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# Common and not so common symbiotic entry

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Great advances have been made in our understanding of the host plant's common symbiosis functions, which in legumes mediate intracellular accommodation of both nitrogen-fixing bacteria and arbuscular mycorrhiza (AM) fungi. However, it has become apparent that additional plant genes are required specifically for bacterial entry inside the host root. In this opinion article, we consider *Lotus japonicus nap1* and *pir1* symbiotic mutants within the context of other deleterious mutations that impair an intracellular accommodation of bacteria but have no impact on the colonization of roots by AM fungi. We highlight a clear delineation of early signaling events during bacterial versus AM symbioses while suggesting a more intricate origin of the plant's ability for intracellular accommodation of bacteria.

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## Beneficial root symbioses

Since conquering the terrestrial landscape some 475 million years ago (mya), plants have evolved a myriad of ways to deal with limitations of water and essential nutrients in the environment. Arguably the most fascinating are the endosymbiotic relationships, which provide mutual benefits for both the host plant and the microsymbiont.

The vast majority of plant species (>80%) are able to benefit from interactions with fungi of the phylum *Glomeromycota* [1]. This ancient symbiosis, termed the arbuscular mycorrhiza (AM) symbiosis, improves the plant's efficiency for uptake of water and nutrients, particularly phosphates [2] and there is fossil evidence of AM associations from the earliest of land plants [3]. Extracellular hyphae produced by the fungus gain access to the plant root by means of specialized structures called hyphopodia (see [Glossary](#)), which form at the root surface [4]. The plant root actively prepares for intracellular accommodation of the fungus by forming trans-cellular cytoplasmic bridges, which are often referred to as 'prepenetration apparatus' [4]. These guide fungal hypha through the root epidermis and within the cortical cell layers, eventually leading to systematic colonization of the host root and the formation of intracellular arbuscules [5].

In a presumed more recent evolutionary event (ca. 60 mya), a confined group of plants evolved a means to obtain atmospheric nitrogen via a symbiotic relationship with either nitrogen-fixing Gram-negative soil bacteria, commonly referred to as rhizobia [6] or Gram-positive *Frankia*. This interaction, termed the root-nodule symbiosis (RNS), required at least two plant adaptations: (i) intracellular accommodation of the bacteria and (ii) formation of a specialized organ known as the root nodule [1]. This sophisticated relationship, which is restricted to members of only four orders within all of the known angiosperms [7], allows the host plant to thrive under nitrogen-limited soil conditions.

In many legumes, nitrogen-fixing rhizobia gain access to the host root by a network of plant plasma membrane-derived tubular structures, called infection threads (ITs) [8]. Similar to fungal hyphae,

ITs are guided within the root cortex by a preordained network of cytoplasmic bridges, called ‘pre-infection threads’ (PIT) [9]. Thus, in both symbiotic interactions, the host plant actively controls the passage of microorganisms inside root tissues [10] and [11].

Perception of chemical signals produced by the rhizobia in the form of lipochito-oligosaccharides, also known as nodulation factors (NF), induces the initial host-plant response pathway, which is required for bacterial colonization at the root epidermis and also PIT formation and cell divisions within the root cortex. This is achieved by activation of specialized plant receptors, named *NFR1* and *NFR5* in the model legume *Lotus japonicus* [12] and [13]. As a result, the root nodule structure is formed, wherein the bacteria are housed and fix nitrogen [14]. Recent unprecedented progress in understanding fundamental signaling events that mediate reprogramming of plant cells for RNS [15] offers important impetus for additional research and also hope for its future exploitation in sustainable agriculture.

## **The common symbiosis pathway is required but not sufficient for bacterial entry**

It is widely accepted that a subset of legume plant genes which mediates early root responses to NF signaling has been recruited from a pre-existing plant mechanism. This proposal is based on the identification of the ‘common symbiosis pathway’ that acts immediately downstream from NF perception and is required for both the RNS and AM. This pathway is presumed to have evolved during the evolution of terrestrial flora to support AM symbiosis of early land plants and subsequently, to be co-opted to mediate an intracellular accommodation of bacterial symbionts during RNS [16, 17] and [18].

In *L. japonicus*, the common symbiosis pathway is composed of at least eight genes [19, 20, 21, 22, 23, 24, 25] and [26]. Deleterious mutations in any of these genes in *L. japonicus* as well as in equivalent loci of other legumes, such as *Medicago truncatula* and *Pisum sativum*, severely affect the plant's ability to initiate and/or maintain both symbioses ([17] and references therein). These observations were interpreted, therefore, as being reflective of a plant mechanism, wherein functioning of the common symbiosis pathway is necessary for induction of accommodation programs for both, fungal (for AM) and bacterial partners (for RNS) [1]. However, the accumulating data indicate that in addition to this pathway, other plant functions are specifically required for the intracellular accommodation of the bacterial partner. Thus, what could have been the origin of these AM-dispensable symbiotic plant functions?

## **Actin cytoskeleton rearrangements at the core of bacterial entry**

The *Nap1* and *Pir1* loci of *L. japonicus*, which we recently characterized as presumed components of the *SCAR/WAVE* complex, are responsible for regulating actin rearrangements during polar tip growth [27]. In addition to a non-symbiotic phenotype related to aberrant trichome formation, deleterious mutations in either *Nap1* or *Pir1* cause severe inhibition of *Mesorhizobium loti* infection, leading to uncolonized, ineffective nodules [27]. With the exception of rare successful colonization events, bacteria remain unable to penetrate the *nap1* or *pir1* roots and do not enter dividing cells of growing nodule structures. Instead, they often accumulate in ‘patches’ on the surface of the exposed nodule cortex or in aborted ITs, thus indicating the essential role for *Nap1* and *Pir1* in intracellular accommodation of bacteria in *L. japonicus* (Figure 1a and b). By contrast, AM colonization remains intact in *nap1* and *pir1* mutants, thus highlighting functional distinction in

the pertinent accommodation processes ( [Figure 1c](#)). Importantly, the specific defect in internalization of the bacterial partner within root tissues of *nap1* and *pir1* mutants occurs in the presence of epidermal calcium spiking [\[27\]](#), which constitutes one of the earliest physiological root responses to bacterial and fungal signaling that necessitates the function of the common symbiosis pathway [28](#) and [29](#). Thus, although required, the common symbiosis pathway and associated epidermal calcium spiking are not sufficient for the IT-dependent accommodation of *M. loti* in *L. japonicus*.

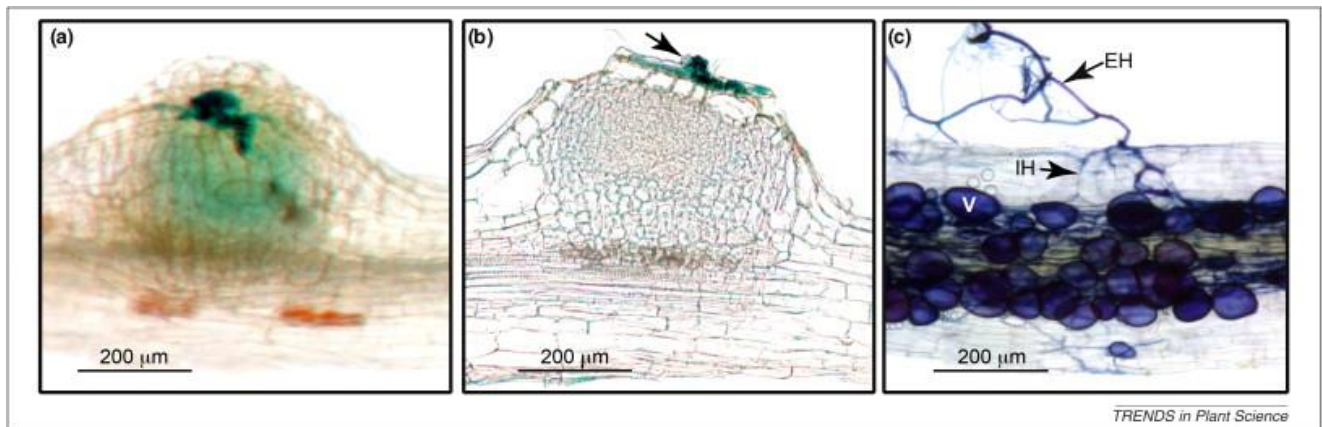


Figure 1. Symbiotic phenotypes of the *L. japonicus nap1-2* mutant. (a) *M. loti* bacteria expressing the *lacZ* reporter gene accumulating at the surface (dark blue patch) but failing to colonize the interior of the growing nodule structure. (b) Longitudinal-section through a *nap1-2* nodule showing *M. loti* colonizing dislodged epidermal cells but failing to enter the nodule structure (arrow). (c) Wild-type colonization of *nap1-2* roots by AM fungus, *Glomus intraradice*. EH: extraradical hyphae; IH: intraradical hyphae; and V: vesicle.

*nap1* and *pir1* mutants are not at all unique in this respect. It has been demonstrated through the characterization of many independent mutants that bacterial and fungal entry can be uncoupled ( [Table 1](#)). For example, the *L. japonicus CERBERUS* locus was recently characterized as encoding a U-box protein with WD-40 repeats. The protein was shown to be essential for the uptake of *M. loti* within susceptible root hairs, but was dispensable during AM symbiosis [\[30\]](#).

Table 1. Selected examples of mutant lines in *L. japonicus* and *M. truncatula*, where mutations impair bacterial infection without impacting on arbuscular mycorrhiza symbiosis (AM<sup>+</sup>)<sup>a</sup>

Mutant allele	Plant	Gene function	Rhizobial infection phenotype	AM Phenotype	Refs
<i>alb1-1</i>	<i>L. japonicus</i>	ND	Short, thick ITs arrested in the epidermis	AM <sup>+</sup>	<a href="#">49</a> , <a href="#">50</a> and <a href="#">51</a>
<i>nin</i>	<i>L. japonicus</i>	Putative transcription factor	No IT formation	AM <sup>+</sup>	<a href="#">[52]</a>
<i>hcl</i>	<i>M. truncatula</i>	LysM-receptor kinase (LYK3)/microtubule network	No IT formation (strong allele)	AM <sup>+</sup>	<a href="#">53</a> and <a href="#">54</a>
<i>crinkle</i>	<i>L. japonicus</i>	ND	ITs arrested at the base of epidermal cells	AM <sup>+</sup>	<a href="#">51</a> , <a href="#">55</a> and <a href="#">56</a>

Mutant allele	Plant	Gene function	Rhizobial infection phenotype	AM Phenotype	Refs
<i>lin</i>	<i>M. truncatula</i>	U-box/WD40 protein/predicted E3 ubiquitin ligase	IT formation significantly reduced	AM <sup>+</sup>	<a href="#">57</a> and <a href="#">58</a>
<i>latd/nip</i>	<i>M. truncatula</i>	NRT1(PTR) transporter	Infection abnormal and reduced, no release of bacteria	AM <sup>+</sup>	<a href="#">59</a> and <a href="#">60</a>
<i>lot1</i>	<i>L. japonicus</i>	ND	IT formation significantly reduced	AM <sup>+</sup>	<a href="#">[61]</a>
<i>itd 1, 3 and 4</i>	<i>L. japonicus</i>	ND	Rare ITs arrested in the epidermis	AM <sup>+</sup>	<a href="#">[62]</a>
<i>bit1-1</i>	<i>M. truncatula</i>	ERN transcription factor	Limited number of ITs; epidermis/cortex block	AM <sup>+</sup>	<a href="#">[63]</a>
<i>api</i>	<i>M. truncatula</i>	ND	Defect throughout the entire infection process/strong block at the outer cortex	AM <sup>+</sup>	<a href="#">[64]</a>
<i>nap</i>	<i>L. japonicus</i>	SCAR/WAVE complex; actin polymerization	Enlarged microcolonies, disintegrated ITs, block in epidermis/cortex	AM <sup>+</sup>	<a href="#">[27]</a>
<i>pir</i>	<i>L. japonicus</i>	SCAR/WAVE complex; actin polymerization	Enlarged microcolonies, disintegrated ITs, epidermis/cortex block	AM <sup>+</sup>	<a href="#">[27]</a>
<i>cerberus</i>	<i>L. japonicus</i>	U-box/WD40 protein	Enlarged microcolonies, disintegrated ITs, epidermis/cortex block	AM <sup>+</sup>	<a href="#">[30]</a>

Abbreviations: AM<sup>+</sup> wild-type colonization of roots by arbuscular mycorrhiza. ND not determined.

Although the phenotypic variation among the mutant lines affected specifically in bacterial entry encompasses a wide range of early to relatively late root colonization stages, all of these lines remain wild-type with respect to AM symbiosis ([Table 1](#)).

Based on these observations it is apparent that, in addition to common symbiosis functions, other plant genes and associated signaling events must have been recruited and/or formed *de novo* specifically in support of intracellular bacterial entry [\[31\]](#). Severe early defects in root colonization by bacteria in several independent mutant lines, such as *L. japonicus nap1*, *pir1*, *itd* and *cerberus*, indicate that at least some of these plant functions ( [Table 1](#)) could have been essential during the



presumed transition from the default plant infection by bacteria, where they remain extracellular, to intracellular accommodation [8].

We speculate that plants have initially utilized some of these functions to accidentally internalize bacteria through a direct mechanism resembling phagocytic events (Figure 2). Alternatively, assuming the previously hypothesized parasitic origin of symbiotic rhizobia [32 and 33], plant functions such as *Nap1* and *Pir1* that regulate actin cytoskeleton might have been pirated by the bacteria for their intracellular invasion in a manner analogous to the behavior of some enteropathogenic bacteria [34 and 35]. Further refinements by, for example, the integration of cellular responses that engage the plant cytoskeleton with additional signaling elements such as those constituting the common symbiosis pathway and subsequent co-evolution have elaborated on this to give rise to a range of accommodation processes and structures, as represented by extant symbiotic interactions. Interestingly, the direct uptake of bacteria by plant cells has persisted and is predicted to operate in approximately 25% of legume genera, including *Arachis* and an aquatic legume, *Aeschynomene* [8]. The *Aeschynomene* plant was shown to be colonized by nitrogen fixing and photosynthetic *Bradyrhizobium* ORS0278 in a NF-independent manner in the absence of trans-cellular ITs [36]. Thus, the invention of NF-signaling likely reflects one of the elaborations in the symbiotic interaction that has been essential in bringing sophistication of the PIT formation and IT-dependent root colonization by bacteria (Figure 2). This sophisticated mode of root infection, where a trans-cellular path for bacterial migration is carefully laid down by the host plant machinery, is known to operate in the majority of extant legumes [8].

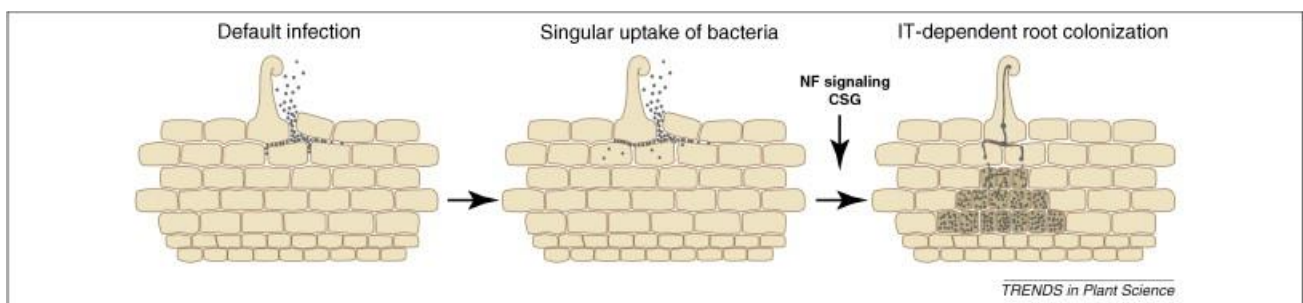


Figure 2. A hypothetical sequence of events in the evolution of the plant intracellular accommodation of bacteria. Default infection: extracellular colonization of roots by bacteria. Singular uptake of bacteria: an intracellular colonization of roots by bacteria through a presumed singular phagocytic-like uptake. Infection thread (IT)-dependent root colonization by bacteria; note that although infection through a root hair is depicted, this model incorporates other modes of IT-dependent infection, such as the extracellular ‘crack-entry’, which is followed by the formation of trans-cellular ITs within the root cortex.

In this context, it is tempting to consider ITs as products of a highly modified phagocytic process. Instead of forming large membrane-bound vacuoles known as phagosomes, the process has evolved to give rise to membrane and cell wall-limited conduits. These inversely growing structures, the ITs, traverse a single cell body to deliver bacteria to subtending intercellular spaces. From there, subsequent infection events will ensue in a similar, cell-autonomous manner, thus assuring highly controlled passage of symbiotic bacteria through root tissues towards their final destination in the interior of nodule cells (Figure 2). It is noteworthy that PIT formation that precedes the IT-dependent root colonization by symbiotic bacteria has been hypothesized to be the result of the modified cell-division process [37]. This modification is likely brought about as a function of NF-dependent signaling [38]; however, additional studies are needed to better understand this process.

As described above, the *Nap1* and *Pir1* genes encode proteins which control rearrangements of the actin cytoskeleton in root hairs. Therefore, these genes are presumed essential for endocytosis and deleterious mutations in the function of these genes would be predicted to impair both the direct uptake and IT-dependent plant cell colonization by bacteria; indeed, this is consistent with the corresponding *L. japonicus* mutant phenotypes (see below). This is also congruent with the involvement of at least two flotillins in supporting the IT-dependent infection by nitrogen-fixing *Sinorhizobium meliloti* in *M. truncatula* [39]. Flotillins belong to a family of lipid raft-associated integral membrane proteins and constitute one determinant of a clathrin-independent endocytic pathway in mammalian cells [40]. They are postulated to act by triggering the recruitment of internal vesicles for the addition of bulk membrane and membrane proteins to the growing processes [41]. Assuming their analogous function in plants [40], this further highlights the importance of endocytic pathways in the evolution of IT-mediated symbiotic bacterial entry.

## From direct to IT-mediated entry

Whether the events described above have indeed contributed to the evolution of RNS remains uncertain. However, recent data appear to bring significant support to such a formulated opinion by showing that the particular developmental conditions in the single host plant might determine the mode of root colonization by bacteria [42]. Importantly, in *L. japonicus* this can range from a direct, IT-independent uptake to a more typical, IT-mediated invasion.

In the *L. japonicus roothairless* mutant, colonization of nodule primordia involves either cortical (as opposed to epidermal) root hair-dependent infection or intercellular crack-entry [43]. These results showed that even in species such as *L. japonicus* where epidermal root hair IT-dependent colonization clearly predominates, alternative modes of infection are possible (Figure 3). Even more surprising, recent analysis of the *L. japonicus snf1* spontaneous nodulation phenotype [44] showed that direct, IT-independent single cell colonization can be supported in this mutant independent of NF signaling [45]. Significantly, this [45] and a parallel study [46] strongly suggest that the integration of signaling through common symbiosis genes and another transduction pathway, which is thought to diverge early upon the perception of NF and to encompass such functions as encoded by the *Nap1* and *Pir1* genes, is required for successful IT-dependent infection. This further highlights a more intricate origin of the plant accommodation program for bacterial endosymbioses, as outlined above.

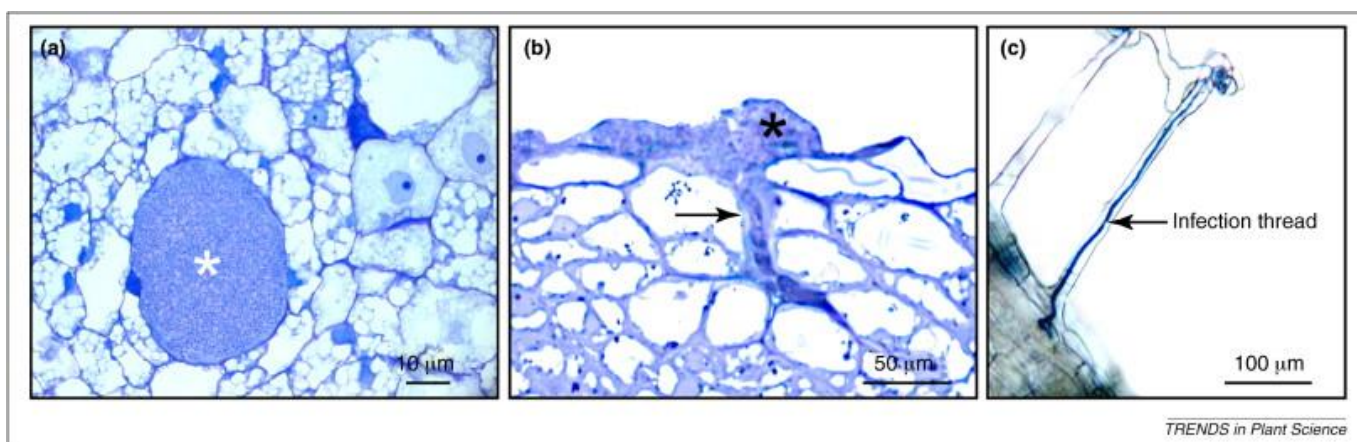


Figure 3. Different modes of *L. japonicus* root colonization by *M. loti*. (a) IT-independent, single cell (\*) colonization by *M. loti* in the *L. japonicus spontaneous nodule formation 1 (snf1)* mutant, in the background of homozygous *nfr1* and *nfr5* Nod factor receptor mutations. (b) An intercellular crack-entry colonization of nodule surface by *M. loti* in the *L. japonicus roothairless* mutant. (c)



Infection thread-dependent (arrow) intracellular penetration of root hairs by *M. loti*, as typically observed in the wild-type *L. japonicus* Gifu.

## Concluding remarks

The apparent support for various root colonization mechanisms by bacteria in a single plant species, such as *L. japonicus*, suggests a more dynamic and also unifying picture of the intracellular accommodation program of microbes by plants. Pursuing this should further clarify the type of processes and associated functions involved, based on which the ability for intracellular accommodation of bacteria by plants evolved. As molecular characterization of currently available mutants in *L. japonicus* and *M. truncatula* continue to aid this process, expanding this research to incorporate plant species that are predominantly inhabited by symbiotic bacteria in the absence of transcellular ITs will be essential [47]. Exploring such avenues will also likely contribute to future crop improvements through the identification of novel targets for enhancement of beneficial association of plants with growth promoting bacteria. As a single bacterium, such as *Bradyrhizobium* sp. ORS0278, can be both an extracellular endophyte of rice (*Oryza sativa*) and an intracellular symbiont of *Aeschynomene* [36], uncovering the underlying feature of the host plant that determines this different behavior of the microsymbiont will likely bring us closer to the understanding of an elusive predisposition event [7 and 48] that shaped the ‘capacity’ of selected plant genomes for bacterial endosymbioses.

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## **Glossary**

### **Arbuscule**

dichotomously branched and terminally differentiated hyphae that is formed inside the plant cell but is separated from the plant cell cytoplasm by an extension of the plant plasma membrane that surrounds the fungus. The arbuscule–cortical-cell interface is presumed to constitute the main region of nutrient exchange between the fungus and the host plant.

### **NAP1**

the Nck-associated protein 1, which constitutes a subunit of the SCAR/WAVE regulatory complex.

### **PIR1**

the 121F-specific p53-inducible RNA, which constitutes a subunit of the SCAR/WAVE regulatory complex.

### **SCAR/WAVE**

suppressor of cyclic AMP repressor (SCAR) also called Wiskott–Aldrich syndrome protein (WASP)-family verpolin-homologous protein (WAVE). SCAR/WAVE is the class 1 actin nucleation promoting factor (NPF) which forms a larger regulatory protein complex that includes NAP1 and PIR1.

### **Nod factor**

lipochito-oligosaccharide signaling molecules (nodulation factors) produced by symbiotic rhizobia and required for the formation of root nodule symbiosis in many, but not all, leguminous plants.

### **NFR1 and NFR5**

Nod-factor specific LysM receptor kinases identified in *L. japonicus* as required for activation of the common symbiosis gene pathway and NF-dependant symbiosis.

### ***snfl***

a mutant allele of *L. japonicus* *CCaMK* gene encoding an auto-activated form of calcium and calmodulin-dependent protein kinase (CCaMK). *Lotus japonicus* plants carrying *snfl* develop spontaneous nodules in the absence of rhizobia.

### **Phagosome**

a membrane-derived vacuole containing particles (e.g. bacteria) absorbed by phagocytosis.

### **Hyphopodia (singular: hyphopodium)**

a flattened tip of a hyphal branch by which AM fungi attach to and penetrate the host plant root.