Insights into the complex role of GRAS transcription factors in the arbuscular mycorrhiza symbiosis

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To improve access to limiting nutrients, the vast majority of land plants forms arbuscular mycorrhizal (AM) symbioses with Glomeromycota fungi. We show here that AM-related GRAS transcription factors from different subgroups are upregulated during a time course of mycorrhization. Based on expression studies in mutants defective in arbuscule branching (ram1-1, with a deleted MtRam1 GRAS transcription factor gene) or in the formation of functional arbuscules (pt4-2, mutated in the phosphate transporter gene MtPt4), we demonstrate that the five AM-related GRAS transcription factor genes MtGras1, MtGras4, MtGras6, MtGras7, and MtRad1 can be differentiated by their dependency on MtRAM1 and MtPT4, indicating that the network of AM-related GRAS transcription factors consists of at least two regulatory modules. One module involves the MtRAM1- and MtPT4-independent transcription factor MtGras4 that activates MtGras7. Another module is controlled by the MtRAM1- and MtPT4-dependent transcription factor MtGras1. Genome-wide expression profiles of mycorrhized MtGras1 knockdown and ram1-1 roots differ substantially, indicating different targets. Although an MtGras1 knockdown reduces transcription of AM-related GRAS transcription factor genes including MtRam1 and MtGras7, MtGras1 overexpression alone is not sufficient to activate MtGras genes. MtGras1 knockdown roots display normal fungal colonization, with a trend towards the formation of smaller arbuscules.
GRAS transcription factors belonging to the GIBBERELLIN-INSENSITIVE (GAI)25, REPRESSOR of gall-1-3 (RGA)26, or SCARECROW (SCR)27 families form a subgroup of plant TFs. Based on their specific domains, 592 or 692 members of the GRAS TF family were predicted in *M. truncatula*. Prominent GRAS TFs have been shown to play a vital role in symbiotic signaling29, e.g. NSP1 and NSP2 that have a key role in the early transduction of signals during rhizobial and mycorrhizal symbioses. In response to the elicitation by Nod-factors (NFs), NSP1 and NSP2 form a heterodimer that binds to cis-regulatory elements in the promoter of the ENOD11 gene30. NSP1 and NSP2 also mediate other early Nod- and also Myc-factor induced responses, a process that incorporates the GRAS TF RAM131,32. RAM1 was initially shown to be required for early mycorrhizal signaling31, but is now known to control arbuscule branching in *Medicago truncatula*, *Lotus japonicus*, and *Petunia hybrida*33,36. Transcription profiling of *ram1-1* mutants in pre-symbiotic signaling37 and in AM roots38 revealed several hundred potential targets of RAM1, including many members of the carbohydrate and lipid metabolism16. In *M. truncatula*, a major task of this GRAS TF is the induction of the *MtRam2* gene, encoding a glycerol-3-phosphate acyltransferase involved in the production of fatty acid precursors required for the formation of the peri-basal membrane34. In addition, MtRAM1 is required for the expression of the AM-induced phosphate transporter gene *MtPi4* and genes encoding other membrane transporters and membrane proteins essential for arbuscule function35. Additional targets of RAM1 also include genes of the WRI family (*MtWRI5a*, *MtWRI5b*, and *MtWRI5c* in *M. truncatula*36 and *CBX1 in Lotus japonicus*37), all encoding AP2-domain TFs that regulate genes related to fatty acid biosynthesis. Expression of *MtRam1* and *MtWri5a* was shown to be interdependent, forming a regulatory feedback loop between the encoded TFs37.

The activation of *MtRam1* transcription34 is controlled by DELLA proteins, which form a subgroup of GRAS-TFs. DELLA proteins, being inactivated at high GA levels, were thus shown to link the level of plant hormones with arbuscule formation36,37.

RAD1, a second prominent member of the AM-related GRAS TF family, was in addition to RAM1 shown to be required for arbuscule development in *M. truncatula*42 and *L. japonicus*36. Interestingly, RAD1 was also shown to interact with RAM136 as well as TF80 and TF124, two additional AM-related GRAS TFs38, suggesting that these regulators interact to control arbuscule development33,36.

A mycorrhiza-inducible clade of GRAS TFs (MIG) has recently been shown to be already activated by *Rhizophagus* spore exudates. Amongst these TFs, MIMIG1 was shown to be crucial for radial cell expansion and arbuscule development by interacting with MtDELLA1, thus intersecting the GA-pathway in mycorrhizal roots43.

GRAS TFs have not only been reported to separately control regulatory processes during mycorrhization33,36. Several studies have in addition identified a direct interaction of GRAS proteins e.g. MtNSP1-MtNSP230, MtRAM1-MtTF8033, MtRAM1-MtRAD133, MtRAM1-MtNSP231, MtRAD1-MtNSP243, MtMIG1-NSP138, and MtMIG1-DELLA143, supporting the idea that networks of GRAS TFs interact to control mycorrhization and in particular arbuscule development17.

Based on genome-wide expression profiling of mycorrhizal and non-mycorrhizal tissues44,49, several *M. truncatula* genes encoding GRAS TFs (MtGRAS genes) were found to be upregulated in AM symbioses, including the *MtRad1*38 and *TF80* genes38 mentioned above. For our study, we selected both those MtGRAS genes exclusively activated during mycorrhization and those that were AM-induced but not AM-specific, since they are also expressed in non-symbiotic conditions or in different tissues. To shed light on the contribution of the AM-activated MtGRAS family to the development of AM symbioses and arbuscule formation, we performed comparative gene expression studies and *in situ* localizations of promoter activities in wild type plants and in the *ram1-1* mutant lacking a key transcriptional regulator of arbuscule branching35 as well as the *pht4-2* mutant49, characterized by a defective phosphate transporter required for the formation of active, phosphate-transporting arbuscules. Together with functional studies in either Tnt1 mutants or RNAi-mediated knockdown roots, we propose a model where we position five AM-related GRAS TFs relative to the well-studied AM-related regulator MtRAM1 in the regulatory circuit that controls arbuscule development. With MtGRAS1, we provide evidence that an AM-related GRAS TF is part of a feedback loop with MtRAM1 to sustain arbuscule formation.

**Results**

**AM-related GRAS TFs of *Medicago truncatula* belong to different subgroups.** Based on genome-wide expression profiles recorded by GeneChip hybridizations44, a core set of AM-activated GRAS TF genes, namely MtGRAS1 (designated *TF80* in44), MtGRAS4, MtGRAS6, MtGRAS7, and MtRAD142 was selected. In addition, the *MtRam1* gene31, encoding a GRAS TF that controls arbuscule branching31, was included. The corresponding identifiers from the *Medicago truncatula* genome31, the *Medicago Gene Expression Atlas*32, and the literature are listed in Supplementary Table S1. Analyses of gene expression data from GeneChip hybridizations stored in the *Medicago Gene Expression Atlas*32 and from studies on *MtRam1*35 revealed two different subgroups among the selected MtGRAS genes. Whereas MtGRAS1, MtGRAS7, MtRAD1, and MtRAM1 are specifically expressed in mycorrhizal roots and are only activated at background levels in non-mycorrhizal controls, MtGRAS4 and MtGRAS6 display a low but detectable expression in non-mycorrhizal roots (Supplementary Fig. S1), yet showing a significant upregulation upon mycorrhization47. This corresponds to the data shown in Fig. 1, where MtGRAS4 and MtGRAS6 are expressed at significant levels at time point 0. Although being AM-induced, MtGRAS4 and MtGRAS6 transcription also occurs in other tissues52. We nevertheless selected these genes in addition to the AM-specific MtGRAS1, MtGRAS7, MtRAD1, and MtRAM1 genes, because arbuscule formation responds to a range of nutritional, physiological, and environmental factors16 that obviously are relevant in non-symbiotic tissues as well.

In a phylogenetic tree of the deduced amino acid sequences of these and selected other symbiotic GRAS TF genes, specific groupings were evident (Supplementary Fig. S2). Interestingly, MtGRAS4 and MtGRAS7, two GRAS TFs belonging to the MIG1 family defined by45 share the highest sequence similarities, suggesting
a functional relationship although only *MtGras7* is specifically expressed in AM roots. In addition, the close relation of *MtRAM1* and *MtRAD1* is of special interest, since these TFs control arbuscule branching as well as arbuscule development and since mutual protein-protein interactions were reported.

AM-related GRAS TF genes are differentially upregulated in the course of mycorrhization. To reveal the timing of GRAS TF action during the development of an AM symbiosis, a gene expression time course study was performed (Fig. 1). Histological analyses of the harvested mycorrhizal roots indicated the predominant presence of *R. irregularis* spores and extraradical hyphae until 7 days post inoculation (dpi), while significant intraradical colonization and arbuscule development started from 10 dpi.

Since root material at 0 dpi already contained germinated *R. irregularis* spores, the AM fungal marker genes *GiTefa* (encoding a translation elongation factor alpha) and *GiaTub* (encoding an a-tubulin) are already expressed, whereas transcription of *MtPt4*, encoding an arbuscule-specific phosphate transporter, is only detected upon arbuscule presence. While the expression of *GiTefa* and *GiaTub* showed a linear increase over time, *MtPt4* activation rose almost exponentially, mirroring the quick and ongoing process of arbuscule build-up.

During mycorrhization, the transcriptional activation of *MtGras4* and *MtGras6*, being upregulated appr. 16-to 17-fold between 0 dpi and 42 dpi (Fig. 1) resembled that of *GiTub* and *GiTefa*, whereas *MtGras1*, *MtGras7*, *MtRad1*, and *MtRam1* (appr. 180- to 1200-fold upregulation from the time point of their first expression, Fig. 1) followed the strong rise in *MtPt4* transcription. These patterns indicate two different types of activation, with *MtGras4* and *MtGras6* being already markedly expressed at 0 dpi, probably due to their weak expression in non-mycorrhized roots (Medicago Gene Expression Atlas and), while the upregulation of *MtGras1*, *MtGras7*, *MtRad1*, and *MtRam1* follows the ongoing build-up of functional arbuscules in *MtPt4*-expressing cells.

AM-related GRAS TF genes differ in their dependency on the GRAS TF *MtRAM1*. To study the dependency of AM-related GRAS TF gene expression on *MtRAM1*, a GRAS TF required for arbuscule branching, comparative gene expression analyses were carried out in *R. irregularis*-mycorrhized roots of wild type plants and *ram1-1* mutants.

Whereas real-time RT-PCR measurements (Fig. 2) of mycorrhizal roots showed a strong decrease in fungal gene expression (*GiTefa*, *GiaTub*; down to 1–2%) as well as a complete lack of *MtRam1* and *MtPt4* transcription in *ram1-1* mutants, indicating the absence of highly branched, symbiotically active arbuscules, the reduction of GRAS TF gene transcription appeared diverging. While expression of *MtGras1* and *MtGras7* strongly decreased

**Figure 1.** Time course of AM-responsive gene expression. 28 days after germination, plants were mycorrhized and harvested at the time points indicated. Plants at 0 days post inoculation were harvested after 3 h of inoculation with *R. irregularis* spores. At each time point, three biological replicates were harvested, each consisting of 6 pooled root systems. Relative gene expression levels were determined by real-time RT-PCR. The standard error of the mean is indicated.

**Figure 2.** Relative expression of selected *MtGras* and AM marker genes in mycorrhized *M. truncatula* A17 wild type (white) and *ram1-1* (black) roots. Transcript amounts are shown relative to *MtTefα*. Roots were harvested at 36 days post inoculation with *R. irregularis*. *n* = 8 biological replicates, error bars represent standard deviations. Numbers indicate the percental expression level compared to the wild type. The following genes were analyzed in addition to the *MtGras* genes listed in Supplementary Table S1: *MtRam1*33, *MtPt4*, *GiTefa*69, *GiaTub*70, *MtRad1*42, and *MtMyb1*18. ***p < 0.001 (Student’s t-test).
to 0.3–0.6% of the level in wild type roots, transcription of \textit{MtRad1}, \textit{MtGras4}, and \textit{MtGras6} was less affected (reduction to 13–35%). A strongly decreased expression in \textit{ram1-1} mutants was also observed for the AM-specific \textit{MtMyb1} gene, encoding a key regulator of arbuscule degradation\textsuperscript{18}.

Since fungal colonization is impaired in \textit{ram1-1} mutants and since expression of AM-induced marker genes as well as \textit{MtGras1} and \textit{MtRad1} was lower in \textit{ram1-1} mutants in response to colonization by \textit{Glomus versiforme}\textsuperscript{33}, a reduced expression of the AM-induced GRAS TF genes studied here was expected. Thus, expression levels of the genes studied (Fig. 2) were divided by the \textit{GiTefa} transcription level in order to adjust gene expression to the amount of fungal tissue. This procedure revealed that lower transcription levels of \textit{MtGras1} (24.3%), \textit{MtGras7} (0.01%), and \textit{MtMyb1} (0.03%) were still evident in \textit{R. irregularis} colonized \textit{ram1-1} in comparison to wild type roots, indicating that the expression of these genes is not just reduced in \textit{ram1-1} mutants as a consequence of lower fungal colonization.

To achieve a cellular resolution of GRAS TF gene expression, their promoter regions were fused to the \textit{gusA} reporter gene, and the resulting transcriptional fusions were expressed in transgenic \textit{M. truncatula} roots. These studies demonstrated a clearly AM-induced promoter activity for \textit{MtGras1}, \textit{MtGras4}, \textit{MtGras6}, \textit{MtGras7}, and \textit{MtRad1} in wild type roots, with a predominant or exclusive activation in the arbuscule-containing cells (Fig. 3). In \textit{ram1-1} mutants, promoter activities of \textit{MtGras1} and \textit{MtGras7} were completely abolished, even after prolonged staining. In contrast, \textit{MtGras4}, \textit{MtGras6}, and \textit{MtRad1} promoters are still AM-induced in the \textit{ram1-1} mutant background. These findings were in line with our gene expression studies (Fig. 2) and suggested a position of MtGRAS1 and MtGRAS7 downstream of MTRAM1 in the regulatory cascade leading to arbuscule formation, while MtGRAS4, MtGRAS6, and MtRAD1 have to be placed either upstream or parallel to MTRAM1.

**Expression of AM-related GRAS TF genes differs in the dependency on morphologically fully developed arbuscules.** To study the dependency of GRAS TF gene expression on the presence of

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**Figure 3.** Histochemical localization of the promoter activities of selected \textit{MtGras} genes. The promoter activities of \textit{MtGras1} (A–D), \textit{MtGras4} (E–H), \textit{MtGras6} (I–L), \textit{MtGras7} (M–P), and \textit{MtRad1} (Q–T) were recorded in transgenic, mycorrhized roots of \textit{M. truncatula} A17 wild type (A,B,E,F,I,J,M,N,Q,R) and \textit{ram1-1} roots (C,D,G,H,K,L,O,P,S,T). GUS stainings were performed for 4–8 hours. Alexa WGA Fluor 488 stainings are shown to visualize fungal colonization.
mutants (Fig. 5). On the other hand, since the expression is not just reflecting a lower degree of colonization in comparison to wild type roots, respectively, indicating that this downregulation of a lower degree of colonization in pt4-2 roots.

Similar to the results for ram1-1 mutants (Fig. 3), the promoters of MtGras1 and MtGras7 are inactivated in pt4-2 roots, while the MtRad1, MtGras4, and MtGras6 promoters are still functional in root areas containing the typical prematurely degrading, stunted arbuscules that were regularly observed for pt4-2 mutants (Fig. 5). On the other hand, since the pt4-2 stunted arbuscule phenotype was not absolutely stable in our growth conditions, some infection units from pt4-2 roots, that in other areas showed typical premature arbuscule degeneration, occasionally developed WT-like arbuscules. Interestingly, wild-type-like expression of MtGras1 and MtGras7 promoter activities were now observed (Fig. 5Ba-h). This local phenomenon suggests that MtGras1 and MtGras7 activation is dependent on a particular stage of arbuscule development that, when modulated by endogenous or exogenous conditions, as e.g. demonstrated for N-starvation (Fig. 5); can alleviate the phenotype of premature arbuscule degeneration. It thus appears that MtGras1 and MtGras7 activation does not simply depend on the pt4-2 genotype, but on the existence of a symbiotic interface beneficial for the plant, enabling the development of mature arbuscules.

A summary of MtGras activity in ram1-1 and pt4-2 mutants is presented in Supplementary Figure S3. In both mutants, MtGras1 and MtGras7 expression is hardly detectable by real-time RT-PCR experiments or histological studies of promoter activity, while MtGras4, MtGras6, and MtRad1 expression is comparable to mycorrhizal wild type roots is observed. With respect to the results from our mutant studies, AM-related GRAS TF genes can thus be divided into two groups, being either MtRAM1- and MtPT4-dependent or -independent.

MtGRAS4 and MtGRAS7 form a regulatory module within the network of AM-related GRAS TFs. As one representative of an MtRAM1- and MtPT4-independent GRAS TF gene, MtGras4 was further characterized. To understand its role in AM formation, the Tnt1 transposon insertion line NF4813 was identified in the Medicago truncatula mutant database (Fig. 5A). Real-time RT-PCR measurements revealed strongly reduced levels of the MtGras4 transcript region up- or downstream of the Tnt1 insertion site, respectively (Fig. 5C,D). Among all tested GRAS TF and AM marker genes, the MtGras4 knockout line showed a reduced expression of MtGras7, both on the transcript level (to 22%, Fig. 6E) and the activity of the promoter (Fig. 6L), whereas transcription of all other AM-related GRAS TF genes was unchanged. On the phenotypical level, both the mycorrhization rate and the arbuscule size distribution are unchanged in MtGras4 knockout mutants (Fig. 6G). Complementation of the M. truncatula R108-based Tnt1 line with the M. truncatula A17 MtGras4 gene led to a restoration of MtGras7 expression (Fig. 6H), confirming that MtGras7, being strongly upregulated during later stages of mycorrhization (Fig. 1), is a direct or indirect target of MtGRAS4. Since MtGras4 expression was independent of MtRAM1 and MtPT4, we conclude that the MtGRAS4/MtGRAS7 regulatory module operates parallel to the formation of highly branched, functional arbuscules.

MtGras1 knockdown affects the expression of other AM-related GRAS TF genes. As an example for a GRAS TF gene dependent on MtRAM1 and MtPT4, MtGras1 was functionally studied in transgenic RNAi roots exhibiting an MtGras1 knockdown, since homozygous knockout lines were not available. Due to the fact that RT-PCR measurements indicated effects of an MtGras1 knockdown on AM-related gene expression, a global transcriptomics approach was pursued.

A comparative genome-wide gene expression study of R. irregularis-mycorrhized RNAi: MtGras1 and RNAi: gusA control roots identified 1020 genes that were at least 2-fold (p < 0.05) downregulated in MtGras1
Figure 5. Histochemical localization of the promoter activities of selected MtGras genes. The promoter activities of MtGras1 (A: a–s), MtGras4 (A: e–h), MtGras6 (A: i–l), MtGras7 (A: m–p), and MtRad1 (A: q–t) were recorded in transgenic, mycorrhized roots of M. truncatula A17 wild type (A: a,b,e,f,i,j,m,n,q, and r) and pt4-2 roots (c,d,g,h,k,l,o,p, and s). The promoter activities of MtGras1 (B: a–d) and MtGras7 (B: e–h) were furthermore recorded in transgenic, mycorrhized M. truncatula pt4-2 roots, showing a premature arbuscule degeneration (PAD; B: a,b,e,f) or Non-PAD phenotype (B: c,d,g,h). GUS stainings were performed for 4–8 hours. Alexa WGA Fluor 488 stainings are shown to visualize fungal colonization. Close-up views of PAD and Non-PAD arbuscules are shown inside red angles.
Figure 6. Molecular and phenotypical analysis of the MtGras4 Tnt1 insertion-carrying line NF4813. (A) Schematic illustration of the Tnt1 insertion site (red), primer position for genomic PCR-amplification (blue and red arrows), and position of real-time RT-PCR primers (orange) in the exon of MtGras4 (black). (B) Leaf disc multiplex PCR identification of homozygous, MtGras4 Tnt1 insertion-carrying NF4813 (Tnt1), MtGras4-segregating NF4813 wild type (sWT), and R108 wild type (WT) plants. A 50 bp DNA ladder shown as standard. A full-length gel is presented in Supplementary Fig. S4. (C,D) Relative expression of selected genes in homozygous MtGras4 Tnt1 mutant (black) and segregating wild type plants (white). Transcript amounts are shown relative to MtTefα expression. Roots were harvested at 35 dpi with R. irregularis. n = 8 biological replicates, error bars represent standard deviations. Numbers indicate the percental expression level compared to control roots. (F) Quantification of fungal structures in homozygous, MtGras4 Tnt1 mutant (black) and segregating wild type plants (white). Roots were harvested at 42 dpi with R. irregularis. Root systems were grouped into four biological replicates each containing a pool of four roots. Standard deviations are indicated as error bars. EH = External hyphae only; IH = Internal Hyphae only; Arb = arbuscules; V = Vesicles (no arbuscules). (G) Distribution of arbuscule sizes in mycorrhized homozygous MtGras4 Tnt1 mutant (black) and segregating wild type plants (grey). Roots were harvested at 42 dpi with R. irregularis. Root systems were grouped into four biological replicates each containing a pool of two roots. Standard deviations are indicated as error bars. (H) Quantification of MtGras4, MtGras7, and MtPt4 in a homozygous MtGras4 Tnt1 mutant complemented with a −1206/+2086 genomic region of the M. truncatula A17 MtGras4 gene (black), and empty vector control roots (white). N = 6 biological replicates, error bars represent standard deviations. Numbers indicate percental
expression levels compared to control roots. (I) Comparison of pMtGras7-gusAint activity in mycorrhizal roots of homozygous MtGras4 Tnt1 mutant and the corresponding segregating wild type (sWT) plants. GUS stainings were performed for 8 hours. Alexa WGA Fluor 488 stainings visualize AM fungal colonization. ***p < 0.001 (Student's t-test).

knockdown roots, indicating the potential of MtGRAS1 to participate in the regulation of gene expression in AM (Supplementary Table S2). A selection of MtGnas genes differentially expressed in the MtGras1 knockout roots is shown in Fig. 7. In line with initial real-time RT-PCR measurements, the RNA interference construct led to a reduction of MtGras1 expression to 29% of the wild type level. This 71% MtGras1 knockdown was able to reduce transcription of the AM-related GRAS TF genes MtGras6, MtGras7, MtRat1, MtRam1, and also MtTF124, while expression of the fungal marker gene GfTefa as well as the M. truncatula MtGras4, MtPt4, and MtMyb1 genes were not significantly affected (Fig. 7).

Especially the only slight, non-significant (p = 0.11; Fig. 7, Supplementary Table S2) reduction of MtPt4 expression in MtGras1 knockout roots indicates that the formation of active, phosphate-transporting arbuscules is not impaired by a reduced MtGras1 expression. This was also true for the transcription of the MtMyb1 gene, encoding a transcription factor controlling arbuscule degeneration18, which is not reduced in MtGras1 knockout roots (Fig. 7). This indicates that MtGRAS1 does not participate in the initiation of arbuscule degradation, e.g. by activating MtMyb1.

Since MtRAM1 was shown to be required for MtGras1 activation33, it is intriguing that MtRam1 expression is downregulated to 24% in mycorrhized MtGras1 knockdown roots (Fig. 7). This finding suggests that similar to the observation for MtRAM1 and MtWRI5a in the regulation of fatty acid biosynthesis39, MtRAM1 and MtGRAS1 are part of a regulatory feedback loop that maximizes MtRam1 transcription and in this case thus might enhance AM-correlated gene expression.

The transcription profile of MtGras1 knockdown roots differs substantially from that of ram1-1 mutants. Facing the large number of genes downregulated in MtGras1 knockdown roots (Supplementary Table S2), the question arose, to what extent these patterns of transcriptional changes resemble those in an MtRam1 knockout. To study this, genome-wide expression was recorded in wild type vs. ram1-1 roots. This experiment revealed that the expression of 836 genes were at least 2-fold lower in ram1-1 knockout roots, including several marker genes for arbuscule function (e.g. MtPt4, being transcribed at a 689-fold lower level, Supplementary Tables S3 and S4). Although a limited set of 250 genes is at least 2-fold lower expressed (p < 0.05) in RNAi:MtGras1 as well as in ram1-1 roots (Fig. 7B), the major pattern of gene expression regulation is characteristic of either the MtGras1 knockdown or the ram1-1 knockout. Specifically, when looking into the detailed pattern of regulation of 31 well-defined AM marker genes (Supplementary Table S4, Fig. 7C), it is evident that most of these are either only (e.g. MtMyb1) or much stronger (e.g. MtPt4) downregulated in ram1-1 mutants, indicating that the core gene expression program activated in arbuscule-containing cells is strongly affected in ram1-1 mutants, but not or only moderately in the MtGras1 knockdown roots. This finding suggests that although MtGras1 expression depends on MtRam133 (Figs 2, 3), MtGRAS1 is not simply a direct target of MtRAM1 to activate downstream genes related to arbuscule formation and function.

MtGras1 overexpression does not activate other AM-related GRAS TF genes. The massive transcriptional effects resulting from an MtGras1 knockdown prompted us to investigate the effect of MtGras1 overexpression in transgenic M. truncatula roots. In these experiments, the arbuscule-specific MtPt4 and the general ubiquitin3 promoters were used to drive MtGras1 expression in mycorrhized and non-mycorrhized roots, respectively. Although a 7.4- and 1523-fold MtGras1-overexpression was achieved, respectively, leading to comparable amounts of MtGras1 transcripts in mycorrhized and uninoculated roots (Supplementary Fig. S5), no activation of other GRAS TF or AM marker genes such as MtPt4 was detected (Supplementary Fig. S5), suggesting that MtGRAS1 is not only a terminal position of a regulatory cascade or requires co-expressed interaction partners to activate transcription of target genes.

MtGras1 knockdown roots show a trend towards the development of smaller arbuscules. To address the question, whether an MtGras1 knockdown influences the fungal colonization of roots or the maturation of arbuscules, phenotypical studies were performed in comparison to control roots.

While no changes in the mycorrhization rate or the frequencies of arbuscules and vesicles were observed (Fig. 7D) and the arbuscule length measurements of mycorrhized RNAi:MtGras1 in comparison to RNAi:GusAint control roots indicated a shift in the distribution of arbuscule sizes (Fig. 7F). Specifically, MtGras1 knockdown roots tend to contain a higher proportion of smaller and a lower proportion of large arbuscules, suggesting a delayed or less sustained arbuscule development. In line with the fact that the expression of most marker genes for arbuscule formation and function is not markedly affected in mycorrhized MtGras1 knockdown roots (Fig. 7B–C), this effect nevertheless appears subtle and is probably part of a fine-tuning of the arbuscule life-cycle.

Discussion

Colonization of roots by AM fungi ultimately leads to the formation of intracellular arbuscules, functioning as a nutrient exchange interface between plant cells and fungal hyphae. Arbuscule development requires a fundamental transcriptional reprogramming of root cortical cells44–49,55, being governed by a suite of AM-activated regulators, including several GRAS TFs39,33,34,36,40,42,47,55.
We show here that the five AM-activated GRAS TF genes MtGras1, MtGras4, MtGras6, MtGras7, and MtRad1 can be classified based on their dependency on MtRAM1, a key transcription factor controlling arbuscule branching, and MtPT4, the major AM-specific phosphate transporter. While MtGras4, MtGras6, and MtRad1 are still expressed in the absence of MtRam1 and MtPt4, MtGras1 and MtGras7 transcription is abolished in the ram1-1 and pt4-2 mutants (Figs 2–5). In our growth conditions, pt4-2 mutants occasionally developed not only prematurely decaying but also apparently vital arbuscules (Fig. 5B). This phenomenon demonstrates the dependency of MtGras1 and MtGras7 transcription not on the genotype, but on the presence of fully developed arbuscules. Obviously, prematurely decaying arbuscules do not reach the phase of MtGras1 and MtGras7 expression, while fully developed arbuscules do.

Figure 7. Molecular and phenotypical effects of an MtGras1 knockdown in mycorrhizal roots. (A) Expression of MtGras1 and selected AM marker genes in transgenic, mycorrhized RNAi: MtGras1 and RNAi: gusA control roots. Roots were harvested at 35 days post inoculation with R. irregularis. Medicago Transcriptome Assay hybridizations were performed, using three biological replicates per experimental group. The standard error of the mean is shown. Percental values shown are based on the calculated linear signal intensities. Genes shown are defined in the Fig. 2 legend. (B) Transcriptional response of M. truncatula ram1-1 and MtGras1 RNAi roots to colonization with R. irregularis. Comparison of gene expression in M. truncatula ram1-1 mutant as well as MtGras1 RNAi roots in relation to control roots. Numbers indicate genes downregulated at least 2-fold (p < 0.05) in the ram1-1 mutant line or the MtGras1 RNAi knock-down roots. (C) Comparative visualization of fold changes in ram1-1 (blue) and MtGras1 RNAi (yellow) roots in comparison to the corresponding control roots. A selection of 31 well-characterized AM-induced marker genes, downregulated at least 2-fold (p < 0.05) in relation to control roots were compared in both mutants. The expression data and identifiers corresponding to the genes studied are listed in Supplementary Table S4. (D) Quantification of fungal structures in MtGras1 RNAi (black) and control roots (white). Roots were harvested at 35 days after inoculation with R. irregularis. Standard errors are indicated as error bars. MYC, colonized root fragments; ARB, arbuscules; VES, vesicles. (E) Alexa WGA Fluor 488 stained mycorrhizal MtGras1 RNAi (I) and control roots (II). (F) Distribution of arbuscule sizes in MtGras1 RNAi (black) and control roots (grey). Sizes were measured for nine biological replicates of independent MtGras1 RNAi, and control root systems, respectively (appr. 3000 arbuscules in total for each group). Roots were harvested at 35 days post inoculation with R. irregularis. Bars represent standard errors. *p < 0.05; **p < 0.01 (Student's t-test).
While analyses of MtGras7 and MtGras4 upregulation during the AM time course revealed different patterns (Fig. 1). MtPT4-dependency of MtGras7 expression was already detectable from the onset of fungal colonization. In line with the MtRAM1- and MtGras4 stages of arbuscule development, when maturation already took place. In this respect, the dependency of MtGras7 on the MtRAM1- and MtPT4-independent GRAS TF MtGras4 is even more remarkable, since it demonstrated a connection between more early and MtRAM1-independent (MtGras4) and more later, MtRAM1-dependent stages (MtGras7) of arbuscule formation. The somewhat outstanding position of the MtGras4/MtGras7 module within the GRAS TF network is also reflected on the sequence level, where MtGras4 and MtGras7 share a common clade, being isolated from the rest of the GRAS TFs investigated (Supplementary Fig. S2).

In spite of its dependency on MtRAM1 and MtPT4 (Supplementary Fig. S3), MtGraS1 was also found to regulate components placed more upstream of arbuscule development, namely MtGras6, MtRad1, and remarkably also MtRam1 (Fig. 7A). In addition, the AM-related GRAS TF gene and MtRam1-homologue MtTj124 appears downregulated in MtGras1 knockdown roots (Supplementary Table S4). Due to the fact that MtGraS1 regulates MtRam1 expression (Fig. 7A), while MtRam1 on the other hand is required for MtGras1 activation (Figs 2 and 3), we propose the existence of a regulatory feedback loop, where MtGraS1 enhances MtRam1 transcription, thus stimulating its own activation as well as the expression of MtRAM1-regulated target genes, ultimately leading to the establishment of a functional, highly branched arbuscule (Fig. 8). Since it was demonstrated that MtRam1 is not able to directly stimulate MtGras1 expression, the activation of MtGraS1 thus has to be dependent on additional, MtRAM1-dependent regulators. It is likely that currently unknown mediators also have to be assumed for most of the relationships shown in the regulatory network visualized in Fig. 8.

Interestingly, MtGraS1 expression was downregulated in pt4-2 mutants colonized by Rhizophagus irregularis (Figs 4, 5) and in pt4-1 mutants mycorrhizized with Gigaspora gigantea, indicating that the encoded GRAS TF acts downstream of the formation of functional arbuscules. It is thus tempting to hypothesize that MtGraS1 acts as a checkpoint at a certain point of the later stages of the arbuscule life cycle, where the initial signaling for arbuscule development and branching via MtRAM1 is completed, and the morphological expansion of the functional, phosphate-transporting fungal interface needs to be accelerated or sustained. The slight shift towards the formation of smaller arbuscules observed in MtGras1 RNAi roots (Fig. 7D–F) might thus be caused by the lack of MtGras1-activated MtRam1 transcription (Fig. 7A), which would lead to a slower or less sustained, MtRam1-controlled, arbuscule branching. The effect on the arbuscule size distribution is nevertheless small and possibly also suffers from the non-synchronized mycorrhization process, which masks subtle differences in arbuscule sizes.

On the protein level, MtGraS1 was shown to interact with MtRam1 in yeast two-hybrid assays, indicating a joint function of these transcription factors. However, we were able to show that an RNAi-mediated knockdown of MtGraS1 leads to a different molecular phenotype than a knockout of MtRam1. Although there are overlaps in the genes regulated by MtGraS1 and MtRam1, ram1-1 mutants show a much stronger downregulation of AM-induced genes characteristic of the presence of functional arbuscules and a strong effect on arbuscule branching, which is not the case for MtGraS1 RNAi roots (Fig. 7A). The different transcription pattern of mycorrhized MtGraS1 RNAi roots and mycorrhized ram1-1 mutants is thus in line with a modifying function of MtGraS1 during the establishment of a functional arbuscule interface. Although these comparative

![Figure 8. Model of the regulatory circuit of AM-related GRAS TFs evolving arbuscule development.](image-url)
transcription patterns (Fig. 7B) were derived from studies of knockout mutants (MtRam1) and transgenic knockdown roots (MtGras1), the fundamentally different expression pattern observed in ram1-1 and MtGras1 RNAi roots most likely excludes that MtGRAS1 itself acts as a key independent regulator of arbuscle formation, downstream of MtRAM1. An additional hint that MtGRAS1 solely is unable to activate AM-related genes derives from our overexpression experiments, where an upregulated MtGras1 transcription had no effect on selected target genes, probably since MtGRAS1 requires an interaction partner such as MtRAM1.33

In addition to the more upstream components regulating arbuscle formation, MtGRAS1 also regulates MtGras7 (Fig. 7A), which is also MtRAM1- and MtPT4-dependent and is thus located at a similar position relative to the formation of mature arbuscles. Incidentally, the downregulation of MtGras7 in MtGras1 knockdown roots might explain why MtGras7 expression is reduced but still detectable in the MtGras4 insertion mutant (that still expresses MtGras1, Fig. 6).

The effect of MtGras1 on MtGras7 transcription implies that MtGras7 can be activated by both the AM-related MtGras4 and the AM-specific MtGras1 TF in an independent manner, suggesting that MtRAM1-/MtPT4-dependent (MtGRAS1 and its targets including MtGras7) and -independent regulatory modules (MtGras4 and its target MtGras7) are connected. The complex network of AM-related and AM-specific GRAS TFs might thus contribute to the balanced expression of AM-related genes at the arbuscle interface, where different physiological and nutritional conditions have to be integrated.

Conclusions
Relying on gene expression and histological studies in symbiotic mutants defective in arbuscle branching or in the formation of phosphate-transporting arbuscles as well as on functional analyses in transgenic knockdown roots or knockout lines, we provide evidence that the network of AM-activated M. truncatula GRAS TFs consists of interconnected modules, including an MtRAM1-MtGRAS1 regulatory feedback loop. MtGras1 knockdown roots displayed normal colonization by AM fungi, but a trend towards the formation of smaller arbuscles was apparent. Although mutants in the AM-specific GRAS TFs RAM1 and RAD1 display more severe AM-related phenotypes in M. truncatula and L. japonica53,34,36,42, our investigation on additional AM-activated MtGras genes provides evidence that a regulatory circuit of multiple GRAS TFs, showing differential dependencies on arbuscle branching and the formation of phosphate-transporting arbuscles, governs and sustains arbuscule development.

We propose that this regulatory circuit allows a flexible response of the symbiotic interface towards the external (e.g. nutrient availability50,53; and internal (e.g. plant hormones20,41) stimuli that together influence and adapt the arbuscule life cycle under changing conditions.

Methods
Cloning of promoter-gusAint fusions and histological analyses. Promoter sequences of MtGras genes were amplified from genomic DNA of M. truncatula using oligonucleotides specified in Supplementary Table S5. PCR-fragments were cloned into pGUS-INT56, in front of the gusAint reporter gene cassette. The resulting transcriptional fusions were released using Sphi and subcloned into the SmaI-digested binary vector pRed-Root15, after fill-in of 5′ overhangs using the Klenow fragment.

GUS assays were performed by incubating roots in GUS staining buffer56 for 4 to 8 hours at 37 °C, if not stated differently. Counterstaining of fungal material was performed using Alexa WGA Fluor 488 (Thermo Fisher Scientific, Langenselbold, Germany), as described above.

Cloning of knock-down and overexpression constructs. An RNAi construct for MtGras1 was generated by amplification and recombination of a 379bp long fragment of the MtGras1 coding region into pDON-R™221 (Gateway®-System, Invitrogen, Karlsruhe, Germany) via the BP-, and subsequently into the binary vector pK7GWKGW2(II)-Q10:DsRED58 via the LR-reaction. Specificity of the MtGras1 RNAi target sequence was verified via BLAST searches against the current release of the M. truncatula genome51 and by using the siFi software that predicts potential off-targets59.

MtGras1 overexpression constructs were generated by PCR-amplification and cloning of the MtGras1 coding sequence into the vectors 315p9RFP-Pt4-Expr and 917p9RFP-ubi3-Expr15, containing either the M. truncatula MtPt4F or the A. thaliana ubiquitin3 3 promoter (AtUbq360), respectively.

Plant growth, inoculation with AM fungi and staining of fungal structures. Medicago truncatula Gaertn cv Jemalong genotype A17 seeds were surface-sterilized and scarified as reported53. Plants were grown in the climate chamber (relative humidity: 60%; photosynthetic photon flux: 150 μmol m−2 s−1). MtGras1 overexpression experiments were derived from studies of knockout mutants (MtRam1) and transgenic knockdown roots (MtGras1), the fundamentally different expression pattern observed in ram1-1 and MtGras1 RNAi roots most likely excludes that MtGRAS1 itself acts as a key independent regulator of arbuscle formation, downstream of MtRAM1. An additional hint that MtGRAS1 solely is unable to activate AM-related genes derives from our overexpression experiments, where an upregulated MtGras1 transcription had no effect on selected target genes, probably since MtGRAS1 requires an interaction partner such as MtRAM1.33

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Transgenic roots were generated by Agrobacterium rhizogenes ARQua1 mediated transformation of M. truncatula seedlings according to62. Bacteria were grown for two days at 30 °C on selective TY (0.5 g/l tryptone; 0.3 g/l yeast extract; 0.07 g/l CaCl2 × 2H2O) agar plates. Cells were resuspended in 10 ml PS buffer (40 mM Na2HPO4 × 2H2O, 85 mM NaCl, 17 mM KH2PO4, pH 7). The agrobacterium solution was injected into the hypocotyl using a syringe. Seedlings were planted into sterile Seramis® (Seramis GmbH, Mogendorf, Germany), incubated at 18 °C in the dark for 16 hours and were then transferred into a phytocabinet. To detect transgenic roots, plants were screened after 4 weeks for dsRed expression using a stereomicroscope (Leica MZ 10 F, Leica Microsystems, Wetzlar, Germany).
After four weeks of growth (or four weeks after induction of transgenic roots), each plantlet or composite plant was mycorrhized by inoculation in a small amount of water with 2000 spores of germinating *Glomus intraradices* Schenck and Smith DAOM197198 spores (Premier Tech Biotechnologies, Rivière-de-Loup, Québec, Canada), having been reassigned to *Rhizophagus irregularis* (Blaszk., Wubet, Renker, and Buscot) C. Walker & A. Schüßler comb. nov.63. After 3–4 hours of inoculation, plantlets were potted into 8 × 7 × 7 cm (height × width × depth) pots filled with sterile Seramis® (Seramis GmbH, Mogendorf, Germany). Each pot contained two plants, and remaining spore solution was directly pipetted onto the root surface while potting. Mycorrhizal and non-mycorrhizal plants were fertilized with half-strength Hoagland’s solution44 containing 20 μM phosphate. The solution was prepared with deionized water, pH was adjusted to 6.4 with KOH.

To visualize fungal colonization, roots were incubated in 10% (w/v) KOH at 95 °C for 7 min, repeatedly rinsed with water and incubated in 1X PBS buffer (0.14 M NaCl, 2.7 mM KCl, 1 mM NaHPO4, 2H2O, 1.8 mM KH2PO4; pH 7.3) containing 20 μg/ml Alexa WGA Fluor™ 488 (Thermo Fisher Scientific, Langenselbold, Germany) conjugate overnight. Photo documentation was performed using a Leica MZ 10 F® stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with an Olympus XC50 camera (Olympus, Hamburg, Germany), a Zeiss Axio Observer Z1 microscope equipped with an AxioCam ICc1 (Carl Zeiss AG, Oberkochen, Germany), and a confocal microscope (Leica TCS SP8 MP, Sohns, Germany). Quantification of fungal colonization was performed using the gridline intersection method65. For arbuscule size determination, confocal images of arbuscules were analyzed using the Fiji software66. Nine independent roots or four independent pools of two individual roots each were used to determine arbuscule sizes, based on the procedure described by66 and13.

To study gene expression in transgenic *M. truncata* A17 roots expressing an MtGras1 RNAi construct (RNAi: MtGras1 roots) in comparison to a gusAint gene (RNAi:gusAint control roots), composite plants were mycorrhized with *R. irregularis* spores as described above and harvested after 35 dpi. To analyse gene expression in *M. truncata* ram1-1 roots in comparison to *M. truncata* A17 control roots, roots were mycorrhized with *R. irregularis* spores as described above and harvested after 35 dpi. In all experiments, harvesting time points were selected depending on the mycorrhization rate in the different mutants, in order to obtain sufficient and comparable colonization levels.

**Analysis of the Tnt1 insertion line NF4813.** The *Tnt1* insertion line NF4813 (based on *M. truncata* R108), harbouring a *Tnt1* insertion in the exon of *MtGras4* after position +1428, was obtained from the Noble Research Institute (Ardmore, Oklahoma, USA). No stable, homozygous *Tnt1* lines could be obtained for other candidate genes. Plants were screened for the *Tnt1* insertion via direct PCR from leaf discs using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific, Langenselbold, Germany). The PCR was performed using a *Tnt1* binding (*Tnt1*-F) and two gene-specific primers (NF4813_16_for, NF4813_16_rev). Homozygous plants were selected, selfed, and used for seed propagation. Segregating plants being wild type with respect to the *MtGras4* locus were propagated to obtain control plants.

**RNA isolation and real-time RT-PCR.** RNA was isolated using the RNaseasy Plant Mini Kit (Qiagen, Hilden, Germany). Tissue disruption was carried out via FastPrep®—24 (MP Biomedicals, Santa Ana, USA). Real-time RT-PCR analyses were performed using the SensiFAST™ SYBR® No-ROX One-Step Kit (Qiagen, Hilden, Germany), using primers listed in Supplementary Table S6. Primers were tested for specificity before use. 5 ng of total RNA were used as a template in a 20 μl reaction. RT-PCR reactions followed a three-step cycling program: Reverse transcription at 45 °C for 10 min; polymerase activation at 95 °C for 2 min; PCR amplification with 40 cycles at 95 °C for 5 sec, 55 °C for 10 sec, and 72 °C for 8 sec. The housekeeping gene *MtTefa* (Medtr6g021805.1 in the *M. truncata* genome) encoding a translation elongation factor was used for normalization. Each biological replicate was measured in three technical replicates. Average values were used to calculate gene expression levels via the 2−ΔΔCT method with ∆ΔCT = CTgene − CTMtTefa. Statistical significances were calculated using a two-tailed Student’s t-test in MS Excel 2016 (Microsoft Corp., Redmond, Washington, USA).

**Hybridization and data evaluation of GeneChip® Medicago Transcriptome Assays.** Biotinylated aRNA obtained from 100 ng of total RNA for each sample was fragmented as recommended (GeneChip® Medicago Transcriptome Assay, ThermoFisher Scientific, Schwerte, Germany). The size distribution of the fragmented aRNA was assessed via an Agilent bioanalyzer (Agilent Technologies, Böblingen, Germany) using an RNA 6000 assay. Standard hybridization, post-hybridization wash and double-staining as well as scanning was done as specified for GeneChip® Medicago Transcriptome Assays (ThermoFisher Scientific, Schwerte, Germany).

Cel files were analyzed using the Expression Console and Transcriptome Analysis Console software (both ThermoFisher Scientific, Schwerte, Germany). Normalization was performed via the Robust Multi-chip Average algorithm, intensity values for each probe set were log2-transformed and averaged across the three biological replicates using the Tukey’s Bi-weight average algorithm, and expression ratios were evaluated statistically via tools of the Transcriptome Analysis Console (ThermoFisher Scientific, Schwerte, Germany).

Original annotations of the genes represented on the GeneChip® Medicago Transcriptome Assays were updated by annotations from the *M. truncata* genome version 4.045, and mapped to probe sets from the GeneChip *Medicago* Genome Expression Atlas52 as well as to UniProt67. Venn diagrams were drawn using the VENNY software67.

**Data Availability**

All data generated or analysed during this study are included in this published article and its supplementary information files. In addition, GeneChip® *Medicago* Transcriptome Assay data are deposited in the Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/, accession number GSE108867).
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Author Contributions
R.M.H. did in silico analyses, performed gene expression studies, carried out RNAi and overexpression experiments, phenotyped transgenic RNAi and insertion mutant roots, and drafted the manuscript. S.S. cloned an MtGras1 RNAi construct and demonstrated its knockdown efficiency. R.M.H., D.N., A.P. and M.B. cloned promoter-reporter gene fusions and performed histochemical studies in transgenic roots. J.V. participated in real-time RT-PCR experiments. H.K. and N.H. initiated and supervised the project, participated in the interpretation of experimental results, reviewed and edited the manuscript.

Additional Information
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