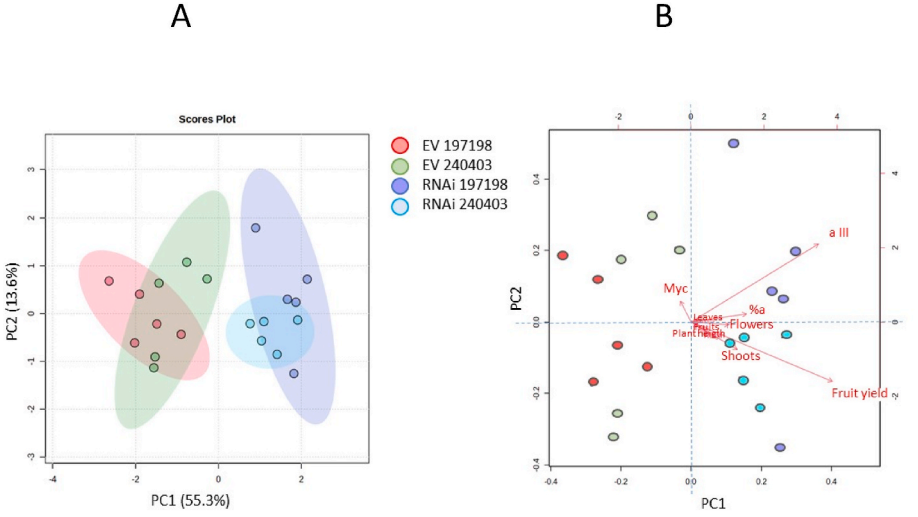

Fig. 7. Principal component analysis (PCA) of physiological traits and mycorrhizal parameters in control and *SlGRAS18* RNAi silenced composite tomato plants. Control (EV) and *SlGRAS18* silenced plants (RNAi) were inoculated with different R. *irregularis* strains (DAOM, 197198 and DAOM 240403) and harvested at 12 weeks after transplantation. The score plot (A) represents the major sources of variability and each point represents one plant, and points of the same treatment are enclosed in a different coloured ellipse. Biplot (B) showing positive and negative correlations for physiological traits (plant height; shoot weight; root weight; leaves number; flowers number; fruits number, fruit yied) and mycorrhizal parameters (Myc = % of mycorrhiza colonization in roots; a% = % of arbuscules in roots; aIII = % of arbuscules type III in roots) and hairy roots genotypes.

homodimers may play an important role in its functionality. Because it does not contain any known DNA binding domains, it has been reported that SCL3 could be involved in a complex nexus together with other transcription factors containing DNA-binding domains in Arabidopsis (Zhang et al., 2011) and Medicago (Seemann et al., 2022). The majority of GRAS proteins do not directly bind to DNA, functioning instead as transcription regulators rather than classical transcription factors (Bolle, 2016). Studies have demonstrated that GRAS proteins, even those belonging to different subfamilies, can interact specifically to form homodimers and heterodimers, which are frequently required for optimal GRAS protein activity (Bolle, 2016). The prerequisite for GRAS18 to form homodimers during mycorrhization is a topic that warrants investigation in the future. It is likely that GRAS18 remains stable and functional in the homodimeric form. This assumption is supported by findings that the Arabidopsis SCL3 protein has been reported to exist as a homodimer in solution, determined by analytical ultracentrifugation (Hakoshima, 2018).

It has been proposed that AtSCL3 positively affects GA signalling and some evidence supports a model in which DELLA, SCL3, and Indeterminate Domain (IDD) family proteins cooperate to in order to control GA signalling in the endodermis by regulating downstream gene expression (Yoshida et al., 2014). A straightforward search of the *SlGRAS18* promoter sequence reveals an AGACAA motif, crucial for IDD DNA binding (Yoshida et al., 2014), at the −790 to −784 position (from the ATG). We have not detected other well-known AM-responsive cis-elements (such as AW-box, CTTC, and P1BS) within the promoter region. This suggests that the AGACAA motif might be indispensable for regulating *SlGRAS18* promoter activity in mycorrhizal tomato roots. According to this, it is tempting to speculate a model in which DELLA regulates *SlGRAS18* expression through interaction with an as yet unknown IDD. IDDs proteins belong to a plant-specific subclass of C2H2 Zinc Finger transcription factors. In a previous study (Ho-Plágaro et al., 2019), we identified 246 genes encoding transcription factors or transcriptional regulators that were significantly upregulated in AM-inoculated roots. Among these, 22 members belong to the C2H2 zinc finger protein family, suggesting that some of these genes could encode IDD proteins involved in the proposed hypothetical mechanism. Further research is imperative to substantiate this stimulating hypothesis and forthcoming studies involving directed mutations in the AGACCA motif are poised to provide the definitive answer.

As in Medicago (Seemann et al., 2022), in this study, we demonstrate that *SCL3* silencing increases the number of arbuscules concomitant with an increase in *SlPT4* expression in tomato mycorrhizal roots, suggesting that, like its orthologue in Medicago, the GRAS18 protein in tomato contributes to restraining arbuscule development. The most notable effect observed in our experiments refers to the relative increase in mature arbuscules in well-colonized roots, suggesting a higher degree of arbuscule maintenance in *SlGRAS18* RNAi roots. In our experiments, *SlGRAS18* ectopic overexpression did not cause any significant alterations in overall mycorrhizal colonization. As for Medicago overexpression of SCL3 did not reduce the frequency or intensity of root colonization by *R. irregularis* (Seemann et al., 2022) and *Pt4* expression was not altered. As proposed by these authors, the action of SCL3 restricting arbuscule size require MIG3 and DELLA. Maybe in our overexpression experiments the levels of DELLA and MIG1 was not sufficient to achieve the desired effect together with SCL3, or perhaps the effect also depends on the plant species.

We focussed on the functional analysis of the *SlGRAS18* RNAi hairy roots genotype in order to evaluate differences in the effectiveness of the AM fungus in the cortex as a consequence of *SlGRAS18* gene silencing. In our experiments, to eliminate any inherent effects of *SlGRAS18* gene silencing, non-inoculated plants were used as control for normal vegetative and reproductive development in tomato and were supplemented with standard nutritional conditions. To determine differences in colonization dynamics and plant responses due to *SlGRAS18* gene silencing we compared sets of control and silenced plants both in the presence and absence of AMF under limited P concentrations and where the demand for nutrients competed between the plant and AMF.

Previous studies have shown that AMF inoculation could advance the flowering of tomato plants and also enhance fruit number, size and quality (Di Fossalunga et al., 2012; Miranda et al., 2015; Bona et al., 2017; Schubert et al., 2020; Wang et al., 2022a, 2022b). Although we have not found a significant effect on shoot growth, our results clearly show that traits, such as leaf chlorophyll content (SPAD units), flowering, fruit numbers and fruit yield, were improved in mycorrhizal *SlGRAS18* RNAi plants with respect to wild-type mycorrhizal plants. This improvement was directly linked to increased production of arbuscules, mainly of the more developed type, in the silenced genotype. Similarly, a functional relationship between occurrence of arbuscules in roots and fruit quality was found in two tomato varieties (Pellegrino et al., 2024).

As described in Medicago (Seemann et al., 2022), this study confirms that the *SCL3* gene acts as a negative regulator of arbuscular development in tomato, thus linking modifications in the morphological phenotype that occur in the silenced plants with alterations in the physiological traits in AM tomato plants. In our study, we used 2 a.m. fungi to validate and reinforce results. While the DAOM 197198 fungus demonstrates more significant results than DAOM 240403, with increased flower numbers and fruit yield, the latter still exhibits the same trend. It is important to consider that colonization dynamics vary among different fungi, and certain responses of the colonized plant, as well as symbiotic efficiency, depend on the genotype of the AM fungus (Munkvold et al., 2004).

Flower formation is a key developmental pivot from vegetative growth to reproductive development and is responsive to a combination of external factors and internal signals. The earlier flowering of plants is mainly caused by large amount of P and other nutrients such as N and K supplied to the host plant after AMF inoculation (Oehl et al., 2011; Kazadi et al., 2021). Furthermore, increased concentrations of photosynthates and phytohormones in mycorrhizal plants could induce flower bud formation and flowering at an earlier stage (Asmelash et al., 2016; Liu et al., 2018). Nutrient uptake rates increase at flowering through to fruiting, which involves both a redirection of minerals into the developing fruits and a redistribution of nutrients from other plant tissues. Interestingly, our in-depth analysis of leaves showed a discernible pattern of mineral nutrients redistribution associated with the root genotype of the mycorrhizal plants closely correlates with the evolution of flowering and fruit numbers in these plants.

Although it is difficult to explain the causes of trends observed in nutrients over time, inoculation of tomato plants with AMF resulted in the following main effects on nutrient redistribution in leaves according to the different root genotypes.

(i) A set of nutrients, including Fe, Cu, K and P, showed a distribution profile in leaves associated with AM formation. Interestingly, P levels in leaves were always higher in AM control plants than in *SlGRAS18* RNAi mycorrhizal plants, thus denoting differences in the redistribution of Pi between mycorrhizal genotypes. AMF may contribute to flowering by supply both phosphorus and potassium (Conversa et al., 2015; Pereyra et al., 2019). The lowest levels of Pi observed in RNAi mycorrhizal plants show that Pi is consumed at flowering in these plants.

(ii) Except for Cu, P and Zn, the amount of nutrients in the leaves of control mycorrhizal plants was lower than in the RNAi genotype before flowering became apparent (first sampling). This could be related to a greater assimilative capacity in RNAi plants whit respect to control plants under growth conditions where the demand of nutrients competes between the plant and the AMF.

(iii) The presence of inflorescences in *SlGRAS18* RNAi mycorrhizal plants (second harvest) entails an evident redistribution of nutrients associated with increased plant investment in floral organs. Around 70% of the minerals analysed showed a reduction in

their concentrations in the leaves of mycorrhizal RNAi plants whit respect to control mycorrhizal plants in which no flowers were detected at this harvest time.

(iv) At the end of the growth period (third sampling), when signs of senescence and crop exhaustion begin to be evident, there was and overall reduction in mineral content in the leaves of mycorrhizal control plants. This may also be related to lower assimilative capacity and greater competition for resources between the AMF fungus and the plant.

The results of our study indicate that silencing *GRAS18* positively influences arbuscule formation. Moreover, this silencing significantly enhances the effectiveness of the symbiosis, particularly in traits such as earlier flowering. As mentioned, the earlier flowering of AM plants is primarily due to the increased availability of nutrients supplied to the host plant following AMF inoculation (Oehl et al., 2011; Kazadi et al., 2021). Based on this hypothesis, the observed earlier flowering could reflect higher nutrient uptake rates in the roots of AM *GRAS18*-silenced plants due to increased arbuscule effectiveness. The redistribution of nutrients in the leaves of AM *GRAS18*-silenced plants (around 70% of the minerals analysed) suggests the translocation of nutrients towards flower production, acting as a nutrient sink organ.

In summary, in this study, we show that the transcription factor SlGRAS18 (SCL3 family) plays a role during arbuscular mycorrhizal formation in tomato. Similar to its orthologue gene in Medicago, SlGRAS18 is a negative regulator of arbuscule formation, whose role is probably associated with its ability to interact with DELLA in transcriptional complexes and to regulate target genes. We also show that inoculation of *SlGRAS18* RNAi tomato plants with AMF leads to an improvement in symbiotic efficiency as measured by increases in of leaf chlorophyll content, flowering, fruit numbers and fruit yield. These improvements in physiological are associated with a discernible pattern of mineral nutrient redistribution in leaves. This study advances our knowledge of plant gene regulators of AM formation and functioning, as well as for how specific genetic alterations can lead to more effective AM symbiosis.

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CRediT authorship contribution statement

JM García Garrido: Conceptualization, investigation, resources and funding acquisition, writing original draft. JD Avilés Cárdenas; N Molinero Rosales; T Rosas-Díaz and J. Pérez Tienda: Conceptualización, methodology and investigation. A G. Castillo: Conceptualization, resources and funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.109019.

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