which should be cited to refer to this work.

A petunia GRAS transcription factor controls symbiotic gene expression and fungal morphogenesis in arbuscular mycorrhiza

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Summary: The petunia GRAS transcription factor ATA/RAM1 regulates symbiotic gene expression and is required for arbuscule development and restriction of fungal colonization in the root tip.

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Abstract

Arbuscular mycorrhiza (AM) is a mutual symbiosis that involves a complex symbiotic interface over which nutrients are exchanged between the plant host and the AM fungus. Dozens of genes in the host are required for the establishment and functioning of the interaction, among them nutrient transporters that mediate the uptake of mineral nutrients delivered by the fungal arbuscules. We have isolated in a genetic mutant screen a petunia GRAS-type transcription factor, ATYPICAL ARBUSCULE (ATA), that acts as the central regulator of AM-related genes and is required for the morphogenesis of arbuscules. Forced mycorrhizal inoculations from neighbouring wild type plants revealed an additional role of ATA in restricting mycorrhizal colonization of the root meristem. The lack of ATA, which represents the orthologue of RAM1 in *Medicago truncatula*, renders the interaction completely ineffective, hence demonstrating the central role of AM-related genes for arbuscule development and function.

Abbreviations:

AM	Arbuscular mycorrhiza
RNS	Root nodule symbiosis
ATA	Atypical arbuscule
RAM1	Required for arbuscular mycorrhiza1
GRAS	GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR of GAI (RGA) and SCARECROW (SCR)

Introduction

Most land plants live in a symbiotic association known as arbuscular mycorrhiza (AM) with soil fungi (*Glomeromycota*), which provide them with mineral nutrients and improve their stress resistance (George, 2000; Pozo and Azcon-Aguilar, 2007; Smith and Read, 2008). In return, the strictly biotrophic fungal partner receives photoassimilates (Douds et al., 2000), and perhaps other vital factors from the host plant. At the core of the interaction is the symbiotic interface, over which the nutrients are exchanged. The interface comprises a highly branched fungal feeding structure, the arbuscule, and the surrounding host membrane in which the arbuscule is accommodated. Establishment of AM interactions requires the mutual recognition of the two partners and the activation of a symbiosis-specific pathway (Harrison, 2012; Gutjahr and Parniske, 2013; Oldroyd, 2013), which relies on a series of so-called common symbiosis (SYM) genes, because their function is required for both, AM and the root nodule symbiosis (RNS) of the legumes (Kistner and Parniske, 2002). The shared signaling pathway entails a nuclear calcium signal, Ca-spiking, which is thought to lead to transcriptional reprogramming of symbiotic cells by a mechanism that involves the calcium and calmodulin-dependent protein kinase (CCaMK), and the transcriptional activator CYCLOPS (Singh and Parniske, 2012; Singh et al., 2014).

The common SYM genes were discovered in legumes by surveying nodulation-defective mutants for their mycorrhizal phenotypes (Duc et al., 1989), however, most plants do not engage in mutual symbioses with bacteria, hence their equivalents of common SYM genes function only in AM, and could therefore be compared with the ancient AM-related signaling pathway before it got adopted by rhizobia (Kistner and Parniske, 2002). Although the leguminous species *Medicago truncatula* and *Lotus japonicus* are among the leading model systems for root symbioses (Udvardi et al., 2005; Jones et al., 2007), it is important to also consider non-nodulating species such as rice (*Oryza sativa*) or petunia *(Petunia hybrida)*, in which the symbiosis-related pathways have not been influenced by the evolution of RNS and therefore may resemble the ancestral mechanisms evolved for AM.

One of the downstream consequences of signalling through the symbiosis signalling pathway is the activation of a symbiosis-related transcriptional program, which involves the activation of dozens of genes that are thought to be required for the establishment of the symbiotic interface, and for symbiotic functioning (Liu et al., 2003; Güimil et al., 2005; Hohnjec et al., 2005; Fiorilli et al., 2009; Gomez et al.,

2009; Guether et al., 2009; Breuillin et al., 2010; Gaude et al., 2012; Tromas et al., 2012; Hogekamp and Küster, 2013). Comparison between transcriptomic studies in dicots (such as *M. truncatula, L. japonicus, P. hybrida, Solanum lycopersicum*), and in the monocot model system rice have revealed a conserved set of AM-induced genes (Breuillin et al., 2010). However, only few of them have been functionally elucidated, among them phosphate and nitrate transporters (Maeda et al., 2006; Javot et al., 2007; Guether et al., 2009), proteases (Takeda et al., 2009; Rech et al., 2013), ABC transporters (Zhang et al., 2010; Gutjahr et al., 2012; Kretzschmar et al., 2012), and transcription factors such as CYCLOPS (Singh et al., 2014), RAM1 (Gobbato et al., 2012) and RAD1 (Xue et al., 2015).

A number of transcription factors involved in AM and/or RNS have been identified in forward genetic mutant screens and some of them are strongly induced in AM interactions. Besides CYCLOPS (Singh et al., 2014), symbiosis-related TFs belong primarily to the large family of GRAS transcription factors (Smit et al., 2005; Gobbato et al., 2012; Xue et al., 2015). Both, RNS and AM rely on specific GRAS-type TFs, which are thought to mediate specific transcriptional read-outs related to AM (Gobbato et al., 2012; Xue et al., 2015) and RNS (Kalo et al., 2005; Smit et al., 2005), respectively. However, detailed characterization of mutants revealed overlapping functions of some GRAS-type TFs in AM and RNS (Kalo et al., 2005; Maillet et al., 2011; Lauressergues et al., 2012).

Here we report the isolation and characterization of a mutant, *atypical arbuscule* (*ata*) in *Petunia hybrida* that is defective in arbuscule development and, as a consequence, cannot establish functional interactions with diverse AM fungi. Infection from colonized wild type plants (nurse-plants) is possible, however, the arbuscule phenotype remains. *ATA* encodes a GRAS-type transcription factor and represents the orthologue of *RAM1* in *M. truncatula*. Gene expression analysis revealed that the induction of all tested arbuscule-related marker genes in petunia depends on ATA/RAM1, with the notable exception of VAPYRIN, which appears to be involved upstream of ATA/RAM1 or in a parallel pathway. Interestingly, *ata/ram1* mutants infected from nurse-plants exhibited exaggerated AM fungal colonization in root tips, indicating that *ATA/RAM1* plays a double role in promoting arbuscule morphogenesis and in preventing colonization of the root meristem.

Results:

Isolation of the *ata* mutant

In a forward genetic mutant screen in the transposon-mutagenized *Petunia hybrida* line W138, an AM-defective mutant has been isolated based on its strongly reduced levels of mycorrhizal colonization in the interaction with the AM fungus *Rhizophagus irregularis* (**Figure 1A,B**). Rare infection events were observed in the mutant at early time points (**Figure 1B**), however, the fungal colonies never reached the same density as in the wild type, and they were characterized by malformed arbuscules (**Figure 1C,D**). In cases where the fungus was able to penetrate the root surface, the first hyphal coil in the epidermis appeared normal, however, in the root cortex the fungus developed profuse hyphal material with many septa, an indication for stress (**Figure 1E**). Based on the prominent defect in arbuscule morphogenesis, the mutant was termed *atypical arbuscule* (*ata*). The *ata* phenotype segregated as a recessive monogenic trait with 98 mutants among 378 segregating F_2 plants (25.93% mutants, p<0.05 chi-square test).

To test whether the AM-defect in *ata* exhibits race- or species-specificity towards different AM-fungi, three fungal isolates with relatively distant phylogenetic relationships were compared for their ability to colonize *ata*, namely *R. irregularis*, *Simiglomus hoi*, and *Acaulospora scrobicularia* (Stockinger et al., 2010; Krueger et al., 2012). All three fungi reached low degrees of transient infection up to 4 weeks after inoculation, but eventually remained unsuccessful in colonizing *ata* (**Figure S1**). Hence, the mutation in the *ATA* gene results in a general defect in AM symbiosis.

The initial transient infections (**Figure 1B,D,E**) indicate that *ata* can be penetrated, but cannot entertain a functional interaction with AM fungi. In such cases, nutrition from a nearby growing wild type plant can potentially enable the fungus to colonize AM-defective mutants, a system known as nurse-plant inoculation. Nurse-plants represent a very strong inoculum, from which highly AM-resistant mutants (Feddermann et al., 2010), and even non-host plants (Veiga et al., 2013) can be colonized. Indeed, nurse-plant inoculation of *ata* resulted in rapid and efficient colonization (**Figure 2A,B**), however, the defect in arbuscule development was retained (**Figure 2C,D**). These results show that the *ATA* gene is essential for arbuscule development, whereas infection and colonization as such are independent
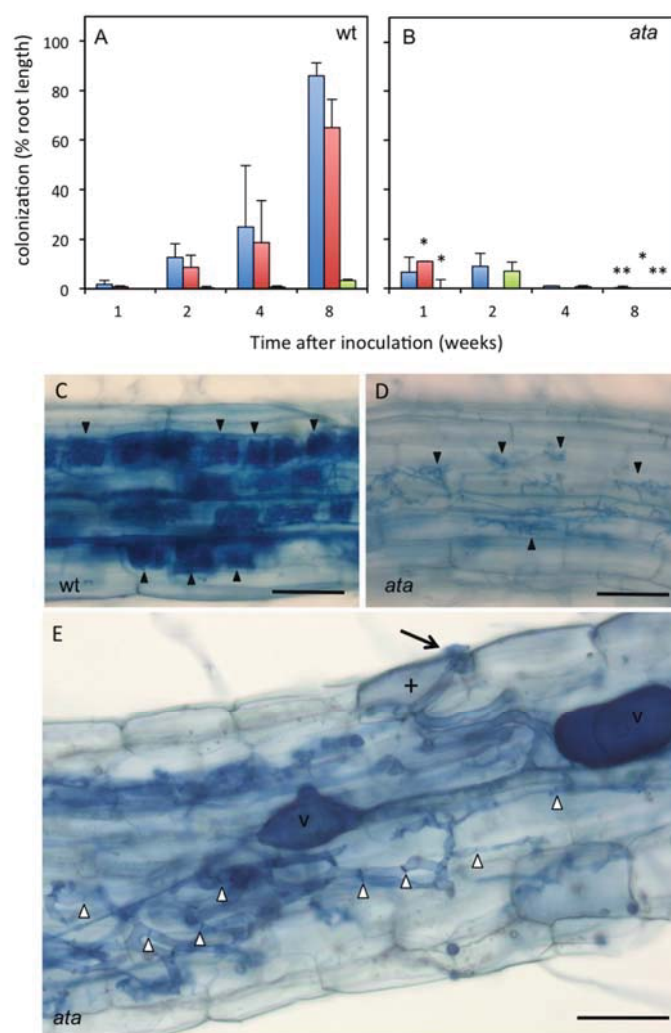
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Figure 1. The *ata* mutant is AM-defective

A) Root colonization in the wild type (*Petunia hybrida* line W138) colonized by *R. irregularis*. Total root colonization (blue), occurrence of arbuscules (red), and of abnormal arbuscules (green) is indicated.

B) Root colonization in the *ata* mutant colonized by *R. irregularis*. Color code as in (A). Asterisks indicate significant differences between the mutant and the wild type.

C) and D) colonization pattern in the wild type (C), and in one of the rare colonization units in the *ata* mutant (D). Arbuscules are indicated by arrowheads.

E) Overview of the developmental stages of *R. irregularis* in *ata*. A hyphopodium (arrow) and an infected epidermal cell with a fungal hypha (cross) mark a successful infection event. Note the frequent septa in the fungal hyphae (white arrowheads), indicative of stress in the fungus.

Columns represent the average of five biological replicates ± standard deviation. Significant differences (Student's t-test) between the mutant and the wild type are indicated with asterisk (*: $p < 0.05$; **: $p < 0.01$). v: vesicles; Size bars: 100 μm in C,D; 50 μm in E.

from *ATA* function. Hence, the function of *ATA* in AM development is relatively late, compared to the function of common SYM genes in infection (Harrison, 2012; Gutjahr and Parniske, 2013).

Characterization of the *ATA* locus and the encoded protein

We carried out transposon display (Van den Broeck et al., 1998; Vandenbussche et al., 2013) to identify the gene mutated in *ata*. Using this technique, we identified and
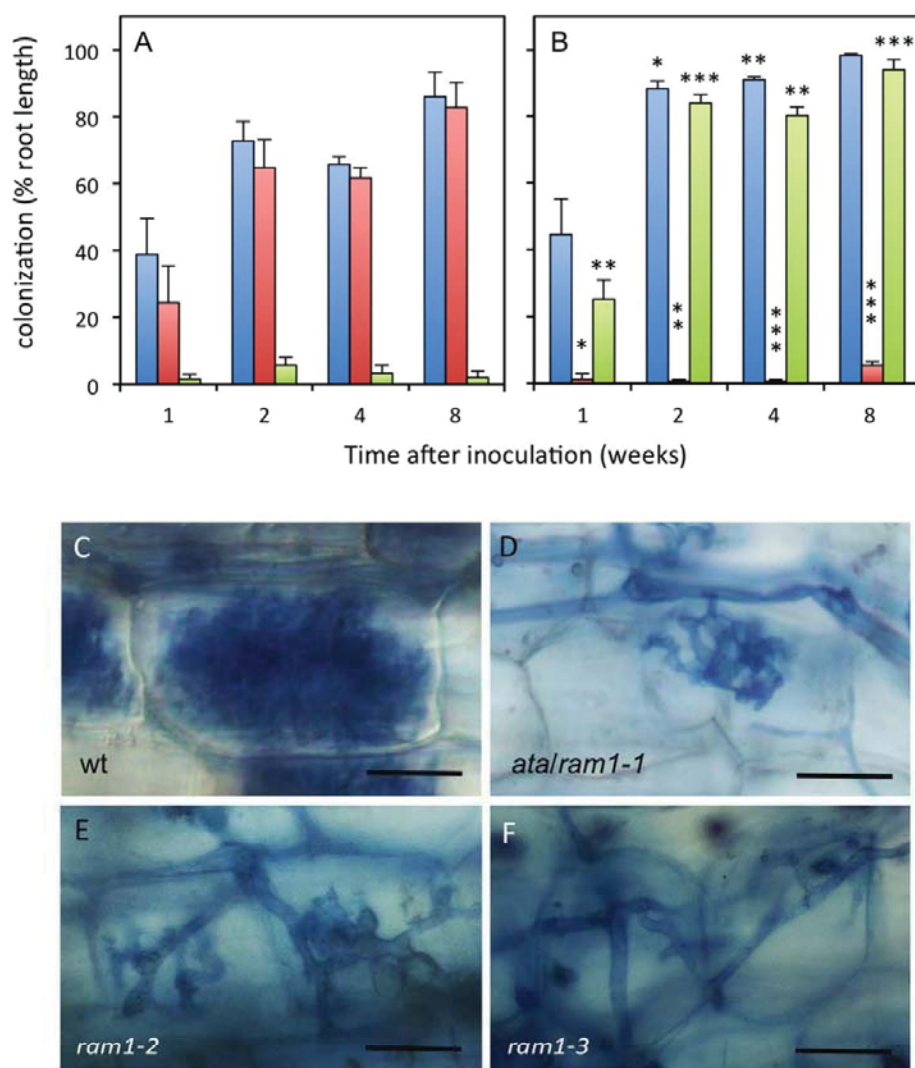
Figure 2. *ata* mutants can be colonized from nurse-plant inoculum but retain the arbuscule phenotype

A) Root colonization in the wild type (*Petunia hybrida* line W138) colonized by *R. irregularis* from nurse-plants. Total root colonization (blue), occurrence of arbuscules (red), and of abnormal arbuscules (green) is indicated.

B) Root colonization in the *ata* mutant colonized by *R. irregularis* from nurse-plants. Color code as in (A). Asterisks indicate significant differences between the mutant and the wild type.

(C-F) Appearance of arbuscules in the wild type (C), *ata* (D), *ram1-2* (E), and *ram1-3* (F).

Columns represent the average of five biological replicates ± standard deviation. Significant differences (Student's t-test) between the mutant and the wild type are indicated with asterisk (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$). Size bars: 25 µm.

cloned a transposon flanking sequence that strictly cosegregated with the *ata* phenotype (**Figure S2A**). The sequence represented a short fragment from the coding region of a GRAS transcription factor gene that contains two exons of 970 and 1121 nucleotides, respectively, separated by an intron of 783 nucleotides, and encodes a

predicted protein of 697 amino acids. The transposon had inserted in the second predicted exon 1384 nucleotides downstream of the ATG start codon (**Figure S2B**). In order to verify that the mutation in this GRAS transcription factor gene is responsible for the AM-defective phenotype in *ata* mutants, we searched for phenotypic revertants in the progeny of a homozygous *ata* mutant plant. Such revertants can result from excision of the transposon and from restauration of the open reading frame. Most transposon excisions leave an 8-bp footprint that results from the target site duplication (TSD) during transposon insertion (Gerats et al., 1990), and therefore cause an irreversible shift in the reading frame. However, in rare cases, the reading frame is restored by partial or complete removal of the TSD, resulting in restored gene function. We isolated a phenotypic revertant plant that carried an altered *ata* allele, in which not only the TSD, but additional 6 bp had been removed at the site of the transposon insertion. Although this results in a deletion of two amino acids, it restores the reading frame and therefore leads to a functional protein (**Figure S2C**). The simultaneous recovery of the *ata* phenotype and the nearly perfect restauration of the gene sequence in the revertant suggest that the *ata* phenotype is caused by the transposon insertion in the GRAS gene. The GRAS transcription factor encoded by the *ATA* gene exhibits close homology to *MtRAM1*, and to predicted GRAS proteins from various dicots and monocots, but no close homologs were found in the non-mycorrhizal species from the Brassicaceae family, such as *Arabidopsis thaliana* and *Brassica rapa* (**Figure S2D**) (Gobbato et al., 2012; Delaux et al., 2014; Favre et al., 2014). Taken together, these results indicate that *ATA* represents the ortholog of *RAM1* in *Medicago truncatula*, therefore we further refer to it as *ATA/RAM1*.

The *ram1-1* mutant in *M. truncatula* was reported to be defective at a very early point of the interaction, before infection (Gobbato et al., 2012), whereas the defect in *ata*/*ram1* mutants in petunia is at a considerably later stage of the interaction, namely at the stage of arbuscule development (see above). In order to verify the mutant phenotype of our first *ata*/*ram1* allele, we obtained two additional mutant alleles (*ram1-2*, and *ram1-3*, respectively) from a large collection of transposon-mutagenized plants characterized by deep sequencing (Vandenbussche et al., 2008). Both new alleles have dTph1 insertions in the first exon after 357 and 381 bp of the coding sequence, respectively (**Figure S2B**), which completely disrupt the conserved C-terminal DNA-binding domain (**Figure S3**). Inoculation with *R. irregularis* revealed a
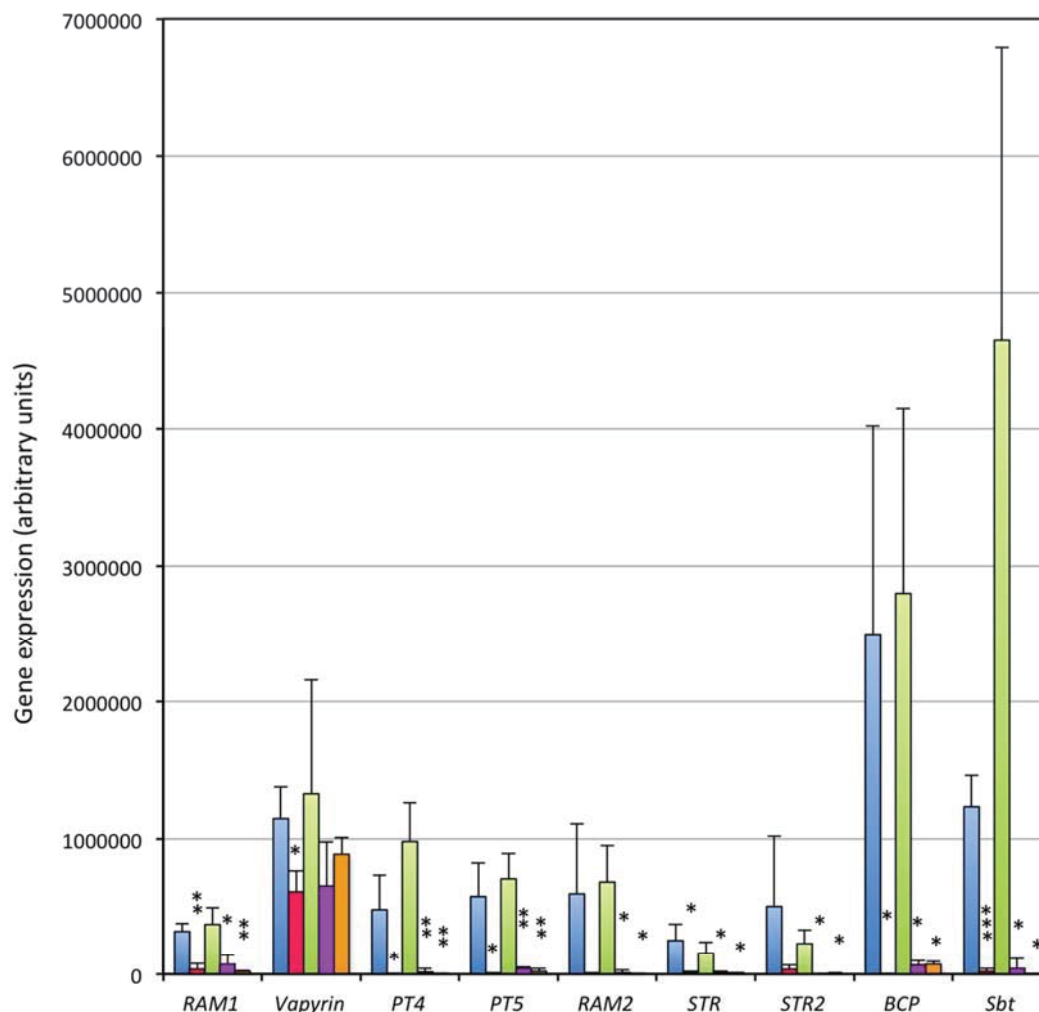
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Figure 3. ATA/RAM1 is required for induction of AM marker genes.
Gene expression was analyzed by quantitative real time RT-PCR one week after inoculation with *R. intraradices* from nurse-plants. Two independent experiments are shown in combination: Firstly, wild type (blue) vs. *ata/ram1-1* (red), and secondly, wildtype (green) vs. *ram1-2* (purple) and *ram1-3* (orange). Columns represent the average of three independent biological replicates ± standard deviation. Gene expression values are expressed relative to the constitutively expressed reference gene GAPDH. Significant differences (Student's t-test) between the mutant and the respective wild type are indicated with asterisk (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

virtually identical phenotype of both new alleles as in *ata/ram1-1*. In particular, arbuscules were poorly developed and colonization was very low and transient with spore inoculum (**Figure S4A**). In contrast, nurse-plant inoculation resulted in efficient colonization (**Figure S4B**), however, the arbuscule-defective phenotype was retained (**Figure 2E,F**). These results corroborate the essential arbuscule-related function of *ATA/RAM1* in petunia, and suggest that all three *dTph1* insertion alleles, which are expressed at considerably lower levels than the wild type allele (**Figure 3**), are strong mutant alleles. This conclusion also follows from the fact that the sequence of *dTph* carries stop codons in all six reading frames, and therefore, proteins encoded by genes

with *dTph* insertions are invariably truncated. Since the three *dTph* insertions in *ATA* eliminate most (*ata/ram1-1*) or all (*ram1-2*, *ram1-3*) of the conserved C-terminal DNA-binding domain (**Figure S3**), and since all three alleles exhibit the same strong AM-defective phenotype, they are likely to represent functional null alleles.

In order to address the arbuscule phenotype of *ram1* mutants in *M. truncatula*, we inoculated *ram1-1* with *R. irregularis* and performed a time course experiment with samplings after 10, 20, and 30 days from inoculation. As in the case of *P. hybrida*, the *M. truncatula* mutant was transiently colonized after 10-20 days (<10% colonization), but at the 30 d time point, the fungus had virtually disappeared, while the wild type had reached >70% colonization (**Figure S5A**). The rare arbuscules exhibited a deviant shape and structure similar to the ones in *ata*/*ram1* mutants of *P. hybrida* (**Figure S5B,C,** compare with **Figure 2C-F**). These results suggest that the function of *RAM1* in arbuscule formation is conserved between *M. truncatula* and *P. hybrida*.

ATA/*RAM1* is required for the induction of several AM-related genes

RAM1 in *M. truncatula* and in *L. japonicus* is known to contribute to the induction of several AM-induced transcripts (Gobbato et al., 2012; Xue et al., 2015). In order to assess the role of the petunia RAM1 in AM-related gene induction, we chose a panel of AM-related marker genes, including *ATA*/*RAM1* itself, the *VAPYRIN* gene that is required for intracellular accommodation of AM fungi (Feddermann et al., 2010; Pumplin et al., 2010; Murray et al., 2011), the two mycorrhizal phosphate transporters *PT4* and *PT5* (Wegmüller et al., 2008; Breuillin et al., 2010), the closest petunia homologues of the G-type ABC transporter genes *STUNTED ARBUSCULE* (*STR*) and *STR2* of *M. truncatula* (Zhang et al., 2010), a glycerol-3-phosphate acyltransferase (*GPAT*), that is closely related to the AM-specific *RAM2* gene in *M. truncatula* (Wang et al., 2012), and two AM markers with unknown function, subtilase and blue-copper-binding protein (BCP) (Breuillin et al., 2010). In order to achieve comparable colonization levels in all genotypes (wt and the three *ram1* alleles), the plants were inoculated with nurse-plants.

Expression of *ATA*/*RAM1* and of all AM marker genes, except for *VAPYRIN*, was undetectable or very low in non-mycorrhizal control roots, but was strongly induced in mycorrhizal wild type roots, whereas the induction was abolished in the *ata*/*ram1-1* mutant (**Figure S6**). To confirm this molecular phenotype, we compared the expression of all AM markers in all three *ram1* alleles in two independent

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experiments. Strong induction ratios were observed in the wild type plants, whereas in all three *ram1* alleles, the AM marker genes remained very low or undetectable (**Figure 3** and **Table S1**). An exception was *VAPYRIN* which is constitutively expressed and becomes further induced in mycorrhizal roots (Feddermann et al., 2010), and who's expression was only marginally affected by the mutations in *ATA/RAM1* (**Figure 3** and **Table S1**). These findings are in contrast to the *vapyrin* mutant, in which AM-marker genes were still induced, despite the strong AM-defective phenotype (Feddermann et al., 2010). These results suggest that *RAM1* is a central regulator of AM-related genes.

Root tip AM colonization in *ata/ram1* mutants

Unexpectedly, nurse-plant inoculation of *ata/ram1-1* resulted in significantly higher colonization levels than in the wild type (**Figure 2A,B**). Visual inspection suggested colonization of the root tips, hence this aspect was quantified. All three mutant alleles and two comparable wild type accessions were scored in two independent experiments for the presence of fungal structures in 100 μm intervals from the root tip. In the wild type, only 10-20% of the roots were colonized up to the root tip (<100 μm), whereas in the three mutant lines, 40-70% of the root tips contained fungal structures (**Figure 4A-E**). Detailed quantification of the different fungal structures in root tips showed that both, total colonization and the formation of vesicles, were increased in the mutants (**Figure 4D-G**). The differences in colonization between mutants and wild type were in all cases significant (**Figure S7**). Taken together these results show that, despite the defect in arbuscule development (**Figure 2**), *ram1* mutants colonized from nurse-plants allow the fungus to grow into the root tip, a region that normally remains devoid of any fungal structures.
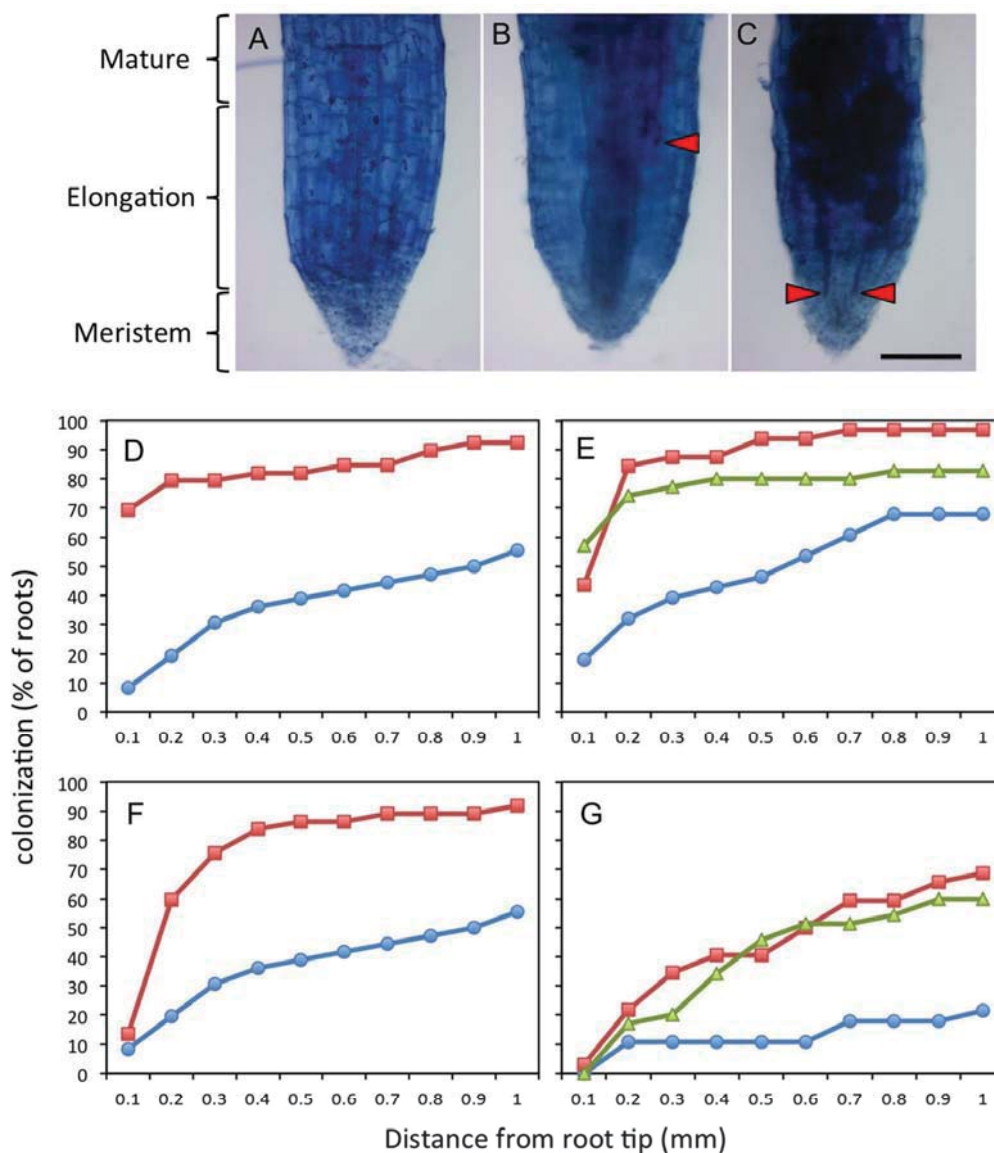
Figure 4. *ata/ram1* mutants allow mycorrhizal colonization of the root tip.
A) Non-colonized wild type root.
B) Colonized wild type root.
C) Colonized *ata/ram1-1* root.
D) Fungal colonization in wild type (blue; n=36) and *ata/ram1-1* roots (red; 39) in the distal 1 mm of the root tip.
E) Fungal colonization as in (D) in wild type (blue; n=28), *ram1-2* (red; n=32), and *ram1-3* (green; n=35).
F) Fungal colonization as in (D), but scored only for vesicles.
G) Fungal colonization as in (E), but scored only for vesicles.
Size bar: 100 µm.

Discussion

Requirement of transcription factors in arbuscular mycorrhiza

Establishment of mutual symbioses such as AM and RNS requires a fundamental reprogramming of root cells. Epidermal and hypodermal cells are involved in early communication and at the early stages of infection (Oldroyd et al., 2011; Harrison,

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2012; Gutjahr and Parniske, 2013). Subsequently, cortical cells are reprogrammed for intracellular accommodation of the endosymbiotic microbes, and for the ultimate goal of the interaction, nutrient exchange. In addition, the establishment of a state of robust and permanent symbiotic compatibility implies that certain defense pathways are kept silent despite the massive AM fungal colonization in mycorrhizal roots (Kloppholz et al., 2011). The acquisition of these multiple new traits entails transcriptional reprogramming of symbiotic plant cells.

For most of the AM-induced transcripts, the mechanism of induction is unknown. Although several predicted transcription factors were identified by their defect in AM (Gobbato et al., 2012; Xue et al., 2015) or RNS (Kalo et al., 2005; Smit et al., 2005; Singh et al., 2014), only few of their downstream targets have been determined. For example, *RAM1* has been shown to be required for induction of *RAM2* (Gobbato et al., 2012; Xue et al., 2015), *NSP1* and *NSP2* induce the strigolactone biosynthetic gene *DWARF27* (Liu et al., 2011), *CYCLOPS* induces the *NODULE-INCEPTION* (*NIN*) gene (Singh et al., 2014), and RAD1 contributes to induction of *STR* and *RAM2* (Xue et al., 2015). Since most of these studies had been carried out in legumes, the question arises to which degree the mentioned transcription factors contribute to the two symbioses, AM and RNS. At least for NSP2 and for CYCLOPS, a dual role in both symbioses has been shown (Yano et al., 2008; Maillet et al., 2011).

Since the majority of AM-competent plants does not engage in RNS, it is important to learn how symbiotic signalling is translated into a transcriptional response in plant species that were not influenced by the evolution of RNS. The example of petunia is ideally suited for this, because its AM-related transcriptome has been characterized in detail (Breuillin et al., 2010). Here, we show that the *ATA*/*RAM1* gene of petunia encodes a central transcriptional inducer of AM-related genes, and an essential component in the development of the fungal arbuscules.

Nurse-plant inoculation reveals a central role of ATA/RAM1 in mycorrhizal gene expression

Reduced induction of an AM-inducible gene in a mutant such as *ata*/*ram1* can have two reasons: It could be caused by the mutation, or it could be due, indirectly, to the reduced levels of fungal colonization. To distinguish between these possibilities, we

used nurse-plant inoculation to achieve comparable colonization levels in all genotypes. Nurse-plants are well colonized wild type plants that grow nearby the mutants and represent a very strong source of inoculum. Such inoculum can even result in colonization of the non-host plant *Arabidopsis thaliana* (Veiga et al., 2013), and in the strongly AM-resistant *vapyrin* mutant, nurse-plant inoculation triggered the induction of the AM-marker gene *PT4* although at reduced levels (Feddermann et al., 2010).

We examined the expression of eight arbuscule-associated marker genes *PT4*, *PT5*, *RAM2*, *STR*, *STR2*, *VAPYRIN* (see references above), as well as subtilase (Takeda et al., 2007), and a blue-copper-binding protein (Paradi et al., 2010), all representing conserved AM-responsive marker genes (Breuillin et al., 2010). Despite efficient colonization from nurse-plants (**Figure 2**), all these genes remained silent in *ata*/*ram1* mutants, with the exception of *VAPYRIN*, which still showed constitutive expression and mycorrhizal induction (**Figure 3, Table S1**). VAPYRIN represents a common symbiosis gene that acts downstream of calcium-spiking (Murray et al., 2011), and is required at earlier stages of AM infection than *ATA*/*RAM1*, namely at cell penetration (Feddermann et al., 2010; Pumplin et al., 2010). In this sence, *VAPYRIN* acts upstream, and therefore independently, of the function of *ATA*/*RAM1*.

ATA/RAM1 prevents AM colonization of the root tips

Nurse-plant inoculation allowed us to discover a second important aspect of the *ata*/*ram1* phenotype. *Ata*/*ram1* mutants tended to be colonized at higher levels than the wild type (**Figure 2**). An obvious aspect of the colonized mutant roots was that they often exhibited fungal colonization into the extreme root tip (**Figure 4**), a region that is normally not colonized. This indicates that *ATA*/*RAM1* is not only involved in the induction of arbuscule-associated genes (**Figure 3**), but also in a negative feedback loop that protects the root meristems from excessive AM fungal colonization. Colonized root tips had a normal overall shape and appearance, indicating that their function was not significantly affected by fungal colonization. The colonization of *ram1* root tips resembles the effect of overexpression of a micro-RNA-resistant version of NSP2 in *M. truncatula*, which also resulted in ectopic AM colonization in root tips (Lauressergues et al., 2012).

<u>Why is arbuscule development inhibited in _ata/ram1_?</u>

In _ram1_ mutants, all the common SYM genes are intact, and accordingly, the initial infection events, such as the formation of hyphopodia on the root surface and hyphal coils in epidermal cells, are normal in _ram1_ mutants (**Figure 1**). Differences in the interpretation of the mutant phenotypes in this study and in previous studies (Gobbato et al., 2012; Xue et al., 2015) may be due to differences in the timing of sampling, and/or in differences in the inoculation procedure. In contrast to the early stages of the interaction, infection in the cortex, and in particular arbuscule development, were strongly affected in _ram1_ mutants (**Figure 2**). The timing of these relatively late defects are consistent with the molecular defect of _ram1_ mutants, which is the lack of induction of genes that are expressed in cells with arbuscules, in particular PT4, STR, STR2, and RAM2. We conclude that _ram1_ has a relatively late defect in AM development, which, however, translates into a strong general AM defect because of the lack of secondary infection events. Therefore, the phenotype of _ram1_ at later stages appears very strong, even stronger than mutants affected in common SYM genes, which act upstream and are affected at early stages of infection, but permit occasional infection events that can develop into almost normal infection units (Bonfante et al., 2000; Novero et al., 2002; Demchenko et al., 2004).

Several of the arbuscule-associated genes were shown to be essential for AM symbiosis. Their mutation results in defective symbiosis which is often associated with incomplete development and/or premature senescence of the fungal arbuscules. This is the case for PT4 (Maeda et al., 2006; Javot et al., 2007), STR and STR2 (Zhang et al., 2010; Gutjahr et al., 2012), subtilase (Takeda et al., 2009), and RAM2 (Wang et al., 2012). The fact that all these genes fail to be induced in _ram1_ mutants (**Figure 3**) can explain the strong defect in arbuscule formation, and, secondarily, the abortion of AM development. Based on the collective evidence described here, we conclude that _ATA/RAM1_ acts as a general transcriptional activator of arbuscule-associated genes in petunia.

Supplemental Material

Supplemental Information and Supplemental Figures are available online at . . . .

Supplemental Materials and Methods

Supplemental Figures

Fig. S1. AM-defective phenotype of *ata* mutant in combination with diverse AM fungi

Fig. S2. *ATA* encodes a conserved GRAS transcription factor

Fig. S3. The GRAS transcription factor encoded by *ATA*/*RAM1* and its closest homologs in *M. truncatula*, rice, and mayze

Fig. S4. Colonization of *ram1-2* and *ram1-3* by *R. irregularis*

Fig. S5. AM-defective phenotype of *M. truncatula ram1-1*

Fig. S6. Expression of AM marker genes in the *ata* mutant

Fig. S7. Statistical analysis of fungal colonization of the root tip

Table S1. Expression of AM marker genes in the *ata*/*ram1* mutants

Table S2. Primers for qPCR

Figure legends

Figure 1. The *ata* mutant is AM-defective

A) Root colonization in the wild type (*Petunia hybrida* line W138) colonized by *R. irregularis*. Total root colonization (blue), occurrence of arbuscules (red), and of abnormal arbuscules (green) is indicated.

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C) and D) colonization pattern in the wild type (C), and in one of the rare colonization units in the *ata* mutant (D). Arbuscules are indicated by arrowheads.

E) Overview of the developmental stages of *R. irregularis* in *ata*. A hyphopodium (arrow) and an infected epidermal cell with a fungal hypha (cross) mark a successful infection event. Note the frequent septa in the fungal hyphae (white arrowheads), indicative of stress in the fungus.

Columns represent the average of five biological replicates ± standard deviation. Significant differences (Student's t-test) between the mutant and the wild type are indicated with asterisk (*: $p < 0.05$; **: $p < 0.01$). v: vesicles; Size bars: 100 µm in C,D; 50 µm in E.

Figure 2. *ata* mutants can be colonized from nurse-plant inoculum but retain the arbuscule phenotype

A) Root colonization in the wild type (*Petunia hybrida* line W138) colonized by *R. irregularis* from nurse-plants. Total root colonization (blue), occurrence of arbuscules (red), and of abnormal arbuscules (green) is indicated.

B) Root colonization in the *ata* mutant colonized by *R. irregularis* from nurse-plants. Color code as in (A). Asterisks indicate significant differences between the mutant and the wild type.

 (C-F) Appearance of arbuscules in the wild type (C), *ata* (D), *ram1-2* (E), and *ram1-3* (F).

Columns represent the average of five biological replicates ± standard deviation. Significant differences (Student's t-test) between the mutant and the wild type are indicated with asterisk (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). size bars: 25 µm.

Figure 3. *ATA/RAM1* is required for induction of AM marker genes.
Gene expression was analyzed by quantitative real time RT-PCR one week after inoculation with *R. intraradices* from nurse-plants. Two independent experiments are shown in combination: Firstly, wild type (blue) vs. *ata*/*ram1-1* (red), and secondly,

wildtype (green) vs. *ram1-2* (purple) and *ram1-3* (orange). Columns represent the average of three independent biological replicates ± standard deviation. Gene expression values are expressed relative to the constitutively expressed reference gene GAPDH. Significant differences (Student's t-test) between the mutant and the respective wild type are indicated with asterisk (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Figure 4. *ata/ram1* mutants allow mycorrhizal colonization of the root tip.

A) Non-colonized wild type root.

B) Colonized wild type root.

C) Colonized *ata*/*ram1-1* root.

D) Fungal colonization in wild type (blue; n=36) and *ata*/*ram1-1* roots (red; 39) in the distal 1 mm of the root tip.

E) Fungal colonization as in (D) in wild type (blue; n=28), *ram1-2* (red; n=32), and *ram1-3* (green; n=35).

F) Fungal colonization as in (D), but scored only for vesicles.

G) Fungal colonization as in (E), but scored only for vesicles.

Size bar: 100 µm.

<u>Supplemental Figures</u>

Figure S1. AM-defective phenotype of *ata* mutant in combination with diverse AM fungi.

Wild type and *ata* mutants were inoculated with *R. irregularis*, *Simiglomus hoi*, and *Acaulospora scrobiculata*. Total root colonization was assessed after 1, 2, 4, and 8 weeks. Columns represent the average of three biological replicates ± standard deviation. Significant differences (Student's t-test) between the mutant and the wild type are indicated with asterisk (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Figure S2. *ATA* encodes a conserved GRAS transcription factor.

A) Transposon display revealed a PCR amplicon that cosegregated with the *ata* mutant phenotype (arrow).

B) Map of the wild type *ATA*/*RAM1* locus and the three *ram1* insertion alleles. Thick lines represent exons, the thin line represents the single intron. Arrows indicate the insertion sites of dTPh1 in the alleles *ata*/*ram1-1*, *ram1-2*, and *ram1-3*, respectively.

C) Analysis of the revertant allele of *ata*/*ram1-1* in comparison with the respective sequence stretch of the wild type allele in *Petunia axillaris*, as well as the closest homologues from *M. truncatula*, rice (*Oryza sativa*), and mayze (*Zea mays*) (compare with **Figure S3**). Note a deletion of 2 amino acid residues in the revertant allele (Pa-ATA-rev).

D) Phylogram of the predicted ATA protein from *P. axillaris* and RAM1 from *M. truncatula* together with the closest homologs from various monocot (*Zea mays*, *Brachypodium distachyon*, *Setaria italica*, *Sorghum bicolor*), and dicot species (*Capsella rubella*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Lotus japonicus*, *Cucumis sativus*, *Solanum lycopersicum*, *Vitis vinifera*, *Populus trichocarpa*, and *Ricinus communis*).

Figure S3. The GRAS transcription factor encoded by *ATA*/*RAM1* and its closest homologs in *M. truncatula*, rice, and mayze.

The predicted petunia ATA/RAM1 protein sequence was aligned with MtRAM1 and the closest homologs of rice (*Oryza sativa*), and mayze (*Zea mays*) using multalin (http://multalin.toulouse.inra.fr). Arrows indicate the insertion sites of dTph1 in the alleles *ata*/*ram1-1* (arrow 1), *ram1-2* (arrow 2), and *ram1-3* (arrow 3), respectively.

Figure S4. Colonization of *ram1-2* and *ram1-3* by *R. irregularis*.

A) Total root colonization in the wild type (blue), *ram1-2* (red), and *ram1-3* (green), colonized from *R. irregularis* spore inoculum.

B) Total root colonization in the wild type (blue), *ram1-2* (red), and *ram1-3* (green), colonized from nurse-plants with *R. irregularis*.

Columns represent the average of five biological replicates ± standard deviation. Significant differences (Student's t-test) between the mutant and the wild type are indicated with asterisk (*: $p < 0.05$).

Figure S5. AM-defective phenotype of *M. truncatula ram1-1*.

A) Total root colonization of *R. irregularis* in *M. truncatula* wild type (Line A17; blue) and in *ram1-1* (red).

B) Wild type root (line A17) colonized by *R. irregularis*. Arbuscules are indicated by arrow-heads.

C) Root of *M. truncatula ram1-1* mutant inoculated with *R. irregularis*. Note the aberrant arbuscules (arrowheads). Size bars: 50 µm in B,C.

Figure S6. Expression of AM marker genes in the *ata* mutant.

Gene expression was determined by real time RT-PCR in non-mycorrhizal control roots (-), and in colonized roots of the wild type (grey bars), and in *ata* mutants (black bars). Each bar represents an individual replicate plant.

Figure S7. Statistical analysis of fungal colonization in the root tip.

The data shown in Figure 4D,E was transformed into survival curves. Log-rank test was performed to assess the significance of the difference between the curves. A p-value <0.05 is considered statistically significant. From top left to bottom right: *ata*/*ram1-1* vs. wt1, *ram1-2* vs. wt2, *ram1-3* vs. wt2, mean mutants vs. mean wt, wt1 vs wt2. Blue curves represent the wt, red curves represent the mutants. In (E), the blue curve represents wt1, the red curve wt2.

Table S1. Expression of AM marker genes in the *ata*/*ram1* mutants.

Expression of the indicated genes was analyzed in plants inoculated for 1 week with *R. irregularis* nurse-plant inoculum. Gene expression was analyzed in the wild type and the three petunia *ram1* alleles relative to GAPDH expression. Gene expression values represent the mean of three independent biological replicates. Comparison between the mutants and the respective wild type plants revealed to which degree gene expression was reduced due to the respective *ram1* mutation (fold less), and p-values (t-test) indicate significant differences.

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Rich et al., Supplemental Materials and Methods

Material and Methods

Plant material, growth conditions, and inoculation

Petunia seeds were surface-sterilized with ethanol (70%, 30 sec) followed by sodium hypochlorite (7%, 10 min), rinced five times in sterile water and germinated on seedling substrate (Klasmann, http://www.klasmann-deilmann.com). After four weeks, plantlets were transferred to a sterilized mixture of 75% sand with 25% unfertilized soil (further referred to as sand substrate) and inoculated with around 10g of soil inoculum of *Rhizophagus irregularis* (MUCL 43204). Seeds of *Medicago truncatula* were scarified in concentrated sulfuric acid (10 min) and surface sterilized as described above before germination on seedling substrate. 20-day-old plantlets were transferred to sand substrate for inoculation. Nurse-plant inoculation was carried out by coculturing petunia plants with leek plants (*Allium porrum*) that had been inoculated at least four weeks before. Plants were grown in growth chambers with a day/night cycle of 12h (25°C)/12h (20°C). Plants were fertilized weekly with a solution containing 3 mM $MgSO_4$, 0.75 mM KNO_3, 0.87 mM KCl, 0.2 mM KH_2PO_4, 1.52 mM $Ca(NO_3)_2$, 0.02 mM NaFeEDTA, 11 µM $MnSO_4$, 1 µM $ZnSO_4$, 30 µM H_3BO_3, 0.96 µM $CuSO_4$, 0.03 µM $(NH_4)_6Mo_7O_{24}$, and 0.01 µM Na_2MoO_4.

Staining procedure and mycorrhizal quantification

Roots were harvested five weeks after inoculation if not stated otherwise, washed and cleared in 10% KOH (30 min at 95°C), washed with water and stained for 5 minutes in Trypan Blue staining solution (20% glycerol, 30% lactic acid, 0.01% Trypan Blue, Fluka, Buchs, Switzerland) at 95°C, and rinsed twice with 30% lactic acid (Phillips and Hayman, 1970). Root colonization was quantified with a modified grid-line intersection method (McGonigle et al., 1990). Briefly, roots were horizontally distributed over a grid of vertical yellow stripes and 50 intersections were scored for the presence of fungal structures. Images were acquired using a Zeiss Axiocam (http://www.zeiss.com) mounted on a Leica DMR. Colonization of root tips was assessed five weeks after introduction in the nurse chamber system by measuring the

distance between the tip of the root and the first visible fungal structure in ImageJ (open source http://imagej.nih.gov/ij/)

Mutant screen, cloning of the *ata* locus, and revertant analysis

The *ata*/*ram1-1* mutant allele was isolated from the transposon line W138 of *Petunia hybrida* as described (Sekhara Reddy et al., 2007). Briefly, eight individuals from segregating families were assessed for mycorrhizal colonization after 5 weeks of colonization with *R. irregularis*. Root samples were taken from inoculated plants, stained as described above and screened visually for the presence of AM fungal structures. Families with AM-defective individuals were further grown for seed production and additional seeds of the respective family were sawn for phenotypic analysis and assessment of the segregation pattern. Cloning of the ata allele was carried out by transposon display as described (Van den Broeck et al., 1998). The alleles *ram1-2* and *ram1-3* were identified *in silico* from flanking sequences obtained by large scale sequencing on a population of transposon-mutagenized plants (W138) as described (Vandenbussche et al., 2008).

RNA isolation and gene expression analysis

Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). cDNA synthesis was performed on 1µg of RNA with the Omniscript RT kit (QIAGEN) using a mix of oligo dT and random primers (Promega). Quantitative real time RT-PCR was performed with the SensiMix™ SYBR® Hi-ROX Kit (Bioline) on a Corbett life science Rotor-Gene with the primers listed in Table S2. Gene expression levels were calculated relative to GAPDH as described (Sekhara Reddy et al., 2007). Each individual plant was assessed with duplicate measurements.

Phylogenetic analysis

Phylogenetic analysis was performed with Phylogeny.fr (Dereeper et al., 2008), involving previously described tools (Castresana, 2000; Guindon and Gascuel, 2003; Edgar, 2004; Anisimova and Gascuel, 2006; Chevenet et al., 2006; Dereeper et al., 2010). Phylograms were produced with the "Advanced" mode (see http://www.phylogeny.fr) using the bootstrap function to calculate support for branch separation. Bootstrap values reflect 100 replicates.

Accession numbers

Sequence data from this article can be found in GenBank under the following accession numbers: *ATA*/*RAM1*, KR612264; *STR*, KR612265; *STR2*, KR612266; *RAM2*, KR612267; *BCP*, KR612268; *Subtilas*e, KR612269; *PhPT4*, EU532763; *PhPT5*, EU532764.

Statistical analysis

In general, the significance of differences between treatments or between genotypes was tested by Student's t-test. Asterisks indicate significant differences with *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. The difference in the colonization of the root tip by *R. irregularis* was tested as follows. The growth of the fungus from a reference point (1 mm behind the root tip) towards the root tip was compared with the survival of individuals in a population analogous to cancer trials. To this end, the fungal colonization curves were first transformed into the equivalent of survival curves with a script in R Survival package version 2.37-7 in R version 3.1.0 (2014-04-10) (see below). Conceptually, the AM fungus represents the "patients" and the growth from the 1 mm reference point towards the root tip represents ist life span, scored in 100µm intervals (see Figure S7). Survival curves between two treatments are usually tested by a log-rank test known as the Kaplan-Meier (K-M) method (Kaplan and Meier, 1958; Mantel, 1966), which tests for the null hypothesis (no significant difference between the survival curves). Curves with $p<0.05$ are considered significantly different.

R script:

```
library(survival)
#small function to invert the results of counts into a survival curve
invert_curve<-function(a) {
j=a[1];
d=0;
e=1;
x=0;
b=0;
```

```
for (i in 1:length(a)) {
  for (k in e:j) {
    b[k]=length(a)-i;
    x[k]=d;
  }
  d=1;
  e=j+1;
  j=a[i+1];
}
return(list(time=b,status=x));
}

#mutant 566
wtdata=c(5,9,11,12,13,15,17,19,19,19,28)
wt=invert_curve(wtdata)
mutdata=c(14,27,28,28,30,30,31,31,31,31,32)
mut=invert_curve(mutdata)

fit1 <- survfit(Surv(wt$time, wt$status) ~ 1)
fit2 <- survfit(Surv(mut$time, mut$status) ~ 1)
plot(fit1,conf.int="none", col ='blue', xlab = 'Inverse root tip distance (10mm-x)', ylab
= '% presence of mycorrhize')
lines(fit2, conf.int="none",col = 'red')
legend(8,1,c('Group 1 (WT)', 'Group 2 (Mutant)'), col = c('blue','red'), lty = 1)
title(main='KM-Curves for Mycorrhize Data mutant 566')

wt$group = 1
mut$group = 2
wt.df=data.frame(wt)
mut.df=data.frame(mut)
data = rbind(wt.df, mut.df)
survdiff(Surv(time,status) ~ group, data=data)

#mutant 568
```

```
mutdata2=c(20,26,27,28,28,28,28,29,29,29,35)
mut=invert_curve(mutdata2)


fit1 <- survfit(Surv(wt$time, wt$status) ~ 1)
fit2 <- survfit(Surv(mut$time, mut$status) ~ 1)
plot(fit1,conf.int="none", col ='blue', xlab = 'Inverse root tip distance (10mm-x)', ylab
= '% presence of mycorrhize')
lines(fit2, conf.int="none",col = 'red')
legend(8,1,c('Group 1 (WT)', 'Group 2 (Mutant)'), col = c('blue','red'), lty = 1)
title(main='KM-Curves for Mycorrhize Data mutant 568')


wt$group = 1
mut$group = 2
wt.df=data.frame(wt)
mut.df=data.frame(mut)
data = rbind(wt.df, mut.df)
survdiff(Surv(time,status) ~ group, data=data)



#mutant ata
wtdata=c(3,7,11,13,14,15,16,17,18,20,36)
wt=invert_curve(wtdata)
mutdata3=c(27,31,31,32,32,33,33,35,36,36,39)
mut=invert_curve(mutdata3)


fit1 <- survfit(Surv(wt$time, wt$status) ~ 1)
fit2 <- survfit(Surv(mut$time, mut$status) ~ 1)
plot(fit1,conf.int="none", col ='blue', xlab = 'Inverse root tip distance (10mm-x)', ylab
= '% presence of mycorrhize')
lines(fit2, conf.int="none",col = 'red')
legend(8,1,c('Group 1 (WT)', 'Group 2 (Mutant)'), col = c('blue','red'), lty = 1)
title(main='KM-Curves for Mycorrhize Data mutant ata')


wt$group = 1
```

```
mut$group = 2
wt.df=data.frame(wt)
mut.df=data.frame(mut)
data = rbind(wt.df, mut.df)
survdiff(Surv(time,status) ~ group, data=data)


#mean values (3 mutants, 2 wt)
wtmean=c(4,8,11,13,14,15,17,18,19,20,32)
wt=invert_curve(wtmean)
mutmean=c(20,28,29,29,30,30,31,32,32,32,35)
mut=invert_curve(mutmean)


fit1 <- survfit(Surv(wt$time, wt$status) ~ 1)
fit2 <- survfit(Surv(mut$time, mut$status) ~ 1)
plot(fit1,conf.int="none", col ='blue', xlab = 'Inverse root tip distance (10mm-x)', ylab
= '% presence of mycorrhize')
lines(fit2, conf.int="none",col = 'red')
legend(8,1,c('Group 1 (WT)', 'Group 2 (Mutant)'), col = c('blue','red'), lty = 1)
title(main='KM-Curves for Mycorrhize Data mutant Mean values')


wt$group = 1
mut$group = 2
wt.df=data.frame(wt)
mut.df=data.frame(mut)
data = rbind(wt.df, mut.df)
survdiff(Surv(time,status) ~ group, data=data)



wtdata1=c(3,7,11,13,14,15,16,17,18,20,36)
wt=invert_curve(wtdata1)
wtdata2=c(5,9,11,12,13,15,17,19,19,19,28)
mut=invert_curve(wtdata2)


fit1 <- survfit(Surv(wt$time, wt$status) ~ 1)
```

```
fit2 <- survfit(Surv(mut$time, mut$status) ~ 1)
```

plot(fit1,conf.int="none", col ='blue', xlab = 'Inverse root tip distance (10mm-x)', ylab = '% presence of mycorrhize')

lines(fit2, conf.int="none",col = 'red')

legend(8,1,c('Group 1 (WT)', 'Group 2 (Mutant)'), col = c('blue','red'), lty = 1)

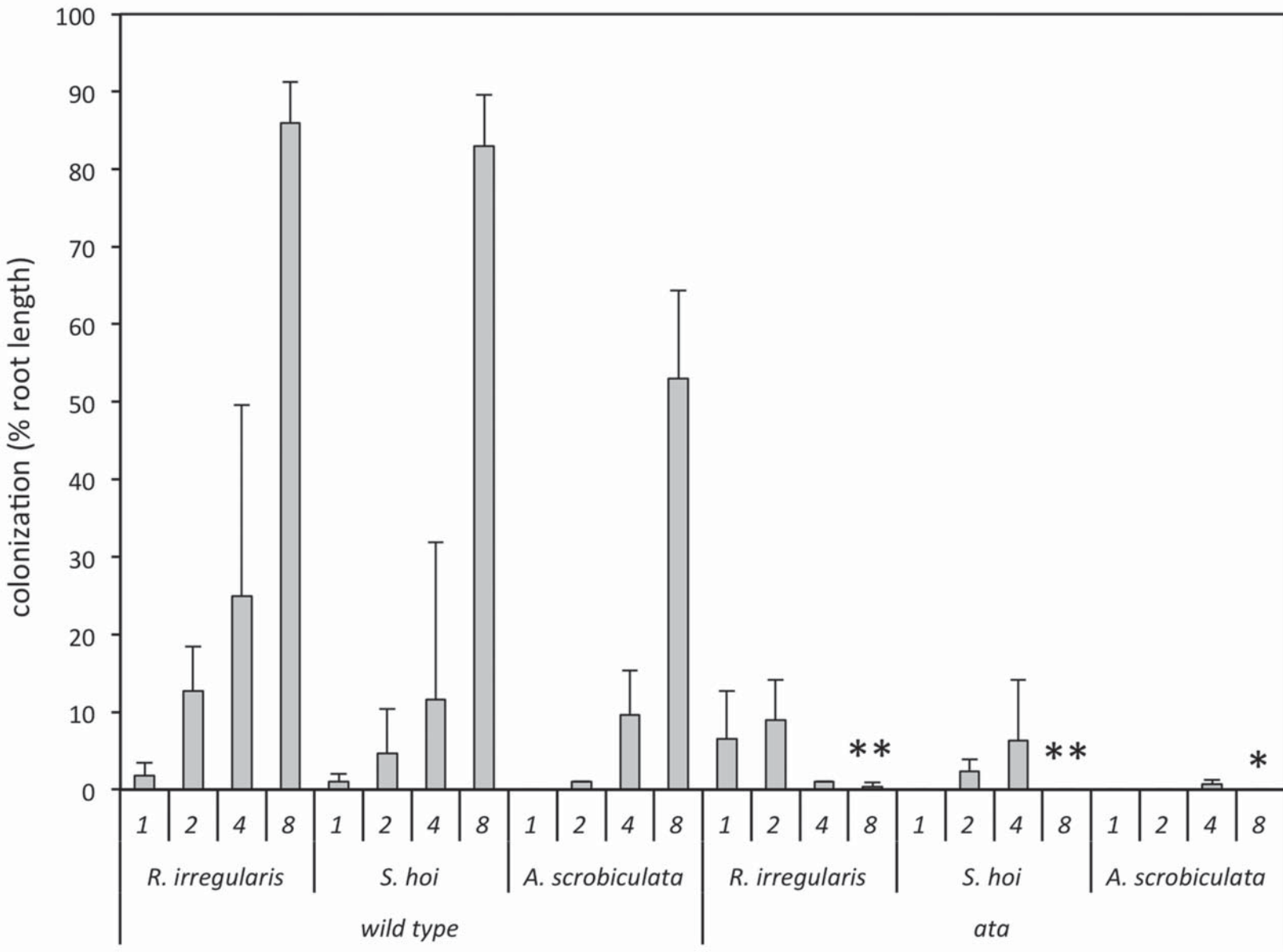title(main='KM-Curves for Mycorrhize Data wt1 vs wt2 comparison')

wt$group = 1

mut$group = 2

wt.df=data.frame(wt)

mut.df=data.frame(mut)

data = rbind(wt.df, mut.df)
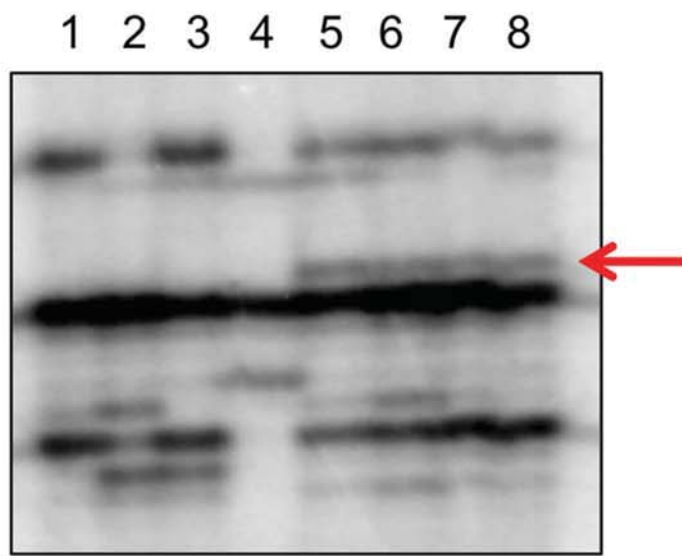
survdiff(Surv(time,status) ~ group, data=data)

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A

1 2 3 4 5 6 7 8

B

ram1-2   ata/ram1-1

ATA/RAM1

ram1-3

C

```
        551        561        571        581        591
         |--------+--------+--------+--------+--------|
Pa-ATA-rev  RVHIIDLDILQGYQWPAFMQALA--PGGAPFLRITGYGPS
P_axillaris RVHIIDLDILQGYQWPAFMQALAARPGGAPFLRITGYGPS
M_truncatula RVHYIDLDILQGYQWPAFMQALAARPGGAPFLRITGYGPC
Z_mays      RVHYVDLDILQGYQWPAFLQALAARPGGPPTLRLTGYGHP
O_sativa    RVHYVDLDILQGYQWPAFLQALAARPGGPPTLRLTGYGHP
Consensus   RVH!!DLDILQGYQWPAF$QALAarPGGaPfLRiTGYGp.
```

D

```
                          0.9 ─ C_rubella
                     0.78 ┤
                          └ A_thaliana_RGL1
                0.86 ┤       A_lyrata
                     └── E_salsugineum
          1 ┤        B_rapa
                          Z_mays
                     1 ┤ B_distachyon
                  0.49 ┤
                   0.67 ┤ S_italica
                          S_bicolor
                     0.7 ┤ M_truncatula_RAM1
                          L_japonicus
              0.91 ┤   C_sativus
                          0.7 ┤ 1 ┤ S_lycopersicum
                          0.39 ┤ P_axillaris_ATA
                     0.84 ┤ V_vinifera
                     0.67 ┤ P_trichocarpa
                          R_communis
```

0.2

35

```
            1        10        20        30        40        50        60        70        80        90        100       110       120       130
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PhATA                                    MGSLNNEVGSIKCEDGSLKS----------ENSFTKLPSEESLASESKKVTPVSSDFELNCG----SLAPTSLSFPAMKFE--DDIEIQSPD
MtRAM1                                    MINSLCGSSNSLK----------EKCLQPNSSNQTNTHSKKNATNSCGDLE-QIN----VLTPQSLNLPSLKFDLDGDVEVQSPD
LOC_Os11g31100.1       MAGGGAKLQ-QQQAPTSPTASVSESNIVASTASADPEANDALAGLQALRFDGGGDIDDVEIQSPDIALHESIFADQIGVSGAGADFLLSMSS--AAASP-RRDFMACSPKRDYMVTTSSPK
Z_mays     MGMLNCVDTTSGAGAKLQHQQQAPTSPTASVSESNIVAPSVSADIDANDALASLQALRFDG--DID-VEIQSPDIAMHESLFAEQMGAAGGDFLMLSPRRDFMAAGSPSRRDFMVSSPKRDYMVS-SSPK
Consensus  .............s...e.ns...s..sLk.............E........s.#sl..e.k.at....dfel.........l.P.sl.fpa.kf..d.d!e.qSPd

            131      140       150       160       170       180       190       200       210       220       230       240       250       260
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PhATA      -----NSIWESFFADHLEGDFMISSPIRNLPSPQPASSFSSSHNNSNNNNNNYNIYAHRQGIHGQDMMMCSPPR--SPLGLYN-HPSHKGKGLSPLQKVF----------------NSPNNQFMQ----
MtRAM1     ------SSMWEAFFNDHLDNDFMISSPIRNI---NPNSPQASTYNNCN----------YNYAQGMQIQSLSGCSPPRFASQIGSLNSNNQQKGKGLSPLHRVF----------------NSPNNQYMQ----
LOC_Os11g31100.1 RDYMVTSSPKRDYMVSSPKREYMVTSPRREMATSPRRATFSNLYTSSHGGGGGGGHHLHHQSYVHGGGMEGGGGGGHGAQPQYGGLAGH-GKGKAQSPLHKVYINNVGGGSGGGGVKSNTPSTLSCRSSYV
Z_mays     RDYMM-SSPMRDLMASSPKREYMVSSPRRDSSSSRRSPFSPNLFSTSGGG---------HQHGYAAHGPTDGGVGGAG-QPLYGGLANHHGKGKSQSPLHKGYINN------SGNKQSTGPSSLSCSSSYG
Consensus  .......sS.we.ffadhl..#%M!sSPiR#..s..p.s.fss.ynnsn.........y.h.qg.hgq.m.gcsppr..splg..n.n...KGKglSPLhkv%.............nsPnnq.mq...

            261      270       280       290       300       310       320       330       340       350       360       370       380       390
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PhATA      -----IESFNLPALESFLDDDY-----DKEEDLASSYSTLKVSGTGVAGSSSESFDALSVVPDDLLECLALPSSLSNTSGGFMESLL--SDTSAVQVINPDDEIFRTGSIAP--LSQQLHQERQQEKQQK
MtRAM1     ----HVENLSLPAIEEFLED-F-----QGDVD---HFSSTKVS---------SECFDMETPI-STILDSLTHQNSSSYGASVNEESTLLHGGNSSSQISQESDIYHQMGSMASASLSQALQQERYQEKHQK
LOC_Os11g31100.1 VHGGESGLPSLPSMDPFLEEGYLG-SYQLPEK---AAGGVGGGGGGDINRSGASVSVVTAPASSQLLPTLSECLAMPEPAAYRGGGDEAVAAAMAVAGELPVGAFVQPEMYYGGGGEFGGEGMTPPLQHQM
Z_mays     HADNDLPSMDTCFLDDYKDGGYIGYQQMPGKQQAAPGIMVNNNGGCSTAVTTVGVAPSPSSHQL-PTLSECLAMPEPAFV--GGEEAAAGGLQMGVGLPSDLYYAGQFAGGGGG--GLTTTSSSLQHQM
Consensus  .......es.slpal#.%l#d.%.....#..ed.....s..kvsg.g...s.se.fda.sp..s.ll..L....s.s..a.v...es.l....g.savqv..psd.y.q.gs.a...lsq.l.qer.qekhQk

            391      400       410       420       430       440       450       460       470       480       490       500       510       520
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PhATA      QRPVTHARPQQQQIINHNVLVPLAAAPDQEQDSGLQLVHLLLACAEAVSKEDYMLARRYLHHLNRVVTPLGDSMQRVASCFTEALSARLAATLATKPST-------------SARKPFNPFPPNSLEILKI
MtRAM1     MQA------QQQSLT-----VPIQIGIEQEQDSGLQLVHLLLACAEAVAKGEYMLARRYLHQLNRVVTPLGDSMQRVASCFTESLSARLAATLTTKSSSTKKLAPSSLSSSSSSSCLSTFPSNPMEVLKI
LOC_Os11g31100.1 A--------ADSSLHSMLGSVIQSEA-EQEQDSGLQLVHLLLACADLVSKGDHPAALRHLHLLRRVASPLGDSMQRVASHFADALAARLSLLSSPTSASPS-------PRAAAAAAPYPFPPSP--ETLKV
Z_mays     AKSDHQWAAAESSLHSMLGSVIQTEADEQEQDSGLQLVHLLLACADFVSKGDQPSALRHLHLLRRVASPLGDSMQRVASYFADALAARLS--SNNPSSSAG-------AGAGAGVAPYTFPPSP--DTLKV
Consensus  .........q#qsl......Vp...a..#QEQDSGLQLVHLLLACA.aVsKg.#ynlArRyLH.LnRVvtPLGDSMQRVAScFt#aLsARLaatl.tksss.............sa.....pFPpnp..#.LK!

            521      530       540       550       560       570       580       590       600       610       620       630       640       650
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PhATA      YQILYQACPYVKFAHFTANQAIFEAFEAEERVHIIDLDILQGYQWPAFMQALAARPGGAPFLRITGVGPSPEAVRETGRCLTELAHSLHVPFEFHP-VGEQLEDLKPHMFNRRVGEALAVNSVNRLHRVP
MtRAM1     YQIVYQACPYIKFAHFTANQAIFEAFEAEERVHVIDLDILQGYQWPAFMQALAARPGGAPFLRITGVGPCIESVRETGRCLTELAHSLRIPFEFHP-VGEQLEDLKPHMFNRRVGEALAVNTVNRLHRVP
LOC_Os11g31100.1 YQILYQACPYIKFAHFTANQAIFEAFHGEDRVHVVDLDILQGYQWPAFLQALAARPGGPPTLRLTGVGHPPAAVRETGRHLASLAASLRVPFEFHAAAADRLERLRPAALHRRVGEALAVNAVNRLHRVP
Z_mays     YQILYQACPYIKFAHFTANQAIFEAFHGEDRVHVVDLDILQGYQWPAFLQALAARPGGPPTLRLTGVGHPAAAVRETGRHLASLAASLRVPFEFHAAVADRLERLRPAALHRRVGEALAVNAVNRLHRVP
Consensus  YQIlYQACPY!KFAHFTANQAIFEAFeaE#RVH!!DLDILQGYQWPAF$QALAARPGGaPfLRiTGVGp.peaVRETGRcLteLAhSLr!PFEFHp.vg#qLEdLkPhmfnRRVGEALAVN.VNRLHRVP

            651      660       670       680       690       700       710       720       730       740       750       760       770       780
            |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PhATA      VNCIGNLLAMIRDQAPNIVTIVEQEASHNGPYFLGRFLEALHYYSAIFDSLDATFPADSSQRAKLEQYIFAPEIMNIVSCEGQERNVRHERLEKWRRLMEGKGFKGVALSANAVTQSKILLGLYSC-DGY
MtRAM1     GNHLGNLLSMIRDQAPNIVTLVEQEASHNGPYFLGRFLEALHYYSAIFDSLDATFPVESAPRAKVEQYIFAPEIRNIVACEGEEERIERHERLEKWRKIMEGKGFKGVPLSPNAVTQSRILLGLYSC-DGY
LOC_Os11g31100.1 SSHLPPLLSMIRDQAPKIITLVEQEAHNGPYFLGRFLEALHYYSAIFDSLDATFPAESTARMKVEQCLLAPEIRNVVACEGAEAVRGGSAAVGQSQVLLGLYGAGDGY
Z_mays     AVHLGPLLSMIRDQAPKIMTLVEQEAHNGPYFLGRFLEALHYYSAIFDSLDATFPDSAQRMKVEQCLLAPEIRNVVACEGAERVARHERLDRWRRIMEGRGFEPVPLSPAAVAQSQVLLGLYGAGDGY
Consensus  .nhlgnLLsMIRDQAPnIvTlVEQEAsHNGPYFLGRFLEALHYYSAIFDSLDATFPa#SaqRakvEQyifAPEIrN!VaCEG.ER..RHERL#kWRriMEGkGFkgVpLSanAVtQS.!LLGLYsc.DGY

            781      790       800    806
            |--------+---------+-----|
PhATA      KLTEDDGCLLLGWQDRAILAASAWRC
MtRAM1     RLTEDKGCLLLGWQDRAIIAASAWRC
LOC_Os11g31100.1 RLTEDSGCLLLGWQDRAIIAASAWRC
Z_mays     RLTEDRGCLLLGWQDRATIAASAWRC
Consensus  rLTED.GCLLLGWQDRAiiAASAWRC
```
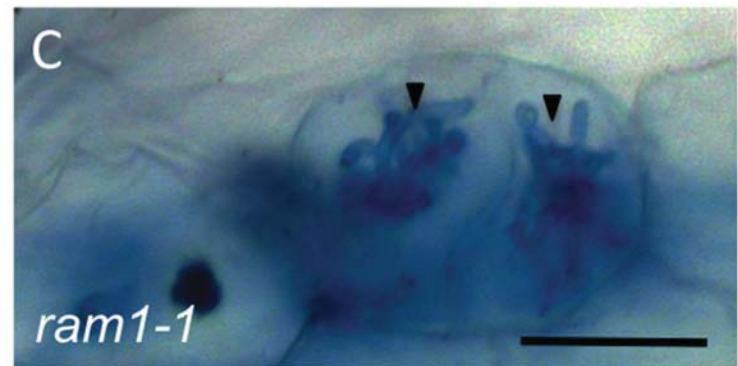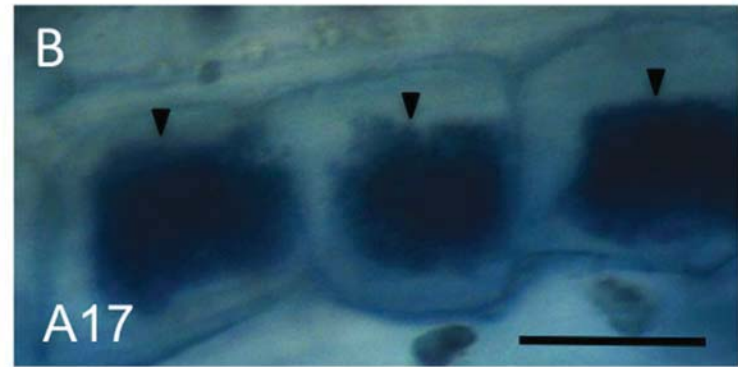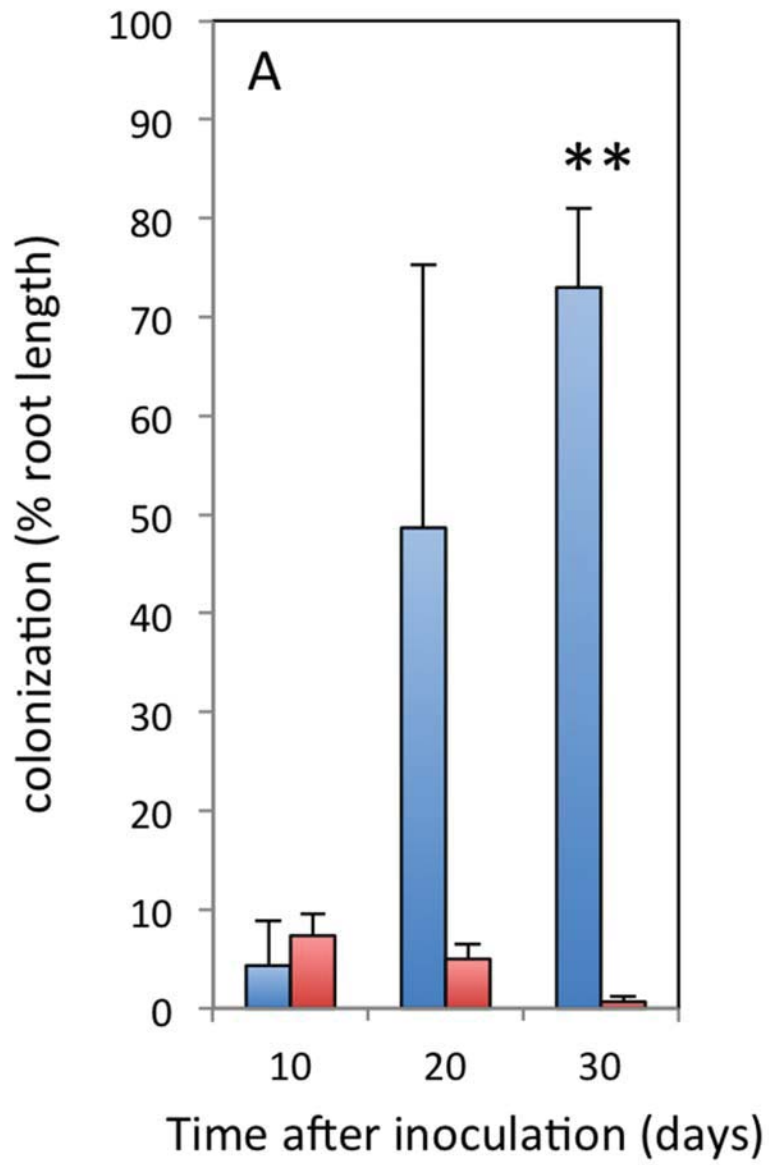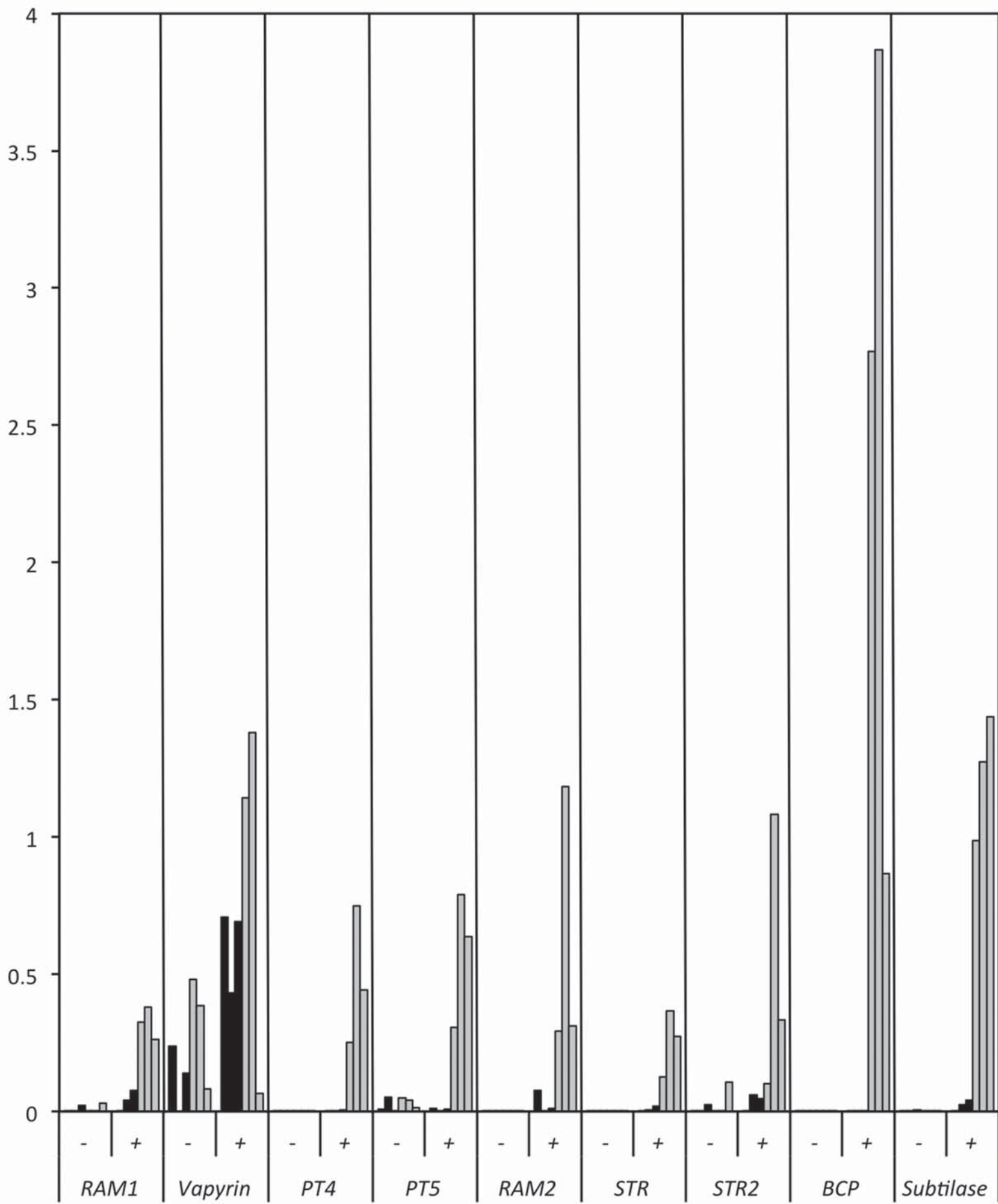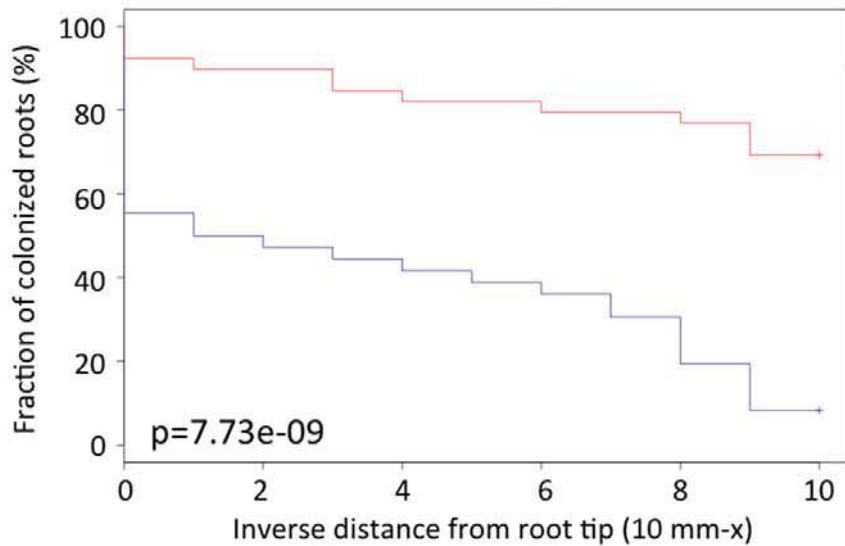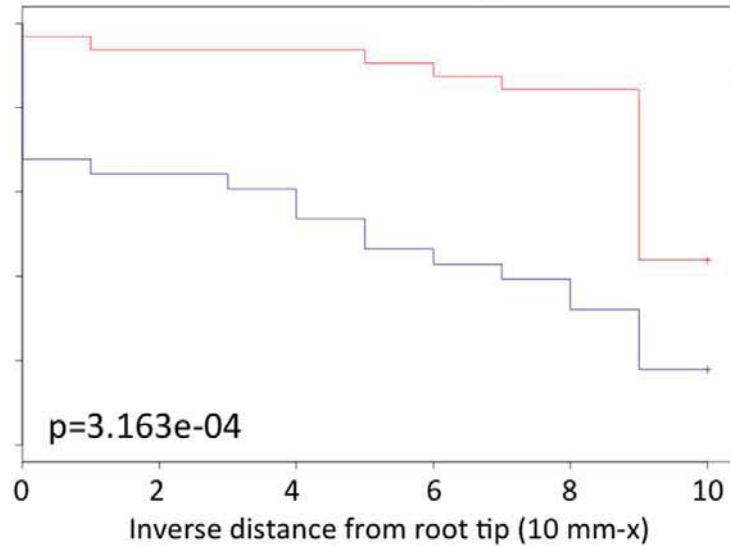
ata vs. wild type1

p=7.73e-09

ram1-2 vs. wild type2

p=3.163e-04

ram1-3 vs. wild type2

p=5.4e-04
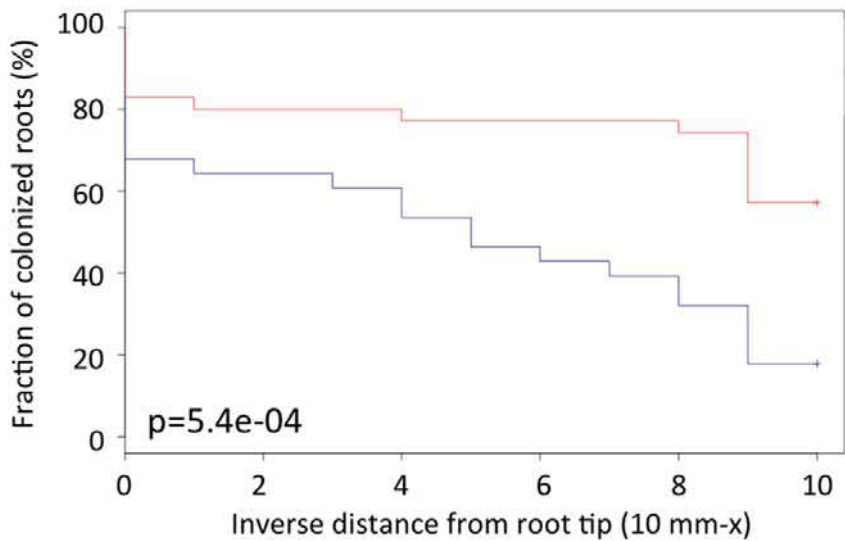
Mean *ram1* mutants vs. mean wild type

p=4.4e-06
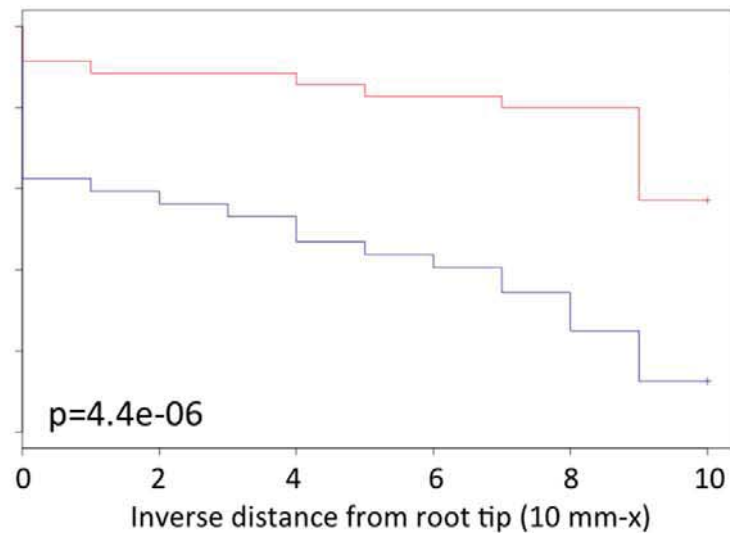
wild type1 vs. wild type2

p=0.221
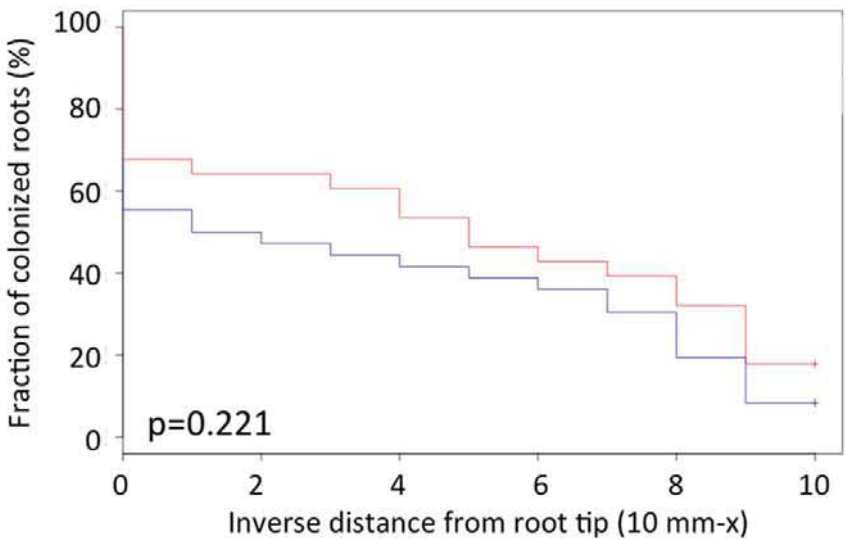
	W115-a	ata/ram1-1			W115-b	ram1-2			ram1-3		
	expr.	expr.	fold less	p-value	expr.	expr.	fold less	p-value	expr.	fold less	p-value
RAM1	321,851	39,087	8	0.0022	373,127	75,857	5	0.0220	22,255	17	0.0081
Vapyrin	1,146,643	609,847	2	0.0290	1,327,435	652,040	2	0.2606	885,166	1	0.4145
PT4	481,117	2,078	231	0.0302	979,081	16,216	60	0.0042	2,009	487	0.0040
PT5	577,052	6,933	83	0.0161	706,846	43,167	16	0.0036	19,208	37	0.0032
RAM2	597,126	3,554	168	0.1136	683,175	11,277	61	0.0126	1,410	485	0.0119
STR	255,319	8,981	28	0.0247	161,650	10,121	16	0.0315	8,422	19	0.0298
STR2	505,049	35,359	14	0.1887	232,375	63	3718	0.0148	4,083	57	0.0158
BCP	2,501,280	593	4215	0.0463	2,800,029	67,416	42	0.0247	71,418	39	0.0248
Sbt	1,232,482	22,039	56	0.0008	4,642,693	42,972	108	0.0207	980	4,737	0.0201

Table S2. **Primers used for qPCR analysis.** Indicated are the gene annotation, the forward (extension F)
and reverse (extension R) primer.

Gene annotation	Primer name	Primer sequence
GAPDH	PhGAPDH-F3	GGAATCAACGGTTTTGGAAGAATTGGGCG
	PhGAPDH-R4	GGCCGTGGACACTGTCATACTTGAACA
RAM1	PhRAM1-F	ACTGGTGCACCTTCTCCTTG
	PhRAM1-R	GGGTCACGACACGATTCAG
RAM2	PhRAM2-F	GCCCATGAGCCAAGACAAG
	PhRAM2-R	CATGCCAAGAGGAAACCAAC
PT4	PhPT4-F	ATCTTTGCAGGGCTGGTTTC
	PhPT4-R	ATCTTTGCAGGGCTGGTTTC
PT5	PhPT5-F	ACATTTGTGCTCCCTGCTGA
	PhPT5-R	CTAAGAGCGTGGCATGTTGA
STR	PhSTR-F	CCAACGCGAAAGCTAATTCC
	PhSTR-R	TCACCTCTCAAGGCTTGTCC
STR2	PhSTR2-F	TGGTGAACGTCGTAGGGTCT
	PhSTR2-R	ACACTGTGAGCACTGGTGGA
Vapyrin	PhVapyrin-F	CATTGTGCAGTGGAATCTGG
	PhVapyrin-R	CTTTTGTTGCACCACCTTGC
Subtilase	Sbt_CL206	TTGGCAAGTCCAGGCATAGT
	Sbt_CL206	ATGTCAGATCGGTGCCAGTT
BCP	BCP_CL85_F	TGGAGGAGGAGCTATGCAGTT
	BCP_CL85-R	GGCTGGACCATTGATGTTGA