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Report

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*)*2*," 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 lysophosphotidylcholine 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 biosythesis involving *GPAT6* [11, 12], not suberin biosynthesis involving *GPAT5* [8, 11]. To analyze further the relative GPAT

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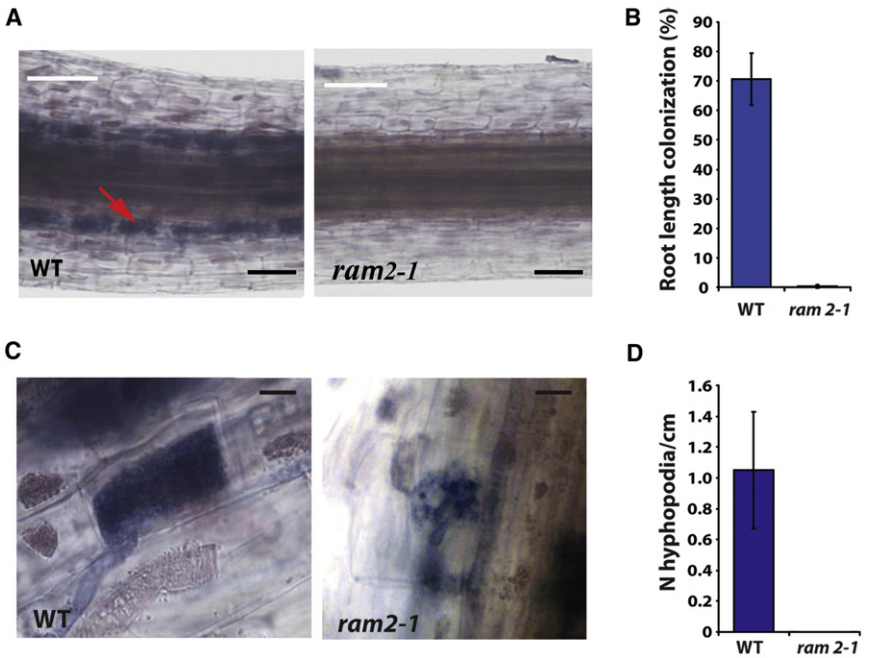

Figure 1. *RAM2* Is Required for Mycorrhizal Colonization

(A) Ink-stained roots of *M. truncatula* reveal a greatly reduced level of infection in *ram2* by *G. intraradices* 6 weeks post inoculation. The arrow indicates an arbuscule in a wild-type root. Scale bars represent 50 μm.

(B) Quantification of *G. intraradices* colonization levels 6 weeks post inoculation (n = 12 plants). This is a representative experiment that was repeated three times. Error bars are standard error.

(C) Occasional colonization events in *ram2* reveals defective arbuscules compared to arbuscules in wild-type plants. Scale bars represent 10 μm.

(D) Quantification of *G. intraradices* hyphopodia formation on the surface of *ram2* roots at 6 weeks post inoculation with purified spores (n = 12 plants). This is a representative experiment that was repeated three times. Error bars are standard error.

activity of *RAM2*, we tested if *Arabidopsis GPAT5* and *GPAT6*, driven by the *RAM2* promoter, could complement *ram2* 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 *GPAT6M* 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 indispensible. 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 α,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, we found no changes in the nature of the *ram2* root surface, using scanning electron microscopy (Figure S3C), and furthermore, we found no differences 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

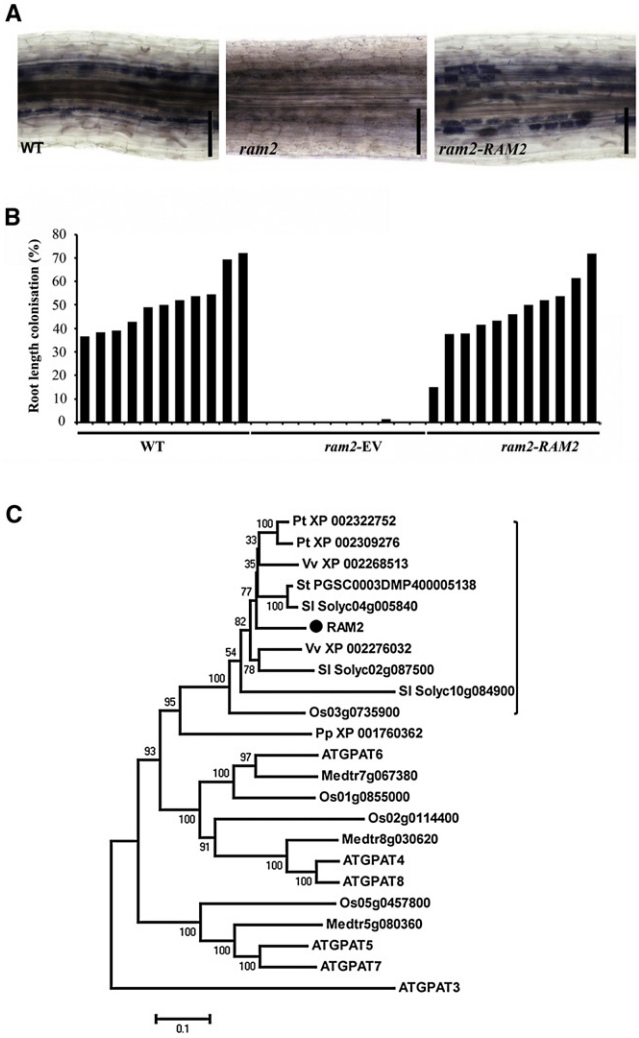

Figure 2. *RAM2* Encodes a *Glycerol-3-Phosphate Acyl Transferase*

(A) *G. intraradices* colonization, measured following ink staining of wild-type (WT) and *ram2* roots transformed either with the empty vector (EV) or with a BAC subclone containing only the *GPAT* (*RAM2*). Scale bars represent 150 μm. This is representative of four independent experiments.

(B) Quantification of *G. intraradices* colonization in wild-type, empty vector (EV)-transformed ram2 and complemented ram2 roots. Each bar shows colonization in a single independently transformed root. This is a representative experiment that was replicated four times.

(C) A phylogenetic tree of GPATs from *Arabidopsis* (AT) and *M. truncatula* (Medtr), as well as RAM2 homologs from poplar (Pt), vine (Vv), *Solanum lycopersicum* (Sl), *Solanum tuberosum* (St), *Physcomitrella patens* (Pp), and rice (Os). Branch support was obtained from 1000 bootstrap repetitions. *RAM2* appears to define a unique group of *GPATs* within plant species that enter the arbuscular mycorrhizal association, indicated with a bracket, but absent from *Arabidopsis*. Note that *Arabidopsis GPATs* appear to have orthologs in *M. truncatula*.

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* appressoria 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

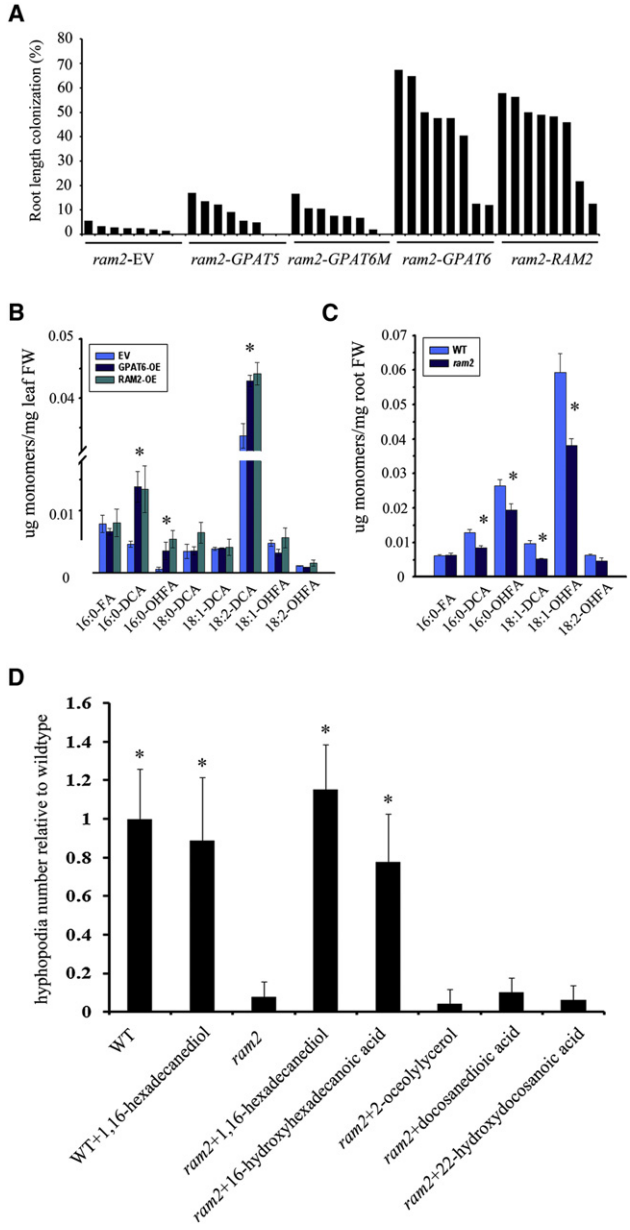

Figure 3. *RAM2* Is Involved in Cutin Biosynthesis

(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.

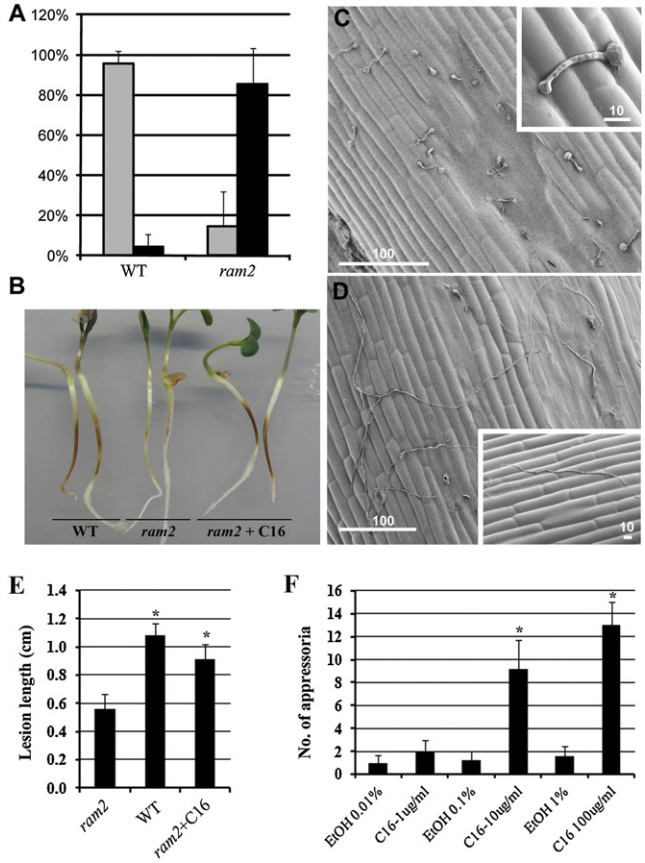

Figure 4. *RAM2* Is Required for *P. palmivora* Colonization

(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 and D) 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.

(E) 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.

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

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* [19, 22], to allow recognition of the plant surface and the promotion of infection structures. The emergence of mycorrhizal fungi and the production of a cuticular surface layer were both adaptations that emerged as plants transitioned from an aquatic to a terrestrial lifestyle. It is tempting to speculate that as these two processes coevolved, common functions emerged, with components of the cuticle layer evolving as fungal signaling molecules to facilitate mycorrhizal colonization.

Accession Numbers

The GenBank accession number for *RAM2* gDNA is JN572682; the GenBank accession number for *RAM2* cDNA is JN572684.

Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.09.043.

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