A Common Signaling Process that Promotes Mycorrhizal and Oomycete Colonization of Plants

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Summary

The symbiotic association between plants and arbuscular mycorrhizal fungi is almost ubiquitous within the plant kingdom [1], and the early stages of the association are controlled by plant-derived strigolactones acting as a signal to the fungus in the rhizosphere [2–4] and lipochito-oligosaccharides acting as fungal signals to the plant [5]. Hyphopodia form at the root surface, allowing the initial invasion, and this is analogous to appressoria, infection structures of pathogenic fungi and oomycetes. Here, we characterize RAM2, a gene of Medicago truncatula required for colonization of the root by mycorrhizal fungi, which is necessary for appropriate hyphopodia and arbuscule formation. RAM2 encodes a glycerol-3-phosphate acyl transferase (GPAT) and is involved in the production of cutin monomers. Plants defective in RAM2 are unable to be colonized by arbuscular mycorrhizal fungi but also show defects in colonization by an oomycete pathogen, with the absence of appressoria formation. RAM2 defines a direct signaling function, because exogenous addition of the C16 aliphatic fatty acids associated with cutin are sufficient to promote hyphopodia/appressoria formation. Thus, cutin monomers act as plant signals that promote colonization by arbuscular mycorrhizal fungi, and this signaling function has been recruited by pathogenic oomycetes to facilitate their own invasion.

Results and Discussion

The genetic dissection of plant components required for the establishment of the mycorrhizal association has been mostly limited to components that are also required for the interaction with nitrogen-fixing rhizobia [1, 6, 7]. To identify loci specifically involved in mycorrhizal signaling, we undertook a genetic dissection in Medicago truncatula, looking for mutants defective in mycorrhizal colonization but normal for nodulation. We identified a locus that we named “Required for Arbuscular Mycorrhization (RAM)”, which had severely reduced levels of mycorrhizal fungal colonization (Figures 1A and 1B), with both Glomus intraradices and Glomus hoi (see Figure S1A available online), but showed normal nodule development (Figures S1D and S1E). Promotion of spore germination and induction of hyphal branching were apparent in fungi treated with ram2 root exudate (Figures S1B and S1C), indicating that strigolactone production was normal in ram2. However, there was a dramatic reduction in the number of hyphopodia that formed on ram2 roots (Figure 1D). Occasionally ram2 was colonized by mycorrhizal fungi, and in these rare cases we observed defective arbuscules in ram2, with only limited penetration into the inner root cortical cells (Figure 1C). This work indicates that RAM2 is required for promoting the colonization of both epidermal and cortical cells by mycorrhizal fungi, and this may reflect a signaling, structural, or nutritional function.

Using Affymetrix transcriptomic analysis, we identified a deletion in the fast neutron-generated ram2 mutant and found that the deletion cosegregated with the mycorrhizal defect. The deletion spanned approximately 120 kb, removing 23 predicted genes. To verify which deleted gene caused the mutant phenotype, ram2 roots were transformed, using an Agrobacterium rhizogenes strain carrying subcloned regions from a BAC spanning the deletion (Figure S2A). The transformed roots were inoculated with purified G. intraradices spores and quantified for fungal colonization. One subclone that could complement ram2 (Figures 2A and 2B) was predicted to contain only a single full-length gene, encoding a glycerol-3-phosphate acyl transferase (GPAT), indicating that RAM2 encodes a GPAT.

RAM2 is part of a family of GPATs involved in cutin and suberin biosynthesis (Figure 2C) [8]. This suggests a role in cutin/suberin biosynthesis, rather than a function in phospholipid biosynthesis associated with lysophosphatidylcholine production [9]. RAM2 appears to define a unique group of GPATs present in plant species associating with mycorrhizal fungi, but absent from the Brassicaceae that are unable to support mycorrhization. The Arabidopsis gpat5 mutant shows defects in root suberin and in seed coat depositions that affect seed color (Figure S3) and permeability [10], and these are not present in gpat6 mutants that are defective in cutin biosynthesis [11]. We found that the ram2 mutant also has a seed coat defect (which cosegregated with the mycorrhizal defect in 60 F2 plants), which closely resembles the gpat5 mutant of Arabidopsis: ram2 seeds are darker and show greater permeability to water than wild-type seeds (Figure S3A). Considering the seed coat similarities, we tested whether RAM2 could function as GPAT5 by transforming gpat5 Arabidopsis mutants with RAM2. RAM2 complemented the seed color defect (Figure S3B), but did not affect the seed coat permeability defect in the gpat5 mutant. We conclude that RAM2 encodes a GPAT with overlapping functions to GPAT5 of Arabidopsis, both producing seed coat deposits that affect seed color and seed permeability.

RAM2 is closely related to both GPAT5, which functions in suberin deposition [10], and GPAT6, which functions in cutin deposition (Figure 2C) [11, 12]. Importantly, RAM2 is predicted to have a functional phosphatase domain (Figure S2B), and this has been reported to be a feature of cutin biosynthesis involving GPAT6 [11, 12], not suberin biosynthesis involving GPAT5 [8, 11]. To analyze further the relative GPAT

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activity of RAM2, we tested if Arabidopsis GPAT5 and GPAT6, driven by the RAM2 promoter, could complement ram2 mutant roots. GPAT6 fully complemented the ram2 mutant for mycorrhizal colonization, whereas GPAT5 showed only weak complementation of ram2 for mycorrhizal colonization (Figure 3A). To test the relative importance of the phosphatase domain for the mycorrhizal function, we used a GPAT5M mutant defective in phosphatase activity [12] and found that this mutated GPAT6 complemented ram2 to a low level, comparable to GPAT5 (Figure 3A). We conclude that the mycorrhizal functionality of RAM2 reflects a GPAT with functions comparable to GPAT6, with the phosphatase domain being dispensable. In contrast, in the seed coat, RAM2 mirrors more closely the function of GPAT5, in which the phosphatase domain is dispensable. Despite the genetic similarities to GPAT6, ram2 showed none of the floral defects reported for gpat6 mutants of Arabidopsis [8–12]. It would appear that there are subtle differences between M. truncatula and Arabidopsis in the relative functions of these GPATs, and variations in substrate specificity as well as functionality of the phosphatase domain may contribute to these differences [11].

GPAT6 has been shown to be involved in cutin biosynthesis, based on loss-of-function and overexpression lines [11], and to further assess the role of RAM2, we undertook a comparable analysis of aliphatic cutin/suberin monomers. Overexpressing RAM2 in Arabidopsis had the equivalent effect to overexpressing GPAT6 [11], leading to increased levels of ω-hydroxy fatty acids (OHFA) and ω,ω-dicarboxylic acids (DCA) (Figure 3B). However, the effect was limited to specific monomers, notably 16:0-DCA, 16:0-OHFA, and 18:2-DCA, and this profile was identical between lines overexpressing RAM2 and GPAT6 (Figure 3B). In a comparable fashion, we found that the M. truncatula ram2 mutant roots showed reduced levels of these same monomers, notably 16:0-DCA and 16:0-OHFA, but also reduced levels of 18:1-DCA and 18:1-OHFA (18:2-DCA was not detectable in M. truncatula roots). Genetic dissection of cutin/suberin has discriminated between wax production and cutin/suberin production [8, 11, 13]. Consistent with this, in the composition of surface waxes in wild-type or ram2 roots, which is equivalent to what has been reported for gpat6 mutants of Arabidopsis [11]. Based on the genetic analyses and the quantification of aliphatic cutin/suberin monomers, we conclude that the biochemical function of RAM2 is equivalent to that of GPAT6, revealing that the synthesis of long-chain OHFAs and DCAs associated with cutin is essential for mycorrhizal colonization of M. truncatula roots.

Cutin/suberin are proposed to be synthesized as monomers, but are laid down at the cell surface in an esterified form. The defect in mycorrhizal perception of the ram2 root surface may be associated with differences in physical features of the cell surface or may be due to the absence of a chemical signal. To discriminate between these two hypotheses, we assessed whether addition of cutin/suberin monomers could compensate for the absence of RAM2. We found that addition of the C16:0 monomer, but not longer-chain lipids, allowed hyphopodia formation on the surface of ram2 roots at levels equivalent to wild-type plants (Figure 3D). Both acidic and alcoholic forms of cutin monomers exist [8], and we found that the addition of C16 OHFA and 1,16-hexadecanediol could complement the ram2 mutant (Figure 3D). The fact that the lipid monomers alone can complement ram2 suggests a signaling, rather than structural, role with a degree of specificity for the molecules that can promote hyphopodia formation.

As discussed in the accompanying manuscript by Gobbato et al. in this issue of Current Biology [14], analysis of RAM2 expression from the Medicago gene atlas [15] reveals induction upon mycorrhizal colonization and expression in flowers. Surprisingly, we did not see strong expression of RAM2 in the pod, where the developing seeds occur; this may reflect a very early function for RAM2 during seed development or a function restricted to a minor tissue. RAM2 induction in the root upon mycorrhizal colonization reaches a peak at 30 days post inoculation (Figure S4A). Considering that mycorrhizal fungi can produce hyphopodia on isolated cell walls [16] we presume that RAM2 or an analogous gene must have a constitutive
function that promotes hyphopodia formation at the root surface. The induction of *RAM2* upon mycorrhizal colonization would imply the synthesis of OHFAs and DCAs, and consistent with this, at 5 weeks post inoculation with mycorrhizal fungi, we observed a 29.0% increase in the overall root levels of 16:0-FA, a 20.0% increase in 16:0-DCA, and a 31.7% increase in 18:1-DCA (p < 0.05 for all these differences) (Table S1).

Mycorrhizal hyphopodia fulfill a similar function to appressoria of pathogenic fungi and oomycetes, and to assess whether the requirement for *RAM2* was specific to mycorrhizal fungi, we assessed whether *ram2* roots were altered in colonization by the pathogenic oomycete *Phytophthora palmivora*, which is a broad host-range oomycete [17], with the capacity to colonize *M. truncatula* roots. Whereas wild-type plants showed necrosis 3 days after inoculation with *P. palmivora* zoospores (Figure 4B), *ram2* mutants appeared resistant to this colonization, being asymptomatic at this stage. After 7 days wild-type plants were dead, while *ram2* mutants still survived. To assess whether this defect in *ram2* colonization by *P. palmivora* was due to a defect in appressorium formation, we imaged *P. palmivora* on the surface of *ram2* and wild-type roots, using scanning electron microscopy. On wild-type roots, *P. palmivora* zoospores germinated and rapidly developed appressoria (Figure 4C); on *ram2* roots zoospores germinated, and the hyphae grew extensively on the surface of the root but appressoria did not form (Figure 4D).

Quantifying the percentage of zoospores resulting in appressoria revealed that the majority (96%) of zoospores on wild-type roots led to appressorium formation, whereas only 15% of zoospores gave rise to appressoria on *ram2* roots (Figure 4A). In an analogous fashion to what we observed in mycorrhizal colonization, the addition of the C16:0 monomer upon *P. palmivora* inoculation compensated for the *ram2* mutation, leading to wild-type levels of disease symptoms (Figures 4B and 4E). Phytophthora species can form appressoria on synthetic surfaces, and we found that addition of the C16:0 monomer gave a 7-fold enhancement in appressoria formation of *P. palmivora* grown on a polypropylene surface, and this increase was significant (p < 0.01) (Figure 4F). We conclude that *RAM2* defines a signaling function that is necessary for both mycorrhizal hyphopodia formation and for *P. palmivora* appressorium formation.

*RAM2* is induced upon mycorrhizal infection, and we wanted to assess whether *RAM2* is also induced in *P. palmivora* infection. Affymetrix gene chip analysis revealed a 7-fold induction of *RAM2* in *M. truncatula* roots 16 hr post inoculation with *P. palmivora* zoospores. Considering that the mycorrhizal induction of *RAM2* is regulated by the GRAS-domain transcription factor *RAM1* [14], we assessed whether *RAM1* also plays a role during *P. palmivora* colonization, but found no defects in the *ram1* mutant. This suggests differential mechanisms for the induction of *RAM2* during mycorrhizal and *P. palmivora* colonization. *P. palmivora* is related to *Phytophthora infestans*, a potent pathogen of potatoes and tomatoes. Affymetrix profiling [18] revealed that close homologs of *RAM2* are induced in potato upon *P. infestans* infection (Figure S4B), suggesting that a comparable function may exist during *P. infestans* colonization.

Strigolactone and lipochito-oligosaccharides are signaling molecules involved in the interplay between plants and mycorrhizal fungi [2–5], and here we reveal that aliphatic monomers, associated with cutin, are also essential components to promoting mycorrhizal colonization. We conclude that these act as signaling molecules, because their abundance specifically affects the promotion of two developmental stages, hyphopodia and arbuscules (Figures 1C and 1D), but does not seem to affect presymbiotic stages (Figure S1). This signaling function appears to be associated with the recognition of the chemical structure, because addition of the C16:0 monomer, but not the longer-chain fatty acids, was sufficient to allow mycorrhizal hyphopodia formation and *P. palmivora* infection, suggesting a degree of specificity for recognition of this signal. These findings are consistent with research in fungal pathogens that has
suggested a signaling role for cutin in promoting appressoria formation [19, 20]. Our work suggests that cutin monomers are inducibly produced by the root upon mycorrhizal colonization, and considering the arbuscule defect in ram2, this is likely to occur in both cortical cells and at the root surface. Interestingly, an ABC transporter with similarity to lipid transporters is involved in cutin biosynthesis.

Figure 3. RAM2 Is Involved in Cutin Biosynthesis
(A) Quantification of G. intraradices colonization of ram2 roots transformed with empty vector (EV), RAM2, Arabidopsis GPAT5, GPAT6, and GPAT6 carrying a mutation in the phosphatase domain (GPAT6M), all expressed from the RAM2 promoter (n = 8 plants). Arabidopsis GPAT6 can fully complement ram2 for G. intraradices colonization, while GPAT5 and GPAT6M can only partially complement the ram2 mutant. Each bar shows colonization in a single independent transgenic root. This is a representative experiment that was replicated twice.

(B) Quantification of cutin monomer levels in the leaves of Arabidopsis plants transformed with empty vector (EV), GPAT6, or RAM2 expressed from the 35S promoter. The data represent the average of three independent experiments. The asterisks indicate statistically significant (p < 0.05) increases in the levels of these molecules in the overexpressing lines as compared to the empty vector lines.

(C) Quantification of cutin monomer levels in M. truncatula wild-type (WT) and ram2 roots. The data represents the average of three independent experiments. The asterisks indicate a significant (p < 0.05) decrease in ram2 roots as compared to wild-type roots.

(D) Addition of the C16 cutin monomers complement ram2, allowing hyphopodia formation on the surface of ram2 roots at levels equivalent to wild-type plants. The asterisk indicates a significant increase relative to untreated ram2 (p < 0.05). Error bars in (B), (C), and (D) are standard deviation.

Figure 4. RAM2 Is Required for P. palmivora Colonization
(A) The relative frequency of appressoria formation on wild-type and ram2 mutants in two independent infections with P. palmivora. The percentage indicates the number of germinated zoospores present on the root surface that form or do not form appressoria (164 spores in WT and 118 spores in ram2 were assessed). Gray bars, hyphae that form appressoria; black bars, hyphae lacking appressoria. Error bars represent standard deviation.

(B) ram2 mutants are immune to P. palmivora infection showing an absence of necrotic symptoms at the infection site, seen in wild-type plants 3 days post inoculation. Application of the C16 cutin monomer at the time of inoculation can rescue ram2, allowing disease progression.

(C) Scanning electron microscopy of WT (C) and ram2 (D) roots infected with P. palmivora 2.5 days post inoculation. Insets depict a single germinated spore. Note that the oomycete forms elongated hyphae lacking appressoria on ram2 roots. Scale bars are in μm as indicated.

(D) Quantification of the length of disease lesions in (B) reveals a significant increase (p < 0.05, indicated with an asterisk) in ram2 plants treated with the C16 cutin monomer. Each measurement is the average from at least 12 plants.

(F) Cutin promotes appressorium formation in P. palmivora grown on a polypropylene surface. The asterisks indicate significant increases (p < 0.01) relative to the 0.01% ethanol treatment. This is a representative experiment that was repeated three times.
induced in arbuscule-containing cortical cells and required for appropriate arbuscule development [21]. It has been suggested that this may transport strigolactones, but it may instead be involved in delivering cutin monomers to the developing arbuscule.

The mycorrhizal symbiosis is extremely ancient, dating back at least 400 million years to the earliest land plants, and this suggests that the utility of signaling by cutin monomers to promote fungal colonization dates back at least to this time. We propose that this signaling process has been usurped both by pathogenic oomycetes, as revealed here, and possibly also by the pathogenic fungi *Magnaporthe grisea* and *Ustilagos maydis*, which may transport strigolactones, but it may also be involved in delivering cutin monomers to the developing arbuscule.