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Review

Plant signals and fungal perception during arbuscular mycorrhiza establishment

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Abstract

Arbuscular mycorrhizal (AM) fungi are obligate symbionts that need their plant hosts to complete their life cycle. In the absence of the plant, germlings arrest growth after a few days and retract most of their cytoplasm back into the multinuclear spores. The spores can germinate again during more favorable conditions. How AM fungi recognize compatible host roots and activate their symbiotic program is not yet understood. However, research in this field in the last years has shed light into this topic. We, and others, have approached some of these aspects by studying changes in fungal gene expression observed at early stages of development, before and at the plant recognition stage in an attempt to identify genes and proteins featuring as key regulators in the switch between the asymbiotic and symbiotic style of life. The molecular bases of this recognition process are now starting to be understood and point to common signaling pathways shared with other microbe-plant associations and to arbuscular mycorrhiza specific signaling pathways.

Keywords: *Glomus mosseae*; Arbuscular mycorrhiza; Symbiosis; Signal molecules; Transcript profiling

Contents

1. The arbuscular mycorrhiza symbiosis

Arbuscular mycorrhizal fungi are soil inhabitants belonging to a recently new ascribed phylum, Glomeromycota with a presumed origin at least 460 million years ago (Redecker et al., 2000; Schüßler et al., 2001). Glomeromycota fungi live in permanent association with roots of the majority of the plants on this planet among Angiosperms, Gymnosperms, Pteridophytes and some Bryophytes (Smith and Read, 1997). Perhaps due to this ancient association with plants, AM fungi have lost their ability to live and

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complete their life cycle in the absence of the green partner. This symbiosis has important consequences for the cycling of nutrients in the soil, since AM fungi provide plants with essential nutrients, such as phosphate, when they are scarce or have low mobility in the soil solution. In turn, photosynthetic carbon is transported into the soil via the transfer of sugar from the root to their endosymbiotic fungi, which later translocate this carbon in form of lipids and sugar into the external mycelium spreading in the soil (Bago et al., 2003). Despite the importance of the AM symbiosis for the sustainability of the terrestrial ecosystem, many aspects of the mycorrhizal symbiosis functioning are surprisingly unknown. The principal reasons for this are the underground nature of the association with a great part of the fungal biomass hidden inside of the root, and the obligate biotrophy of the fungal partner. However, significant progress has been made in the last decade with the arrival of new molecular and biochemical techniques. High throughput genetic analyses parallel to gene expression, protein and metabolite profiling have literally changed our view of the arbuscular mycorrhizal symbiosis (Bestel-Corre et al., 2004; Franken and Requena, 2001; Küster et al., this issue). These methods have helped to unravel many aspects of the AM physiology, including several aspects of the early communication between plant and fungus. In this review we will concentrate on recent advances in the understanding about the nature of plant signals and their perception by the fungal partner during early stages of the symbiosis.

1.1. Arbuscular mycorrhizal life cycle

The establishment of the AM symbiosis begins with the colonization of a compatible root by the hyphae produced by AM fungal soil propagules, asexual spores or mycorrhizal roots. Even dead roots from annual plants might be a good source of inoculum because they protect the fungus from environmental hazards until the time when new hyphae can grow out of the roots and colonize other plants (Requena et al., 1996). After attachment of a hypha to the root surface by means of an appressorium, the fungus penetrates into the cortex and forms distinct morphologically specialized structures: Inter- and intracellular hyphae, coils and arbuscules. Arbuscules are specialized hyphae, similar to haustoria from plant pathogenic fungi, formed as intercalary structures between coil hyphae. They are presumed to be the main site of nutrient exchange between symbionts. However, there are many reports of Paris type mycorrhiza where arbuscules are completely absent. This opens the debate to what extent other plant–fungal interfaces are also involved in the nutrient exchange (Smith and Smith, 1990).

After host colonization, the fungal mycelium grows out of the root exploring the soil in search of mineral nutrients, and it can also colonize other susceptible roots. The fungal life cycle is completed after formation of asexual chlamydospores on the external mycelium. Distinct morphological stages can be therefore identified during the life cycle of arbuscular mycorrhizal fungi (Fig. 1; Requena and Breuninger, 2004).

1.2. Early stages of the symbiosis: fungal asymbiotic growth

Arbuscular mycorrhizal fungi are obligate biotrophs, unable of completing their life cycle during asymbiosis (Bonfante and Bianciotto, 1995). AM fungal spores are the only plant-independent phase of the mycobiont. They are round-shaped with a thick cell wall and average diameters

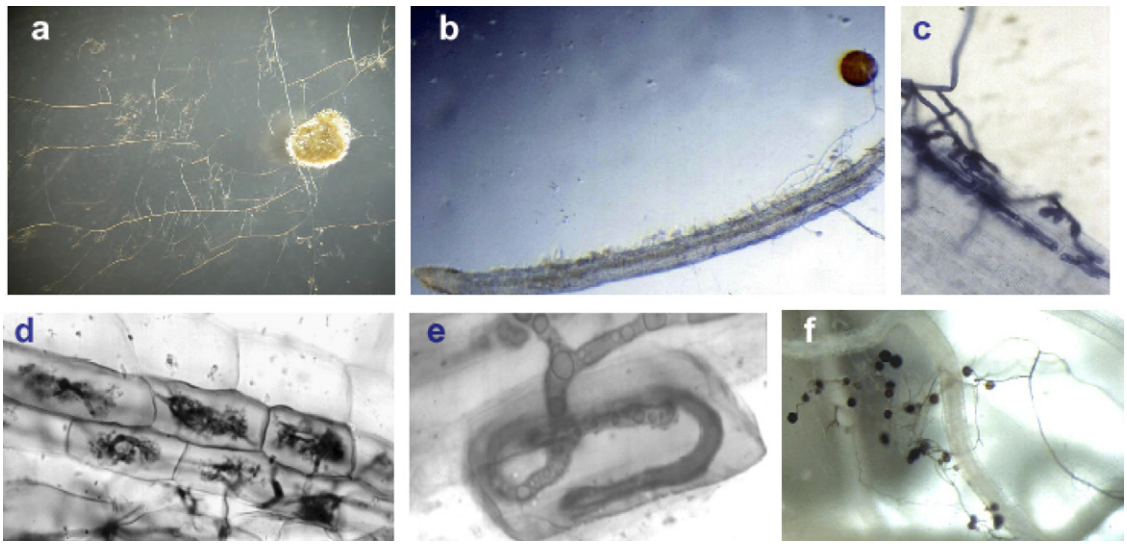

Fig. 1. Life cycle of an arbuscular mycorrhizal fungus. (a) Spore germination and asymbiotic growth on water-agar. (b) Host recognition and presymbiotic growth in the proximity of a host root. (c) Appressoria formation on the root epidermis and colonization of the first root cortex layer. (d) Arbuscules in inner cortical cells. (e) Detail of a intracellular hyphae, the so-called coil, in a cell of the root cortex. Observe the big lipid droplets within the fungal hypha. (f) Extraradical mycelium exploring the soil and forming the next spore generation.

between 50 and 100 μm. They contain a very large number of nuclei, up to 2000 per spore (Bécard and Pfeffer, 1993). After germination, hyphae are always coenocytic. This fact makes AM fungi not amenable for traditional genetic approaches. Studies on two AM fungal species have shown that these are haploids with an unusual high genetic variation (Hijri and Sanders, 2004; Hosny et al., 1997). Assessment of the genome size of these fungi have shown extreme variations between different species ranging from about 16.5 Mb in *Glomus intraradices* (Hijri and Sanders, 2004) up to 1058.4 Mb in *Scutellospora gregaria* (Hosny et al., 1998). *Glomus intraradices* with its small genome has been selected as the AM model fungus for genome sequencing (http://darwin.nmsu.edu/~fungi/).

However, the most striking feature of AM spores is their particular physiology. Different to other soil-borne fungi, these spores have the ability to germinate and arrest growth many times if plant-derived signals are missing (Koske, 1981; Mosse, 1959). AM spores germinate under appropriate water and temperature conditions and hyphae grow for about 2–3 weeks. Several nuclei from the spore move into the extending mycelium and some of them undergo mitosis (Bianciotto and Bonfante, 1993; Bianciotto et al., 1995; Requena et al., 2000). During this time the fungal colonies extend a few centimeters, showing a characteristic growth pattern with marked apical dominance and infrequent hyphal branching. In the absence of a host root, growth ceases after about 2–4 weeks and hyphal septation from the apex occurs (Mosse, 1988). The apical septation is accompanied by extensive vacuolization and retraction of the protoplasm, including most of the nuclei, towards the spore (Logi et al., 1998). During this asymbiotic phase, the fungus is living mainly from its triacylglyceride reserves. The presence in the germination-growth medium of different carbon and nitrogen sources has little effect on the length or extension of this development (Hepper, 1979). Nevertheless, perception of these nutrients takes place, as some of them induce changes in the fungal gene expression and enzyme activity (Breuninger et al., 2004; Requena et al., 2003; Ocón, Hampp and Requena, unpublished results). Growth arrest occurs long before the spore reserves are depleted. Probably it is induced by the absence of (a) host-derived signal/s. This phase of growth in the absence of signal from the plant is what it is known as asymbiotic stage. Cytological studies have shown that although nuclear division occurs at this stage, most nuclei remain arrested in the S phase or between G2-M phases (Bianciotto and Bonfante, 1993; Bianciotto et al., 1995). A gene homologous to the cell cycle check-point TOR2 from *Saccharomyces cerevisiae* has been isolated from *Glomus mosseae*. The anti-inflammatory drug rapamycin, known to interfere with the role of TOR2 controlling arrest of cell cycle in G1 was found to decrease hyphal growth during asymbiosis, although it did not affect spore germination (Requena et al., 2000). This indicates that DNA replication is not necessary for germination but for asymbiotic hyphal elongation. Attempts to cultivate AM fungi *in vitro* led to the interesting discovery that certain soil microorganisms could significantly increase the saprotrophic growth of the mycelia during this stage (Mosse, 1959; Hepper, 1979). Some of them improved spore germination but most of the organisms reported had a beneficial effect on hyphal growth, branching and the production of vegetative spores. Little is known about the mechanisms by which these microorganisms are able to improve asymbiotic growth. However, molecular studies have shown that the fungus is able to perceive these microorganisms and change its gene expression pattern in response to them (Requena et al., 1999).

2. Plant signals and fungal perception

2.1. Plant-induced morphogenesis of AM fungi

There is increasing evidence showing that fungus and plant start to recognize each other long before the first colonization structures on the root epidermis appear. It is known since a long time that germinating hyphae from spores respond to the presence of roots in their vicinity (Mosse and Hepper, 1975). Although no directional growth has been observed towards the root, several experiments showed that exudates from host root elicit growth stimulation in contrast to non-host exudates (Bécard and Piché, 1989; Gianinazzi-Pearson et al., 1989; Giovannetti et al., 1993a,b, 1996; Nair et al., 1991). Using a membrane sandwich system to separate plant and fungus, Giovannetti et al. (1996) showed that only compatible host roots were able to elicit hyphal branching and that a molecule smaller than 500 Da could be responsible for this change in fungal morphology.

Given the fact that in many plant–microbe interactions the dialogue between partners is initiated by the presence of plant phenolic substances, in particular flavonoids, they were obvious candidates to investigate as plant signals during mycorrhiza formation. Interestingly, there are many reports showing that, indeed, flavonoids [exogenously applied to spores] exert a positive effect on hyphal growth during asymbiosis (Bécard and Piché, 1989; Gianinazzi-Pearson et al., 1989; Poulin et al., 1997; Tsai and Phillips, 1991). These effects range from increased spore germination to enhanced hyphal growth, hyphal branching and formation of secondary spores. In addition, studies on mycorrhizal plants showed that AM mycorrhiza formation changed the flavonoid profile of roots extracts quantitatively and qualitatively through modifications of the expression patterns of genes from the phenylpropanoid, flavonoid and isoflavonoid metabolism (Harrison and Dixon, 1993, 1994). Some of the flavonoids induced in mycorrhizal roots but absent in non-mycorrhizal roots and in Myc^- mutant roots (unable to form mycorrhiza) (Harrison and Dixon, 1993), were previously shown to be inducers of fungal spore germination *in vitro* (Tsai and Phillips, 1991). Akiyama et al. (2002) showed that a glycosylated

derivative of a flavonoid, *de novo* synthesized in non-mycorrhizal phosphate starved roots, was able to stimulate mycorrhiza formation, even under conditions of high phosphate concentration, where mycorrhizal colonization is usually very reduced. However, contradictory results about the effect of flavonoids on AM growth and development have been published. And thus, a single flavonoid might exert a positive, negative or neutral effect on different fungi (Nair et al., 1991; Siqueira et al., 1991; Poulin et al., 1997; Vierheilig et al., 1998). This could be explained by a species-specific effect of each flavonoid as recently reported (Scervino et al., 2005a,b). However, flavonoids might not be essential for the plant–fungal recognition since a study using maize mutant plants impaired in flavonoid production showed that they were able to form mycorrhiza similarly to wild type plants (Bécard et al., 1995). Therefore it is likely that their role is limited to a stimulatory effect on AM fungal growth, thereby facilitating host root encounter as it is also hypothesized for the role of stimulating microorganisms.

Search for other plant signals stimulating AM fungal growth revealed that several hydrophilic as well as hydrophobic components of root exudates exerted that effect (Nagahashi and Douds, 1999, 2000). However, the branching effect often observed when applying root exudates to germinating hyphae was related to the presence of a lipophilic molecule that Buee et al. (2000) succeeded in partially purifying from host root exudates. Application of this semi-purified fraction to spores of three *Gigaspora* species promoted hyphal branching of the asymbiotic mycelium within 5–8 h after injection into the growing medium. Akiyama and coworkers recently succeeded in isolating the active compound triggering this branching effect on germinated spores. The compound is a strigolactone, a sesquiterpene lactone, that interestingly belongs to a family of compounds known to activate germination of parasitic plants like *Striga* (Akiyama et al., 2005). In concentrations as small as 0.2 μM applied to the primary hypha, 5-deoxy-strigol induced branching after 24 h.

Despite all this accumulating evidence of the positive effect of root exudate compounds in stimulating hyphal growth during pre-symbiosis, it is remarkable that root exudates do not allow per se the AM fungus to sustain asymbiotic growth indefinitely, neither to induce appressoria formation. This indicates that only an intact compatible root is able to overcome fungal growth arrest. It is likely that other signals, such as thigmotropic signals from the plant surface or secondary metabolites produced *in planta* after perception of the fungus, are required for appressoria formation and symbiosis progression. In contrast to many plant pathogenic fungi, appressoria in AM fungi are not induced by fake root surfaces such as nylon, polyamide, silk, cellulose or glass threads, even when additionally stimulated with host root exudates (Giovannetti et al., 1993a). An experiment performed with isolated cell walls of carrot roots showed that appressorium formation can occur without prior hyphal branching, and does not require a signal secreted from the host root nor the presence of intact host cytoplasm (Nagahashi and Douds, 1997). Appressoria were formed on isolated epidermal cell, although further fungal penetration was not observed. Therefore, it appears that appressoria formation can be triggered by structural features of the rhizodermis, but other signals from living cells are necessary for further colonization. This is in agreement with the findings of Bonfante et al. (2000) who showed that discrete steps in the root colonization are controlled by the plant at each cell layer.

The precise mechanism how plant signals are perceived by AM fungi is unknown. Akiyama and co-workers proposed that strigolactones interact with a putative fungal receptor, and that the active molecule is quickly inactivated after docking by removing its D ring (Akiyama et al., 2005). Although it is unknown which receptor or signaling pathway is targeted by flavonoids, Poulin et al. (1997) showed that the flavonoid effect could be mimicked by estrogens and blocked by anti-estrogens. This is strong evidence that a specific receptor/interacting protein with a binding site for flavonoid or structurally related compounds (estrogens and antiestrogens) exists in AM fungi (see Fig. 2).

2.2. Molecular responses of AM fungi to plant signals

As we have seen above, AM fungi modulate their growth pattern during the life cycle according to the developmental situation they face. In response to chemical or/and thigmotropic signals from the plant, the germinating hypha is able to release its preset cell growth arrest and form appressoria on the rhizodermis. It is obvious that the fungus undergoes a reorganization of its cellular program to accomplish this developmental switch. A few studies have been carried out to dissect fungal transcriptional changes induced by development or by plant signals. First attempts tried to identify fungal genes up-regulated *in planta* during the symbiosis. However, due to the low amount of fungal biomass within the root, especially during the colonization phase, these approaches were not as successful as in other plant–microbe interactions such as the pathogenic interactions with the biotrophic fungi *Uromyces fabae* or *Ustilago maydis* (Hahn and Mendgen, 1997; Kahmann and Basse, 2001). Thus, while those techniques allowed successful identification of plant genes induced or repressed in response to AM fungi, the few fungal genes identified corresponded mainly to highly constitutively expressed fungal genes (Harrier et al., 1998; Martin-Laurent et al., 1997). To circumvent this problem and to identify fungal genes regulated during development, Requena et al. (2002) used suppressive subtractive hybridization (SSH) to create a subtractive cDNA library from *G. mosseae* enriched in genes induced during the asymbiotic phase. With this approach a novel gene (GmGin1) encoding a two-domain protein with a putative role in signaling was identified. Expression analyses showed that

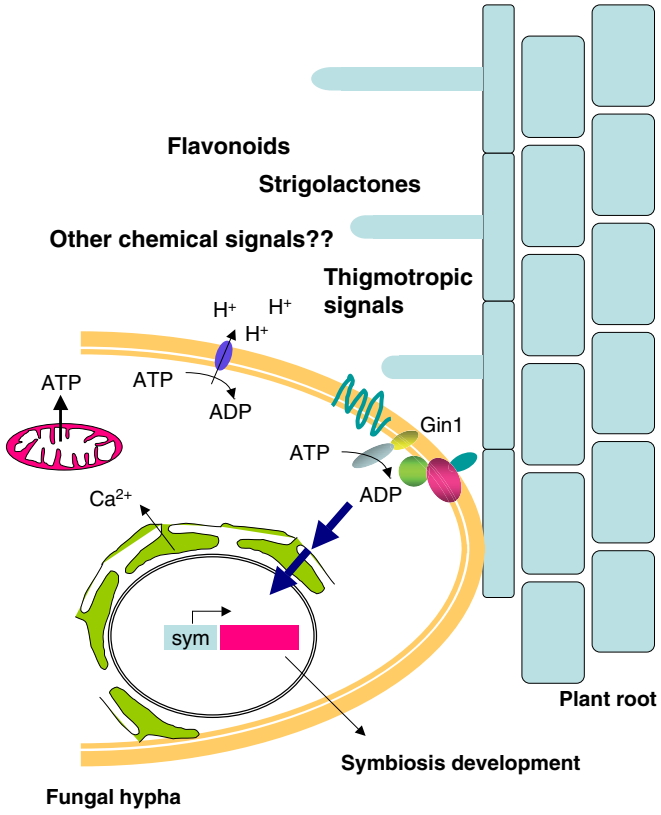

Fig. 2. Schematic representation of AM fungal perception of plant signals during mycorrhiza establishment. Chemical signals exuded by the plant, such as flavonoids and strigolactones, together with surface or thigmotropic signals from the rhizodermis are possibly recognized by receptor proteins associated to the fungal plasma membrane. Upon signal perception receptor proteins are modified and possibly interact with downstream components. Gin1 might be one of the downstream components, located at the plasma membrane where it is covalently modified by plant signals. Through its ATPase activity, Gin1 might interact/modify other membrane proteins to transmit the signal towards the nucleus. Calcium, released from cellular organelles such as the endoplasmatic reticulum, might act as a second messenger. Activation of mitochondrial respiration and increased ATPase activity causing membrane hyperpolarization occurs after transcriptional induction of the corresponding genes. Some of the fungal genes activated in response to plant signals are instrumental in the developmental decision which disable programmed growth arrest and allows the fungus to enter into the symbiotic modus.

GmGin1 was down-regulated upon entry into symbiosis, suggesting it could play a role at the plant recognition stage (Requena et al., 2002). Given the similarity of the amino terminus of GmGin1 (possibly released as mature protein) with plant and animal GTPases involved in the control of apoptotic processes, we envisaged two possible scenarios for GmGin1 function. In one, GmGin1 responds to a signal from the plant and stops the pre-set growth arrest, which takes place in the absence of a compatible host. In a second scenario, GmGin1 might sense the absence of a plant signal and induce an apoptosis-like process with hyphal septation from the apex and degradation of protoplasm components within the septated fragments. In this context, a recent paper has shown that autophagic fungal cell death is required for functional appressorium formation in the plant pathogenic fungus *Magnaporthe grisea* (Veneault-Fourrey et al., 2006). If the gene required for autophagy is knocked out, appressoria are still formed but they do not penetrate the leaf epidermis. Recent data from our laboratory seem to confirm the hypothesis that GmGin1 could be a sensor for plant signals. We have evidence that this protein is located at the cell membrane and that by means of its carboxy terminus, which is homologous to the carboxy terminus of Hedgehog self-splicing proteins from animals, GmGin1 undergoes splicing in response to signals from the plant (Serrano, Ocon and Requena, unpublished results). After splicing, the amino terminus will remain covalently attached to the plant signal acting as nucleophile. We are currently trying to identify the plant molecule/s which induce splicing of Gin1. It is likely, that a modified Gin1 is able to exert a signaling function through its ATPase activity and modulate other downstream signaling proteins. These interesting results show that the chemical communication with the plant symbiont does not only modify fungal gene expression but it is also able to induce post-transcriptional modification of fungal proteins.

Using differential RNA display, Tamasloukht et al. (2003) used the lipophilic fraction isolated by Buee et al. (2000) to show that extensive hyphal branching observed by *Gigaspora rosea* spores in response to that fraction correlated with an increased expression of several mitochondrial-related genes. The authors could show that these transcriptional changes were followed by an induction in the respiration rate as well as by changes in the mitochondrial dynamics. These results prove that fungal perception of chemical plant signals, possibly strigolactones, induces transcriptional changes followed by physiological and morphological responses.

Because it was known that not only chemical but also physical plant signals are required for induction of appressoria formation, Breuninger and Requena (2004) designed an *in vitro* system to study transcriptional changes taking place during that developmental stage. *G. mosseae* germinated sporocarps challenged with seedlings of parsley plants brought together under a microscope coverslip were visualized during appressorium formation. Chitin detection showed *G. mosseae* hyphae approaching towards the root epidermis at 24 and 48 h. After 72 h, hyphae started to run parallel to the root epidermis with first contacts with epidermal cells after 96 h. First, appressoria were observed at 120 h as dichotomously branched hyphae attached to an epidermal cell. At this stage, a SSH library was constructed enriched in appressoria expressed genes (Breuninger and Requena, 2004). Transcript profiling of AM fungi during appressoria formation showed that plant contact induces the activation of genes from different categories, including several components related to Ca^{2+}-signaling, including a putative P-type Ca^{2+}-ATPase, a calmodulin, a leucine zipper EF-hand protein, and a Ca^{2+}-induced Ras inactivator (CAPRI) (Breuninger and Requena, 2004). This suggests the involvement of Ca^{2+} as a second messenger in the

transmission of plant-derived signals leading to appressorium formation. Several publications have shown the involvement of calcium- and calmodulin-dependent signaling during appressorium formation in several plant-interacting fungi, including the plant pathogen *Magnaporthe grisea* (Ahn et al., 2003; Lee and Lee, 1998; Liu and Kolattukudy, 1999; Poulin et al., 1997; Shaw and Hoch, 2000; Warwar et al., 2000). At the plant side, symbiosis with nitrogen fixing bacteria induces an increased Ca^{2+} influx, a subsequent K^+ efflux, plasma membrane depolarization and intracellular alkalinization in root hairs upon perception of bacterial Nod factors (Cardenas et al., 1999, 2000). In support of a similar signaling cascade during perception of plant signals by AM fungi, we have shown an increase in the expression of a H^+-ATPase gene upon appressorium formation (Requena et al., 2003). This correlates with previous observations showing an increase in ATPase activity (Lei et al., 1991) and membrane depolarization (Ayling et al., 2000) when germ tubes of AM fungi were in contact with host roots. It is therefore tempting to speculate that Ca^{2+} could play a significant role in transmitting plant signals to appressoria formation in the AM symbiosis (see Fig. 2).

3. Where to go now?

Molecular results show that in the mycorrhiza symbiosis novel signaling components as well as conserved ones are directing the molecular dialogue between the fungus and plant. Major goals in the future will be to identify all players of these signaling networks, particularly the signals and receptors that open the door to symbiosis formation.

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