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Signalling and Communication in the Early Developmental Phase of Ectomycorrhizal Symbiosis

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Ectomycorrhizas (ECM) are interact and mutualistic associations by which tree roots and soil-borne fungi interact and form, which are typical dominate the global terrestrial ecosystems. Nutritional physiology and resistance function of host plant may be enhanced by fungal symbiont for diversification ways. However, to achieve this physiological functions encompasses a series of complex and fine control mechanisms in the colonizing mycelium and roots of host trees, indicating that both partners evolved unique molecular mechanisms to establish and maintain this kind of mutualistic relationships. The development processes of an effective ECM symbiosis, in comparison with some other plant-microbe organizations, such as rhizobia or arbuscular mycorrhiza (AM) symbiosis, little is known about the mechanistic details of the signalling between partners. Current studies have provided crucial insights into multiplicity of signal molecules, diversity of signalling pathways and the regulation of symbiosis-specific genes expression during the ECM symbiosis establishment, based on existing knowledge from the interaction of symbiosis organizations, we will gradually identify this mechanism of signal recognition and transduction related with ECM development on a molecular level.

Key words: Ectomycorrhizal symbiosis, Rhizosphere signals, Signalling pathway, Gene expression.

In natural ecosystems, plant tissue can be used as a niche for many micro-organisms colonization, especially in roots, that always with numerous harmful, beneficial and neutral microbes living together, and have a direct or indirect contact. An adaptive mechanism has been developed by plants for this colonization in a long-term process of co-evolution, which able to identify signal molecules from microorganisms and make appropriate physiological reactions, including compatible interactions and incompatible interactions. In order to adapt complex ecological environment, plant evolved into many forms of plant-microbe symbiotic associations. One successful association is the plant root tissue and soil-borne fungi formed mutually symbiotic - mycorrhizae, this symbiotic relationship has existed as early as 400 million years ago and plays an important role in the evolution of plants from aquatic to terrestrial[1]. About 95% of the world's living species of vascular plants belong to families that are characteristically mycorrhizal, which to be the norm in terrestrial plant nutrition[2-4]. Different plant species with different types of mycorrhizal symbiosis, but our main interest focused on ECM. While only around 3% of seed-bearing plants establish the ECM symbiosis with fungi, this group plant species majority includes the dominant tree

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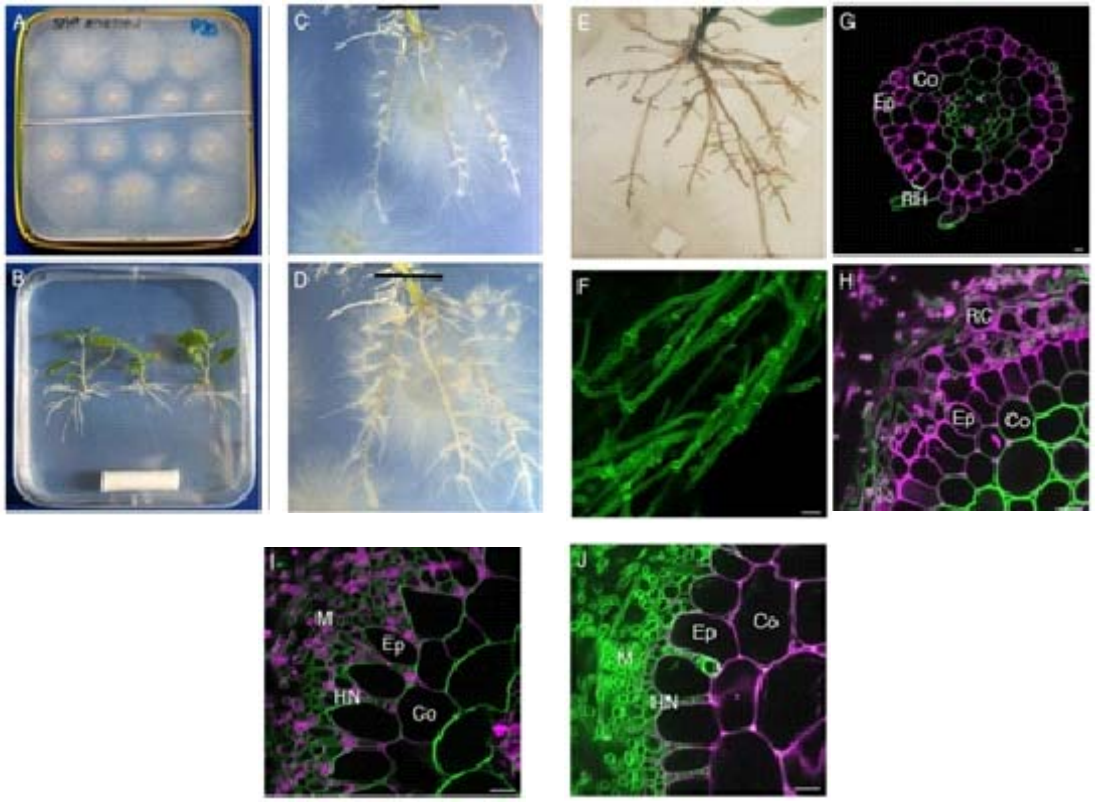
species in forests and woodlands of temperate, boreal, and partially tropical latitudes[5], take into consideration the greatly area in the land surface and the highly economic value of forests, ECM trees indeed overwhelmingly dominant the global terrestrial ecosystems. Establishment of functional ECM can increase absorption of nutrients in soil, promote tree growth, enhance resistance ability and improve living conditions, ECM symbiosis plays an important role in physiology, ecology, resistance, production, and other aspects of life of a single trees, populations, and ecosystems[6].

The establishment of ECM symbiosis is an ancient interaction form between roots and fungi, results from in a time-regulated sequence of highly coordinated events. Both partners release an array of various metabolites (morphogens and signalling molecules), recognize each other by identifying mechanisms, induce specific functional genes expression, cause a series of complex morphological and physiological changes, and finally complete the specific development programs on the basis of self-regulation. In this process, the mycobiont has the ability to recognize and escape the host defense surveillance, being able to become associated with host roots and establish bidirectional nutrient transfer. Plant cells also carry out so-called "accommodation program" in favor of mycelium infection and organization development. Despite representing an active research area, the current comprehension of plant-fungi recognition, establishment and function of ECM are still rather limited, and only few genes and proteins associated to symbiosis have been identified. Recent studies on signal transduction mechanisms are relatively clear for the development of leguminous plant rhizobia or arbuscular mycorrhiza(AM) symbiosis, suggest that the formation of symbiotic relationship between plants and microbes with great conservative. More importantly, the *Populus* genome is the first tree genome that has been completely sequenced, annotated and publicly released[7], subsequently the full genomic sequence of the basidiomycete ECM fungi *Laccaria bicolor* strain S238N-H82 has been resolved[8]. These achievements are currently been used for genome-wide expression profiles at different stages of ECM development, and generating an immense amount of valuable data. Scientists have provided crucial insights into specific phases and signalling pathways of ECM development, and encourage future studies at the molecular level, the mystery of symbiotic mechanism began to be gradually unveiled.

Formation and development of the ECM symbiosis

The functioning structure defined as an ECM is characterized by a basic pattern including the presence of a dense mass of fungal hyphae forming a pseudoparenchymatous tissue around the fine root tips (the mantle), and a labyrinthine inward growth of hyphae colonization between epidermal (angiosperms) and also cortical (gymnosperms) cells, called the Hartig-net. These well-conserved features can be used as mechanical barrier to protect the roots from pathogenic organisms, and drive the photosynthate, water and essential nutrients flow between the two partners. ECM play a key role in maintaining and enhancing the health of a broad range of plants[5].

In the formation of symbiosis, the partners recognize each other and cause a series of complex morphological and physiological changes, suggesting that ECM development involves abundant signalling events of multifaceted participation and fine coordination. To elucidate such events, most research has employed simplified in vitro systems that are conducted to separate sequential phases, and usually divided the development into three main phases (Fig. 1, quote from Judith Felten *et al.*, 2009)[9]: (p™) presymbiotic phase - signals dialogue before physical contact; (q™) early symbiotic phase - hyphae adhere to the root epidermis and progressively colonize in the intercellular space; (r™) mature symbiotic phase - formation the functional mycorrhiza and the nutrient exchanges take place between both partners. These phases in chronological order describe a series of evens in an infection site. Rhizosphere signals communication between the partners before physical contact is commonly called the presymbiotic phase, after perceived the key metabolites released from host roots, fungi is attracted towards the potential partner[10] by increasing hyphal branching and inducing spore germination[11]. Meanwhile, ECM fungi also secrete the corresponding signal molecules be identified by host plants, which futher initiate a series of genes expression and regulate the growth of roots.



A and B, *L. bicolor* and poplar precultures, respectively. C to E, Root development of poplar at 3 DODI (C), 10 DODI (D), and 30 DODI (E). Note the increasing LR number starting at 10 DODI. Root swelling at the LR basis started at 10 DODI, and LR arrest was observed at 30 DODI. F, *L. bicolor* hyphae from precultures after UVitex staining. G, Transverse root section after propidium iodide UVitex dual staining (green, UVitex; magenta, propidium iodide). H to J, Dual fluorescence-stained transverse root sections at 3 DODI (H), 10 DODI (I), and 30 DODI (J). Co, Cortex; Ep, epidermis; HN, Hartig net; M, mantle; RC, root cap cells; RH, root hair. Note hyphae attachment at 3 DODI and mantle and Hartig net development from 10 to 30 DODI. In H, single, magenta-colored cells surrounding the epidermis are detached root cap cells. Images shown are representative from a series of three experiments. Bars = 10 µm.

Fig. 1. Time course of colonization of poplar roots by *L. bicolor* in an sandwich culture system (quote from Judith Felten *et al.*, 2009 [9])

When hyphae grow around the host roots and physical contact the roots surface, they entered the early symbiotic phase. Hyphae aggregate on epidermal cells near the root tip formation an "adhesion pad" through secreted adhesion molecules[12], their penetration between epidermal cells colonization in roots, meanwhile the roots secrete hormones to promote hyphae branching. At this stage, like other pathogenic fungi, ECM fungi elicitor also induce the host defense responses, but this intensity is weak and the enzyme or metabolite accumulation is only temporary or partial[13].

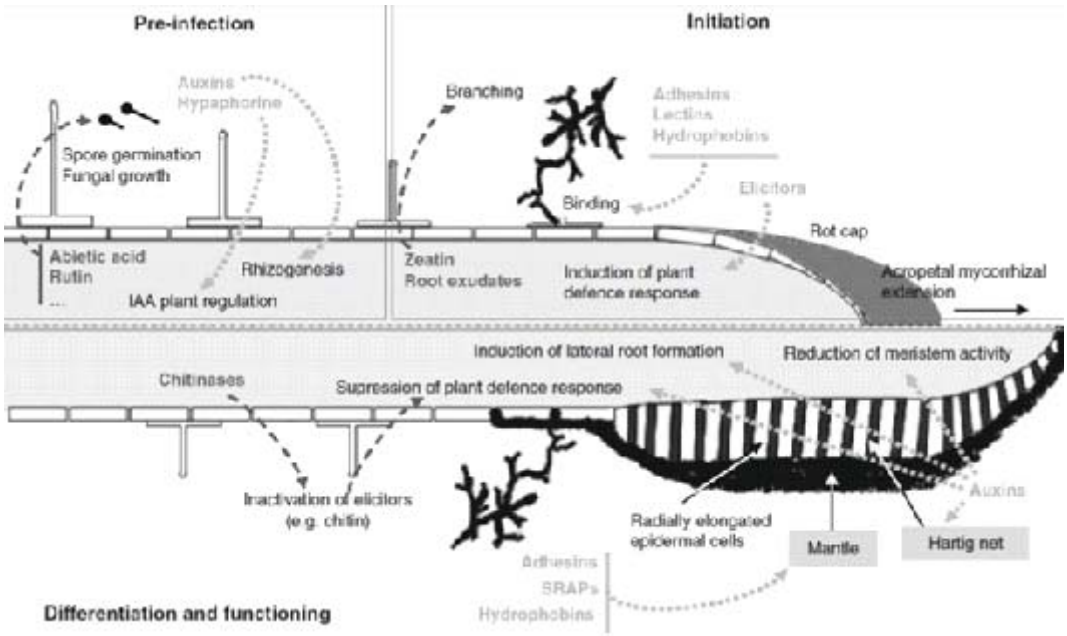
In the mature symbiotic phase, roots surface hyphae proliferate multiple layers that surround the roots and differentiate into the mantle, while the internal hyphae penetration between epidermal (angiosperms) and also cortical (gymnosperms) cells develops the Hartig-net. Once mantle and Hartig net are well developed in roots, the bidirectional transfer of nutrients take place between both symbionts, signs of formation the functional ECM. At the same time, surface hyphae of mantle constantly extending and winding in soil to form extraradical hyphae and rhizomorphae, the both connected into a huge underground mycelium network to improve nutrients and water uptake for circulation and redistribution between plant - fungi.

Furthermore, fungi does not penetrate any further when the colonization is accomplished,

which occurs efficient nutrient exchange until the ECM senescence. It has been shown that ageing mycorrhizas may lose their fungal mantle, even if the Hartig net is maintained, and that their contribution to host plant - fungi nutrients exchange diminishes. The fungi can return back to a saprophytic lifestyle and invade senescent root cells[14].

Rhizospheric signals dialogue during the presymbiotic phase

The period before any physical contact, the recognition and exchange of appropriate signals between mycorrhizal fungi and host roots are considered to be the key links in symbiotic development. The identification process of diffusible elicitors between symbiotic partners has always been the scientists interested questions. By contrast to some other plant-microbe interactions, the nature of the signal molecules in ECM presymbiotic phase are unknown (Fig. 2, quote from Paula Baptista *et al.*, 2011)[15].



Root and fungal exudates are the source of specific signals during the presymbiotic stage that lead to the perception and recognition of compatible partners. The morphological changes that occur for forming the symbiotic structures (in *gray boxes*) and the main signals that induce them are outlined. Signal molecules produced by the plant are represented in *dark gray bold* and the developmental processes in which they are involved are indicated by *dashed gray arrows*. Fungal signals are represented in *light gray bold* and the processes controlled by them pointed out by *dotted arrows*.

Fig. 2. Signals required for the ectomycorrhizal development (quote from Paula Baptista *et al.*, 2011 [15])

Fungi responses to plant-derived signals

Early studies have suggested that the plant secretion of certain signal molecules into the rhizosphere seems to promote spore germination and hyphal growth[16,17]. Baptista *et al.*[18] confirmed that the root extracts have ability to regulate ECM fungal growth during the early stage of *Pisolithus tinctorius* - *Castanea sativa* association. Abietic acid extracted from *Pinus* roots was able to induce spore germination at a very low concentration (10^{-7} M) and this effect seems to be specific to the genus *Suillus*[11]. The mycelium of *P. tinctorius* and *Paxillus involutus* were attracted toward the root cap under the conditions to create a physical barrier between *Eucalyptus globules* and ECM fungi[10].

Due to the complex composition and low concentration of root exudates, it is difficult to determine which compounds have characteristic of signal molecules. Based on current knowledge of the molecules released in other plant-microbe interactions, speculated the secondary metabolites

by host roots secreted into the rhizosphere may act as signal molecules. In *Eucalyptus*, roots release the flavonol rutin and cytokinin zeatin, that have been identified to significant change the hyphal morphology of the *P. tinctorius* at only a picomolar in concentration, rutin stimulated growth of hyphae, whereas the zeatin modified the hyphal branch angle[19, 20]. In the case of AM interactions, believed that hyphal branching is the fungi first response for rhizosphere signals. Akiyama *et al.*[21] found that AM fungi can be induced branch by a sesquiterpene compound, which isolated from *Lotus japonicas* root secretions - strigolactones. Subsequently, studies have revealed the role of strigolactone as a signalling compound to the hyphae aiding in branching and establishment of the mycorrhizae[22]. However, similar molecules have not been identified in the initial stage of ECM development.

Plant responses to fungi-derived signals

Numerous studies have shown that host plant also perceives and response the first fungi diffusible factor before contact, that induction in the morphology change of roots and plays a key role in the development of ECM[9,23,24]. Phytohormones are metabolites that exist in extremely low quantities in plants, or also in microorganisms, their participation in all aspects of root development. Therefore, under the uncertain of the real identities of ECM fungi symbiotic signals, phytohormones are the best signal molecules to initiate symbiotic relationship.

These conclusions have been confirmed that the hyphae of *Cenococcum geophilum*, *Laccaria laccata*, *Hebeloma crustuliniforme*, *Tuber melanopsorum* and *Tuber borchii* are able to produce volatile phytohormone ethylene[23, 25, 26]. Reference ethylene is known for its stimulatory effect on lateral root(LR) development[27, 28], surmised it may be the key actor during the presymbiotic phase. However, Splivallo *et al.*[23] considered ethylene mimicked fungi-induced LR stimulation only when applied together with exogenous auxin in feeding experiments. It is worth noting the exact content of fungi-derived volatile phytohormone has not been detected, fungi-derived ethylene levels likely mixing with plant released[25], and the *P. tinctorius* which do not produce ethylene but also stimulate LR development. This suggests that ethylene not be the only fungi-induced signals during the early phase of interaction.

Several research groups have deemed the fungi-derived auxin as a signal molecule operating in the presymbiotic phase of the ECM formation[9, 23, 29, 30]. Previous researches have found that the addition of an inhibitor of auxin transport (2,3,5-triiodobenzoic acid, TIBA) blocked the colonization of the Norway spruce root by *Laccaria bicolor*, restricting the hyphal growth between cortical cells and limiting the Hartig-net formation[31], and the levels of indole-3-acetic acid (IAA) produced by the fungi also seem to control the production of elicitors by the host plant[32, 33]. Although the fungi-derived auxin appears too low to be responsible for early plant responses such as LR stimulation[34, 35], when both fungi and host plant in a symbiotic system, *L. bicolor* has resulted in a visible LR stimulation for secretes only about 10 nM IAA[31]. Recently, Felten *et al.*[14, 24] have Suggested that fungi signals and auxin could work in synergy to induce LRs, fungi-derived auxin in its very low quantity is unlikely to be the trigger of the early plant-fungi signalling.

Furthermore, fungi produce the hypaphorine also appears to influence the ECM establishment. In *E. globules* roots and *P. tinctorius* co-culture system using membrane separation technology after 24h, the fungal hypaphorine levels increased three to five fold when compared to the pure mycelium culture[36], and seems to regulate the activity and levels of plant auxins[37,38]. Ditengou and Lapeyrie[39] have proposed the reduction in the root hair growth is indeed a marker feature of ECM roots, and hypaphorine seems also to display a morphogenetic effect on host roots by reducing root hair elongation[36, 39-41].

Signalling in the early developmental phase of ECM symbiosis

Host plant transduct the symbiotic signals after recognizing of fungi effectors, initiate a series of genes expression, make the adjustment in the physiology and morphology, finally preparing for the fungi infection and colonization. Thanks to the *Populus trichocarpa* genome has been completely sequenced, annotated and publicly released[7], and the full genomic sequence of the *L. bicolor* has been resolved[8], the signal transduction in the early symbiosis stages becomes an interesting problem to the scholars in recently years. Due to the true identity of effector is still

scarce, greatly hindered the understanding of signal recognition and transduction mechanism, despite a few genes and proteins associated to ECM symbiosis have been recognized and a small number of signal molecules have been chemically identified, their specific function remain largely unknown.

In comparison with bacterial or fungal endosymbiosis, the clear scheme of factors involved in exchange, recognition, and colonization are still black box for ECM signalling. However, the recognition mechanism (ligand/receptor pair) between plant-fungi and parts of the downstream signalling cascade have been revealed in nitrogenfixing root nodule symbiosis and AM symbiosis, where the genetic programs have a partial overlap. They all require three plant signalling components involved in AM development[42-45], these genes encoding the SYMRK/NORK/DMI2 receptor kinase, the DMI1 ion channel and DMI3 calcium- and calmodulin-dependent kinase are collectively referred to as the "common" *SYM* genes[46], and associate with the Ca^{2+} signalling pathway, suggesting that this genetic programs is universally exist in different types of symbiotic system.

Different research groups on ECM signalling pathways have different speculation, Francis Martin *et al.*[46] have suggested that the younger bacterial symbiosis (which first evolved around 60 million years ago)[47]has recruited perception functions from the ancient AM symbiosis (about 400 million years ago)[48] through evolutionary analysis. Considering these *DMI* genes are found in the genome of *P. trichocarpa*[7], a tree hosting both AM and ECM symbioses, therefore a tempting speculation is proposed that the recent ECM symbioses (about 180 million years ago)[49]have also recruited the AM symbiosis *SYM* genes for signalling and the early steps of the symbiosis[46]. Conversely, many scholars have believed that the independent evolution of endomycorrhizal and ECM symbiosis may account for the difference in their signalling mechanisms, these require using RNAi technology to verify whether or not perennial tree species and legumes have evolved similar mechanisms to interact with mycorrhizal fungi.

ECM fungi trigger the typical plant defense responses

With the exchange and recognition of symbiotic signalsÿECM fungi produces elicitors commonly trigger the typical plant defense responses at the early symbiosis stages, which exhibit biochemical resistance responses similar to those described for incompatible pathogen attack, such as the changes in protein phosphorylation status[50], modifications in ion fluxes[51], transient accumulation of reactive oxygen species (ROS)[18], increase the activity of antioxidative enzymes[52]and strengthening the cell-wall[53]. Moreover, different transcriptome studies have confirmed the transient increase in plant defense responses which have been interpreted as an initial reaction of plant to restrict fungal growth[52, 54-56].

It is interesting to note that these nonspecific, broad-spectrum defenses are repressed at later stages of colonization, suggests the ECM fungi suppress defense responses through yet unknown mechanisms. Take account of microbes and pathogens can release effectors (polysaccharide, proteins, phytotoxins, etc.) that repress this defense response, we may suggest that it takes place upon root-ECM fungi interaction[14]. The relevant reports say that the auxins produced by the ECM fungi *H. crustuliniforme* during the colonization process seem to play a role in declining the defense capacity of plant cells[32], and the high chitinase amounts produced by the host plant that inactivates elicitors also seem to inhibit the defense[57]. Future research should explore the molecular mechanisms that orchestrate the escape of the symbionts from the host defense system[46].

Quest for master symbiotic genes in the ectomycorrhizal symbiosis

Gene that control traitsÿand is the molecular basis of all interactions process in the symbiosis between ECM fungi and host roots. In recent years, highthroughput technology, genome sequencing of fungi, plants and associated microbes, transcriptomic analyses, availability of mutant collections, RNA interference lines and plants transformed with fluorescent tags have all provided new insight into the signalling pathway and action mechanism and consequentially great progresses have been made[58]. At the different developmental stages studied, have led to the transcriptome analysis of several symbiotic couples includes *Pisolithus* - *Eucalyptus* / *Populus*

/ *Castanea*[56, 59-61], *Paxillus* - *Betula*[54, 55, 62], *Tuber* - *Tilia*[63, 64], *Laccaria* - *Populus* / *Pseudosuga* / *Pinus*[60, 65, 66] and *Quercus* / *Piloderma*[67]. These studies have identified hundreds of symbiosis-regulated genes for plant and fungi that are preferentially expressed to required for fungi attachment, plant defense and symbiosis-related metabolism, but no symbiosis-specific genes were detected.

These reviewed data indicates the changes in morphology associated with mycorrhizal development were accompanied by changes in transcript patterns, symbionts use of different genetic programs during different ECM phases, related genes change expression levels during formation and maintenance of symbiotic relationships, but all of them are basally expressed in most symbiotic conditions. Thus, Martin *et al.*[46] have suggested that ontogenic and metabolic programs that lead to the development of symbiosis be driven by the differential expression of pre-existing transcription factors and/or transduction pathways. However, a more detailed study is needed in order to clarify this question, completion of larger sets of ECM expression profiles on a wider range of associations using whole-genome microarrays and high-throughput quantitative PCR of transcription factors.

These studies have not revealed any mycorrhiza-specific genes but have described a large set of differentially expressed symbiosis-regulated plant and fungi genes from different time points of mycorrhiza formation. Recently, only a few genes and proteins associated to ECM symbiosis have been recognized in the early stages of interaction[52, 68-70], but there are still many genes have not shown similarity to known sequences, some of these genes may represent unidentified mycorrhiza specific genes[66], or the potential specific genes with very rare transcripts that have not been previously identified. In the wake of the first demonstration of RNA silencing in *L. bicolor*[71], the function of ECM-regulated genes that could be potential participants in the development of the symbiosis will gradually clear by using reverse genetics.

DISCUSSIONS

The development of mutualistic symbiosis organization is a long-term and complex delicate process, between fungi and host plants have a series of signals exchange, identification, transduction and response. In the wake of the developpement of model plant genomics and functional genomics, as well as the application of efficient state-of-the-art molecular and genetic tools, the genome and transcriptome analysis of several symbionts offers new insights into our understanding of the mechanisms that govern the establishment and functioning of ECM symbioses.

Dissecting the molecular mechanisms of symbiosis mutualistic requires both the identification of the functions of individual genes and knowledge of how genes interact to form complex traits. However, currently the puzzling question is that the real identities of symbiosis signals are still unknown, this led to plant and fungi genetic switches that are necessary for ECM development remain unidentified to date. In addition, there are still many pieces of the puzzle remain to be elucidated, it seems inescapable that the cross-talk between rhizospheric metabolites, hormonal balance and signalling networks involving roots morphological changes in coordinating the execution of the each symbiotic events. These facts highlight a knowledge gap concerning the mechanism of development and functioning in symbiosis and encourage future researches at molecular level to ECM associations.

There are three routes for further work. Firstly, we should be on the basis of existing knowledge, focus on the research of chemical nature and functional characteristics for root exudates and fungi-derived signal molecules, such chemicals are potential biological regulation factors occurring in naturally, as a “green elements”, that will available for use to optimized and enhanced mycorrhizal function in forestry and ecological systems. Secondly, continue quest for master regulatory genes, or symbiosis-specific genes, a more detailed study is needed to completion of larger sets of ECM expression profiles on a wider range of associations using whole-genome microarrays and high-throughput quantitative PCR of transcription factors, that could help in understanding the molecular basis of the early events in plant-fungi interaction. Thirdly, symbiosis-regulated genes need to be studied in order to determine their function in the development of the symbiosis by using reverse

genetics, confirming these genes are essential for symbiosis development. We can expect that new components of the transduction pathways will soon be identified, facilitating an understanding of the cross-talking between signalling networks and the early gene regulation processes involved in ECM development, lead to a better understanding of plant-microbe interactions and evolution of plant-fungi associations.

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