A Novel Putative Microtubule-Associated Protein Is Involved in Arbuscule Development during Arbuscular Mycorrhiza Formation

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The formation of arbuscular mycorrhizal (AM) symbiosis requires plant root host cells to undergo major structural and functional reprogramming to house the highly branched AM fungal structure for the reciprocal exchange of nutrients. These morphological modifications are associated with cytoskeleton remodelling. However, molecular bases and the role of microtubules (MTs) and actin filament dynamics during AM formation are largely unknown. In this study, the tomato tsb (tomato similar to SB401) gene, belonging to a Solanaceae group of genes encoding MT-associated proteins (MAPs) for pollen development, was found to be highly expressed in root cells containing arbuscules. At earlier stages of mycorrhizal development, tsb overexpression enhanced the formation of highly developed and transcriptionally active arbuscules, while tsb silencing hampers the formation of mature arbuscules and represses arbuscule functionality. However, at later stages of mycorrhizal colonization, tsb overexpressing (OE) roots accumulate fully developed transcriptionally inactive arbuscules, suggesting that the collapse and turnover of arbuscules might be impared by TSB accumulation. Imaging analysis of the MT cytoskeleton in cortex root cells OE tsb revealed that TSB is involved in MT bundling. Taken together, our results provide unprecedented insights into the role of novel MAP in MT rearrangements throughout the different stages of the arbuscule life cycle.

Keywords: Arbuscular mycorrhiza • Cytoskeleton • Hairy roots • Microtubule-associated protein • Mycorrhiza-related genes • Tomato.

Introduction

Mutually beneficial interactions between plants and microorganisms, particularly at the rhizosphere level, have a major impact on plant growth and development (Berendsen et al. 2012, Curl and Truelove 2012). In this respect, the arbuscular mycorrhizal (AM) symbiosis formed between particular kinds of soil-borne fungi (Glomeromycotina subphylum) and the roots of most higher plants is of great interest, and AM formation substantially increases the plant growth of many crop plants, especially under stress conditions (Smith and Read 2008). The formation of AM symbiosis requires plant root host cells to undergo major structural and functional modifications to house the arbuscule, a new highly branched AM fungal structure for the reciprocal exchange of nutrients (Gutjahr and Parniske 2013). The functionality and development of these arbuscules are regulated by the plant at the molecular, cellular and physiological levels, and knowledge of these basic regulatory processes is essential to understand these interactions.

Penetration and growth of AM fungi in roots activates symbiotic programs for functional mycorrhization, resulting in a complex sequence of morphological and physiological changes, including plasma membrane invagination, central vacuole fragmentation, movement of the nucleus and other organelles toward the centre of the cell, de novo formation of the so-called periarbuscular membrane, location of new transmembrane proteins in this membrane and deposition of specific cell wall molecules and proteins at the interface compartment between the two partners (Reinhardt 2007). As pointed out by Timonen and Peterson (2002), it is important to highlight the possible key role played by cytoskeleton in carrying out all these modifications in the host cells during mycorrhization. Microtubule (MT) and actin filament remodelling have actually been observed in host cells colonized by AM fungi, leading to a basket-like structure around the arbuscule (Genre and Bonfante 1998, Blancaflor et al. 2001).

The cytoskeleton is responsible for the subsequent establishment of cell polarity, plasma membrane expansion, membrane differentiation, secretion, cell wall formation and membrane recycling (Gutierrez et al. 2009, Onelli et al. 2015, Lehman et al. 2017, Thyssen et al. 2017). All these processes are reported to take place in host cells invaded by AM arbuscules and to be necessary for functional symbiosis (Balestrini and Bonfante 2014, Harrison and Ivanov 2017, Ivanov et al. 2019, Ivanov and...
Insights into cytoskeleton rearrangements during mycorrhization were initially obtained in tobacco cortex cells infected with the AM fungus Gigaspora margarita (Genre and Bonfante 1997, Genre and Bonfante 1998). In these cells, an overall increase in the α- and γ-tubulin synthesis occurs (Bonfante et al. 1996, Genre and Bonfante 1997, Genre and Bonfante 1999), and the distribution of the MT-organizing center changes from a perinuclear location to a diffuse occupation, exclusively surrounding the thin fungal branches (Genre and Bonfante 1999). Later, Blancaflor et al. (2001) reported the MT cytoskeleton pattern in Medicago root cells occupied by arbuscules of the mycorrhizal fungus Glomus versiforme and observed extensive remodelling of the MT cytoskeleton from the early stages of arbuscule development up to arbuscule collapse and senescence. Further research carried out by Genre et al. (2005) also revealed a novel cytoskeletal organization of MTs and microfilaments in epidermal cells when the pre-penetration apparatus is assembled.

As previously suggested by Timonen and Peterson (2002), the best way to gain a better understanding of the cause and effect relationship between cytoskeletal remodelling and mycorrhizal development and/or function in AM colonized roots is probably to study MT-associated proteins (MAPs) and actin-binding proteins. Interestingly, two microarray hybridizations that compare AM tomato roots and non-mycorrhizal roots (García Garrido et al. 2010, López-Ráez et al. 2010) revealed that a tomato gene called tsb (tomato similar to SB401; gene ID: Solyc01g067370), encoding a lysine-rich protein, was upregulated in roots colonized by the AM fungus Rhizophagus irregularis. The induction of tsb gene expression was reduced by over 90% in the inoculated roots of tomato mutants with impaired AM formation (the ABA-deficient sítens mutant) relative to the inoculated wild-type roots, suggesting that this gene may play an essential role in AM formation and functioning (García Garrido et al. 2010). Surprisingly, tsb has previously been described as a gene exclusively expressed in pollen (Zhao et al. 2003). The tsb gene encodes a protein, which is homologous to several pollen-specific proteins from the Solanaceae family that have been reported to function as MAPs able to bind and bundle MTs and involved in cytoskeleton remodelling during pollen development: SBgLR and ST901 from Solanum tuberosum and SB401 from Solanum berthaultii (Zhao et al. 2006, Huang et al. 2007, Liu et al. 2013).

Similarly, as cytoskeleton plays a crucial role in pollen tube development (Cai et al. 2017), cytoskeletal reorganization of plant root cells must play a similar role in the mycorrhization process. In this study, we attempt to gain an understanding of the pivotal role played by MT cytoskeletal remodelling during arbuscular mycorrhization through a functional analysis of tsb, a mycorrhizal-regulated Solanum lycopersicum gene encoding a novel putative MAP.

### Results

**TSB, a member of a Solanaceae family-specific group of MAP proteins**

To find potential homologs in plants for the TSB protein, we used BLASTP. Ten homolog proteins were obtained for TSB, with an E-value of $<10^{-12}$, all of which belonged to species from the Solanaceae family (Supplementary Fig. S1), as the other matching proteins from the BLASTP query had an E-value of $>0.03$. Thus, TSB-like proteins seem to constitute a Solanaceae family-specific group of proteins. Of the 10 putative homolog proteins, three have been previously functionally characterized and, curiously, all have been reported to function as MAPs able to bind and bundle MTs and to be involved in cytoskeletal remodelling during pollen development: SBgLR and ST901 from S. tuberosum and SB401 from S. berthaultii (Zhao et al. 2006, Huang et al. 2007, Liu et al. 2013) (Fig. 1A). The expression pattern of the tsb gene in different tomato organs, which was analysed using RT-qPCR (Fig. 1B), showed that this gene is strongly upregulated in flowers, particularly after anthesis, suggesting that tsb, like its SBgLR, st901 and sb401 homologs, could play a role in pollen development. Low expression levels were found in other organs, including non-mycorrhizal roots.

Like its close potato homologs st901 and SBgLR, the tsb gene contains three exons and two introns (Supplementary Fig. S2), and protein alignment revealed several features common to TSB and its putative homologs (Fig. 1C). TSB, ST901, SBgLR and SB401 proteins contain certain imperfect repeated motifs of the V-V-K-N/E-E sequence, a domain that resembles the repetitive K-K-E-I/V motifs, demonstrated to be responsible for in vivo interaction between the mouse neural protein MAP1B and MTs (Noble et al. 1989). In addition, many potential casein kinase II phosphorylation sites and all putative glycosylation sites were found to be conserved (Fig. 1C). Although considerable variability was detected among the various servers used for the predictions of potential casein kinase II phosphorylated sites, we suggest that residues S-162 and S-95 may play an important role in the regulation of TSB by phosphorylation. S-162 is the only phosphorylation site consensually predicted by all the servers used (Supplementary Fig. S3) and is actually situated within the V-V-K-N/E-E repeats and conserved in ST901 and SB401 proteins (Fig. 1C). Another residue that could play an important role in the regulation of TSB by phosphorylation is the amino acid S-95, which is conserved across all the TSB homologs and situated next to a V-V-K-N/E-E repeat (Fig. 1C).

**Tsb gene expression is associated with arbuscule-colonized cells in mycorrhizal roots**

To confirm that tsb shows an expression pattern associated with AM symbiosis, as previously characterized in the microarray hybridizations carried out by García Garrido et al. (2010) and López-Ráez et al. (2010), the expression pattern of the tsb gene in mycorrhizal and non-mycorrhizal tomato roots was quantified in a time-course experiment at 32, 42, 52 and 62 days post-inoculation (dpi) using RT-qPCR. As expected, tsb
gene expression was higher in mycorrhizal plant roots than in non-mycorrhizal plant roots at all times evaluated, and tsb expression was also significantly upregulated as root length colonization by R. irregularis increased over time (Fig. 2A, B), reaching its maximum expression (18.6-fold higher) at the final time of 62 dpi. The expression pattern of tsb gene was parallel to
that of the arbuscule-related genes PT4 and RAM1 (Supplementary Fig. S4). Data from similar experiments were analysed together to compare the percentage of mycorrhizal colonization and tsb gene expression for each biological replicate, between which a very significant positive correlation \( (r = 0.759, P < 0.0001) \) was found (Fig. 2C). Altogether, RT-qPCR data suggest that this gene is tightly regulated during AM development and thus could play an important role in the colonization process.

To strengthen the argument that tsb expression is associated with AM and not with the different inorganic phosphate (Pi) nutrient levels between non-mycorrhizal and mycorrhizal plants used in our experiments, we analyzed the expression of tsb in non-mycorrhizal plants under different Pi nutritional statuses (Supplementary Fig. S5). Our data ruled out a possible tsb gene induction by phosphate nutritional conditions. This result is in agreement with previous transcriptome analysis experiments using similar nutrient levels for mycorrhizal and non-mycorrhizal plants where tsb gene was identified as AM responsive (García Garrido et al. 2010, López-Ráez et al. 2010).

To analyze tsb promoter activity in roots, composite tomato plants were generated. The 1.5-kb upstream region of tsb was fused to a GUS reporter gene system (ptsb:GUS). We observed blue dye corresponding to GUS activity in non-mycorrhizal transgenic hairy roots only in some regions restricted to the central cylinder, although this observation could be unspecific

Fig. 2  Tsb gene expression is induced in tomato AM-inoculated roots and is associated with arbuscule-containing cells. After 32, 42, 52 and 62 dpi, the percentage of total root length colonized by R. irregularis was measured (A) and tsb gene expression was analysed by RT-qPCR (B) in the AM-inoculated (I) and non-inoculated (NI) root systems (\( n = 3 \)). For each sample, fold change for tsb gene expression was calculated with respect to non-mycorrhizal plant roots at 32 dpi. The scatter plot depicting the relationship between tsb expression levels and mycorrhizal colonization is shown (C), and the Pearson correlation coefficient \( (r) \) determining the relationship between two variables is indicated. GUS activity in A. rhizogenes-transformed tomato roots expressing the tsb promoter \( \beta \)-glucuronidase fusion was assessed in 54-day-old NI control plants (D) or after inoculation with R. irregularis (E, F). Vibratome-sectioned GUS-stained inoculated roots where labelled with WGA-Alexa488 to detect AM fungus structures (G–I). Bright-field image (G) exclusively shows GUS activity (deep grey colour), and green fluorescence (H) illustrates the R. irregularis hyphae stained with WGA-Alexa Fluor 488. Merged image (I) resulted from the GUS staining combined with the WGA-Alexa 488 green fluorescence. Asterisks indicate arbuscules in the host cortical cells showing GUS activity. Images were obtained using an inverted microscope (D–F) and by CLSM (G–I).
and needs to be study in more depth (Fig. 2D). In addition, mycorrhizal roots examined 54 days after inoculation also showed GUS activity in the cortex zones affected by AM fungal root colonization (Fig. 2E), mainly in cells containing arbuscules (Fig. 2F), together with the staining again found in the central cylinder (Fig. 2E). The promoter of tomato phosphate transporter 4 (SlPT4), previously described to be specifically expressed in arbuscule-containing cells (Nagy et al. 2005), was used as a positive control for the GUS staining associated with arbuscules (data not shown). Longitudinal root sections were stained for GUS activity and counterstained with WGA-Alexa 488 to visualize fungal structures (Fig. 2G–I). Colonization by R. irregularis redirected tsb expression to cortex cells colonized by AM fungal arbuscules, while neighbouring non-colonized cells remained unstained.

Surprisingly, RT-qPCR analyses revealed that only SbgLR is slightly (but not significantly) induced by mycorrhization in potato roots (Supplementary Fig. S6), a result that is in agreement with the microarray carried out by Gallou et al. (2012). We therefore hypothesize that certain additional motifs must be exclusively present in the promoter region of the tomato tsb gene to enable its specific induction in mycorrhizal colonized cells. Actually, promoter alignment analysis showed that the tsb promoter has two unique sequences (−1,132 to −996 bp and −417 to −163 bp upstream of the starting codon) not present in the SbgLR and st901 promoters, while many putative pollen-specific elements were effectively conserved among the tsb, SbgLR and st901 promoters (Supplementary Fig. S7).

To check if other genes, apart from tsb, could be induced in pollen development and mycorrhizal colonization processes in tomato, we performed a comparison based on previous transcriptomic data revealing that, from a total of 1,252 genes induced in tomato pollen development (Keller et al. 2018), 108 of these genes (6.62%) are also significantly induced (fold change >2, P-value <0.05) in roots upon colonization with the AM fungus R. irregularis (NCBI Bioproject PRJNAS09606) (Supplementary Fig. S8A). As expected, tsb is part of this set of 108 commonly upregulated genes. Moreover, gene ontology (GO) enrichment analyses (Supplementary Fig. S8B) revealed that only the specific GO terms related to the endomembrane system, vacuole and plasma membrane were significantly enriched (False Discovery Rate, FDR < 0.01) in this set of commonly upregulated genes, thus pointing towards transcriptional similarities related to the formation of new membranes occurring in both root mycorrhizal colonization and pollen tube development.

Tsb is required for proper arbuscule development and activity

Silencing (RNAi) and overexpressing (OE) tsb composite tomato plants were used to perform a functional analysis of tsb at different stages of AM development. We first analyzed a possible effect of tsb at earlier stages of AM development. For that purpose, mycorrhizal colonization and AM-associated transcriptional activity were assessed in tsb OE and tsb RNAi roots inoculated with the AM fungus R. irregularis at 43 dpi. Down- and upregulation of tsb gene expression were successfully achieved in RNAi and OE transgenic roots, respectively (Supplementary Fig. S9). Tsb RNAi roots as compared to the control roots presented a slight but significant increase (P < 0.05) in the percentage of root length colonized by the AM fungus (Fig. 3A), and the mycorrhizal parameters showed a tendency towards induction, although %a was the only parameter that was significantly induced by tsb silencing (Fig. 3B).

However, overexpression of tsb significantly affected neither root length colonization (Fig. 3C) nor the colonization parameters (Fig. 3D). These root samples were additionally submitted for the expression analysis of AM-related genes. In particular, we selected the R. irregularis RiEF constitutive gene and the S. lycopersicum PT4 gene which encodes an arbuscule-specific phosphate transporter, together with several other tomato genes, which are putative orthologs to previously described markers of arbuscule functionality, including RAM1, Vapyrin, EXO84, RAM2, AMT2.2 and STR (Zhang et al. 2010, Genre et al. 2012, Gobbato et al. 2012, Pumplin et al. 2012, Wang et al. 2012, Breuillin-Sessoms et al. 2015, Zhang et al. 2015).

Curiously, despite the observable increase in AM colonization in the tsb RNAi roots (Fig. 3A), we did not observe a significant alteration in gene expression (Fig. 3E). Rather, all genes related to arbuscule functionality showed a tendency (not significant) toward downregulation in the tsb RNAi roots (Fig. 3E). To provide more representative data on the real expression of these genes at the arbuscule level, where they are specifically expressed, we decided to normalize the value of gene expression according to arbuscular abundance by expressing the gene expression results as a ratio to the mycorrhizal parameter %A, which better represents arbuscule abundance in the total root system. In this manner, a clearly significant repression of all genes in the arbuscules of the tsb RNAi roots was obtained (Fig. 3E), suggesting that arbuscule activity, measured here as transcriptional activity, was effectively repressed in tsb RNAi hairy roots. In addition, RiEF gene expression was normalized to the mycorrhizal parameter %M, which more faithfully depicts the total AM fungal amount. Although differences were not significant, AM fungal activity measured in terms of RiEF gene expression also appeared to be negatively affected in tsb-silenced roots (Fig. 3E).

As done for the tsb RNAi hairy roots, the expression of a subset of genes required for arbuscule functionality was quantified in the tsb OE hairy roots at 43 dpi. The molecular phenotype in OE plants was just the opposite of that observed in RNAi plants. A significant increase in the expression of all marker genes was obtained for the tsb OE mycorrhizal hairy roots at 43 dpi inoculated with R. irregularis, which, in most cases, was at least twice that achieved in the control mycorrhizal hairy roots (Fig. 3F). Moreover, this trend was maintained when relativizing gene expression to the arbuscule abundance parameter %A, or to %M in the case of RiEF (Fig. 3F). To check if the results obtained are reproducible for other AM fungus species, in parallel, we also analyzed the effect of tsb overexpression in Funneliformis mosseae inoculated roots. In our experiments, F. mosseae showed a slower colonization dynamic than R. irregularis, and the results obtained for F. mosseae–colonized roots at 65 dpi (20–30% mycorrhizal colonization; Fig. 3G) were...
comparable to those for *R. irregularis*-inoculated roots at 43 dpi, with the *F. mosseae*-inoculated *tsb* OE roots depicting a similar induction in the expression of all marker genes (Fig. 3H).

As the results obtained for AM colonization and arbuscule abundance in *tsb* RNAi plants were appeared to be inconsistent with arbuscule-associated gene expression, we wondered whether arbuscule morphology was altered by *tsb* silencing and/or overexpression in a more coherent way. An examination of roots subjected to trypan blue or WGA-Alexa Fluor 458 staining of AM fungal structures was performed. We observed that, compared to the control roots transformed with the corresponding empty vectors (Fig. 4A–D), highly intense arbuscules, which completely occupied the whole cytoplasm of the arbuscule-hosting cell, were more abundant in the *tsb* OE roots (Fig. 4E–H). By contrast, in the *tsb*-silenced roots, most of the arbuscules seemed to be incompletely formed and we hardly ever found root areas full of mature arbuscules (Fig. 4I–L), which is in agreement with the lower gene expression associated with arbuscule activity found in these roots.

To statistically confirm differences observed in arbuscule morphology upon *tsb* silencing or overexpression, the percentage of root intersects with a prevalence of arbuscules at three different morphological phases of arbuscule formation was quantified. As similarly described in Herrera-Medina et al. (2007), three arbuscule classes were defined as follows: class a, arbuscules in formation (or degradation), with no fine branches, partially occupying the plant cell; class b, arbuscules with intermediate intensity of trypan blue stain occupying almost all of the plant cell; class c, arbuscules with a high intensity of trypan blue stain.
occupying the whole of the plant cell. As expected, the comparison of \( \text{tsb} \) OE roots with respect to the control roots transformed with the empty vector revealed that, in the \( \text{tsb} \) OE hairy roots, fully developed arbuscules (class c) were significantly more abundant (3.5-fold), while small- and medium-sized arbuscules (classes a and b) were under-represented (Fig. 4M). Accordingly, \( \text{tsb} \) RNAi hairy roots showed a 5-fold increase in small and unbranched arbuscules (class a), while the presence of mature arbuscules was impaired in these roots, exhibiting a 3-fold decrease in class c arbuscules (Fig. 4N).

Fig. 4 Analysis of arbuscule morphology in control, \( \text{tsb} \) OE and \( \text{tsb} \) RNAi roots at earlier stages of AM development (43 dpi). Images correspond to representative pieces of tomato control hairy roots transformed with the empty vector (A–D), as well as \( \text{tsb} \) OE (E–H) and \( \text{tsb} \) silenced (I–L) hairy roots at 43 d after inoculation with \( R. \) irregularis, and subjected to trypan blue or WGA-Alexa Fluor 488 staining. Images of trypan blue-stained roots were taken with an optical microscope (scale bars = 50 \( \mu m \)) (A, B, E, F, I, J) and WGA-Alexa Fluor 488 fluorescent staining were visualized by CLSM (scale bars = 100 \( \mu m \)) (C, D, G, H, K, L). Arrowheads indicate incompletely formed arbuscules. Graphs show the percentage of root intersects with a prevalence of arbuscules from three different morphological types: class a (small and unbranched), class b (medium-sized) and class c (highly intense, occupying the whole plant cell) and the percentage of root intersects in which AM fungal vesicles were present (n = 4; 120 root intersects per biological sample), measured in \( \text{tsb} \) RNAi (M) and \( \text{tsb} \) OE (N) plants and compared with their respective control. Values correspond to mean ± SE. Significant differences (Student’s t-test) between the plants transformed with the corresponding empty vectors (EV) and RNAi or \( \text{tsb} \) OE-transformed plants are indicated with asterisks (*\( P < 0.05 \); ***\( P < 0.001 \); ****\( P < 0.0001 \)).
These results as a whole indicate that the tsb gene is clearly involved in the proper development of arbuscules, as well as in correct arbuscular activity and functionality, measured here as transcriptional activity.

**Tsb OE triggers an accumulation of senescent arbuscules at late stages of AM development**

To determine whether the effects on arbuscular development are maintained over time, *R. irregularis*-colonized composite plants from the same tsb OE and RNAi experiment described above were collected at a second harvesting point (65 dpi). RNAi lines maintained a similar trend, with a slight increase in the percentage of root length colonized by the AM fungus with respect the control (empty vector), no changes in the arbuscule abundance in the total root system and no significant alteration in gene expression, except that due to the tsb silencing (Supplementary Fig. S10). Again, control and tsb OE plants did not show significant differences in root mycorrhizal colonization, which, this time, reached levels of ～50% (Fig. S1A). However, when analysing gene expression, we obtained a very interesting result. Surprisingly, a generalized significant repression of arbuscule functionality in all marker genes upon tsb overexpression occurred at these advanced stages of AM colon- ization (65 dpi) (Fig. 5B), in contrast with the induction observed of these genes in tsb OE plants at earlier stages of mycorrhization (43 dpi) (Fig. 3F). A second independent experiment, where roots were harvested at 77 dpi, confirmed that at later stages of mycorrhizal colonization, tsb OE gives rise to an overall downregulation of arbuscule marker genes (Supplementary Fig. S11).

Interestingly, a detailed histological examination of arbuscule morphology showed that, in tsb OE hairy roots, fully developed (class c) arbuscules were significantly more abundant, while small and unbranched (class a) arbuscules were under-represented (Fig. 5C). The repression of arbuscule marker genes in tsb OE roots (Fig. 5B) appeared to contradict the predominant abundance of highly intense arbuscules occupying the whole cell in these roots (Fig. 5C), as arbuscules with this morphology are typically associated with mature and highly active arbuscules. To better determine the arbuscule phenotype in the tsb OE roots at later stages of mycorrhization, the viability of arbuscules was analyzed by measuring succinate dehydrogenase (SDH) activity (Schaffer and Peterson 1993). We found that the percentage of arbuscules with SDH activity was reduced by nearly a half in tsb OE roots, with only 46% of arbuscules showing SDH activity in these roots (Fig. 5D). In addition, the root samples were submitted to co-staining of the neutral lipid fatty acids (NLFAs) using the fluorescent probe Nile red (NR) to visualize NLFA accumulation in cells containing arbuscules, a feature described to be typical of senescent arbuscules (Kobae et al. 2014, Feng et al. 2020). While NR staining was virtually absent in the control mycorrhizal roots at 65 dpi, tsb OE roots at 65 dip showed a large number of arbuscules accumulating NLFA (Fig. 5E). Both the decrease in arbuscules showing SDH activity and the increased number of arbuscules with NLFA upon tsb overexpression at later stages of mycorrhizal colonization are in agreement with the lower expression of arbuscular genes observed in these tsb OE roots.

**Tsb overexpression leads to the bundling of cortical MTs**

MT immunostaining was carried out to check if TSB, as a putative MAP, plays a role in MT dynamics. The control hairy roots containing the empty vector showed the typical helicoidal arrangement of MTs in the mature cortex cells described elsewhere for tomato roots (Genre and Bonfante 1997, Genre and Bonfante 1998) (Fig. 6A). By contrast, non-inoculated roots overexpressing the tsb gene appeared to show a differential
array of MTs, consisting of wider spaced MTs following a random organization (Fig. 6B). A detailed analysis of MT fibre metrics was performed using image processing with CSK Morphometrics software (Flores et al. 2019). Interestingly, in the tsb OE root cortex cells, fibre thickness was found to be over 2-fold thicker than that for the control roots, with a very high statistical significance ($P < 0.0001$) (Fig. 6C), clearly indicating that bundling of MTs occurs upon tsb overexpression. Moreover, this effect of tsb OE on MT bundling seems to affect, in a general manner, all MTs, as thickness variance was reduced in these roots (Fig. 6D). In addition, the number of fibres measured per cell was greatly reduced in the tsb OE cortex cells (Fig. 6E), and fibre length showed a tendency to increase (Fig. 6F), which is consistent with the generalized MT bundling in the root cortex cells overexpressing the tsb gene. Regarding the organization of MTs, the angular variance in tsb OE cells showed a tendency (although not significant) to increase, suggesting that MTs may have a more random organization in
these cells (Fig. 6G). No significant differences were found among the many other cytoskeletal fiber metrics recorded (Supplementary Fig. S12), including total fluorescence intensity, a negative control that enabled us to rule out possible variabilities due to the immunostaining procedure.

MT immunostaining was similarly performed in mycorrhizal roots to analyse possible alterations in MT organization from variabilities due to the immunostaining procedure. MT pattern consisted in a net of MTs enveloping the arbuscule and create a very complex 3D pattern with denser and sparser areas and short bundling spots could be lost in the crowd, making difficult to quantify MT cytoskeleton structures.

**Discussion**

Tsb encodes a putative MAP belonging to a Solanaceae-specific group of pollen-related proteins, herein named TSB-like proteins (Zhao et al. 2006, Huang et al. 2007, Liu et al. 2013). In this study, we show that a very significant positive correlation exists between mycorrhizal colonization levels and tsb gene expression. We also demonstrate that tsb gene expression is particularly associated with arbuscule-containing cells, suggesting that this gene could play an important role in the AM colonization process at the arbuscule level.

Substantial MT cytoskeletal remodelling has been observed in arbuscule-containing cells (Genre and Bonfante 1999, Blancaflor et al. 2001). Nevertheless, little is known about the specific role of MTs during mycorrhization and their possible involvement in directional growth or the specific plasma membrane and cell wall formation in arbuscule-hosting cells. In this study, we addressed this issue through a functional characterization of the tsb tomato gene induced during mycorrhization and encoding a putative MAP. We investigated the arbuscule morphology and functionality of tsb OE and tsb RNAi hairy roots by analyzing histological features and by measuring the expression of several arbuscule-specific genes, including RAM1, PT4, STR, AMT2.2, Vapyrin, EXO84 and RAM2. Our results suggest that tsb participates in MT rearrangements in arbuscule-hosting cells and is required for proper arbuscule development and activity.

At earlier stages of colonization, tsb overexpression clearly enhances arbuscule-specific gene expression for all the marker genes used. Accordingly, a general repression of all these genes was observed in the tsb silenced roots at the arbuscule level. A detailed histological examination of AM fungal development also revealed differences in arbuscule morphology. The mature and compact arbuscules occupying most of the cell volume were found to be predominantly present in the tsb OE roots, while small unbranched arbuscules prevailed in the tsb RNAi roots. Thus, at the earlier stages of AM development, tsb expression levels in the different composite plants used (control, tsb OE and tsb RNAi plants) positively correlated with the degree of arbuscule formation and with the expression of genes encoding for specialized periarbuscular transporters and proteins reported to provide a specific exocytosis capacity necessary for membrane proliferation. Taking into account previous evidence on the role of MTs in membrane and cell wall formation in polarized cells (Sieberer et al. 2005, Muller and Jurgens 2016, Cai et al. 2017), we speculate that a TSB is an MAP that could be responsible for developing a suitable MT arrangement in arbuscule-containing cells for vescicle transport, as well as for the proper delivery, embedding and positioning of specific compounds in the periarbuscular membrane and interface compartment that facilitates correct arbuscule development and functionality.

It is worth mentioning that, although small unbranched arbuscules predominated in the tsb RNAi plants, the percentage of total arbuscules was quite high as compared to those in control plants. In our view, this confusing observation is probably due to a mechanism used by the AM fungus to compensate for the impossibility of establishing functional symbiotic structures. Similarly, with respect to *Rhizobium*-legume symbiosis, Rogato et al. (2008), while also pointing to a compensation mechanism, observed a higher nodulation capacity accompanied by an inhibition of nodule activity in AMT RNAi Lotus lines.

As the cortical MT arrangements of arbuscule-containing cells are modified during arbuscule maturation, senescence and collapse (Blancaflor et al. 2001), we decided to expand the study of tsb OE roots to more advanced stages when mycorrhizal colonization is well established. In these roots, we observed the transcriptional repression of arbuscule marker genes, a lower percentage of metabolically active arbuscules with SDH activity, together with an enormous increase in the number of root cells with arbuscules that accumulated neutral fatty acids, a typical sign of senescent arbuscules (Kobae et al. 2014). Altogether, our data suggest that an accumulation of transcriptionally and metabolically inactive senescent arbuscules occurs upon TSB protein accumulation in tsb OE composite plants at advanced stages of mycorrhization. Moreover, we hypothesize that tsb OE gives rise to an accumulation of apparently mature highly developed arbuscules, many of which must be inactive and senescent, suggesting that their collapse and turnover are impaired in tsb OE. Maybe, fragmentation and unbundling of these MTs might be required for arbuscule collapse. In this respect, TSB accumulation in tsb OE roots might hamper these MT cytoskeleton rearrangements that naturally occur after arbuscule development, making it impossible for senescent arbuscules to collapse, with a pronounced accumulation of fully developed metabolically and transcriptionally inactive arbuscules being observed.

In a previously proposed model of MT dynamics during mycorrhization, MT bundling is reported to occur during arbuscule formation, with MT bundles appearing along the arbuscule trunk and throughout the arbuscule branches, together with additional MT bundles connecting arbuscule branches to each other (Genre and Bonfante 1997, Genre and Bonfante 1998, Blancaflor et al. 2001). In completely mature arbuscules, all these MT fibers were found to get thinner (less bundled) and...
more fragmented (Blancaflor et al. 2001). Finally, during arbuscule senescence and collapse, new bundles of longer MTs connecting the arbuscule to the cell periphery transiently appear in parallel to the reconstruction of the helicoidal array of cortical MTs typical of non-mycorrhized cortex cells (Blancaflor et al. 2001). Our data of MT immunostaining of tomato root cortex cells show a trend toward more random MT organization and a sharp increase in fibre thickness in the \( \text{tsb}\) OE roots, suggesting a strong MT-bundling effect on \( \text{tsb}\) overexpression and \( \text{tsb}\) accumulation. Although images of immunostaining mycorrhizal \( \text{tsb}\) OE and silencing roots did not show macroscopic alteration of periarbuscular MT organization, we cannot exclude the occurrence of local bundling (as suggested by individual images in non-inoculated OE roots), and possibly more refined techniques can be required to observe subtle differences. Actually, MT binding and bundling ability has previously been demonstrated for the close \( \text{tsb}\) homologs SBgLR from potato and SB401 from wild potato (Huang et al. 2007, Liu et al. 2013). The \( \text{tsb}\) homolog SB401 is able to bind and bundle both MTs and actin filaments, which is regulated through phosphorylation by specific kinases (Liu et al. 2009, Komis et al. 2011).

According to this model and given the results obtained with the \( \text{tsb}\) RNAi and \( \text{tsb}\) OE plants, we suggest that \( \text{tsb}\) could act as a MAP, which is required for the formation of MT bundles surrounding the arbuscule. We also hypothesize that these bundles are essential for proper arbuscule development and activity and probably contribute to the specific exocytosis capacity necessary to form the specialized cell wall and membrane surrounding the arbuscule (Fig. 7).

In conclusion, this research shows the involvement of \( \text{tsb}\), acting as a MAP protein, in the MT-cytoskeletal remodeling required for arbuscule development and functionality.

The study also provides initial evidence of the role of MT-cytoskeletal rearrangements during different stages of the arbuscule life cycle, from its formation to its senescence and collapse. However, further research is needed for effective in vivo confirmation that \( \text{tsb}\) is an MT-binding protein and to elucidate the specific \( \text{tsb}\)-mediated changes in cytoskeletal remodelling in arbuscule-containing cells. Also, the possible role of \( \text{tsb}\) in actin filament dynamics cannot be ruled out, since many plant MAPs, including the \( \text{tsb}\) homolog SB401 (Huang et al. 2007), bind both MTs and actin filaments (Wang et al. 2007, Deeks et al. 2010, Li et al. 2011, Zhu et al. 2013, Qin et al. 2014). This study also provides an insight into the existence of specific biological machinery commonly shared by arbuscule-containing cells and pollen tube cells that, as previously suggested by other authors, undergo strong membrane polarization (Nguema-Ona et al. 2012, Nouri and Reinhardt 2015, Ivanov and Harrison 2019).

**Materials and Methods**

**Plant growth and AM inoculation**

*Solanum lycopersicum* (Moneymaker cv) seeds were surface sterilized with a 5-min soaking using 2.35% w/v sodium hypochloride, subjected to shaking for 1 d in the dark at room temperature (RT) and germinated on sterilized moistened filter paper for 3 d at 25°C in the dark. Germinated seeds were deposited on vermiculite for hypocotyl elongation for 1 week. 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 fiber (2:2:1, by volume). In the AM inoculated treatments, the plants were inoculated with a piece of monoxenic culture in Gel-Gro medium produced according to the method described by Chabot et al. (1992), containing 50 *R. irregularis* (DAOM 197198) (Schüller and Walker 2010) spores and infected carrot roots. For the non-inoculated treatment, a piece of Gel-Gro medium containing only uninfected carrot roots was used. Plant growth took place in a growth chamber (day:
night cycle, 16 h, 24 °C, 65% humidity, 50% with an average photosynthetic photon flux density of 350 μmol m⁻² s⁻¹.

Fumellaforms mosseae (Schüller and Walker 2010) inoculum was obtained from the EEZ germplasm collection. The fungus was propagated using sorghum (Sorghum bicolor) as the host, and the infected roots, hyphae, spores and substrates were collected and used as inoculum (40 g per pot). An inoculum filtrate (washed and filtered twice through a 20-μm mesh) was added to all non-inoculated pots to create a uniform microbial community in all treatments.

One week after planting and weekly thereafter, 20 ml of a modified Long Ashton nutrient solution (Hewitt 1966) containing 0.325 mM of P concentration was added to the pots to prevent mycorrhizal inhibition due to excess of phosphorus. In the case of non-mycorrhizal plants, a complete Long Ashton solution (1.3 mM P) was used in the same manner. Plants were harvested at different developmental stages, as well as the presence or absence of vesicles. For a detailed imaging of arbuscule morphology, the inoculated roots were embedded in 4% agarose blocks, and 60 μm transverse sections were cut on a Leica VT1200S vibratome. Root sections were vacuum-infiltrated with 10 μg ml⁻¹ Wheat Germ Agglutinin (WGA)-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR, USA) in Phosphate-buffered saline (PBS 1x) for 60 min in the dark. Z-stack images were obtained using a laser scanning confocal fluorescence microscope (C-1; Nikon).

Dual labeling of SDH activity and neutral lipids was also conducted to investigate the presence of metabolically active arbuscules and the accumulation of neutral lipids in AM fungal structures, based on Kobae (2014). One-centimeter root fragments were digested in enzyme solution (0.05 M pH 9.2 Tris/citric acid buffer, 50 mg ml⁻¹ sorbitol, 3 μl ml⁻¹ cellulase, 3 μl ml⁻¹ pectinase) for 1 h at room temperature. Root samples were washed and covered with a solution for staining an SDH-active hyphae containing 0.2 M Tris/HCl buffer (pH 7.4), 5 mM MgCl₂, 1 mg ml⁻¹ nitro blue tetrazolium and 0.25 μM sodium succinate. Roots were submitted to a vacuum infiltration for 20 min followed by an overnight incubation at 37°C. After incubation in the reaction medium, roots were soaked with 1% sodium hypochlorite for 15 min and washed generously with water. For subsequent fluorescent staining of neutral lipids, NR was used. Root segments were incubated for 1 h in the dark in the NR solution (2 μg ml⁻¹ NR in lactic acid).

RNA extractions and gene expression quantification

For the RT-qPCR experiments (reverse transcription and quantitative real time polymerase chain reaction), representative root samples from each root system were collected and immediately frozen in liquid nitrogen. Eventually, other tomato organs were also collected. Tissues were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was isolated from 0.2 g samples using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions and treated with RNase-Free DNase. One microgram of DNase-treated RNA was reverse-transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the supplier’s protocol. For the quantitative real time PCR (qPCR), a 20 μl PCR was prepared containing 1 μl of diluted cDNA (1:10), 10 μl 2X SYBR Green Supermix (Bio-Rad) and 200 nM of each primer using a 96-well plate. The PCR program consisted of a 3-min incubation period at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 58–63°C and 30 s at 72°C. The specificity of the PCR amplification procedure was checked using a melting curve after the final PCR cycle (70 steps of 30 s, from 60 to 95°C, at a heating rate of 0.5°C). Experiments were carried out on five biological replicates, and the threshold cycle (Ct) was determined in triplicate. Relative transcription levels were calculated using the 2⁻ΔΔCt method (Livak and Schmittgen 2001). The Ct values of all genes were normalized to the geometric mean of Ct values from the LeEF-1α (accession X14449) and actin (NM_001321306.1) housekeeping genes.

The RT-qPCR data for each gene were shown as the relative expression with respect to the reference treatment to which an expression value of 1 was assigned. The reference treatment generally corresponded to the non-AM inoculated treatment or control plants transformed with the empty vector. All genes, whose transcript abundance was measured by RT-qPCR, and the corresponding primers used are listed in Supplementary Table S1.

Plasmid construction and hairy root transformation

The tsb gene and the tsb RNAi fragment were amplified from S. lycopersicum cDNA of roots infected by the AM fungus R. irregularis. The putative promoter of tsb (a 1,484-bp fragment immediately upstream of the tsb start codon) was amplified from genomic DNA of S. lycopersicum cv MoneyMaker. Amplifications were carried out by PCR using iProof High Fidelity DNA-polymerase (BioRad) and specific primers (Supplementary Table S1). PCR fragments were introduced into the pENTR/D-TOPO (Invitrogen) vector and sequenced. pENTR/D-TOPO containing the tsb gene, an RNAi tsb fragment and the tsb promoter were subsequently recombined into the pUBICGFP-DR (Kiryuworthco et al. 2016), pKGWIIWG2-RRdRedRoot (http://gateway.psb.ugent.be) and pBGFW57: pARTub10:DsRed (modified from Karimi et al. 2002) vectors, respectively, using GATEWAY technology (Invitrogen).

For hairy root transformation, Agrobacterium rhizogenes MSU840 cultures harbouring the corresponding overexpression, RNAi and promoter-GUS constructs were used to transform S. lycopersicum cv MoneyMaker plantlets according to the protocol described by Ho-Plagaro et al. (2018). Composite plants were transferred to pots under the same plant growth conditions as explained previously. Screening and selection of DsRed (transformed) roots were carried out by observation under a fluorescent Leica M165SF stereomicroscope.

Gene expression localization

To localize the tsb gene expression, AM inoculated and non-inoculated transgenic roots carrying the tsb promoter-GUS fusion were used, based on a technique originally developed by Jefferson (1989). Pt4 promoter-GUS fusion transformed roots were used as control. Hairy root fragments (1 cm) carrying the promoter-GUS fusions were vacuum-infiltrated with a GUS staining solution composed of 0.05 M sodium phosphate buffer, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 0.05% Triton X-100, 10.6 mM EDTA-Na and 5 μg ml⁻¹ X-gluc cyclodextrin sodium salt (previously dissolved in N,N-dimethylformamide) for 30 min to improve substrate penetration. The tissues were then incubated in the dark at 37°C from 1 h to overnight until staining was satisfactory in the same staining solution.

For co-staining of the AM fungus, GUS-stained AM inoculated roots were submitted to WGA staining following the procedure explained in the Staining of the AM fungus and neutral lipids section.

MT immunofluorescence staining

Staining of the MT cytoskeleton of tomato hairy roots was carried out based on the protocol used by Blanchaff et al. (2001). Hairy roots were selected under a stereomicroscope and fixated for 2 h with PMA buffer (50 mM piperazine-diethanesulfonic acid, 2 mM MgCl₂ and 10 mM EDTA pH7) containing 3.7% formaldehyde and 5% dimethyl sulfoxide (DMSO). Roots were washed with PMA buffer, attached to an aluminum block using cyanoacrylate and cut longitudinally with a vibrotome (Vibrotome 1000 Plus; Vibrotome Company, St. Louis, MO, USA). The 60-μm sections were transferred onto glass cover slips and secured with a thin film of agar. Root sections were digested for 10 min with...
1% cellulose Y-C (Karlan Research Products, Santa Rosa, CA, USA) in PME buffer, washed, incubated for 20 min in 1% Triton X-100 and also in PME buffer and washed again. Sections were subjected to a 2-h incubation with monoclonal rat anti-yeast 138 alpha tubulin (clone YOL1/34; 1:50 dilution; Accurate Chemicals, Westbury, NY, USA), washed, incubated for 2 h with goat anti-rat IgG (H + L) conjugated with Alexa Fluor 488 (1:100 dilution; Molecular Probes, Life Technologies, Eugene, OR, USA) and washed again. All sections were mounted in 20% Mowiol 4-88 (Calbiochem, La Jolla, CA, USA) and observed under a Perkin Elmer Ultraview ERS spinning disk confocal microscope (Norwalk, CT, USA). Single-cell quantification of MT cytoskeleton structures was performed using the CSKMorphometrics algorithm (Flores et al. 2019).

**Bioinformatics analysis**

The TSB amino acid sequence was subjected to a homology search against the Viridiplantae database (taxid: 33090) using the on-line BLAST server at NCBI (www.ncbi.nlm.nih.gov) with all default settings on. From the resulting blastp output, putative TSB homologs were subjected to phylogenetic analysis. Phylogenetic relationships were determined using Geneious software (Biomatters Ltd.) to create a neighbour-joining tree using the Jukes-Cantor model. Possible homologs previously characterized in the literature were selected for further alignment using Clustal Omega (Sievers et al. 2011). The imperfect repetitive V-V-E-K-K-N/E-E motifs were identified. Putative N-linked glycosylation sites were predicted using the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/). Potential casein kinase II phosphorylation sites were detected using the KinasePhos 2.0 (Wong et al. 2007) and NetPhos 3.1 (Blom et al. 2004) servers and the plant-specific phosphorylated site predictors PlantPhos (Lee et al. 2011) and PhosPhAt 4.0 (Durek et al. 2010).

Putative functional promoter elements were identified using the PlantCARE (Plant Cis-Acting Regulatory Element) database (Lesot et al. 2002).

**Statistical analysis**

When two means were compared, data were analysed using a two-tailed Student’s t-test and significantly different means were labelled with asterisks (*P < 0.05; **P < 0.01; ***P < 0.001). For comparisons of all means, one-way or two-way analysis of variance (ANOVA) was performed followed by the Least Significant Difference (LSD) multiple comparison test and significant differences (P < 0.05) were labelled with different letters. Graphpad Prim version 6.01 (Graphpad Software, San Diego, CA, USA) was used to determine statistical significance. Data represent the mean ± SE.

**Disclosures**

The authors have no conflicts of interest to declare.

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