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Ectopic activation of cortical cell division during the accommodation of arbuscular mycorrhizal fungi

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Title page

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Title

Ectopic activation of cortical cell division during the accommodation of arbuscular mycorrhizal fungi

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Summary

• Arbuscular mycorrhizas (AM) between plants and soil fungi are widespread symbioses with a major role in soil nutrient uptake.

• In this study we investigated the induction of root cortical cell division during AM colonization by combining morphometric and gene expression analyses with promoter activation and protein localization studies of the cell plate-associated exocytic marker TPLATE.

• Our results show that TPLATE promoter is activated in colonized cells of the root cortex where we also observed the appearance of cells that are half the size of the surrounding cells. Furthermore, TPLATE-GFP recruitment to developing cell plates highlighted ectopic cell division events in the inner root cortex during early AM colonization. Lastly, transcripts of TPLATE, KNOLLE, and CYC1 are all upregulated in the same context, alongside endocytic markers AP2A1 and CHC2, known to be active during cell plate formation. This pattern of gene expression was recorded in wild-type Medicago truncatula roots but not in a CSSP mutant where fungal colonization is blocked at the epidermal level.

• Altogether, these results suggest the activation of cell division-related mechanisms by AM hosts during the accommodation of the symbiotic fungus.

Introduction

The majority of extant plants, including both wild and domesticated crop species, is supported in its uptake of soil nutrients by Glomeromycotina fungi (Lin *et al.,* 2014) - in a beneficial symbiosis called arbuscular mycorrhiza (AM). The broad diffusion of this interaction among plant species is considered a consequence of its ancient evolutionary origin (Bonfante & Genre, 2008). Indeed, 400-million-year old plant fossils already hosted hyphal structures that closely resemble the arbuscules produced by living AM fungi (Remy *et al.,* 1994; Strullu-Derrien *et al*., 2018). Arbuscules are highly branched hyphae that develop inside living cells of the root cortex (Gutjahr & Parniske, 2013). In fact, arbuscules are accommodated inside a novel cell compartment enveloped by the perifungal (or periarbuscular) membrane, a specialized extension of the host plasmalemma (Pumplin & Harrison, 2009). This symbiotic interface compartment assembles within the pre-penetration apparatus (PPA), a broad, nucleus-associated cytoplasmic aggregation, where the secretory process is focused and coordinated (Genre *et al*., 2005; 2008).

Both PPA organization and fungal accommodation depend on the activation of a conserved signal transduction pathway (Delaux *et al*., 2015), the so-called Common Symbiotic Signaling Pathway, or CSSP (see Oldroyd, 2013 for a comprehensive review) and represent the earliest visible response of plant cells to AM fungi (Genre *et al*., 2005). The CSSP is believed to transduce diffusible AM fungal signals through the sequential activation of a number of proteins, starting from receptor-like kinases in the plasma membrane and culminating with the nuclear localized kinase DOESN'T MAKE INFECTIONS 3 (DMI3; Lévy *et al.*, 2004), which mediates the regulation of gene expression by activating a set of transcription factors (Oldroyd, 2013; Zipfel and Oldroyd, 2017; Floss *et al*., 2017). Altogether, CSSP activation is essential for root colonization (Novero et al., 2002; Lévy et al 2004, Kistner et al., 2005; Parniske, 2008), as demonstrated by the block of PPA formation (Genre *et al*., 2005) and epidermal penetration in *dmi3* mutants (Morandi *et al.,* 2005).

Biogenesis of the perifungal membrane has been described as a massive exocytic process that involves cytoskeletal rearrangements as well as the preemptive accumulation of an extensive quantity of secretory membranes, such as trans-Golgi network (TGN) compartments and vesicles (Genre *et al*., 2012). Alongside secretion-related endomembrane compartments, transmission electron microscopy imaging of PPA aggregates has shown the abundant presence of multivesicular bodies - or late endosomes - indicating that endocytic processes are likely also in place during interface biogenesis (Genre *et al*., 2012), even if their role has not been investigated further.

Such intense and targeted membrane dynamics have few analogs in plant cells and possibly the most notable similarity is with cell division. This process initiates with vacuole partitioning by fusion of multiple trans-vacuolar cytoplasmic bridges (phragmosome) and nuclear positioning in the cell center

(Smith, 2001; Lloyd & Buschmann, 2007). Phragmosome formation is followed by regular mitosis and cell plate assembly through the progressive fusion of TGN-derived cytokinetic vesicles (Segui-Simarro *et al.*, 2004; Smertenko *et al.*, 2017). Intriguingly, Siciliano *et al.*, (2007) reported that histone H2B1 gene was induced in root segments of Medicago on which hyphopodium formation had occurred, hinting at the possibility that cell cycle determinants are active during early AM colonization. The involvement of cell division-related processes has also been proposed for the symbiotic nitrogen fixation, where infection thread growth is coupled to cell cycle reactivation and cell division events (Timmers *et al.*, 1999; Fournier *et al.*, 2008; Breakspear *et al.*, 2014; Downie, 2014).

During plant cell division, the massive exocytic process directed by cytoskeletal fibers in the phragmoplast (Lee & Liu, 2013; Boruc & van Damme, 2015) is associated with endocytic recycling of surplus membrane. In fact, next to cell plate specific markers such as the syntaxin KNOLLE, other plasma membrane proteins also strongly accumulate at the forming cell plate (Van Damme *et al.*, 2006; Richter *et al.*, 2014), among which, the adaptin-related protein TPLATE has been shown to accumulate on both the cell plate membranes and plasmalemma at the cortical division zone, where the cell plate will eventually fuse (Van Damme *et al.*, 2006; Van Damme *et al.*, 2011; Gadeyne *et al.*, 2014). TPLATE is the founding member of an octameric protein complex, the TPLATE complex (TPC), which acts largely together with Adaptor Protein complex 2 (AP-2) and other components (such as Dynamin-related proteins) and the clathrin scaffolding proteins for the assembly of the clathrin endocytic machinery at the plasma membrane (Gadeyne *et al.*, 2014). The accumulation of TPC members at the cortical division zone has been proposed to facilitate membrane reorganization during cell plate fusion with the peripheral plasmalemma (Boutté *et al.*, 2010; Van Damme *et al.*, 2011). In short, TPLATE appears to be a promising marker to investigate the occurrence of both cell division- and endocytosis-related processes during the accommodation of AM fungi.

Here, we report that *TPLATE* expression is activated in cortical root cells colonized by arbuscular mycorrhizal fungi, where we also observed the appearance of arbusculated cells with half the size of the surrounding cortical cells. Combined with TPLATE-GFP recruitment to forming cell plates, this allowed us to identify ectopic cell division events that are induced in the inner root cortex during AM colonization of the outer root tissues. Lastly, *TPLATE* transcripts, alongside other cell division and endocytic markers, are upregulated in the colonized area of wild-type roots but not in a CSSP mutant where fungal colonization is blocked at the epidermal level. Taken together, these results suggest that extant plants activate cell division-related mechanisms during AM fungal accommodation.

Materials and Methods

Plant materials

The generation of composite *L. japonicus* plants was performed using *Agrobacterium rhizogenes* strain Ar1193 carrying the*pLjTPLATE:GUS* vector, according to the protocol by Stougaard (1995) on wild-type seedlings of MG20 genotype (see below).

Medicago truncatula Jemalong A17 and *Lotus japonicus* MG20 plants were grown from surface sterilized seeds in pots containing sterile quartz sand and respectively inoculated with *Gigaspora margarita* or *Funneliformis mosseae* and watered with Long-Ashton solution (Hewitt, 1966).

Agrobacterium rhizogenes-transformed root organ cultures (ROCs) expressing the *35S::AtTPLATE-GFP* vector (Van Damme *et al.,* 2004) were obtained from *Medicago truncatula* Jemalong A17 wild-type and *dmi3-1* plants (Sagan *et al.,* 1995; kindly provided by M. Chabaud, LIPM, INRA, Toulouse, France), according to Boisson-Dernier *et al.* (2001). ROC generation was repeated in two independent experiments for both lines, with overlapping results in terms of GFP fluorescence pattern. ROCs from *Daucus carota* var Sativus expressing the same vector were obtained according to Bécard & Fortin (1988). For both species, transformed roots with a high level of fluorescence were selected 21 days after transformation, decontaminated and subcultured on M medium (Bécard & Fortin, 1988) at 25°C in the dark for subsequent use as hairy roots. In each case, a single representative clone was chosen for further studies. Transformation efficiency and expression of *35S::AtTPLATE-GFP* was checked with GFP specific primers (Supporting Information Table S1) on both genomic DNA and cDNA obtained from all the selected lines. Wild type and *dmi3-1* mutant *M. truncatula* ROCs expressing a nuclear cameleon YC2.1 probe were already available in the lab (Chabaud *et al.,* 2011; Genre *et al*., 2013) and were used for all gene expression analyses.

Fungal materials

Different AM fungi were chosen depending on how their characteristics suited the experimental setup. The AM fungus *Funnelliformis mosseae* (strain BEG 12), supplied by MycAgroLab (http://www.mycagrolab.com/), was used to inoculate *L. japonicus* composite plants with a 10% ratio of inoculum to total substrate volume; *Gigaspora margarita* (BEG34, International Bank for Glomeromycota, University of Kent, Canterbury, UK) was used to inoculate *M. truncatula* whole plants (50 spores per pot) and ROCs (Chabaud *et al*., 2002) for gene expression analyses. *Gigaspora gigantea* (isolate HC/FE30, Herbarium Cryptogamicum Fungi, University of Torino, Italy), which is characterized by strong cytoplasmic autofluorescence (Genre *et al*., 2005), was used to inoculate ROCs for live confocal imaging. Spores of both *G. gigantea* and *G. margarita* were collected from

pot cultures in sand (with leek and clover, respectively), surface-sterilized and stored at 4°C according to Bécard & Fortin (1988).

GUS assay

A *LjTPLATE* promoter fragment of 2073 bp (Supporting Information Fig. S1) was PCR-amplified from genomic DNA by using specific primers (Supporting Information Table S1). The resulting promoter fragment was cloned into pDONR221 (Invitrogen) and recombined upstream of the GUS gene into pKGWFS7.0 (Gateway vector), modified to contain the red fluorescent marker DsRed under the control of the constitutive *Arabidopsis* UBIQUITIN10 promoter (pUBQ10; Limpens *et al*., 2005).

Half of the 30 *L. japonicus* composite plants selected for DsRed fluorescence were inoculated with *F. mosseae* and the remaining were used as controls. Root sampling was repeated at 14, 21 and 42 days post inoculation.

Hairy roots expressing DsRed fluorescence were selected using a stereomicroscope (Leica M205 FA) from both inoculated and control plants; excised roots were immediately submerged in freshly prepared GUS buffer (0.1 M potassium phosphate buffer pH 7, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 0.3% Triton X-100, 0.3% X-GlcA; Duchefa Biochemie), incubated overnight at 37°C in the dark, destained with 70% ethanol and washed twice in distilled water. Part of the roots were then counter-stained with 0.01% (w/v) acid fuchsin in lactoglycerol (lactic acid:glycerol: water, 14:1:1; Kormanik & McGraw, 1982) to highlight intraradical fungal structures. Samples were then either directly mounted on a microscope slide for observation, or previously embedded in agarose (5%) and cut into 100 µm thick vibratome slices. Observations were done using a bright field microscope (Nikon Eclipse E400).

Confocal microscopy imaging

The targeted AM inoculation technique for studying early stages of the symbiotic association between *Gigaspora* species and transformed root cultures, developed by Chabaud *et al*. (2002) and adapted for confocal observation by Genre *et al.* (2005), was applied to both *Medicago truncatula* and *Daucus carota* ROCs expressing *35S::AtTPLATE-GFP*. An upright Leica TCS SP2 confocal microscope fitted with a long distance 40X water-immersion objective (HCX Apo 0.80) was used for imaging living ROCs directly in the Petri dishes. The argon laser band of 488 nm was used to excite both GFP and *G. gigantea* autofluorescence. The two signals were distinguished using specific emission windows: 500 to 525 nm for GFP and 590 to 630 nm for fungal autofluorescence. The latter channel was then false-colored in red to maximize the contrast in overlapping images.

Morphometric analyses

In order to visualize intraradical fungal structures and perform a quantitative analysis of cortical cell morphology, root samples from 8 weeks old pot-grown plants were harvested and fixed in phosphate-buffered saline (PBS), pH 7.2, containing 1% formaldehyde and 10% dimethyl sulfoxide (DMSO) for 24 hours at 4°C. Fixed roots were sliced using a Vibratome (Oxford Vibratome® sectioning system) and stained with wheat germ agglutinin and propidium iodide (Kojima *et al*., 2014) or acid fuchsin (Kormanik & McGraw, 1982) to highlight intraradical fungal structures and plant cell walls, respectively. The argon laser line at 488nm was used to excite all fluorochromes and emission windows were set at 500-530nm to record FITC, 600-700nm for propidium iodide and fuchsin fluorescence. A total of 42 optical sections (375 x 375 µm) were recorded from 13 independent root segments sampled from 3 different uninoculated plants of *M. truncatula*; 47 optical sections from 8 independent root segments (3 plants) were used for colonized *M. truncatula*; 22 optical sections (6 root segments from 3 plants) were used for uninoculated *L. japonicus* and 23 optical sections (6 root segments from 3 plants) for colonized *L. japonicus*. For all our analyses, 0.5cm-long segments from lateral roots of comparable size, length and position in the root system were sampled from both inoculated and uninoculated plants.

In addition, the number of split cells (Supporting Information Table S2) was counted in the inner cortical layer of live uninoculated or inoculated ROCs of *D. carota* and wild type or *dmi3-1 M. truncatula* expressing *35S::AtTPLATE-GFP*. A minimum of 22 optical sections (375 x 375µm) from at least 5 different roots of comparable size, length and position, belonging to 3 independent ROCs was used for each condition.

Quantitative gene expression analyses

Wild type and *dmi3-1 Medicago truncatula* ROCs were inoculated with *Gigaspora margarita*; fungal development and root colonization were followed under a stereomicroscope to identify hyphopodium development. A few of these samples were further observed in fluorescence microscopy (Leica TCS-SP2) to verify the repositioning of YC2.1 fluorescent nuclei under the hyphopodia - a hallmark of epidermal cell prepenetration responses. After 48/60 hours - required for the development of young colonization units – the corresponding root segments were excised and immediately frozen in liquid nitrogen. All root tips were removed before sampling. Four biological replicates - each consisting of a pool of ten 1cm long root segments - were collected from both uncolonized and colonized wild type ROC lines; and three replicates were collected from the corresponding *dmi3-1* lines. Uncolonized root segments were selected from lateral roots with a similar development and position in the root system architecture as the colonized segments from inoculated ROCs.

Total RNA was isolated from colonized and uncolonized samples and prepared using the RNeasy™ Plant Mini kit (Qiagen, Hilden, Germany). Plant material was processed with a mechanical disruption procedure using a TissueLyser system (Qiagen Retsch GmbH, Hannover, Germany) and further treated following the manufacturer's instructions. RNA quality and quantity were evaluated by photometric measurements and on agarose gels.

To remove traces of genomic DNA before cDNA synthesis, samples were treated with TURBO™ DNase (Ambion) according to the manufacturer's instructions. The RNA samples were routinely checked for DNA contamination by RT-PCR analysis, using primers for *MtTEF* (Supporting Information Table S1). Conventional PCR assays on fungal genomic DNA excluded any cross-hybridization of *M. truncatula* specific primers.

For single-strand cDNA synthesis, about 700 ng of total RNA was denatured at 65 °C for 5 min and then reverse-transcribed at 25 °C for 10 min, 42 °C for 50 min and 70 °C for 15 min. The final volume was 20µl and contained 10µM of random primers, 0.5mM dNTPs, 4µl 5Xbuffer, 2 µl 0.1 M DTT and 1µl Super-Script II (Invitrogen, Carlsbad,CA, USA).

Specific primers for *MtTPLATE, MtAP2A1, MtCHC1* and *MtCHC2* were designed on putative *M. truncatula* CDS sequences from NCBI database (http://www.ncbi.nlm.nih.gov/) and designed using SerialCloner software specifically for qRT-PCR analyses (Supporting Information Table S1). Primer validation was obtained with conventional PCR asssay on *M. truncatula* cDNA to avoid overlapping with unspecified sequences, and testied on fungal genomic DNA.

qRT-PCR experiments were carried out in a final volume of 15µl containing 7.5µl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 1µl of 3µM specific primers, and 10 ng of cDNA. Samples were run in the Rotor Gene apparatus (Qiagen) using the following program: 10min pre-incubation at 95°C, followed by 40 cycles of 15s at 95°C, and 30s at 60°C. Each amplification was followed by melting curve analysis (60–94°C) with a heating rate of 0.5°C every 15s. All reactions were performed with three technical replicates and only Ct values with a standard deviation that did not exceed 0.3 were considered. The comparative threshold cycle method (Rasmussen, 2001) was used to calculate relative expression levels using the plant *MtTEF* as reference genes.

Accession numbers

Sequence data from this article can be found in the NCBI data libraries under the following accession numbers: LjT06B17 (*LjTPLATE* promoter from 47759 to 49830); MTR_5g012010 (*MtKNOLLE*); MTR_7g089730 (*MtCYC1*); MTR_5g082900 (*MtCHCL1*); MTR_3g070940 (*MtCHCL2*); MTR_7g031450 (*MtTPLATE*); MTR_2g084610 (*MtAP2A1*); MTR_1g105120 (*MtBCP*); MTR_6g021805 (*MtTEF*).

Results

TPLATE expression is activated in AM colonized areas of the root

In order to highlight the involvement of the endocytosis and cell division marker TPLATE in the process of AM fungal accommodation, we studied the pattern of *TPLATE* promoter activity by expressing a *pLjTPLATE:GUS* transcriptional reporter (2kb promoter) in *Agrobacterium rhizogenes*-transformed roots from wild type *Lotus japonicus* and applying the GUS assay to excised roots. More than 50 roots were screened in brightfield microscopy and intense GUS-positive staining was observed in most root tips (Supporting Information Fig. S2a,b), consistent with the role of TPLATE in cell division. In roughly 1 out of 5 root tips, the expression pattern was less uniform and limited to small groups of meristematic cells (Supporting Information Fig. S2c,d) or isolated cell pairs (Supporting Information Fig. S2e). Taken together, the diverse meristematic patterns of *pLjTPLATE:GUS* staining appear to highlight a transient upregulation of the TPLATE promoter above a low basal level as previously described in Arabidopsis (Van Damme *et al.,* 2006; Brady *et al*., 2007), even if literature data and public databases indicate that TPLATE mRNA is expressed in virtually all cells due to its constitutive role in endocytic processes, as confirmed by both protein localization specifically in root and hypocotyl epidermal cells as well as functional studies on *A. thaliana* seedlings (Van Damme et al.,2006, Van Damme et al., 2011; Gadeyne et al., 2014, Sanchez-Rodriguez et al., 2018). Moreover, GUS staining was often observed in the root vascular cylinder in both young (Supporting Information Fig. S2a) and differentiated areas of the root (Supporting Information Fig. S2f, h), possibly related to endocytic processes linked to phloem activity (Liesche *et al.,* 2008).

AM colonization induced an evident change in this *TPLATE* expression pattern, with the appearance of intense GUS-positive areas associated with intraradical fungal structures (Supporting Information Fig. S2g), independent of the sampling time (14, 21 or 42 days post inoculation). The observation of over 60 vibratome sections from 10 independent root samples consistently indicated that GUS activity was particularly intense in inner cortical cells containing arbuscules (Supporting Information Fig. S2i). This was further confirmed by the analysis of acid fuchsin counter-stained samples (20 root segments overall), where the precise location of intraradical fungal structures could be detected more easily in longitudinal sections (Fig. 1a-c).

In conclusion, the GUS assay provided strong indications that AM colonization boosts *TPLATE* promoter activity in arbusculated root cortical cells.

Split cells are observed in the cortex of colonized roots

In addition to the correlation between arbuscule presence and *TPLATE* promoter activation, a consistent feature was observed in dozens of longitudinal root sections: the presence of one or more couples of arbuscule-containing cells, half the size of the surrounding (either colonized or uncolonized) cortical cells (Fig. 1d-f). In fact, the roughly square shape of such cells made them stand out very clearly among the more elongated cortical cells.

To better investigate this intriguing observation and exclude that ectopic cell divisions could be caused by our use of *A. rhizogenes*-transformed composite plants, we performed an extensive screening of longitudinal root sections from both *L. japonicus* and *M. truncatula* wild type plants, grown in pots for 8 weeks with or without AM inoculation. Fluorescent staining of fungal and plant walls made split cells easy to recognize by confocal imaging (see materials and methods for details). Representative images of *L. japonicus* samples (from a total of 6 colonized and 6 uninoculated root segments) are shown in Fig. 2. Couples of short cortical cells were only occasionally observed in uninoculated plants (Fig. 2a, b). By contrast, split cells were regularly found in AM colonized roots (Fig. 2c-e), alongside arbusculated and non-arbusculated cortical cells of regular size. In more detail, both unpenetrated (Fig. 2c, d) and arbusculated split cells (Fig. 2c-e) were observed in the colonized area, including couples where one cell contained an arbuscule and the second one did not (Fig. 2c), and couples where the two arbuscules had different (Fig. 2c,d) or comparable stages of development (Fig. 2e).

The table in Fig. 2, resumes our quantitative analysis of split cell occurrence in control and mycorrhizal plants of both *L. japonicus* and *M. truncatula*. We quantified the average number of split cell couples per image and the number of images containing at least one couple of split cells. In both plants, AM colonization significantly increased (t test <0.01) the average number of split cells: over 6 times in *L. japonicus* (from 0.71 to 4.80) and over 4 times in *M. truncatula* (from 0.58 to 2.53). The percent of images containing split cells was also increased in both AM hosts, from 36% to 96% in *L. japonicus* and from 36% to 66% in *M. truncatula*.

In short, the induction of anticlinal cell divisions was confirmed in the inner cortex of colonized areas in untransformed *L. japonicus and M. truncatula* plants, excluding a causal connection with *A. rhizogenes* infection. Secondly, our detailed quantification of split cell occurrence demonstrated a statistically supported relation between AM colonization and inner cortical cell divisions. Lastly, our observations of split cells where each contained differently developed arbuscules, alongside uncolonized split cells next to the arbusculated area, were suggestive of cell divisions occurrence in advance of arbuscule development.

Live imaging of ectopic cortical cell divisions in mycorrhizal roots using TPLATE-GFP

To clarify whether the AM-dependent induction of cortical cell division was indeed triggered before fungal entry, we expressed a *35S::AtTPLATE-GFP* fusion (originally developed in *Arabidopsis thaliana* and tobacco BY-2 cells by Van Damme *et al.*, 2004; 2006), in *A. rhizogenes*-transformed root organ cultures (ROCs). Since *L. japonicus* ROCs can only very rarely be colonized in our experimental conditions, we used *M. truncatula* (legume) and *D. carota* (non-legume) ROCs instead, two more amenable models for live cell imaging of symbiotic interactions (Chabaud *et al*., 2002; Genre et al., 2008; Fournier *et al*., 2015).

In vivo observations of at least 10 uninoculated *M. truncatula* ROCs confirmed the predicted localization of TPLATE-GFP (Van Damme *et al.*, 2004; 2006) with a major accumulation of the construct along newly formed cell walls in the apical meristem (Supporting Information Fig. S3a,b). By increasing microscope sensitivity, a weaker background fluorescence could also be recorded in the cytoplasm (likely related to 35S-driven overexpression) and at the plasma membrane of both meristematic and differentiated cells (Supporting Information Fig. S3c), in agreement with the reported plasma membrane targeting of TPLATE (Van Damme *et al.*, 2011, Gadeyne *et al*., 2014) in non-dividing cells.

Our observation of over 50 *G. gigantea* infection units, intended as root areas underlying the sites of hyphopodium contact, revealed an intense TPLATE-GFP labeling along a few anticlinal cell walls in the inner root cortex of both *M. truncatula* (Fig. 3a,b) and *D. carota* ROCs (Fig. 3c; Supporting Information Fig. S4a,b).

In particular, GFP-marked anticlinal cell walls were observed in the inner cortex of both plant species as soon as hyphopodia developed on the root surface (Fig. 3a,c; Supporting Information Fig. S4a) and until the first hyphae reached the inner cortex, about 2 days later (Fig. 3b; Supporting Information Fig. S4b). Such GFP-labeled walls appeared to separate two square-shaped cells that corresponded in shape to the previously described split cells (Fig. 1; Fig. 2). In fact, couples of split cells were constantly observed in the colonized areas of the inner cortex of TPLATE-GFP-expressing ROCs (Fig. 3d; Supporting Information Fig. S4b,c), although their walls were not as strongly labeled by GFP in more advanced stages of root colonization (Supporting Information Fig. S4c) nor after arbuscule development (Fig. 3d).

Comparable couples of square-shaped cells were only occasionally observed in the inner cortex of corresponding control segments from uninoculated roots of the same age and size as the colonized segments, (Supporting Information Fig. S4d), and their transverse wall was anyway never highlighted by intense TPLATE-GFP accumulation. These observations were strongly supported by consecutive imaging of a single site in an inoculated carrot root with a gap of 24 hours between each observation

(Fig. 3e-h). At the first time point (Fig 3e-g), hyphae developing from a single hyphopodium had colonized the epidermis (Fig. 3e) and outer cortex (Fig. 3f). A deeper focal plane reaching the inner cortex (Fig. 3g) revealed an intensely labeled cell plate being formed. When the same focal plane was imaged 24 hours later (Fig 3h), the TPLATE-GFP signal had significantly faded from the mature cross wall and - in the meantime - four additional inner cortical cells had divided in the same area.
On the same line, quantitative morphometric analyses performed on both *D. carota* and *M. truncatula* confirmed a significant increase in the number of split cells in mycorrhizal compared to uninoculated ROCs (Supporting Information Table S2).
Based on cell morphology and the analogous TPLATE-GFP labeling of recent cell walls in the root meristem and in the colonized area of the mature cortex, we interpreted our observation as evidence of an early, AM-dependent induction of cell division in the root inner cortex.

Ectopic cortical cell division is not induced in *Mtdmi3-1* mutants

Because cortical cell divisions were observed since the early steps of root colonization and often occurred in cells that were not in direct contact with the fungus, we decided to investigate whether this process was induced in the CSSP mutant *Mtdmi3-1*, where fungal colonization is arrested at the epidermis level, with the development of extensive and branched hyphopodia (Catoira *et al*., 2000; Morandi *et al.*, 2005). By expressing the same TPLATE-GFP construct in this genotype, we first confirmed construct functionality observing the expected GFP localization at newly formed cell walls in the root tip meristem (Fig. 4a). Our careful observation of 8 independent hyphopodium-associated root segments (Fig. 4b-e) did not reveal any fungal penetration of the root inner tissues and no statistically significant change was observed in the number of split cells between uninoculated and inoculated *dmi3-1* roots (Supporting Information Table S2). On this basis we concluded that the activation of cortical cell divisions in *M. truncatula* is dependent on fungal presence inside the root - which in turn requires DMI3-mediated signaling within the CSSP.

Cell division and TPC-associated endocytic markers are upregulated in mycorrhizal roots

Based on our observations of cell division induction and TPLATE involvement in AM fungal accommodation processes, we used qRT-PCR to analyse the expression of cell division markers and TPC-interacting proteins that act in endocytic processes associated with cell plate assembly.
Gene expression was analyzed in *M. truncatula* ROCs (Chabaud *et al*., 2011; Genre *et al*., 2013) obtained from both wild type and *dmi3-1* plants (Genre *et al*., 2005).
Two acknowledged cell division markers were chosen. *MtKNOLLE* encodes a syntaxin (homolog to *AtSYP111*) exclusively localizing to cell-plate directed vescicles and essential for cytodieresis (*knolle*

mutants develop incomplete cell walls and multinucleated cells; Lukowitz *et al*., 1996). Our second cell division marker was *MtCYC1*, encoding a putative Cyclin-like F-box protein, previously reported to be upregulated by 3,01 log2 FC in arbusculated vs non-colonized cells (data from the *M. truncatula* genome array performed on a population of root cortical cells colonized with *Rhizophagus irregularis* and isolated through laser capture microdissection by Gaude *et al*., 2012). Both transcripts were significantly upregulated in mycorrhizal root samples compared to uninoculated roots in WT, while no upregulation was recorded in *dmi3-1* mutants (Fig. 5). This result was consistent with our hypothesis that cell division-related mechanisms are involved in AM colonization.

We also analysed the expression of four TPC-related endocytic markers. *MtTPLATE* and the alpha subunit of the *Adaptor Protein complex 2* (*MtAP2A1*) - both involved in clathrin-mediated endocytosis (Gadeyne *et al*., 2014; Di Rubbo *et al*., 2013) - were constantly and significantly more abundant in mycorrhizal root segments compared to controls in wild type *M. truncatula*, while their expression did not change significantly in *dmi3-1* (Fig. 5). A weak but statistically significant upregulation was observed for *Clathrin Heavy Chain* 2 (*MtCHC2*), in mycorrhizal WT samples, while this was not the case for *Clathrin Heavy Chain* 1 (Fig. 5).

Lastly, as a marker of active AM colonization, we analyzed the expression of the AM-specific *Blue Copper-binding Protein MtBCBP* (Parádi *et al.*, 2010) in the same samples. This confirmed that the upregulation of our selected markers was strictly associated with *MtBCBP* expression (Supporting Information Fig. S5). On this basis, we propose that cell division markers and actors of clathrin-mediated endocytosis act during AM fungal accommodation, in line with our microscopic observations.

Discussion

The recruitment of cell division mechanisms in AM

During symbiotic interface biogenesis, a novel membrane-bound compartment is assembled in the middle of the cell, an area that is normally occupied by the vacuole in fully differentiated root cells. Displacing the tonoplast is indeed among the first consequences of PPA-associated nuclear movements in both epidermal and cortical cells (Genre *et al*., 2005; 2008). As the PPA assembly proceeds, the vacuole is further deformed (and reduced in volume), until a mass of cytoplasm (the PPA aggregate) occupies the portion of the cell volume where the symbiotic interface and intracellular hyphae are about to develop (Genre *et al*., 2012). An analogous mechanism is orchestrated when differentiated plant cells undergo mitosis: the vacuole has to be reduced - and eventually split - so that the correct positioning of nucleus, mitotic spindle, phragmoplast and cell plate can generate the cell division plane (Van Damme, 2009; Smertenko *et al.*, 2017). Also in this case, initial vacuole remodeling is obtained by the coordinated movement of the nucleus and the assembly of phragmosome cytoplasmic bridges that cross the vacuole along the equatorial plane and eventually fuse into one central mass of cytoplasm encompassing the nucleus (Lloyd & Buschmann, 2007).

In addition to such similarities in cell dynamics, our results suggest a more direct relationship between the two processes. The upregulation of several cell division markers in colonized root areas and *TPLATE* expression pattern, that indicate an activation of gene expression in colonized cortical cells, are both consistent with a reactivation of cell cycle-related processes. This is reinforced by the recurring presence of split cells in the colonized region of roots from phylogenetically distant hosts and the direct observation of dividing cortical cells in the vicinity of intraradical hyphae and hyphopodia.

Altogether, we interpret our results as indicative of the reactivation of cell cycle-related processes in the inner cortex since early root colonization. The biological meaning of this mechanism remains to be fully clarified; nevertheless, literature on AM fungal accommodation provides intriguing clues: the symbiotic interface materials have been described as very similar to the primordial wall found in cell plates (Balestrini & Bonfante, 2014); a temporary, global re-routing of exocytic processes has been proposed to take place in both cell plate (Lam et al., 2008) and periarbuscular interface deposition (Pumplin et al., 2012); lastly, evidence of ploidy increases in mycorrhizal root systems (Berta *et al*., 2000; Lingua *et al*., 2002; Fusconi *et al*., 2005; Bainard *et al.*, 2011) suggests the onset of endoreduplication (deriving from the arrest of the mitotic cycle) during AM colonization, even if the precise location and timing of such endoreduplication events remains to be defined (Genre et al.,

2008). In the light of our current results, it is tempting to speculate that a mechanistic analogy links the exocytic processes that lead to cell plate deposition and symbiotic interface biogenesis, even if further investigation is needed to test this hypothesis.

Membrane dynamics during fungal accommodation: a role for endocytosis?

Our results on the upregulation of endocytic effectors such as TPLATE, CHC2 and AP2A1 during AM fungal accommodation are suggestive of a role for clathrin-mediated endocytosis (CME)-related processes in this symbiotic interaction.

Even if further investigation is needed to clarify this hypothesis, the involvement of CME complements previous descriptions of the exocytic origin of the symbiotic interface in both AM and nitrogen fixing symbiosis (Ivanov *et al*., 2012; Genre *et al*., 2012; Fournier *et al*., 2015; Zhang et al., 2015; Barker *et al*., 2017): all major exocytic events in the plant cell are associated with endocytic membrane recycling (Samaj *et al.*, 2004; Ketelaar *et al.*, 2008). Significantly, this also applies to cell plate formation (Dhonukshe *et al.*, 2006; McMichael & Bednarek, 2013).

Indeed, CME has recently been shown to play a fundamental role in legume-rhizobium interactions: a 60% reduction in the abundance of *L. japonicus Clathrin Heavy Chain 1* (*LjCHC1*) transcripts (but not *LjCHC2*) has been shown to strongly reduce rhizobial infection and nodule development (Wang *et al.*, 2015). In the light of this finding, the consistent 30% upregulation of *M. truncatula CHC2* (but not *MtCHC1*) during AM colonization opens the intriguing possibility that the two genes act in alternative pathways controlling legume mycorrhization or nodulation, although more detailed analyses going beyond the scope of the present research will be required to clarify this point.

Evolutionary context

The reactivation of the cell cycle is not unique to AM interactions, and has indeed been described much earlier in legume-rhizobium interactions. The study by Xiao and colleagues (2014) provides one of the most thorough descriptions of cell proliferation during nodule development, using *Medicago truncatula* as a model system. In this legume, rhizobium inoculation triggers anticlinal divisions that start in the pericycle and then extend to the endodermis, inner and middle cortical cell layers, with the latter eventually generating the nodule meristem. The authors describe this process as reminiscent of lateral root development, in line with the hypothesis of nodule evolutionary origins from modified lateral roots. In the case of AM, however, cell cycle-related processes (including marker gene regulation and the appearance of split cells) were restricted to a few, often sparse cells in the inner cortical layer. This important difference indicates that the AM-related response is more limited and also lacks the coordination observed in neo-organogenesis of both nodules (Xiao *et al.*,

2014) and lateral roots (Downie, 2014). On this basis, we cannot define a direct relationship between the AM-dependent induction of cortical cell divisions and nodule initiation. Nevertheless, isolated split or endoreduplicated cells are often observed in the vicinity of the infection thread, as it develops across the root cortex. Such cell divisions are clearly unrelated to those that originate the nodule primordium (Xiao *et al.*, 2014), and their observation led to the conclusion that pre-infection thread assembly (a transvacuolar cytoplasmic aggregation that is remarkably similar to the PPA) could involve reactivation of cell cycle (Yang *et al.,* 1994) and cell division-related mechanisms (Downie, 2014). Based on our current results we interpret the triggering of cortical cell divisions as a conserved developmental trait in both symbioses.

The induction of ectopic cell divisions in roots colonized by the evolutionarily more ancient AM symbiosis is particularly intriguing - also in the light of the frequent observation of couples of short arbusculated cells in *Horneophyton ligneri* fossils from the Rhynie Chert (Strullu-Derrien *et al*., 2018) - and suggests that this response is not related to developmental innovations introduced by legumes during their co-evolution with nitrogen-fixing bacteria, but rather a conserved strategy derived from the 400 million year-old AM symbiosis.

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Author contributions

G.R. designed the experiments, developed the transgenic lines, performed microscopy, morphometric and gene expression analyses and wrote the text. G.C. performed gene expression and morphometric analyses, transgenic line production and contributed to the writing. V.F. performed gene expression analyses and developed the *LjTPLATE:GUS* vector. M.C. developed the *LjTPLATE:GUS* vector.

V.V. performed gene expression analyses, transgenic line production and contributed to the writing. D.V.D. provided the *AtTPLATE-GFP* vector and contributed to the writing. A.G. designed the research and experiments and wrote the text.

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Supporting Information

Table S1. Primers used in this work.

Table S2. AM colonization causes the appearance of split cells in the inner root cortex.

Fig. S1. *Lotus japonicus TPLATE* promoter sequence used for GUS analysis.

Fig. S2. Details of *TPLATE* expression pattern in uninfected roots of *L. japonicus* expressing the *pLjTPLATE:GUS* construct.

Fig. S3. *At*TPLATE-GFP localization in uninfected *M. truncatula* ROCs.

Fig. S4. *At*TPLATE-GFP-labeled cell divisions in colonized carrot ROCs.

Fig. S5. Validation of sample mycorrhizal status by BCP expression analysis.

Figure legends

Fig. 1. *TPLATE* expression pattern in mycorrhizal roots of *L. japonicus*. Following the GUS assay reaction (blue), root samples were counterstained with acid fuchsin (purple) to outline *F. mosseae* hyphae (hy) and arbuscules (ar). A young colonization unit is shown in (a) with limited intraradical development from a single hyphopodium (hp) at 14 days post inoculation (dpi); (b) intense TPLATE promoter activity is associated with arbuscules in the inner cortex. (c) Details of GUS-positive staining in arbusculated cells. The largest hyphal branches are indicated by arrowheads. (d-f) GUS staining of arbuscule-containing cells in the inner cortex at 14 dpi (c,d) and 21 dpi (e,f): a few arbuscules have developed inside split cells (white dashed line). Bars = 100 µm in a; 20 µm in (b,d; 40 µm in (c,e,f).

Fig. 2. Qualitative and quantitative analysis of cortical cell divisions in colonized roots of pot-grown *L. japonicus* and *M. truncatula*. (a-d) Representative images of untransformed *L. japonicus* samples where the double staining of *F. mosseae* walls with WGA-FITC (green) and plant walls with propidium iodide (red) indicated an increase in the number of split cells in AM colonized roots. (a,b)

Control uninoculated plants only occasionally displayed couples of cortical cells (white dashed line) that were roughly half as long as the neighboring cells (asterisk). By contrast, split cells were frequent in AM colonized roots (c-e), alongside cortical cells of regular size (asterisks). Four main conditions were found: uncolonized split cells in the vicinity of arbuscules (c, left; d, right); split cells where one cell contained an arbuscule and the second one did not (c, center); split cells containing arbuscules at different stages of development (c, right; d, left); split cells containing arbuscules with comparable morphology (e). Bars = 40µm (a, c); 30µm (b, d, e).

The table reports the results of our quantitative analysis of split cell occurrence in 375x375 µm optical sections (as those shown in a and c) from control and mycorrhizal plants. Two parameters were considered: the average number of split cells per image (reported +/- the standard error) and the number of images containing at least one couple of split cells. For *L. japonicus*, AM colonization significantly increased the average number of split cells over 6 times (t test <0.01) as well as the percent of images containing split cells (from 36% to 96%). An analogous situation was observed in *M. truncatula*, where the average number of split cells per images increased over 4 times (T-test; pvalue <0.01) and the percent of split cell-containing images passed from 36% in controls to 66% in mycorrhizal roots. Overall these analyses correlate the presence of AM colonization with a consistent increase in the number of split cortical cells.

Fig. 3. *At*TPLATE-GFP highlights AM-induced cell divisions in the inner cortex. All panels show longitudinally oriented optical sections cutting through the inner cortex from living *M. truncatula* (a,b) or *D. carota* (c-h) ROCs colonized by *G. gigantea*. In the presence of a hyphopodium (hp) and penetrating hyphae (hy) in the epidermis (a,b), several cell divisions are visible in the *M. truncatula* inner cortex, marked by the intense TPLATE-GFP fluorescence at anticlinal walls (arrowheads); the faint and diffuse GFP fluorescence in the cytoplasm and on the plasma membrane was obtained by increasing the microscope sensitivity to provide a general outline of root cell shapes; asterisks mark neighboring undivided cortical cells. (c) shows an analogous situation in carrot, where bright TPLATE-GFP labeling (arrowheads) highlights recent cell divisions in the inner cortex underlying a hyphopodium; also in this case, the asterisk marks an undivided cortical cell. (d) Following arbuscule development, the compact shape of several divided cells is recognizable (arrowheads), but the lack of intense anticlinal wall labeling by TPLATE-GFP suggests that cell divisions have not occurred in the preceding hours; one cell (arrow) appears to have divided twice, as suggested by the extremely reduced size of two neighboring cells. Serial optical sections recorded at increasing depth from the same carrot root are displayed in (e,f,g). A large hyphopodium is visible on the root epidermis in (e). Several intracellular hyphae extend from the hyphopodium to the outer

cortex (f), where particularly intense accumulation of GFP signal is associated to a cortical PPA (arrowheads). (g) Fluorescence of comparable intensity marks a forming cell wall (arrowhead) in the inner cortex; white dashed lines mark long, undivided cortical cells. (h) displays the same focal plane as (g) 24 hours later; the previously partial cell wall is now completed (arrowhead) and all the cells marked by the dashed line have divided. Bars = 50µm.

Fig. 4. *At*TPLATE-GFP localization in *M. truncatula dmi3-1* mutant ROCs. Recent cell divisions in the root tip meristem were marked by intense TPLATE-GFP accumulation (arrowheads), as shown in (a). In contrast to our observations on WT ROCs, the presence of a *G. gigantea* hyphopodium (hp) on the surface of a *dmi3-1* root (b) did not induce ectopic cell divisions in the underlying inner cortex ((c), asterisk). An analogous example is shown in (d,e) where two optical sections from the same root area are presented, one focused on the root epidermis (d) and one on the inner root cortex (e). Bars = 20 µm in (a); 40µm in (b-e).

Fig. 5. Regulation of cell division and endocytic markers in wild type and *dmi3-1 M. truncatula* upon AM colonization. The expression of the cell divisions markers *KNOLLE* and *CYC1*, alongside *TPLATE* and TPLATE interactors in clathrin mediated endocytosis *AP2A1, CHC1* and *CHC2*, were analyzed by quantitative RT-PCR 48 hours post hyphopodium development on ROC segments. *TPLATE* and *AP2A1* transcripts were significantly upregulated in wild type inoculated root segments (striped histograms) compared to controls (white histograms); no significant upregulation was observed in *dmi3-1* mutants, where symbiotic signaling and intraradical colonization are blocked. A differential regulation was observed for the two *Clathrin heavy chain* genes: while no change in gene expression was observed for *CHC1,* a weak but statistically significant upregulation of *CHC2* was recorded in inoculated wild type roots. Relative gene expression levels (fold change) are normalized to the corresponding control; bars represent standard errors. Different letters indicate statistically significant differences; T-test p value < 0.04; at least three biological replicates were used for each condition.

Table S1. Primers used in this work

Table S2. AM colonization causes the appearance of split cells in the inner root cortex. Two parameters were considered for quantitative analysis of split cell occurrence in 375x375 µm optical sections: the average number of split cells per image (reported +/- the standard error) and the number of images containing at least one couple of split cells. A marked increase in the number of split cells was observed upon fungal colonization in both *D. carota* and wild type *M. truncatula* (T-test; p value

<0.01). By contrast, when the same analysis was done on *M. truncatula dmi3-1* mutants, no significant change was observed.

Figure S1. *Lotus japonicus TPLATE* promoter sequence used for GUS analysis.

Figure S2. *TPLATE* expression pattern in roots of *L. japonicus* expressing the *pLjTPLATE:GUS* construct. (a) Intense blue GUS staining in a root tip meristem (asterisk). While GUS activity often extended to the whole meristem, as shown in (b), alternative expression patterns were occasionally observed (c, d), likely related to differences in meristematic activity: as shown here, *TPLATE* promoter activity was limited to small groups of meristematic cells (arrowheads). (e) Higher magnification of a root tip meristem, showing two pairs of GUS-positive meristematic cells; these observations suggest that *pTPLATE* is upregulated in actively dividing cells. (f) GUS staining in the central cylinder (cc) was much more prominent than in the other tissues of the differentiated zone, where hardly any GUS staining could be detected during the reaction period, indicating much lower expression levels than in the central cylinder. (g) 42 days post-inoculation with the AM fungus *Funnelliformis mosseae*, GUS activity was evident in the colonized areas of the root cortex, where arbuscules (ar) develop. (h, i) Transverse sections from control (h) and mycorrhizal (i) roots showing GUS-staining in the central cylinder and arbuscule-containing cells. Bars = 200 µm in (a, f, g); 100 µm in (b, c, d); 30 µm in (e); 50 µm in (h, i).

Figure S3. *At*TPLATE-GFP localization in uninfected *M. truncatula* ROCs. New-laid cell walls are highlighted in the root apical meristem (a,b) by an intense GFP signal (arrowheads). The nuclei (n) of four recently-divided meristematic cells in (a) are recognizable as dark areas in the faintly fluorescent cytoplasm and allow an easy identification of relative cell positions. A deep longitudinal section from an uninfected root is shown in (c); root cortex layers are recognizable between the epidermis (ep) and central cylinder (cc): the lack of intense labeling in any anticlinal wall and the regularity of cell sizes indicate that no recent cell division has occurred. Bars = 20 µm in (a) and (b); 50µm in (c).

Figure S4. *At*TPLATE-GFP-labeled cell divisions in colonized carrot ROCs. Longitudinal optical sections cutting through the inner root cortex. (a) Intense GFP fluorescence (arrowheads) labels a few anticlinal walls in the inner cortical layers underlying a hyphopodium (hp); such cell walls divide regular cells in two smaller daughter cells. (b) (c) Couples of split cells (dashed line) and *At*TPLATE-

GFP marked walls (arrowheads) are visible in the vicinity of an intraradical hypha (h) reaching the inner cortex. (d) No divided cells are recognizable in uninoculated roots. Bars = 50µm.

Figure S5. Validation of sample mycorrhizal status by *BCBP* expression analysis. The statistically significant upregulation of *Blue copper-binding protein* (*BCBP*) gene - an acknowledged plant marker for AM colonization - confirmed AM establishment in the samples used for gene expression analyses. Relative gene expression levels (fold change) are normalized to the control. Different letters indicate statistically significant differences; T-test p value < 0.04.