Isolation of a Premycorrhizal Infection (*pmi2*) Mutant of Tomato, Resistant to Arbuscular Mycorrhizal Fungal Colonization

Rakefet David-Schwartz,^{1,2} Vijay Gadkar,¹ Smadar Wininger,¹ Roza Bendov,¹ Gad Galili,² Avraham A. Levy,² and Yoram Kapulnik¹

¹Department of Agronomy & Natural Resources, ARO, the Volcani Center, Bet Dagan 50 250, Israel; ²Department of Plant Sciences, the Weizmann Institute of Science, Rehovot 76 100, Israel

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Arbuscular mycorrhizae (AM) represent an ancient symbiosis between mycorrhizal fungi and plant roots which co-evolved to exhibit a finely tuned, multistage interaction that assists plant growth. Direct screening efforts for *Myc*[−] plant mutants resulted in the identification of a tomato (*Lycopersicon esculentum* L. cv. Micro-Tom) mutant, M20, which was impaired in its ability to support the premycorrhizal infection (*pmi*) stages. The *Myc*[−] phenotype of the M20 mutant was a single Mendelian recessive trait, stable for nine generations, and nonallelic to a previously identified M161 *pmi* mutant. The M20 mutant was resistant to infection by isolated AM spores and colonized roots. Formation of *Glomus intraradices* appressoria on M20 roots was normal, as on wild-type (WT) plants, but in significantly reduced numbers. A significant reduction in spore germination was observed in vitro in the presence of M20 exudates relative to WT. Our results indicate that this new mutant shares similar physiological characteristics with the M161 *pmi* mutant, but has a more suppressive *Myc*[−] phenotype response.

Additional keywords: *Agrobacterium rhizogenes*, hairy roots, *Myc*[−] mutants, root organ culture, tomato mutants.

Arbuscular mycorrhizal (AM) fungi belong to the family *Zygomycetes*. They are capable of forming nonspecific associations with roots of more than 80% of the terrestrial plant species (Smith and Read 1997). Conservative estimates suggest that this ancient symbiosis, dating back to the early Devonian age (398 million years ago), was instrumental in the adaptation of aquatic plants to land life (Remy et al. 1994). This long period of symbiosis with the host plants has resulted in a unique status for the fungal partner: the absolute necessity of having the host present of to complete its life cycle. This association also has been found to be mutually beneficial: the plant supplies the fungus with carbon (from its fixed photosynthates), while the fungus assists the plant in its uptake of phosphate and other mineral nutrients, thus increasing the plant's ability to tolerate and survive under adverse conditions (Smith and Gianinazzi-Pearson 1988).

Fungal colonization of the host partner involves a series of events which are highly regulated and controlled by both the fungus and the host. Successful colonization, and the subse-

quent formation of fungal progeny spores, is the result of a cascade of steps in the host roots. The interaction between plant and fungus starts before they come into actual physical contact. Hyphal growth and branching are induced by the roots of the host plant, and are followed by the formation of appressoria, which are thought to be the key feature in a recognition process that leads to hyphal penetration of the roots (Gadkar et al. 2001; Harrison 1999). The nonmycorrhizal character of plants, which probably evolved during the course of evolution (Trappe 1987), is expressed at the higher taxonomic level of the family (e.g., *Cruciferae*, *Chenopodiaceae*, *Proteaceae*). The mechanism for this exclusion is poorly understood, but there is reasonable evidence suggesting that the signals inducing hyphal growth, branching, and recognition at the root surface apparently are switched off (Giovannetti and Sbrana 1998).

In the mycorrhizal symbiosis, growth and differentiation of the plant root and fungal hyphae require reciprocal recognition, either via the exchange of diffusible signals or through direct cell-to-cell interaction (Harrison 1999). In studies aimed at characterizing these processes at the molecular level, abnormal AM phenotypes have been identified in mutant plant lines, most frequently in legumes (Gadkar et al. 2001; Marsh and Schultze 2001). Unfortunately, these mutants were not selected to early recognition stages. The AM fungi underwent the same set of developmental steps, leading to appressorium formation, as in the parental cultivars. The subsequent stages, which lead to penetration by the infection hyphae as they develop from appressoria, were hindered in the pea *Myc*[−] mutants, probably through the induction of the plant defense response (Giovannetti and Sbrana 1998; Gollotte et al. 1993). Thus, AM fungi also can recognize *Myc*[−] plant as hosts, even if these mutants are defective in the early stages of "host recognition" (Giovannetti and Sbrana 1998). Efforts to screen directly for AM mutants that are blocked in the early signaling stages revealed a premycorrhizal infection (*pmi*) tomato mutant (David-Schwartz et al. 2001) which is impaired in its preinfection stages.

In the present study, aimed at isolating additional *pmi* tomato mutants, we have identified another mutant, designated M20, in which AM fungal infection is blocked specifically at the premycorrhizal infection stage. We did this by using the established screening protocol (David-Schwartz et al. 2001) for tomato as a host. The new M20 mutant could genetically complement our previously reported *pmi* M161 mutant (David-Schwartz et al. 2001), with which it shares many common phenotypic properties. Further, an in vitro

Corresponding author: Y. Kapulnik; Telephone: +972-3-9683483; Fax: +972-3-9669642; E-mail: kapulnik@agri.huji.ac.il

model system based on the hairy root culture system enabled us to verify the mutant phenotype of M20, and to study the precolonization stages in greater detail.

RESULTS

Selection of AM mutant and its genetic analysis.

In the reported screening effort, 3,000 fast-neutron-mutagenized plants from an M2 population were used. Each of the 3,000 M2 plants were screened for mycorrhizal colonization by observing stained roots under a binocular microscope. One putative M2 nonmycorrhizal homozygotic recessive mutant, designated M20 (mutant #20), was selected. No significant differences were observed when phenotypic characters of the M20 mutant, including growth parameters, seed germination, and chlorophyll content, were compared with those of the wild type (WT) plant (data not shown).

Stability of the AM mutant phenotype was found to be invariant for nine generations, and all tested plants from the M3 to M10 generations showed the resistance phenotype, confirming the homozygosity of the mutations. Sixteen F_1 progenies from two independent cross events of M20 with its corresponding WT showed root colonization percentages of 79 ± 3 and 65 ± 5 , compared with 87 ± 3 for WT plants. F_2 progenies from these two cross events showed 154 colonized and 44 noncolonized plants—a 3:1 Mendelian ratio of the recessive *Myc*⁻ allele (χ^2 analysis: $P > 0.3$). Taken together, all these results indicate that the *Myc*⁻ phenotype of M20 segregates as a single Mendelian recessive locus.

Because of the close similarity of the M20 phenotype to that of M161, it was necessary to describe its genetic properties and to compare them with what we know about M161 genetics. A complementation test between mutants M161 and M20 was performed by crossing them with each other and scoring the nonmycorrhizal character of the F_1 progeny. M20 and M161 showed no colonization at all, whereas all the WT plants were colonized, at a rate of $87 \pm 3\%$, and eight F_1 progenies from each of the two cross events (H and I) between the two mutants showed 83 ± 6 and $84 \pm 5\%$ root colonization, respectively. The F_1 phenotype implies that the two mutants are nonallelic and are able to complement the *pmi* phenotype between them.

Resistance capacity of the M20 mutant.

To test the level of susceptibility of the M20 mutant to AM fungal infection, *Glomus intraradices* was used as a sole inoculum source in a series of increasing concentrations of whole inoculum, ranging from 0 to 1,000 propagules per plant. The propagules included spores, mycelia, and colonized roots. After 6 weeks of growth, WT roots showed colonization percentages ranging from 0 to 52.7 ± 11.3 , which were correlated with the inoculum levels, whereas there was no significant colonization in the roots of the M20 plants (0 to $2.2 \pm 1.0\%$) (Table 1). These findings indicate that M20 resists infection by whole inoculum, including spores and colonized roots. In order to compare among the infection rates of WT, M20, and M161 mutants, an independent experiment was carried out in which two different inoculum treatments were used. One of them, termed “whole inoculum”, was a mixture of isolated spores (*G. intraradices*) and pieces of infected roots, and the other contained only isolated spores of the same fungus. The whole inoculum was added at a rate of 10 infection units per plant and the isolated spore inoculum was added at a dosage of 400 spores per plant. The results (Table 2) suggest that WT roots were colonized at rates which increased with time in both inoculum treatments. Compared with WT, the percent-

ages of the root length of M161 that were colonized by the whole inoculum resource were statistically significantly smaller at 5 and 9 weeks of growth (Table 2), while the M20 mutant showed no colonization by whole inoculum at any of the sampling dates. No colonization was observed when either mutant was challenged with isolated spores (Table 2). These results suggested that M20 resists AM colonization, and that it has a significantly more suppressive *Myc*⁻ phenotype response than M161.

The ability of extraradical hyphae to infect the M20 mutant was examined using the dual-compartment system in which a 30- μ m mesh (between compartments) restricted the extension of the root, but allowed extraradical hyphae and root exudates to cross to the adjacent compartment (Table 3). When AM fungal spores were placed only in the outer compartment and WT plants were planted in both compartments, roots became colonized at similar rates in both sections. When mutant plants were planted in both compartments, roots in both remained uninfected throughout the experiment. However, when mutant plants were planted in the central compartment and WT plants together with AM spores in the surrounding compartment, the mutant roots showed a normal infection pattern (Table 3). These results indicate that the mutant plants were competent to support AM colonization in this particular experimental setup.

Germination and appressorium formation.

In an attempt to identify and characterize the defective stages in the M20 mutant that may affect *pmi* phenotype, we decided to assess the M20 more precisely under in vitro growth conditions. For that purpose, transformed hairy roots were generated from WT, M20, and M161 plants. The initial transformation event of the mutant and WT cultivars yielded 60 and 72 hairy root clones, respectively. There was no significant difference between the mutant and WT plants in the number of clones obtained, indicating that the mutant phenotype did not have any influence on the transformation frequency. Fifteen clones each from the WT and M20 were selected for similar growth characteristics and were transferred to low-nutrient M-medium (Bécard and Piché 1989). The clones that survived on the nutrient-poor M-medium adapted to it with negative geotropic growth and high lateral branching. The success of the transformation was confirmed by detection by polymerase chain reaction (PCR) of the *rol* gene on the TL-DNA fragment and of the *ags* gene on the TR-DNA fragment (data not shown). The *rol* and *ags* fragments were not detected in non-transformed plants.

To test whether the M20 mutant exhibits the *pmi* phenotype through its exudation, a mesh-based in vitro assay with hairy roots was applied. The spore germination rate was significantly higher in the presence of WT versus mutant roots; the latter significantly ($P < 0.05$) reduced the number of germinated spores (by 37.4%) relative to WT plants (Fig. 1). In comparison, in the M161 mutant, the reduction was 32%

Table 1. Root length colonization rates (%) of “whole inoculum” of *Glomus intraradices* in wild-type (WT) and M20 tomato roots, 6 weeks after sowing^z

Inoculum density (propagules/plant)	WT	M20
1,000	52.7 ± 11.3	2.2 ± 1.0
500	58.7 ± 11.3	2.4 ± 1.1
100	40.5 ± 8.7	0
10	20.1 ± 10.1	0
0	0	0

^z Results are means of seven plants $\pm$ SE.

with respect to the WT. No significant difference was found in germination rate in the case of spores exposed to carrot exudates relative to the WT. Interestingly, there was no germination when the spores were exposed to a nonhost, the sugar beet (Fig. 1).

The “sandwich” experimental system described by Giovannetti and Citeresi (1993) was employed to determine the appressorium development on WT and M20 mutant plants. Germinated spores formed normal appressoria on both WT and M20 roots, but the rate of appressorium formation was significantly lower in M20. The numbers of appressoria found on WT and M20 roots 5 days after the beginning of the incubation were 0.56 ± 0.06 and 0.39 ± 0.03 appressoria/cm, respectively. In comparison, the M161 mutant formed 0.32 ± 0.04 appressoria/cm.

Dual-culture assessment.

To evaluate the colonization in the transformed hairy roots, a root culture was established. The development of extraradical *G. intraradices* mycelium was profuse and it quickly covered the petri dishes, within 4 weeks of symbiosis during the association with WT hairy roots. Hyphae spread in all directions and sometimes anastomosed into lateral branches, with the main hyphae bearing numerous branched absorbing structures (BAS) (Fig. 2A). Sporulation began within 1 week of contact and continued until the plates were harvested. Spores were found inside the WT roots at a late stage, generally 3 months after initiation of the dual association (Fig. 2B). After 3 months of incubation, the dual-culture medium was completely covered with a network of hyphae and arbuscule-like branches, and mature spores were formed at an average of $11,625 \pm 836$ per plate, after 3 months of dual association (Fig. 2C) with a colonization percentage of 22.1 ± 5.6 . Spore formation and its ontogenic development were as described previously by Chabot and associates (1992) for this species. No colonization was detected in the M20 hairy roots after 3 months of dual culture. In the case of the M20 dual-culture plates, the mycelia from the spores used for inoculation formed hollow spores on their branches (Fig. 2D). These hollow spores, which exhibited typical characteristics of abortive spores (average 8 to 15/plate), were formed with no specific preference in terms of location and number. They lacked any lipidic filling and were not able to germinate when transferred to fresh M-medium and exposed to 2% CO₂ at 32°C. Prolonged incubation (6 months) of the M20 mutant dual-culture plates did not alter the hollow and dysfunctional nature of these spores. It also should be noted that these hollow spores were not found in symbiotic

association with the WT hairy roots. To show that the dual culture formed with the WT as the host was viable, we propagated it by transferring a chunk of medium containing colonized roots, mycelia, and spores onto fresh M-medium. Active outgrowth of the mycelia from the plugs was routinely observed, with the formation of healthy, viable progeny spores. No colonization was detected in the mutant roots after staining and assessing them under microscope.

To test the colonization capability of an AM fungus belonging to another genus, *Gigaspora rosea* was used for root organ culture (ROC) establishment; in this case, after the initial contact between the germ tube and the WT root, the first hyphal attachment or intercellular colonization took place on the third day. This attachment came from a ramification of the germ tube, which continued its growth in a negative geotropic direction after bypassing the root. This was followed by widespread proliferation of the fungal mycelia with rapid formation of auxiliary cell clusters (Fig. 2E). Sporogenesis was observed within 4 weeks of the establishment of the dual culture, and the number of spores increased temporally, as observed previously (Fig. 2F) (Diop et al. 1992). The colonization percentage in WT roots was found to be 11.6 ± 3.3 after 3 months of dual association. In the case of the M20 mutant roots, mycelial growth ceased after the initial contact, no proliferation was seen after 4 to 7 days, and very few auxiliary cell clusters formed on the mycelial branches. No colonization was detected in the M20 roots.

DISCUSSION

We identified a tomato mutant, M20, which was defective in its ability to manifest the early stages of AM infection. Contact of AM fungi with this *pmi* phenotype resulted in reduced spore germination, hyphal growth, and appressorium formation. The mutation was a single Mendelian recessive trait and stably inherited over nine generations. Similar to the previously identified *pmi* mutant, M161 (David-Schwartz et al. 2001), M20 plants showed resistance to colonization when AM spores were used as inoculum. Unlike M161, M20 showed resistance to whole inoculum of *Glomus intraradices*, which comprised spores, mycelia, and inoculated root segments. Although both mutants were susceptible to infection when inoculated with extraradical hyphae, the colonization rates of M20 were lower than M161 (Table 3). The current data strongly indicates that the *Myc*⁻ phenotype of the M20 mutant is much more severe than the earlier reported M161 mutant (Table 2). Buee and associates (2000) observed that mutants belonging to the *Myc*⁻¹ and *Myc*⁻² phenotypes do not affect hyphal growth and branching, thus corroborating our present and previous findings (David-Schwartz et al. 2001) that the novel phenotype is

Table 2. Percentages of the root length colonized by wild-type (WT), M20, and M161 tomato roots, following inoculation with two different inoculum sources^y

Treatment, weeks ^z	WT	M20	M161
Isolated spores			
5	0.5 ± 0.3 a	0 a	0 a
7	15.5 ± 3.3 a	0 b	0 b
9	28.3 ± 6.2 a	0 c	1.5 ± 1.2 b
Whole inoculum			
5	16.3 ± 1.4 a	0.2 ± 0.2 c	8.3 ± 1.8 b
7	26.8 ± 5.1 a	0 b	17.5 ± 5.6 a
9	45.0 ± 7.7 a	0 c	27.8 ± 7.2 b

^y Means within the same row followed by different letters are significantly different (Tukey-Kramer test, $P > 0.05$).

^z Treatment (type of inoculum) and weeks after sowing. Inoculum designated as “Isolated spores” contained only spores, and was applied at a rate of 400 spores per plant. Inoculum designated as “Whole inoculum” comprised isolated spores inoculum supplemented with infected roots (10 infection units per plant). Roots were harvested and stained 5, 7, and 9 weeks after sowing, and the results are means of five replicated plants $\pm$ SE.

Table 3. Resistance capacity of M20 plants when inoculated with a hyphae-based inoculation system (dual-compartment system)^z

Plant origin	Root length colonization (%)
Surrounding compartment (+AM)	
M161	0
M20	0
WT	45.2 ± 9.3
WT	54.6 ± 11.0
WT	53.6 ± 7.7
Central compartment (–AM)	
M161	0
M20	0
WT	53.8 ± 10.7
M161	63.0 ± 14.5
M20	42.5 ± 3.9

^z Plants were harvested 6 weeks after sowing. Values represent means of four plants $\pm$ SE; AM = arbuscular mycorrhizae; WT = wild type.

endemic to mutants belonging to the *pmi* category. This observation strongly supports our hypothesis that the currently known *Myc*⁻ mutants (Marsh and Schultze 2001) are not blocked in their pre-infection stages, whereas mutants able to affect hyphal branching would logically belong to the *pmi* class of mutants.

In many cases, research into the isolation of *Myc*⁻ mutants of legumes involved indirect screening methods, whereby a pre-screened *Nod*⁻ mutant population was used as the starting material. The reason that a mutant that was defective specifically in the *pmi* phenotype was not isolated in legumes could be attributed to the fact that no direct screening or selection methodology had been employed for this purpose (Marsh and Schultze 2001; Peterson and Guinel 2000). Isolation of the M161 and, subsequently, M20 *pmi* mutants reinforces our argument for the very existence of this novel phenotype. Complementation analysis of these two mutants indicated that they are nonallelic; therefore, the *pmi* phenotype is genetically controlled by at least two different loci. The ability of M20 to support the successful formation of appressoria, albeit in smaller numbers than on the WT, suggests that the mutation does not affect the process of appressorium formation per se. Nagahashi and Douds (1997), using isolated cell walls of carrot, demonstrated that appressorium formation is dependent on host epidermal cell wall factors. Interestingly, appressorium formation processes on excised nonhost roots (Giovannetti and Citeresi 1993) were similar to those that took place on the host roots (Logi et al. 1998). In the case of *Myc*⁻¹ mutants, Bradbury and associates (1991) routinely observed the formation of appressorium structures that were complex and somewhat abnormal compared with those on the WT, which did not happen with our *pmi* mutants.

The exudate of M20 hairy roots was able to reduce spore germination of *G. intraradices*, but this reduction was not as intense as that observed for the nonhost plant, sugar beet (Fig. 1). Spore germination has been shown to be stimulated by the exudates of host plants (Gianinazzi-Pearson et al. 1989), but inhibited by those of nonhost (sugar beet) plants (Schreiner and Koide 1993), suggesting the involvement of an inhibitory factor in the exudate (Fig. 1) (Vierheilig et al. 1996). Further efforts now should be focused on testing for the presence of such an inhibitory factor, or the reduced level of a stimulating factor in the exudates of M20 mutant.

Buee and associates (2000) reported on a branching factor, exuded from a host plant, but did not examine its effect on

spore germination or appressorium formation. At present, no evidence has been presented, to the best of our knowledge, for the factor or factors affecting both spore germination and appressorium formation. Interestingly, in *Colletotrichum gloeosporioides* and *C. musae*, both spore germination and appressorium formation have been hypothesized to be induced by host-elicited ethylene (Flaishman and Kolattukudy 1994). Thus, it may be that, in AM fungus-plant interactions, root exudates affect more than one of the pre-infection stages. The present plant material would serve as an ideal tool to evaluate this hypothesis.

The ROC technique, using *Agrobacterium rhizogenes*-derived hairy roots, has found useful application in AM fungal biology (Fortin et al. 2002). Despite the artificial nature of the in vitro system, the susceptibility of the WT hairy roots and the resistance of M20 hairy roots to infection by *Gigaspora rosea* or *Glomus intraradices*, belonging to two separate genera, was consistently observed (Fig. 2). Our results are in line with earlier observations of Balaji and associates (1994), who found that the *Myc*⁻ phenotype of two *Nod*-Fix⁻ pea mutants remained intact when the Ri T-DNA hairy root cultures derived from this cultivar were used in vitro. The production of spores is the ultimate indication, from a fungal point of view, of a successful mycorrhizal establishment (Bécard et al. 1995) and has been found to correlate with degree of root colonization (Bécard et al. 1995; Diop et al. 1992). The propensity of the M20 mutant hairy roots to induce the production of abortive spores by *G. intraradices* (Fig. 2) is an interesting and not fully understood phenomenon; it remains to be clarified whether this also occurs in nature or is confined only to dual associations under in vitro conditions. Spore abortion may result either from a lack of a critical signal from the host plant to proceed into the next stage of the ontogenic cycle, as observed in the case of *G. clarum* Nicolson and Schenck (de Souza and Berbara 1999). The abortive spores, which were termed “juvenile spores” by the authors, were formed only under nonsymbiotic conditions, and were suggested to be the result of a deficiency in critical signal from the host. The abortive spores formed by *G. intraradices* in our present study and those formed by *G. clarum* (de Souza and Berbara 1999) did not show any leakage of their contents into the surrounding medium, as noted earlier by Pawlowska and associates (1999) in AMF *G. etunicatum* in association with Ri T-DNA carrot as the host.

The recent publication of the cloning of a gene, SYMRK (symbiosis receptor-like kinase), from a *Lotus japonicus*

Fig 1. Percent germination of spores after exposure to root exudates from the wild type (WT), M20 mutant, M161 mutant, carrot, sugar beet, and nontreatment (blank) were used for comparative analysis. Carrot and sugar beet, respectively, were used as host and nonhost controls. Values are means of seven plates each containing at least 30 isolated spores. Values followed by different letters differ at $P < 0.05$ according to a multiple range test.

Nod⁻Myc⁻ mutant, and NORK, from *Medicago sativa* Nod⁻Myc⁻, opens up exciting possibilities for unraveling the specific mechanism of the AM fungus-host symbiosis establishment (Endre et al. 2002; Stracke et al. 2002). The accumulating identification of new mutants of nonlegumes that are impaired in their support of AM formation, and in which nodulation and mycorrhization events are therefore uncoupled, should provide us with ideal complimentary tools for dissecting the stages of AM establishment, formation, and functioning; specifically, the early stages of infection, of which very little is known at present.

MATERIALS AND METHODS

Plant and fungal material.

Mutation screening of 3,000 fast-neutron-treated M2 seeds of the miniature tomato (*Lycopersicon esculentum* L.) cultivar Micro-Tom (Micro-Tomato) was performed as previously described (David-Schwartz et al. 2001). The fungal material for screening comprised sand-based inocula, prepared at the Volcani Center and used essentially as described by David-Schwartz and associates (2001). Alternatively, *G. intraradices* N. C. Schenck & G. S. Sm. (DAOM 181602), grown in a bi-compartmental system in association with Ri T-DNA-transformed *Daucus carota* L. (clone DC2) roots as host, was used (St-Arnaud et al. 1996). The AM fungi *Gigaspora rosea* Nicolson & Schenck (DAOM 194757) were harvested from pre-established dual-culture plates of transformed carrot (clone DC2). Spores were isolated according to a protocol established by Doner and Bécard (1991). Root-length coloni-

zation percentage was assessed by the gridline intersection method (Giovannetti and Mosse 1980); the slides were examined with a light microscope (Olympus 1X70; Olympus Optical Co. Ltd., Tokyo) and were photographed with a Camedia C-2000 Z camera (Olympus Optical Co. Ltd.).

Complementation analysis.

The nonmycorrhizal mutant M20 was crossed as a female parent with WT or with the nonmycorrhizal mutant M161. Mycorrhizal root length colonization percentage by *Glomus intraradices* was examined by means of the above screening procedure; it was performed essentially on F₁ and F₂ of the cross with WT and on 16 F₁ progenies from two independent crosses (H and I) between M20 and M161, and compared with that on WT and mutant lines, separately.

Dual-compartment system.

The dual-compartment pot system for hyphal-based inoculation was used as described in detail by David-Schwartz and associates (2001). In brief, 200 spores of *G. intraradices* per plant were placed 5 cm below the soil surface in a 1-cm-thick layer. One plant was grown in the central compartment and four plants in the surrounding compartment. Roots were harvested after 6 weeks, and were stained and observed under the binocular microscope for fungal colonization.

Transformation procedure for hairy root induction.

Agrobacterium rhizogenes strain ATCC 15834 was used for the generation of hairy roots from WT and M20 mutant plants, by means of the protocol described for other plant species

Fig 2. Colonization events of arbuscular mycorrhizae (AM) fungal inoculation under in vitro conditions. **A**, Progeny spore formation in case of *Glomus intraradices* in association with wild-type (WT) roots (bar = 140 μ m). **B**, Spores of *G. intraradices* formed intraradically after 3 months of dual symbiosis. Note the large amount of root hairs characteristic of tomato (bar = 105 μ m). **C**, Large number of daughter spores being formed in petri dish after 3 months of association with WT hairy roots (bar = 0.84 mm). **D**, Abortive spores being formed in association with M20 hairy roots. Note that the spores are hollow with no lipidic filling (bar = 94 μ m). **E**, Large numbers of auxiliary cell clusters of *Gigaspora rosea* being formed in association with WT roots, indicating a successful symbiotic event (bar = 9 μ m). **F**, Progeny spores of *G. rosea* being formed after 4 weeks of dual association with WT roots (bar = 350 μ m).

(Balaji et al. 1994; Tepfer and Casse-Delbart 1987). The hairy roots were repeatedly transferred to fresh Murashige-Skoog medium containing antibiotic (Claforan, 0.5 g/liter) until visible signs of bacteria were eliminated. The roots then were transferred onto M-medium with the same antibiotic for a few cycles of growth, and were maintained until the roots became accustomed to the low-nutrient regime on M-medium. Finally, they were maintained on M-medium without any antibiotic, pending use for dual-culture establishment.

Integration of the TL-DNA and TR-DNA regions of the pRi plasmid was confirmed by PCR analysis of the genes located in this region. For the TL-DNA region, the presence of *rolA* (ATGGATTAGCCGGACTAAACG) and *rolB* (ATGGATCCCAAATTGCTATTCC) fragments, and for the TR-DNA region, the presence of the *ags* gene (CGGAAATTGTGGCTCGTTGTGGAC and AATCGTTCA-GAGAGCGTCCGAAGTT) was used as templates. For the positive control TL- and TR-DNA, the cosmids pLJ 1 and pLJ 85, respectively (Jouanin 1984) were used as templates during the PCR.

Establishment of root organ culture system.

Dual cultures of *G. intraradices* were established by taking a cube of spores, containing approximately 50 to 60 spores with mycelia, from the root-free compartment of a previously grown carrot-*G. intraradices* dual culture (St. Arnaud et al. 1996). WT and M20 hairy roots were introduced into petri dishes (9 cm in diameter) containing 35 ml of M-medium (minus phosphorus) with 0.3% Phytigel, and the dishes were sealed, inverted, and incubated at 26°C in the dark to promote fungal spread and colonization. A dual culture of *Gigaspora rosea* was established by using spores from transformed carrot roots. The establishment of a dual culture with the hairy roots of WT and M20 was according to Diop and associates (1992). Petri dishes (4 cm in diameter) containing 15 ml of M-medium were used and two pregerminated spores per dish were used for inoculation.

After 3 months of incubation, the dual-culture plates were harvested and the colonization data were evaluated by gridline intersection method (Giovannetti and Mosse 1980). In the case of *Glomus intraradices*, the spore count was done by scanning in all possible fields in the in vitro petri dish under a dissecting microscope.

Evaluation of pre-infection stages.

A thin layer of sterile 0.3% (wt/vol) Phytigel with 0.2% (wt/vol) MgSO₄ was poured into standard petri dishes. One layer of a precut sterile mesh was placed on top of the medium and a thin layer of M-medium was poured on top of it. An actively growing root of WT or M20 was placed on the top layer and the dishes were sealed and incubated in the dark at 26°C for 6 weeks. The upper layer of medium, containing the grown roots, was harvested and the mesh was removed. Single spores (approximately 30) of *G. intraradices*, which had been harvested from a carrot dual culture, were inserted in the gel layer. The plates (total: seven per treatment) were incubated in a 2% CO₂ atmosphere at 32°C for 5 days. Trypan blue staining (0.47 g trypan blue in 250 ml of lactic acid, 417 ml of glycerol, and 250 ml of H₂O) was applied and the germination percentages were evaluated under a dissecting microscope. Another host, *Daucus carota* L. carrot (DC2 clone), and a nonhost, *Beta vulgaris* L., were used as controls for comparative analysis.

Appressorium formation was analyzed by means of the "root sandwich" approach as described by Giovannetti and Citeresi (1993). Experimental details are described in David-Schwartz and associates (2001).

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