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Transcriptional Regulation of Arbuscular Mycorrhiza Development

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Arbuscular mycorrhiza (AM) is an ancient symbiosis between land plants and fungi of the glomeromycotina that is widespread in the plant kingdom. AM improves plant nutrition, stress resistance and general plant performance, and thus represents a promising addition to sustainable agricultural practices. In return for delivering mineral nutrients, the obligate biotrophic AM fungi receive up to 20% of the photosynthetically fixed carbon from the plant. AM fungi colonize the inside of roots and form highly branched tree-shaped structures, called arbuscules, in cortex cells. The pair of the arbuscule and its host cell is considered the central functional unit of the symbiosis as it mediates the bidirectional nutrient exchange between the symbionts. The development and spread of AM fungi within the root is predominantly under the control of the host plant and depends on its developmental and physiological status. Intracellular accommodation of fungal structures is enabled by the remarkable plasticity of plant cells, which undergo drastic subcellular rearrangements. These are promoted and accompanied by cell-autonomous transcriptional reprogramming. AM development can be dissected into distinct stages using plant mutants. Progress in the application of laser dissection technology has allowed the assignment of transcriptional responses to specific stages and cell types. The first transcription factors controlling AM-specific gene expression and AM development have been discovered, and *cis*-elements required for AM-responsive promoter activity have been identified. An understanding of their connectivity and elucidation of transcriptional networks orchestrating AM development can be expected in the near future.

Keywords: Cell plasticity • *cis*-element • Root • Symbiosis • Transcription factor • Transcriptomics.

Abbreviations: AM, arbuscular mycorrhiza; AMT, ammonium transporter; Cbf, CAAT-box transcription factor; CCa MK, calcium and calmodulin-dependent kinase; CO, chitin oligosaccharide; CoIP, co-immunoprecipitation; DIP1, DELLA INTERACTING PROTEIN; DIS, DISORGANIZED ARBUSCULE; DMI, DOES NOT MAKE INFECTIONS; ERF, ETHYLENE RESPONSE FACTOR; GID1, GIBBERELLIN INSENSITIVE DWARF 1; IDD, INDERTERMINATE DOMAIN; JKD, JACKDAW; LCO, lipochitooligosaccharide; LOM, LOST MERISTEMS; MAMI, MERISTEM AND MYCORRHIZA INDUCED; MIG1, MYCORRHIZA INDUCED GRAS 1; NARK, NODULATION AUTOREGULATION RECEPTOR KINASE; NSP, NODULATION SIGNALING PATHWAY; NUP, NUCLEOPORIN; PAC, paclobutrazol; PAM, peri-arbuscular membrane; PAS, peri-arbuscular space; PPA, pre-penetration apparatus; PT, phosphate transporter; RAD1, REQUIRED FOR ARBUSCULE DEVELOPMENT 1; RAM, REDUCED ARBUSCULAR MYCORRHIZA; SCR, SCARECROW; SHR, SHORT ROOT; STR, STUNTED ARBUSCULE; SYMRK, SYMBIOSIS RECEPTOR KINASE; WRI5b, WRINKLED 5b; Y2H, yeast-two hybrid.

Introduction

Arbuscular mycorrhiza (AM) is a symbiosis between the majority of land plants and fungi of the monophyletic glomeromycotina (Parniske 2008, Spatafora et al. 2016). AM enhances mineral nutrient acquisition, mainly phosphate, from soils. The nutrients are collected by a fungal extraradical hyphal network, which develops around the root. They are transported into the root and released at highly branched fungal structures, the arbuscules, which form inside root cortex cells (Smith and Smith 2011, Garcia et al. 2016). In return, plants provide up to 20% of their photosynthetically fixed carbon in the form of hexoses and lipids to the obligate biotrophic fungi (Shachar-Hill et al. 1995, Pfeffer et al. 1999, Bago et al. 2000, Helber et al. 2011, Bravo et al. 2017, Jiang et al. 2017, Keymer et al. 2017, Luginbuehl et al. 2017). In addition to nutrient supply, AM also increases plant resistance to certain pathogens and to abiotic stresses such as drought, salinity or heavy metals, thereby enhancing the overall fitness of the host plant (Augé 2001, Ruiz-Lozano 2003, Göhre and Paszkowski 2006, Liu et al. 2007). AM is an essential component of ecosystems as it contributes to biodiversity and ecosystem productivity (Janos 1980, Van Der Heijden et al. 1998). It also participates in the global carbon cycle by sequestering atmospheric carbon dioxide fixed by the host plant in the soil, thereby increasing soil organic carbon pools (Bago et al. 2000, Rillig et al. 2001).

Root colonization by AM fungi is tightly regulated by the plant depending on its physiological and developmental status (Carbonnel and Gutjahr 2014, Gutjahr 2014). It can be dissected into several steps by plant mutants which are perturbed in AM development (Gutjahr and Parniske 2013, McLean et al. 2017).

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Prior to colonization, the symbionts reciprocally exchange diffusible signaling molecules (Nadal and Paszkowski 2013). Plants, which grow under low phosphate or nitrogen conditions, exude strigolactones from their roots into the rhizosphere (Yoneyama et al. 2007). These are recognized by AM fungi and induce spore germination, and enhance fungal metabolic activity, hyphal growth and branching, and exudation of short chain chitin oligosaccharides (chitotetraose and chitopentaose) (Akiyama et al. 2005, Besserer et al. 2006, Besserer et al. 2008, Genre et al. 2013). Along with chitin oligosaccharides (Myc-COs) AM fungi also secrete lipochitooligosaccharides (Myc-LCOs) (Maillet et al. 2011). Myc-COs and Myc-LCOs are collectively called 'Myc factors' and trigger plant symbiotic responses, such as transcriptional activation of plant genes, nuclear calcium spiking in rhizodermal cells, starch accumulation and lateral root formation (Kosuta et al. 2003, Olah et al. 2005, Kosuta et al. 2008, Gutjahr et al. 2009, Kuhn et al. 2010, Mukherjee and Ané 2010, Chabaud et al. 2011, Maillet et al. 2011, Genre et al. 2013, Sun et al. 2015). The definition of 'Myc factors' is derived from the 'Nod factors', which are exuded by nitrogen-fixing rhizobia that form root nodule symbioses with legumes (Oldroyd 2013). Equivalent to the Nod factors, the Myc factors activate the so-called common symbiosis signaling cascade, which is required for AM formation as well as nodulation (Oldroyd 2013). Upon physical contact with the host root, the fungal hyphae differentiate into attachment structures called hyphopodia (Bonfante and Genre 2010). Following hyphopodium formation, fungal hyphae penetrate the epidermis and grow towards the inner cortex. In the most commonly studied *Arum*-type symbiosis, the hyphae mostly grow intercellularly and infrequently also across cells (Harrison 2005). Furthermore, the fungal hyphae spread longitudinally in the cortical apoplast and form highly branched tree-shaped structures called arbuscules inside cortical cells. Arbuscules and their host cells are the primary site of mineral nutrient exchange between the two symbionts (Luginbuehl and Oldroyd 2017, McLean et al. 2017). Some fungal species mostly belonging to the Glomerales such as the widely used model fungus *Rhizophagus irregularis* also form vesicles—ballon-shaped structures, which are filled with lipids (Schüβler et al. 2001).

To accommodate the fungal structures, the host cell undergoes drastic subcellular changes. Prior to fungal entry into the epidermis, the host cell forms a so-called pre-penetration apparatus (PPA), a cytoplasmic bridge across the vacuole, which is surrounded by endoplasmic reticulum, cytoskeleton and plasma membrane (Genre et al. 2005). PPA formation is preceded and accompanied by nuclear movements and nuclear calcium spiking (Genre et al. 2005, Sieberer et al. 2012). Once the fungus enters the PPA, the frequency of calcium spiking increases. Simultaneously, the underlying outer cortical cell already responds with low frequency nuclear calcium spiking, nuclear movement and cytoplasmic aggregation probably to prepare for the passage of the fungal hypha (Sieberer et al. 2012). Thus, during symplastic growth, the fungal hyphae are guided from cell to cell by intracellular structures that resemble the PPA (Sieberer et al. 2012). PPA formation is also observed during arbuscule development in cortical cells (Genre et al. 2008). The developing arbuscules are continuously surrounded by a plant-derived membrane, called the peri-arbuscular membrane (PAM) (Gutjahr and Parniske 2013), which prevents direct contact of the fungal hyphae with the plant cytoplasm and serves as the interface for nutrient exchange between the symbionts (Luginbuehl and Oldroyd 2017, McLean et al. 2017).

The protein composition of the PAM differs from that of the peripheral plasma membrane as it harbors several proteins such as transporters, which have so far been exclusively observed in the PAM (Harrison et al. 2002, Kobae and Hata 2010, Kobae et al. 2010, Zhang et al. 2010, Breuillin-Sessoms et al. 2015). Accumulating evidence indicates a predominant role for exocytosis in the construction of the PAM and delivery of PAM-resident proteins (reviewed in Harrison and Ivanov 2017). The space between the fungal plasma membrane and the PAM creates an isolated apoplastic compartment called the peri-arbuscular space (PAS) (Bonfante and Perotto 1995).

The development of arbuscule-containing cells is accompanied by the fragmentation of the large central vacuole into a tubular network (Cox and Sanders 1974, Scannerini and Bonfante-Fasolo 1983, Toth and Miller 1984, Gianinazzi-Pearson 1996, Pumplin and Harrison 2009). The cytoskeleton reorganizes extensively not only in arbuscule-containing cells but—possibly as a symptom of PPA formation—also in cells adjacent to them or cells in contact with intercellular hyphae (Genre and Bonfante 1998, Blancaflor et al. 2001, Genre et al. 2008). Actin filaments and microtubules form a dense array around the arbuscule branches (Carling and Brown 1982, Balestrini et al. 1992, Blancaflor et al. 2001), which may be essential for fragmentation of the vacuole, nuclear movement, vesicle trafficking and membrane protein localization. Plastids and mitochondria increase in number and assemble around the arbuscule (Fester et al. 2001, Hans et al. 2004, Lohse et al. 2005). The nucleus moves from the cell periphery to the center of the branched arbuscule. It increases in size, which is interpreted as a sign of endoreduplication and/or chromatin de-condensation, needed for the massive transcriptional reprogramming, which is observed prior to and during arbuscule formation (Balestrini et al. 1992, Fusconi et al. 2005, Genre et al. 2008, Bainard et al. 2011, Gaude et al. 2012, Ivanov and Harrison 2014).

AM Development is Accompanied by Largely Stage-Specific Transcriptional Changes

Root colonization by AM fungi is accompanied by drastic local as well as systemic transcriptional changes (see, for example, Liu et al. 2003, Güimil et al. 2005, Hohnjec et al. 2005, Liu et al. 2007, Fiorilli et al. 2009, Guether et al. 2009, Gaude et al. 2012, Schaarschmidt et al. 2013, Handa et al. 2015). The major portion of activated genes is involved in signaling, transcriptional regulation, protein synthesis, nutrient transport, plant metabolite biosynthesis, cell wall synthesis and lipid metabolism, suggesting that cellular restructuring during AM formation is largely driven by changes in transcription. Furthermore, a significant number of accumulating transcripts encoding putative transcriptional regulators (see, for example, Guether et al. 2009,

Table 1 *Cis*-regulatory elements involved in AM symbiosis

	Element name	Sequence	Identified in promoter of	Identified in plant species	Reference
1	AT-rich motifs	TTATT(N)7-12AATAA	*ENOD11*	*Medicago truncatula*	(Boisson-Dernier et al. 2005)
2	Mycorrhiza responsive sequence 1 (*MYCRS1*)	ATATTTGTCCGTGACCAACA AACTCACAGGAAAAG	*GH3.4*	*Solanum lycopersicum*	(Chen et al. 2017)
3	Mycorrhiza responsive sequence 2 (*MYCRS2*)	ACAAAAAAACCCTAGCTGGATA	*GH3.4*	*Solanum lycopersicum*	(Chen et al. 2017)
4	Mycorrhiza transcription factor binding sequence (*MYCS*)/CTTC element	[T/C][T/G][T/A]CTTGTT[/C/T/G][T/C]	AM-related phosphate transporter genes, SNARE *LjVTI12* and several other AM-induced genes	Several species	(Karandashov et al. 2004, Chen et al. 2011, Lota et al. 2013)
5	PHR1-binding site (*P1BS*)	GNATATNC		Several species	(Chen et al. 2011, Lota et al. 2013)
6	–	Between −410 and −198 bp, and between −197 and −31 bp upstream of ATG	*Lb29*	*Vicia faba*	(Fehlberg et al. 2005)
7	–	Between −300 and −150 bp upstream of the transcriptional start site	*Lec5*	*Medicago truncatula*	(Frenzel et al. 2006)
8	p*SbtM1* region I	ACCGTCCAAGGCCAGCA	*SbtM1*	*Lotus japonicus*	(Takeda et al. 2011)
9	p*SbtM1* region II	AACAGCGGGTATTGCTATGC			
10	*AMCYC-RE*	GTGAACAGATGGGCCGG CCCAAAAAGTGGG	*RAM1*	*Lotus japonicus*	(Pimprikar et al. 2016)
11	*Myc1**	G[G/A]GGTT[C/T][G/A]AACC[C/T]C	Several AM-induced genes	Several species	(Favre et al. 2014)
12	*Myc2**	TGAG[C/T]T[T/A]A[G/A]CTCA			
13	GCC-box*	GCCGGC			

*Motifs which have been found by computational analysis have and not been confirmed *in planta*.

Gaude et al. 2012, Xue et al. 2015, Rich et al. 2017) indicates that AM development is regulated by a complex transcriptional control network. This is underlined by a growing number of AM-responsive *cis*-regulatory promoter regions or *cis*-elements, which are being identified by promoter deletion studies or computational analysis (**Table 1**; Karandashov et al. 2004, Boisson-Dernier et al. 2005, Fehlberg et al. 2005, Frenzel et al. 2006, Chen et al. 2011, Takeda et al. 2011, Lota et al. 2013, Favre et al. 2014, Pimprikar et al. 2016, Chen et al. 2017). In cereal root systems, some of the transcriptional changes occur in a root type-specific fashion or at divergent amplitudes in different root types (Fiorilli et al. 2015, Gutjahr et al. 2015b). Nevertheless, there is a high degree of conservation among up-regulated genes across angiosperm species and even in a liverwort (Breuillin et al. 2010, Delaux et al. 2015). Several of the activated genes are present only in the genomes of AM-competent plant species (Delaux et al. 2014, Favre et al. 2014, Bravo et al. 2016). Together with available genetic evidence for the importance of some of these candidates (reviewed in McLean et al. 2017), this indicates that they are likely to be crucial for AM development.

AM development is an asynchronous process, such that all fungal structures (hyphopodia, intracellular hyphae, arbuscules and vesicles) are simultaneously present in the root. Therefore, it is challenging to attribute transcriptional changes to specific stages of AM formation. In recent years, this problem has been partially circumvented by pharmacological application of synthetic fungal signaling molecules to non-colonized roots and by separation of specific colonized cell types using laser microdissection (Balestrini et al. 2007, Gomez et al. 2009, Czaja et al. 2012, Gaude et al. 2012, Hogekamp and Küster 2013, Miyata et al. 2014, Camps et al. 2015, Giovannetti et al. 2015, Gutjahr et al. 2015a, Hohnjec et al. 2015). Transcriptional responses to complex exudates from germinating fungal spores, or to isolated synthetic Myc-COs or Myc-LCOs, have thus been uncovered (Czaja et al. 2012, Miyata et al. 2014, Camps et al. 2015, Giovannetti et al. 2015, Gutjahr et al. 2015a, Hohnjec et al. 2015). However, transcriptional responses to germinating spore exudates (GSEs) have not yet been systematically compared with responses to isolated Myc-LCOs or COs. This would be necessary to distinguish, whether fungal molecules other than canonical Myc factors contribute to the transcriptional response to diffusible fungal signals. The first plant transcriptome associated with hyphopodium formation was examined by subjecting RNA from hand-dissected root pieces carrying hyphopodia from *Medicago* hairy root culture grown on plates to suppressive substractive cDNA library sequencing (Siciliano et al. 2007). The transcriptome of arbuscule-containing cells was determined, using laser microdissection followed by quantitative PCR or microarray hybridization (Fiorilli et al. 2009, Gomez et al. 2009, Gaude et al. 2012, Hogekamp and Küster 2013). The most comprehensive data set capturing several

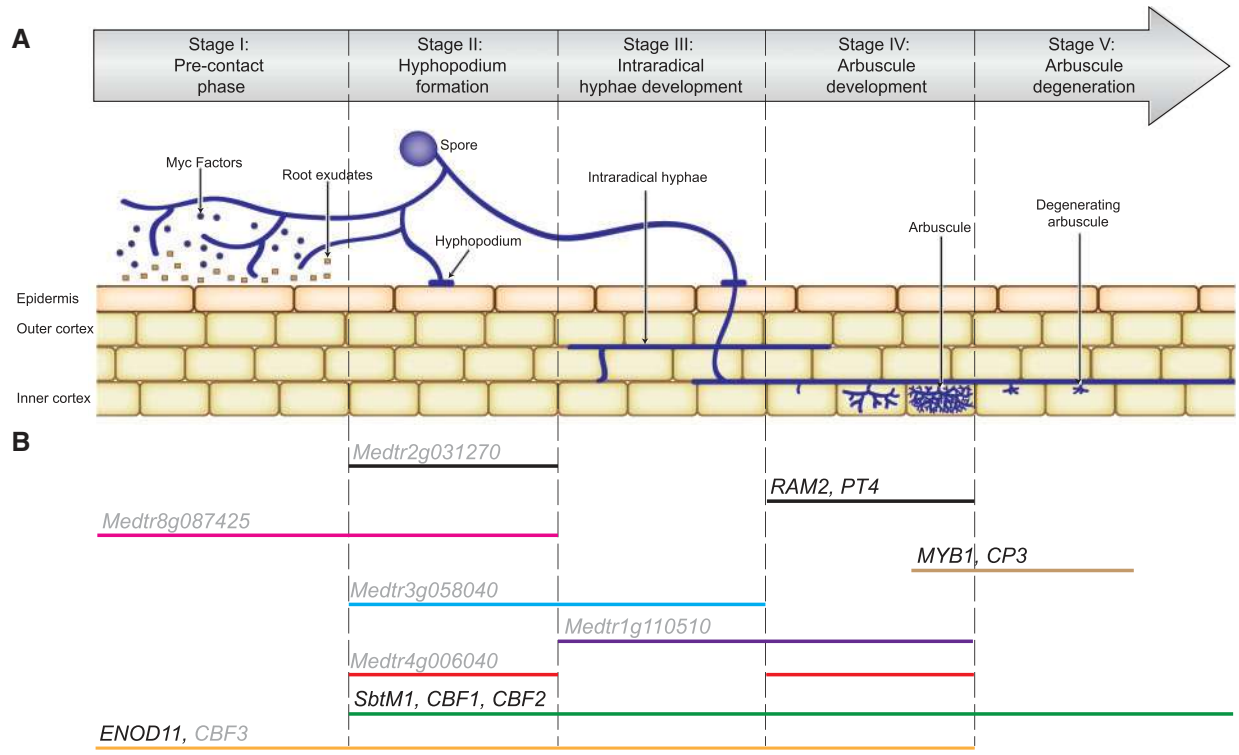

Fig. 1 Stage-specific gene expression during AM development. (A) Schematic representation of AM development. Stage I: in the pre-contact phase, AM fungi release Myc factors (violet circles) and roots exude signals such as strigolactones (brown squares), which induce fungal spore germination, hyphal growth and branching. Stage II: upon reaching the root surface, fungal hyphae differentiate to form hyphopodia to enter the host epidermal cell. Stage III: intraradical fungal hyphae traverse epidermal cells and outer cortical cell layers to reach the inner cortex. Stage IV: highly branched arbuscules form in the inner cortex. Stage V: senescing arbuscules collapse and are subsequently degraded. (B) Stage-specific induction of plant genes during AM development with examples representative of gene sets based on Harrison et al. (2002), Kosuta et al. (2003), Boisson-Dernier et al. (2005), Takeda et al. (2009), Hogekamp et al. (2011), Czaja et al. (2012), Hogekamp and Küster (2013), Camps et al. (2015), Keymer et al. 2017 and Floss et al. (2017). Gene sets induced during one individual AM developmental stage (black arrows), two stages (pink, blue, violet and red arrows), four stages (green and orange arrow) or in mature arbuscule-containing cells and during arbuscule degeneration (brown arrow). The expression of *Cbf1*, *2*, *3* and *MYB1* during arbuscule degeneration is unknown because gene expression data (except for those for *MYB1*) are based on RNA from laser-dissected arbuscule-containing cells containing visible arbuscules (Hogekamp and Küster 2013). For genes written in gray expression patterns are based on data from laser dissection and microarrays. For genes in black these have been additionally confirmed by promoter–reporter assays. The figure was adapted from Hogekamp and Küster (2013).

stages of colonization was produced by Hogekamp and Küster (2013) who used laser microdissection to collect epidermal and directly underlying cortical cells colonized by hyphopodia, as well as epidermal and cortical cells adjacent to fungal hyphae and arbuscule-containing cells. They recorded and compared the transcriptomes of all dissected cell types, and revealed a number of genes, activated across all stages of colonization, such as the CAAT-box transcription factor genes *Cbf1* (*CAAT-box transcription factor*) and *Cbf2* (Hogekamp et al. 2011), and genes which were specifically induced in only one or two stages of AM development (Hogekamp and Küster 2013). This is symptomatic of several regulatory modules which are specific to certain colonization stages, while others may be generally associated with fungal growth through the root tissue (**Fig. 1**). Alternatively, genes expressed across all stages of AM development may be targeted by a number of successively acting, stage-specific transcriptional regulators. The experiment did not include dissected cells from non-colonized roots, which prevented detection of systemically induced genes or genes activated by diffusible fungal molecules (Hogekamp and Küster 2013). The largest number of specifically accumulating transcripts was found in arbuscule-containing cells, and the transcriptome of whole AM roots was dominated by their transcriptome (Hogekamp and Küster 2013), highlighting the profound developmental changes required to host an arbuscule.

According to mutant phenotypes the development of arbuscule-containing cells can be conceptually divided into distinct stages, indicating that the gene products of the corresponding wild-type genes precisely guide the step-wise formation of different parts of the arbuscule (**Fig. 2B;** Gutjahr and Parniske 2013). It starts with the formation of the PPA (stage I) and the insertion of the arbuscule trunk (stage II). This is followed by low-order branching (birdsfoot stage, stage III) and subsequent fine branching (stage IV). After a few days at maturity, the arbuscule collapses (stage V) and finally disappears from the cell. There is compelling evidence that transcriptional changes during development of arbuscule-containing cells occur in successive but overlapping waves (**Fig. 2**). For example, some

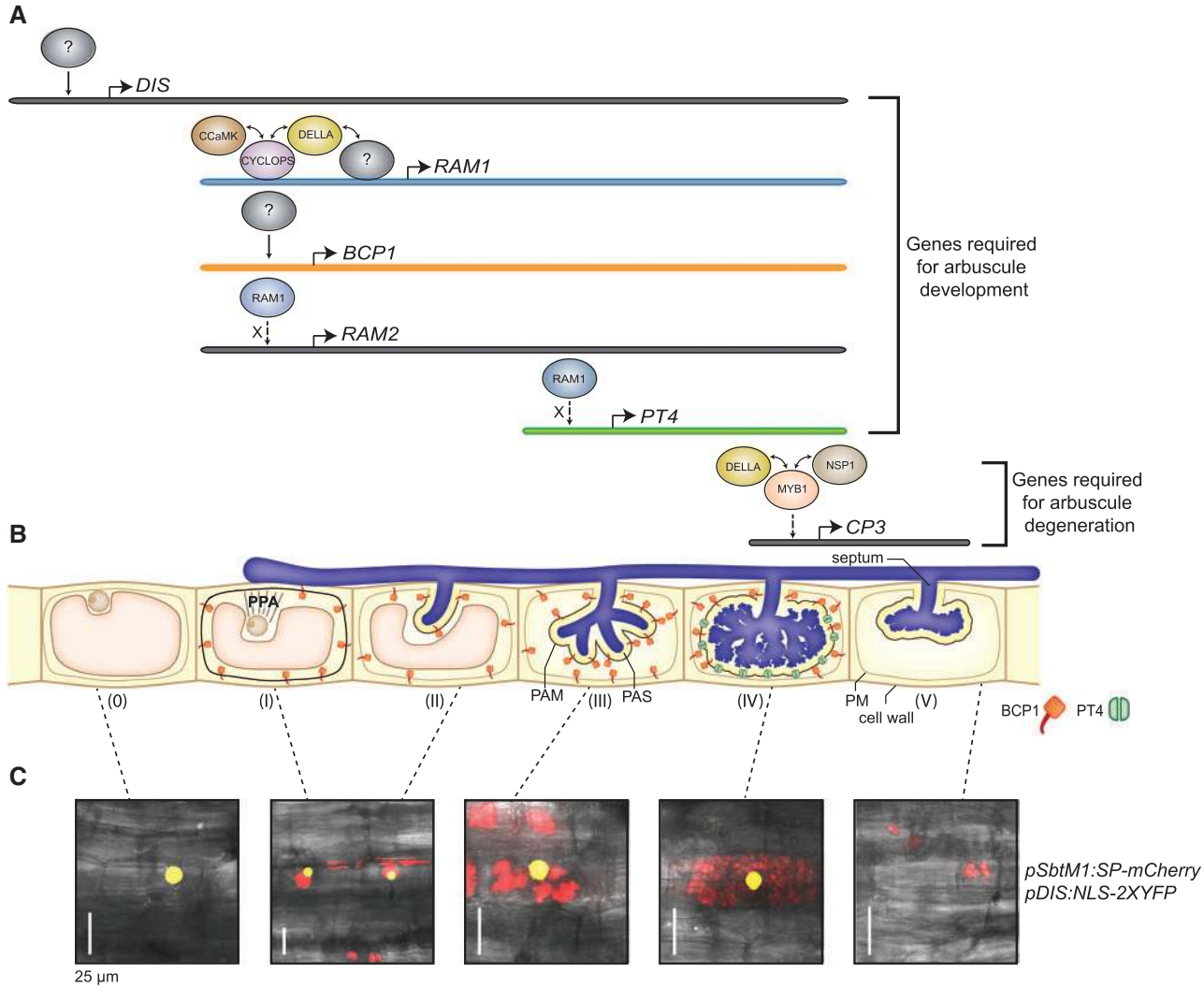

Fig. 2 Correspondence of gene expression and transcription factor activity with stages of arbuscule-containing cell development. (A) Model summarizing the evidence for transcriptional waves occurring during arbuscule development. Genes induced cell specifically during arbuscule formation such as *BCP1*, *DIS*, *RAM1*, *RAM2* and *PT4* (Pumplin and Harrison 2009, Pumplin et al. 2012, Pimprikar et al. 2016, Keymer et al. 2017) and during arbuscule degeneration such as *CP3* (Floss et al. 2017) are representatives of probably larger cohorts of co-regulated genes. The *RAM1* gene required for arbuscule branching is transcriptionally regulated by a CCaMK–CYCLOPS–DELLA complex through direct binding of CYCLOPS to the *AMCYC* response element in the *RAM1* promoter (Pimprikar et al. 2016). Circumstantial evidence suggests that RAM1 participates in the transcriptional activation of *RAM2* and *PT4* (Gobbato et al. 2012, Park et al. 2015, Rich et al. 2015, Pimprikar et al. 2016). An X beside dotted arrows indicates species-specific differences (*M. truncatula* vs. *L. japonicus*) in the dependence of *RAM2* and *PT4* induction on *RAM1*. MYB1 is involved in the regulation of arbuscule degeneration and predicted to regulate the *Cysteine protease* gene *CP3*. MYB1 interacts with DELLA1 and NSP1 in binary interaction assays (Floss et al. 2017). It is possible that all three interact in a trimeric complex. Experimentally determined binary interactions are indicated by double-headed arrows. Unknown transcription factors are indicated by gray circles and a question mark. Dashed arrows indicate that it is unclear whether the transcription factor directly binds to the promoter, whether it binds to a DNA-binding transcription factor in a complex or whether it acts upstream in a transcriptional cascade, which is responsible for the activation of the respective gene. (B) Stages of arbuscule development. Arbuscule development can be separated into six stages (Gutjahr and Parniske 2013): (0) unchanged cortex cell prior to PPA formation; (I) PPA formation; (II) formation of an arbuscule trunk; (III) formation of coarse and low-order branches (birdsfoot stage); (IV) mature arbuscule; and (V) collapsed arbuscule. During arbuscule development, the temporal regulation of transcription determines the subcellular localization of the corresponding secreted protein. For example, *BCP1* is induced during early stages of arbuscule development, and the BCP1 protein localizes to the peripheral plasma membrane, the trunk and the fine branch domain of the PAM. *PT4* is induced during later stages of arbuscule development, and the PT4 protein localizes only to the fine branch domain of the PAM (Pumplin and Harrison 2009, Pumplin et al. 2012, Ivanov and Harrison 2014). PAM, peri-arbuscular membrane; PAS, peri-arbuscular space. (C) *DIS* promoter activity during development of an arbuscule-containing cell (images from Keymer et al. 2017). Promoter activity of *DIS* is indicated by nuclear-localized yellow fluorescent protein (YFP). The PPA (an apoplastic compartment filled with mCherry) and the silhouette of the arbuscules are indicated by red fluorescence protein (mCherry). The dotted line indicates correspondence with the arbuscule developmental stages in (B). It is not visible whether the PPA already contains a growing arbuscule trunk. To indicate this possibility, dotted lines from both stage I and II point to the image displaying the PPA. The figure was adapted and updated from Gutjahr and Parniske 2013.

genes which are activated in arbuscule-containing cells are also up-regulated in cells in their direct neighborhood (Gaude et al. 2012). Although, in fixed tissue samples, arbuscules are visibly absent from these neighboring cells, they are likely to be preparing for arbuscule development by formation of a PPA. Keymer et al. (2017) have recently succeeded in visualizing activation of promoters of two AM-specific lipid biosynthesis genes *DISORGANIZED ARBUSCULE* and *REDUCED ARBUSCULAR MYCORRHIZA2* (*DIS* and *RAM2*) during PPA formation in *Lotus japonicus* hairy roots (**Fig. 2C**). Co-visualization in living roots of nuclear-localized yellow fluorescent protein (YFP) expressed under the control of the *DIS* and *RAM2* promoters with the stage of arbuscule development, showed that these promoters are active during all stages of arbuscule formation but inactive during arbuscule collapse (**Fig. 2** Keymer et al. 2017).

A critical step during development of arbuscule-containing cells is the switch from lower order to higher order branches and fine branches of the arbuscule, because, during fine branching, nutrient transporters accumulate in the PAM, which take up fungus-delivered mineral nutrients into the plant cell and therefore render the symbiosis functional (Harrison et al. 2002, Javot et al. 2007, Yang et al. 2012, Breuillin-Sessoms et al. 2015). Elegant promoter–reporter and promoter-swap experiments showed that the polar localization of membrane proteins and secreted proteins in the arbuscule-containing cell depends on their promoter and thus probably their timing of expression. For example, the *BCP1* promoter drives protein localization to the peripheral membrane of arbuscule-containing cells as well as the PAM, while the *PT4* promoter is responsible for protein localization exclusive to the PAM (Pumplin and Harrison 2009, Pumplin et al. 2012, Ivanov and Harrison 2014). This indicates that during arbuscule development, the *BCP1* promoter is activated first and the *PT4* promoter is induced at a later stage (Pumplin et al. 2012; **Fig. 2**). Thus, we hypothesize that stepwise development of arbuscule-containing cells is controlled by partially distinct and partially overlapping transcriptional modules, which drive transient cellular development from step to step. A number of transcription factors, most prominently GRAS transcription factors, required for arbuscule development, have been identified (Floss et al. 2013, Park et al. 2015, Rich et al. 2015, Xue et al. 2015, Heck et al. 2016, Pimprikar et al. 2016). However, their direct target genes and interconnections in a transcriptional network are still obscure.

Signal Transduction during AM Development

AM development and a large part of the transcriptional response to AM colonization require a conceptual signaling cascade termed 'common symbiosis signaling', which is shared with another root symbiosis, the root nodule symbiosis (RNS) between legumes and nitrogen-fixing rhizobia (Oldroyd 2013, McLean et al. 2017).

Signaling is triggered by the perception of Myc factors at the plasma membrane and can be observed as nuclear calcium spiking (Parniske 2008, Gough and Cullimore 2011, Oldroyd 2013, Sun et al. 2015). In analogy to perception of rhizobial Nod factors, it is hypothesized that Myc factors are perceived by LysM receptor-like kinases (Antolín-Llovera et al. 2012). Although the bona fide Myc factor receptors are still not unequivocally identified, candidates for receptors or co-receptors have been revealed in tomato, *Parasponia* and rice (Op Den Camp et al. 2011, Miyata et al. 2014, Zhang et al. 2015a, Buendia et al. 2016, Carotenuto et al. 2017). It is likely that at least in *Medicago* more than one receptor or co-receptor participates in the perception of Myc factors because treatment with sulfated or non-sulfated Myc-LCOs leads to partially specific transcriptional responses (Czaja et al. 2012, Camps et al. 2015).

A number of common symbiosis genes such as *SYMRK/DMI2* (*SYBIOSIS RECEPTOR KINASE/DOES NOT MAKE INFECTIONS2*), *CASTOR*, *POLLUX/DMI1*, *NUP85* (*NUCLEOPORIN85*), *NUP133*, *NENA* and *CCAMK/DMI3* are required for penetration of AM fungi into epidermal cells (Endre et al. 2002, Stracke et al. 2002, Ané et al. 2004, Levy et al. 2004, Mitra et al. 2004, Imaizumi-Anraku et al. 2005, Kanamori et al. 2006, Tirichine et al. 2006, Saito et al. 2007, Groth et al. 2010). The receptor-like kinase SYMRK interacts with the Nod factor receptors in *Lotus* (Ried et al. 2014). It is required for the generation of Myc factor-induced calcium spiking (Sun et al. 2015) and probably acts as a co-receptor of Nod and Myc factor receptors. SYMRK/DMI2 also interacts with a 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (HMGR1), which is involved in the production of mevalonate (Kevei et al. 2007). Mevalonate application to *Medicago* roots was sufficient to induce nuclear calcium spiking and symbiotic gene expression, suggesting that mevalonate or a downstream metabolite may act as a second messenger in common symbiosis signaling (Venkateshwaran et al. 2015). Calcium spiking is generated by three recently discovered cyclic nucleotide-gated channels (CNGC15s) (Charpentier et al. 2016) in concert with the calcium ATPase MCA8 (Capoen et al. 2011), and the potassium channels CASTOR and POLLUX/DMI1 (Charpentier et al. 2008), which all reside in the nuclear membrane. In addition, the nucleoporins NUP85, NUP133 and NENA are also required for the generation of nuclear calcium spiking (Kanamori et al. 2006, Saito et al. 2007, Groth et al. 2010).

A nuclear-localized calcium and calmodulin-dependent kinase (CCaMK/DMI3) is thought to interpret the rhythmic nuclear calcium elevations (Miller et al. 2013). In addition to the kinase domain, CCaMK contains a calmodulin-binding domain and three EF-hands which, upon calcium and calmodulin binding, release the kinase from autoinhibition (Miller et al. 2013). Ectopic expression of an autoactive version, NLS-CCaMK^{314}, from which all regulatory domains were removed, leaving only the kinase domain, induced cytoplasmic aggregations, resembling PPA-like structures in cortical cells, and expression of AM marker genes in the absence of the AM fungi (Takeda et al. 2012, Takeda et al. 2015). This, together with the impaired AM development in roots of *ccamk* mutants (Levy et al. 2004, Kistner et al. 2005, Banba et al. 2008, Gutjahr et al. 2008), demonstrated the central importance of CCaMK in regulating cellular responses to AM fungi. In addition, a large part of the transcriptional response to Myc-LCOs is CCaMK dependent (Czaja et al. 2012, Camps et al. 2015).

CCaMK interacts with and phosphorylates CYCLOPS/IPD3, a DNA-binding coiled-coil domain-containing transcription factor (Messinese et al. 2007, Yano et al. 2008, Singh et al. 2014). The *cyclops* mutant fails to form arbuscules but establishes intraradical hyphae in *Lotus japonicus* and *Oryza sativa* (Gutjahr et al. 2008, Yano et al. 2008), and displays reduced colonization in *Medicago truncatula* (Horváth et al. 2011). Furthermore, the activation of the AM-inducible gene *RAM1*, by ectopic expression of *NLS-CCaMK*^{314} in the absence of fungus, depends on *CYCLOPS*. CYCLOPS directly binds to a *cis*-element called '*AM-CYC box*' in the *RAM1* promoter (**Fig. 2A; Table 1**; Pimprikar et al. 2016). The *AM-CYC* box contains a sequence (CGGCCG) which has been computationally identified in several AM-induced genes (Favre et al. 2014). Therefore, a number of these genes may be direct targets of CYCLOPS. Interestingly, in heterologous systems, CYCLOPS interacts with the gibberellin signaling repressor DELLA and DELLA enhances the capacity of CYCLOPS to transactivate the *RAM1* promoter (Pimprikar et al. 2016) as well as the promoters of two important nodulation genes *NIN* and *ERN1* (Jin et al. 2016). Furthermore, *della1della2* double mutants of *Medicago* are perturbed in AM development and also in nodulation (Floss et al. 2013, Fonouni-Farde et al. 2016, Jin et al. 2016). Thus, besides its many roles in plant development and environmental adaptation (Davière and Achard 2013, Davière and Achard 2016, Fonouni-Farde et al. 2016), DELLA also qualifies as a common symbiosis gene.

The DELLA–Gibberellin Module Determines Arbuscule Initiation

DELLA proteins are central regulators of gibberellin signaling as they are repressors of gibberellin responses. Active gibberellins are perceived by the soluble α,β-fold hydrolase receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1). This induces the interaction of GID1 with the DELLA and TVHYNP domains of the DELLA protein, recruitment of the SCF$^{SLY/GID2}$ complex, ubiquitylation of DELLA and its degradation via the 26S proteasome (McGinnis et al. 2003, Gomi et al. 2004, Griffiths et al. 2006, Willige et al. 2007). Consistent with the importance of DELLA in AM development, gibberellin treatment inhibits AM formation and leads to phenotypes resembling *della* mutants (El Ghachtouli et al. 1996, Floss et al. 2013, Foo et al. 2013, Yoshida et al. 2014, Takeda et al. 2015). Removal of the DELLA or TVHYNP domain stabilizes DELLA and makes it insensitive to gibberellin treatment (Willige et al. 2007). Also, AM development becomes largely insensitive to gibberellin when a DELLA version from which the N-terminus including the DELLA domain was removed (*della-Δ18*) is ectopically expressed in *M. truncatula* or *L. japonicus* hairy roots (Floss et al. 2013, Takeda et al. 2015). Gibberellin promotes a range of developmental and growth processes in plants, and the DELLA–gibberellin module plays a central role in regulating developmental responses to the environment (Harberd et al. 1998, Achard et al. 2006, Achard et al. 2007, Jiang et al. 2007, Achard et al. 2008). Environmental factors such as light or mineral nutrient availability also have a strong impact on AM development (Carbonnel and Gutjahr 2014, Breuillin-Sessoms et al. 2015, Nagata et al. 2015, Konvalinková and Jansa 2016). Thus, the CYCLOPS–DELLA complex, being an integrator of symbiosis and gibberellin signaling, is a likely target in the adjustment of AM formation, with the plant's physiological state resulting from environmental conditions.

DELLAs appear to act at multiple stages of arbuscule development and, although there are reports on the role of DELLA in AM development also from rice and pea (Foo et al. 2013, Yu et al. 2014), this has been most thoroughly described in *Medicago* (Floss et al. 2013, Floss et al. 2017). The *Medicago* genome contains three DELLA proteins but only two of them appear to be important for AM development because the AM phenotypes of the *della1 della2* double mutant and the *della1 della2 della3* triple mutant are indistinguishable: they both allow the formation of intraradical hyphae but hardly any arbuscules (Floss et al. 2013, Floss et al. 2017). The rare arbuscules show a wild-type-like degree of branching (Floss et al. 2013), indicating that the *della* mutants affect arbuscule initiation rather than branching. To our knowledge, the exact stage at which DELLA acts in arbuscule initiation has not been described and it is as yet unclear whether it is PPA formation (stage I), trunk formation (stage II) or first-order branching (stage III; Gutjahr and Parniske 2013). However, a stage in which the cell prepares for PPA formation, which is visible due to nuclear enlargement and movement towards the fungal hypha, has been observed in the *della* double mutant (Ivanov and Harrison 2014). Interestingly, this initial cellular response is also independent of CCaMK (Genre et al. 2009) and thus probably of the whole CCaMK–CYCLOPS–DELLA complex, possibly because it does not require transcriptional regulation.

In addition to CYCLOPS, DELLA also interacts with the transcription factor MYB1 in yeast and *Nicotiana benthamiana* leaves (Floss et al. 2017). MYB1 regulates arbuscule degeneration (see below), and the rare arbuscules observed in the *della1 2* double mutant display a longer life time than wild-type arbuscules (Floss et al. 2017). Thus, DELLA seems to play an additional role in arbuscule degeneration. This dual role of DELLA at two independent stages of arbuscule development may explain why the promoters of genes involved in gibberellin biosynthesis (*GA20ox*) as well as gibberellin degradation (*GA2ox*) are activated in arbuscule-containing cells, as visualized by the β-glucuronidase (GUS) reporter (Takeda et al. 2015). This observation seems contradictory at first sight. However, under the assumption that the activity of these promoters is indicative of a subsequent activity of the encoded enzymes, it is tempting to speculate that arbuscule formation is accompanied by a cell-autonomous wave of gibberellin accumulation. A low gibberellin level would promote DELLA stability and therefore arbuscule formation or degradation, while a high gibberellin level would trigger DELLA degradation and therefore arbuscule maintenance at maturity. Gibberellin accumulation can be induced by high phosphate levels (Jiang et al. 2007). Therefore, the hypothesis of a high gibberellin content during arbuscule maturity would be consistent with a cell-autonomous high phosphate level, as a result of phosphate delivery by the arbuscule

(Floss et al. 2013). In summary, it appears that gibberellin can play suppressive as well as promotive roles in arbuscule development and maintenance.

The involvement of DELLA in transcriptional activation of AM-induced genes has been consolidated with several independent approaches. Activation of the *RAM1* gene by autoactive NLS-CCaMK^{314} in the absence of fungus was inhibited by gibberellin, indirectly indicating a requirement for DELLA for the activation of *RAM1* (Takeda et al. 2015, Pimprikar et al. 2016). Furthermore, *35S* promoter-driven ectopic expression of *della1-Δ18* induced a range of AM-activated genes including *RAM1* in the absence of fungus (Park et al. 2015, Floss et al. 2016). Also, treatment with the gibberellin biosynthesis inhibitor paclobutrazol (PAC), which promotes accumulation of DELLAs (Feng et al. 2008), activated *RAM1* expression in the absence of fungus. Surprisingly, this also occurred in *ccamk* and *cyclops* mutants, consistent with the finding that expression of *della1-Δ18* and PAC treatment can restore arbuscule formation in *cyclops* (Floss et al. 2013, Pimprikar et al. 2016). This dilemma can be resolved by assuming an additional DNA-binding protein X, which interacts with DELLA and becomes sufficient in the absence of CYCLOPS when DELLA is stabilized (**Fig. 2A;** Pimprikar et al. 2016).

DELLAs belong to the family of GRAS transcription factors, which have so far been exclusively detected in plants (Bolle 2004). Based on electrophoretic mobility shift assays (EMSAs) two GRAS proteins, NODULATION SIGNALING PATHWAY 1 (NSP1) from *M. truncatula* and SCARECROW LIKE 7 (SCL7) from rice, have been suggested to bind to DNA (Hirsch et al. 2009, Li et al. 2016). However, for DELLAs and other GRAS transcription factors, no evidence for DNA binding is available. DELLAs can activate genes through interaction with DNA-binding C2H2 zinc finger transcription factors of the INDERTERMINATE DOMAIN (IDD) family (Fukazawa et al. 2014, Yoshida et al. 2014, Fukazawa et al. 2017). Also, two other GRAS transcription factors SHORT ROOT (SHR) and SCARECROW (SCR) bind to IDD transcription factors. The crystal structure of the trimeric complex of SHR, SCR and the IDD protein JACKDAW (JKD) confirmed that SHR and SCR do not contain DNA-binding domains and that DNA binding is mediated via the IDD transcription factor JKD (Hirano et al. 2017). Thus, also in AM development, DELLA probably acts as a transcriptional cofactor, and transcriptional activation of symbiosis genes by DELLA independent of CYCLOPS may be mediated by an IDD protein. Interestingly, several C2H2 zinc finger transcription factor family members are specifically induced during AM (Gutjahr et al. 2015b, Rich et al. 2015, Takeda et al. 2015, Luginbuehl et al. 2017).

Arbuscule Branching is Regulated by a Number of GRAS Transcription Factors

Forward and reverse genetic approaches in *Medicago*, *Lotus* and *Petunia* have identified four transcription factors involved in the regulation of arbuscule branching: the GRAS proteins RAM1, REQUIRED FOR ARBUSCULE DEVELOPMENT (RAD1), MYCORRHIZA INDUCED GRAS 1 (MIG1) and ETHYLENE RESPONSE FACTOR 1 (ERF1)/WRINKLED 5b (WRI5b) (Devers et al. 2013, Gobbato et al. 2013, Park et al. 2015, Rich et al. 2015, Xue et al. 2015, Heck et al. 2016, Pimprikar et al. 2016, Luginbuehl et al. 2017).

RAM1 is currently best characterized (Gobbato et al. 2013, Park et al. 2015, Rich et al. 2015, Xue et al. 2015, Pimprikar et al. 2016, Luginbuehl et al. 2017, Rich et al. 2017). *ram1* mutant roots fail to support higher order branching of fungal arbuscules and contain only stunted arbuscules with trunks, coiled trunks or coarse low-order branches (Park et al. 2015, Rich et al. 2015, Xue et al. 2015, Pimprikar et al. 2016). In *Lotus*, *Medicago* and *Petunia*, *RAM1* is strongly and specifically induced upon root colonization by AM fungi, and *RAM1* promoter activity is spatially restricted to colonized regions of the root (Gobbato et al. 2012, Gobbato et al. 2013, Park et al. 2015, Rich et al. 2015, Xue et al. 2015, Pimprikar et al. 2016). Transcriptional analyses using qRT-PCR and whole-transcriptome RNA sequencing (RNAseq) with RNA from *ram1* mutants suggest that RAM1 acts as an early transcriptional switch to regulate downstream cascades required for the transition from low-order to high-order fine branching of the arbuscule (Park et al. 2015, Rich et al. 2015, Xue et al. 2015, Pimprikar et al. 2016, Luginbuehl et al. 2017). Several AM-responsive genes, with genetically determined roles in arbuscule branching, were unresponsive to colonization in *ram1*. These included *VAPYRIN* and *Exo70I*, which are both hypothesized to play a role in PAM formation (Feddermann et al. 2010, Pumplin et al. 2010, Zhang et al. 2015b), AM-specific phosphate and ammonium transporters such as *PT4* and *AMT2* family members and lipid biosynthesis genes such as *KASIII* (*β-ketoacyl-acyl carrier protein synthase III*), *DIS*, *FatM* (*FAT REQUIRED FOR ARBUSCULAR MYCORRHIZA SYMBIOSIS*) and *RAM2* (Park et al. 2015, Pimprikar et al. 2016, Jiang et al. 2017, Keymer et al. 2017, Luginbuehl et al. 2017, Rich et al. 2017), suggesting an important role for RAM1 in regulating PAM formation as well as nutrient exchange between the symbionts. In addition, ectopic expression of *RAM1*, using a *35S* or *Ubiquitin* promoter, activated a set of the *RAM1*-dependent genes in the absence of fungus (Park et al. 2015, Pimprikar et al. 2016, Luginbuehl et al. 2017). Interestingly, in *L. japonicus*, RAM1 was sufficient (when ectopically expressed in the absence of fungus) but not required (in colonized *ram1* mutants) for the induction of several genes expressed in arbuscule-containing cells such as *RAM2*, *STR* and *PT4* (Pimprikar et al. 2016), while in *Medicago* and *Petunia* the induction of these genes was absent from *ram1* mutants (Park et al. 2015, Rich et al. 2015). This points to species-specific wiring of transcriptional networks and/or redundancy at the level of RAM1 in *L. japonicus*. RNAseq analysis also revealed a series of transcription factors, which are activated by AM in a *RAM1*-dependent manner (Luginbuehl et al. 2017, Rich et al. 2017). Among them were three *ERF/AP2* (*APETALA2*) domain transcription factor genes (called *WRI5a*, *b* and *c*), which are specific to genomes of AM-competent plants and homologous to the WRINKLED transcription factors in Arabidopsis (Bravo et al. 2016, Luginbuehl et al. 2017). In Arabidopsis, these transcription factors regulate glycolysis and fatty acid biosynthesis, and

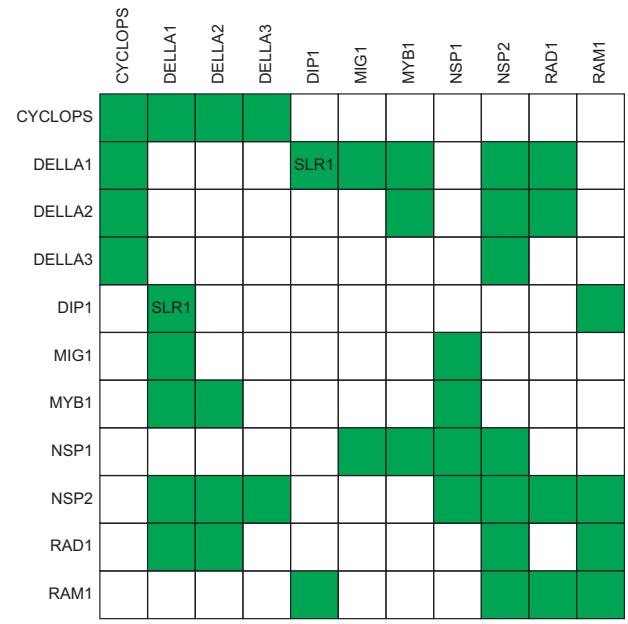

Fig. 3 Summary of described binary interactions among transcription factors involved in AM development. Details on the methods used for detecting the interaction as well as the articles in which the interaction was reported are shown in Supplementary Table S1. SLR1 indicates that the interaction was found with the single DELLA protein from rice SLENDER RICE 1 (SLR1).

Medicago WRI5a–c were also able to increase triacylglycerol accumulation, when ectopically expressed in *N. benthamiana* leaves (To et al. 2012, Luginbuehl et al. 2017). Silencing of *WRI5b* (called *ERF1* in that study) using an artificial microRNA (miRNA) driven by the arbuscule-containing cell-specific *PT4* promoter caused a stunted arbuscule phenotype resembling the phenotype of *ram1* and of mutants perturbed in arbuscule-containing cell-specific lipid biosynthesis such as *dis*, *fatm* or *ram2* (Devers et al. 2013, Bravo et al. 2017, Jiang et al. 2017, Keymer et al. 2017, Luginbuehl et al. 2017). Therefore, the question emerges of whether RAM1 regulates lipid biosynthesis genes directly, or indirectly via activation of *WRI5* genes.

Phylogenetic analysis of GRAS transcription factors revealed that RAM1 and DELLA proteins are highly related as they probably emerged from a common ancestral gene (Gobbato et al. 2012, Park et al. 2015, Pimprikar et al. 2016, Rich et al. 2017). However, despite their high similarity at the protein level, they are functionally distinct because the *ram1* mutant could not be complemented by ectopic expression of *Δ18-DELLA*. However, ectopic *RAM1* expression restored arbuscule formation in the presence of gibberellin (Pimprikar et al. 2016). This indicates that RAM1 acts downstream of *DELLA*. GRAS proteins have the tendency to form hetero-complexes (Hirano et al. 2017) and also RAM1 interacts in yeast and *N. benthamiana* leaves with several other GRAS proteins involved in AM development (**Fig. 3**; Supplementary Table S1). Rice and *Medicago* RAM1 interact with DELLA INTERACTING PROTEIN1 (DIP1), and DIP1 also interacts with DELLA (Yu et al. 2014). The significance of these interactions in AM development is still unclear. Furthermore, it is unknown whether the three proteins form a trimeric complex or whether DIP1 interacts with DELLA and RAM1 separately. The former would imply that DELLA acts at several levels of a transcriptional cascade: upstream of RAM1 as well as in a complex with RAM1. However, a defect in arbuscule branching has not yet been observed in *della* mutants, and *RAM1* overexpression in gibberellin-treated roots restores arbuscule formation (Pimprikar et al. 2016), suggesting that RAM1 does not need to interact with DELLAs to be able to induce the transcriptional program for arbuscule development. RNA interference (RNAi)-mediated silencing of *dip1* and *ram1* as well as mutation of DELLA (*SLENDER RICE1*) in rice led to a strong general reduction of AM formation such that arbuscule branching could not be investigated (Yu et al. 2014). Apparently, the phenotypic consequence of *DELLA*, *DIP1* and *RAM1* deficiency are stronger in rice than in legumes.

The closest homolog of *RAM1*, is *RAD1*, and RAD1 also interacts with RAM1 in heterologous systems (Park et al. 2015, Xue et al. 2015). The stunted arbuscule phenotype of *L. japonicus rad1* mutants is similar to that of *ram1* (Xue et al. 2015). However, in *Medicago*, *rad1* mutants display a somewhat weaker phenotype, a decreased root length colonization but normally developed arbuscules (Park et al. 2015). It appears that the relative importance of RAM1 and RAD1 in supporting arbuscule development differs between *Lotus* and *Medicago*. This may explain the different effects of *RAM1* mutation on expression of downstream genes in the two species (Park et al. 2015, Pimprikar et al. 2016, Luginbuehl et al. 2017) and provides an interesting example for 'micro-diversification' of gene regulatory networks in closely related species such as the legumes *L. japonicus* and *M. truncatula*.

MIG1 belongs to a different clade of GRAS proteins. In *M. truncatula*, the gene is duplicated, and three *MIG1* homologs (*MIG1*, *MIG2* and *MIG3*) are located in tandem on chromosome 2. Transcript accumulation of all three *MIG* genes is increased during AM symbiosis, and the promoter of *MIG1* (p*MIG2* and p*MIG3* were not examined) is specifically active in arbuscule-containing cells (Heck et al. 2016). Simultaneous down-regulation of all three *MIG* genes by RNAi in hairy roots resulted in the formation of smaller and distorted arbuscules, although the total colonization remained unchanged (Heck et al. 2016). It will be interesting to examine whether a triple knock out mutant, defective in all three *MIG* genes, will lead to a stronger phenotype. Overexpression of *Δ18-DELLA* in combination with the RNAi construct targeting *MIG1* and *MIG3* restored arbuscule development (Heck et al. 2016), suggesting that DELLA can compensate for the reduction in *MIG* transcript abundance when it is stabilized. MIG and DELLA interact in yeast-two hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays in *N. benthamiana* leaves (Heck et al. 2016). When ectopically expressed under the control of a 35S promoter, both induce an increase in cell width and number of cortex cell layers, and therefore in root diameter (Heck et al. 2016). It was suggested that MIG recruits DELLA to regulate radial expansion of arbuscule-containing cells, which could be a prerequisite for arbuscule fine branching (Heck et al. 2016). A role for the MIGs in regulating cell size rather than PAM formation or arbuscule functionality is consistent with the finding

that several AM marker genes (*RAM1*, *RAD1* and *Exo70I*), which are induced upon ectopic expression of *Δ18-DELLA* and involved in regulating components of nutrient exchange (Park et al. 2015, Zhang et al. 2015b, Floss et al. 2016), are not induced upon ectopic expression of MIG1 (Heck et al. 2016). The effect of cell size on arbuscule branching is not well studied and it is unclear how the two processes are mechanistically connected. Identification of the MIG target genes will be an important step towards understanding their role and towards enabling further investigations regarding the connection between host cell expansion and arbuscule branching.

MYB1, NSP1 and DELLA are Involved in Regulating Arbuscule Degeneration

Arbuscules are constantly turned over, and it has been shown in rice that a mature arbuscule is maintained for only 2–3 d (Kobae and Hata 2010). However, the lifetime can differ among individual arbuscules and may also depend on the plant and fungal species involved (Brown and King 1982, Alexander et al. 1989, Kobae and Hata 2010). Arbuscule degeneration commences with the collapse of parts of the fine branches followed by gradual collapse of the whole arbuscule (Kobae and Fujiwara 2014, Kobae et al. 2014). This process proceeds rapidly and, within a few hours, the marker for growing and mature arbuscules in rice, PT11–green fluorescent protein (GFP), disappears from the host cell (Kobae and Hata 2010). The biological significance of this rapid arbuscule turnover has not been established; however, based on phenotypes of plant mutants perturbed in AM-specific nutrient transporters, it has been suggested that arbuscule turnover may serve to avoid fungal cheating by enabling the plant to eliminate rapidly any arbuscules, which do not deliver nutrients (Javot et al. 2007, Gutjahr and Parniske 2017). In *Medicago* and rice mutants of AM-specific phosphate transporters, *mtpt4* and *ospt11*, arbuscule degeneration is accelerated, which leads to an accumulation of collapsed arbuscules and a reduction in total root colonization (Javot et al. 2007, Yang et al. 2012). PT4 and PT11 localize to the PAM and most probably import phosphate released by the fungus to the PAS into the host cytoplasm (Harrison et al. 2002, Javot et al. 2007, Kobae and Hata 2010, Yang et al. 2012). Thus, in *pt4* and *pt11* mutants, the arbuscules are compromised in delivering phosphate to the plant cell, suggesting that premature arbuscule degeneration is caused by their insufficient performance. Accelerated arbuscule degeneration in the *Mtpt4* mutant is suppressed under nitrogen deficiency (Javot et al. 2011, Breuillin-Sessoms et al. 2015), indicating that nitrogen delivery via the arbuscule also supports arbuscule maintenance. In *Medicago*, this is dependent on the AM-specific ammonium transporter gene *AMT2.3*, and arbuscule development cannot be restored in *pt4 amt2.3* double mutants by nitrogen limitation (Breuillin-Sessoms et al. 2015). Thus, the efficiency of delivery of two and possibly more nutrients emerges as a crucial factor in determining arbuscule life span when the availability of the specific nutrient is limiting.

Until recently, no molecular players involved in arbuscule turnover were known. Using an elegant RNAi-based suppressor screen for suppressors of the *pt4* phenotype, Floss et al. (2017) found that the transcription factor MYB1 of *Medicago* plays an important role in arbuscule degeneration. Arbuscule lifetime is restored to the wild-type level in *pt4 myb1* double mutants, indicating that MYB1 regulates accelerated arbuscule degeneration in the absence of phosphate delivery. However, it seems to be dispensable for 'normal' arbuscule turnover in the wild-type background because *myb1* single mutants do not show signs of extended arbuscule lifetime (Floss et al. 2017, Gutjahr and Parniske 2017). Colonized *pt4* mutants display increased expression of genes involved in degradative processes, such as a range of hydrolases (proteases, lipases and chitinases) and ripening-related proteins. This indicates an important if not predominant role for the plant cell in arbuscule degradation. A subset of these, including *CYSTEINE PROTEASE 3* (*CP3*; **Fig. 2A**), were used as markers and shown to be induced by ectopic expression of *MYB1* under control of the *35S* promoter (Floss et al. 2017). Consistently, this also increased arbuscule degeneration and affected root colonization in a quantitative manner (Floss et al. 2017). The induction of arbuscule degeneration markers was absent from *nsp1* and reduced in *della1 2* double mutants. Furthermore, both NSP1 and DELLA interacted with MYB1 in Y2H assays and by co-immunoprecipitation (CoIP) from *N. benthamiana* leaves, indicating that MYB1 may interact with NSP1 and DELLA to regulate genes involved in arbuscule degeneration. It is unclear whether MYB1, NSP1 and DELLA interact in the same protein complex or whether subsets of genes are regulated by two distinct MYB1 complexes, which involve either NSP1 or DELLA. In the *myb1* mutant, induction of the arbuscule degeneration markers by colonization was only mildly affected, indicating redundancy at the level of *MYB1* in the presence of *PT4*, consistent with the absence of an increased arbuscule life span in *myb1* mutants (Floss et al. 2017).

It is currently unknown what triggers arbuscule degeneration in wild-type roots and whether it is initiated by the plant as a default mechanism or by a fungus-derived signal, for example by decreasing phosphate delivery as the arbuscule ages. Furthermore, it is unknown how the output of phosphate sensing connects to transcriptional regulation, and whether transcription factors such as MYB1 are regulated at the transcriptional and/or the post-transcriptional level. Clearly, in the wild-type under low nutrient conditions, the regulon governing degeneration of an arbuscule should be active in a temporally controlled manner and only after the mature arbuscule has developed and performed its function. The *MYB1* promoter is specifically active in arbuscule-containing cells, and the *MYB1* transcript accumulation in RNA extracts from whole roots correlates with the quantity of root colonization, consistent with MYB1's function in arbuscule degeneration (Floss et al. 2017). Its transcriptional activation by root colonization was absent from a *ram1* mutant. Furthermore, it could not be induced by ectopic expression of *Δ18-DELLA*, unlike other genes which play a role during earlier stages of the development of arbuscule-containing cells (Floss et al. 2017). This suggests that MYB1 is

induced somewhat later in the plant transcriptional cascade, which orchestrates arbuscule development.

The putative ortholog of *MYB1* in *L. japonicus* was described previously. Its promoter was activated in arbuscule-containing cells and in root meristems, and the gene was consequently named *MERISTEM AND MYCORRHIZA INDUCED* (*MAMI*) in *L. japonicus* (Volpe et al. 2013). An RNAi construct targeting *MAMI* in *L. japonicus* hairy root culture led to a significant reduction in root branching consistent with a putative role in the root meristem and in lateral root primordia (Volpe et al. 2013). However, no AM phenotype was observed, which can now be explained by the findings of Floss et al. (2017). Ectopic expression of *MAMI* under control of the *35S* promoter did not cause premature arbuscule degeneration in contrast to *MYB1* in *Medicago* (Volpe et al. 2013, Floss et al. 2017). This could be due to the low efficiency of the *35S* promoter in *L. japonicus* hairy roots, in which it is mainly expressed in the vasculature and only weakly in the cortex (Maekawa et al. 2008). *MAMI* is duplicated in the *Lotus* genome (Floss et al. 2017) and it remains an additional possibility that the other copy, which has not been used for overexpression, may have been more efficient in triggering arbuscule turnover.

Regulatory Circuits Comprising GRAS Proteins and MicroRNAs Regulate the Amount of Root Colonization

To adjust the symbiosis with their physiological status, plants regulate the progression of AM fungi within the root to control the amount of root colonization. Three GRAS transcription factors, OsAM18, NSP1 and NSP2, have been implicated in supporting quantitative colonization (Liu et al. 2011, Lauressergues et al. 2012, Delaux et al. 2013, Takeda et al. 2013, Fiorilli et al. 2015). *OsAM18* is specifically induced during AM symbiosis (Güimil et al. 2005, Gutjahr et al. 2008), and an *osam18* insertion mutant displays a 50% decrease in root length colonization and in arbuscule abundance (Fiorilli et al. 2015). The exact mechanism of action of OsAM18 in fungal progression within the root is still elusive. *NSP1* and *NPS2* were originally identified as indispensable for root nodule symbiosis (Kaló et al. 2005, Smit et al. 2005). Their role in AM was only discovered later because, initially, research was mainly focused on plant mutants affecting fungal morphology rather than quantity and because strong inocula may have prevented the detection of quantitative phenotypes. In *Medicago*, mutation of both *NSP1* and *NSP2* leads to a reduction in AM colonization, (Lauressergues et al. 2012, Delaux et al. 2013). However, in *Lotus*, this was only observed for *nsp1* and not for *nsp2* mutants (Takeda et al. 2013). This species-specific discrepancy is surprising because, based on interaction studies in yeast and *N. benthamiana*, NSP1 and NSP2 are thought to interact physically and this interaction seems to be important for induction of nodulation-specific promoters (Hirsch et al. 2009, Jin et al. 2016). Perhaps the interaction is not crucial in AM symbiosis. In fact, in heterologous systems, NSP2 also shows the capacity to interact with RAM1, RAD1 and DELLA; and NSP1 with MIG1 (**Fig. 3**; Supplementary Table S1). Although the biological significance of these interactions is unknown, it is tempting to speculate that different GRAS transcription factor complexes including NSP1 and/or NSP2 may regulate distinct groups of genes depending on the context.

Both *nsp1* and *nsp2* mutants are affected in the expression of strigolactone biosynthesis genes and strigolactone production (Liu et al. 2011). Strigolactone activates AM fungi prior to colonization, and AM development is strongly reduced in mutants affected in strigolactone biosynthesis or exudation (summarized in Waters et al. 2017). Therefore, the reduction in strigolactone biosynthesis could explain the lower colonization level of *nsp1* and *nsp2*. However, colonization was not restored by exogenous treatment of *Lotus nsp1* mutants with the synthetic strigolactone GR24 (Takeda et al. 2013), indicating that additional factors reduce colonization in *nsp1*. *NSP1* is activated by Myc-LCOs and is also crucial for transcript accumulation of a large portion of genes in response to Myc-LCO perception (Delaux et al. 2013, Camps et al. 2015, Hohnjec et al. 2015). Some of these genes may contribute to *NSP1*-dependent promotion of root length colonization. Interestingly, the MycLCO-triggered activation of a large cohort of genes was also dependent on *RAM1* (Hohnjec et al. 2015), suggesting that NSP1 and RAM1 may co-operate in the regulation of transcriptional responses to Myc-LCOs.

The transcript of *NSP2* is post-transcriptionally regulated by an miRNA called miR171h (Lauressergues et al. 2012, Hofferek et al. 2014). This has been shown in *M. truncatula*, but, according to in silico analyses, miR171h and its binding site in the *NSP2* mRNA sequence are conserved across AM-competent plant species (Lauressergues et al. 2012). Overexpression of miR171h in *M. truncatula* hairy roots caused a reduction of root colonization, while overexpression of a miR171h-resistant *NSP2* gene lead to its significant increase (Lauressergues et al. 2012, Hofferek et al. 2014). The promoter of miR171h is particularly active just above the root tip in the root elongation zone, which is normally not colonized. Overexpression of a miR171h-resistant version of *NSP2* allows colonization of the elongation zone (Lauressergues et al. 2012). Furthermore, miR171h transcript accumulation depends on the nutrient conditions, with low accumulation at low phosphate and high nitrogen, and high accumulation under the reverse condition (Hofferek et al. 2014). Taken together, this suggests that the *NSP2*–miR171h regulatory circuit is involved in the spatial adjustment of colonization with root developmental zones and in the regulation of colonization quantity in response to environmental stimuli such as nutrients.

Other members of the miR171 family are known to target *LOST MERISTEMS* (*LOM*) genes, which also encode GRAS family proteins and were initially identified in *Arabidopsis thaliana* and *Petunia hybrida* (Schulze et al. 2010, Xue et al. 2014). *LOM* genes are required for the maintenance of shoot and root indeterminacy by regulating meristem cell differentiation (Stuurman et al. 2002, Engstrom et al. 2010, Schulze et al. 2010). In *M. truncatula*, *LOM1* was shown to control the quantity of AM development, as RNAi-mediated silencing of *LOM1* in *M. truncatula* hairy roots resulted in a significant decrease in root

colonization (Couzigou et al. 2017). *LOM1* and *LOM2* share 40–44% homology with *LOM* genes of Arabidopsis and are predicted in silico to be targeted by miR171a–f (Couzigou et al. 2017). Indeed, expression of a miR171-resistant *LOM1* genomic sequence (*rLOM1*) driven by its native promoter led to an increase in *LOM1* transcript accumulation and root colonization. Cleavage of the *LOM1* RNA is induced by miR171a, c, d, e and f at the 13–14 position, similar to Arabidopsis. miR171b forms an exception and displays a mismatch at the cleavage site and enhanced expression of miR171b phenocopied roots expressing *rLOM1* (Couzigou et al. 2017). Thus, miR171b most probably acts as a target mimic, which protects the *LOM1* transcript from other miR171 family members and cleavage by DICER. The mismatch at the cleavage site in miR171b is conserved across AM-competent species but not in *A. thaliana*, indicating that fine tuning of *LOM1* spatio-temporal expression via miR171–target mimic pairs may be specifically advantageous for regulating AM symbiosis (Couzigou et al. 2017). Indeed, the promoters of *LOM1* and some of the miR171 members are active throughout the root in symbiotic and asymbiotic conditions, while the promoter of miR171b is predominantly active in colonized cells (Couzigou et al. 2017). Possibly, *LOM1* mRNA accumulates across the root, but is constantly cleaved, due to the ubiquitous presence of miR171 family members. In this scenario, the precise spatial expression of miR171b would be a critical factor in allowing AM development.

Plants have additionally developed a systemic feedback mechanism to control root colonization by microbes from the shoot. This phenomenon, called autoregulation, involves the CLV-like receptor kinase NODULATION AUTOREGULATION RECEPTOR KINASE (NARK) (Meixner et al. 2005, Schaarschmidt et al. 2013). In roots of asymbiotic *nark* mutants, the transcripts of two genes encoding CCAAT-binding transcription factors of the NFYA family, *NF-YA1a* and *NF-YA1b*, accumulated to higher levels as compared with wild-type roots (Schaarschmidt et al. 2013), indicating that they may be targets of NARK-mediated autoregulation. Suppression of *NF-YA1a* and *NF-YA1b* transcripts by RNAi resulted in a significant reduction in root colonization of wild-type as well as *nark* mutant roots, suggesting that NF-YA1a and NF-YA1b act as positive regulators of quantitative AM colonization downstream of NARK (Schaarschmidt et al. 2013). It will be interesting to identify the target genes of NF-YA1a and NF-YA1b and to learn how they interact with the GRAS transcription factors in a regulatory network.

Conclusions and Perspectives

Extant land plants carry a genetic heritage from their ancestors: the ability to form an intimate interaction with beneficial fungi that deliver mineral nutrients. The earliest land plants did not form roots. The absence of roots was compensated with the help of a far-reaching hyphal network that scavenges nutrients in return for plant-derived carbon sources that enable the fungus to grow (Brundrett 2002). Despite the evolution of roots, AM fungi remained essential to bridge depletion zones of mineral nutrients such as phosphate that form around plant roots due to limited diffusion rates (Smith and Smith 2011). The accommodation of the symbiont inside the plant tissue required remodeling of plant cells. To regulate this cellular reorganization, plants evolved an AM-specific network of transcription factors (Delaux et al. 2014, Favre et al. 2014, Bravo et al. 2016), which is currently being uncovered. In recent years, there has been amazing progress in the identification of transcription factors and *cis*-regulatory elements involved in the transcriptional regulation of AM development (**Table 1**: Supplementary Table S1), in the correlation of transcriptional waves comprising specific stages of AM formation and in the characterization of the spatio-temporal activity of plant promoters during arbuscule development. Furthermore, we now have evidence that this AM-specific regulatory network intersects with transcriptional networks involved in plant development, probably to adjust the symbiosis with the physiological and developmental state of the plant or the individual root (Floss et al. 2013, Gutjahr 2014, Heck et al. 2016, Pimprikar et al. 2016, Floss et al. 2017). However, for a comprehensive understanding of regulons driving AM development, the full set of target genes, DNA-binding sites and interaction partner need to be determined for all known transcription factors. The range of transcription factor genes up-regulated in AM roots suggests that more transcriptional regulators involved in AM formation remain to be discovered and characterized. Furthermore, we need to understand how individual regulons interact in a larger transcriptional network.

It emerges that GRAS proteins play a predominant role in AM symbiosis. Although the structure of some representatives has been solved (Li et al. 2016, Hirano et al. 2017) and the activity of GRAS proteins in transcriptional regulation firmly established, their exact molecular function in activating transcription is still obscure. DNA binding by GRAS proteins has only exceptionally been observed (Hirsch et al. 2009, Li et al. 2016). Therefore, it is likely that most of them do not bind DNA. Probably, they rather act as transcriptional cofactors in complexes with DNA-binding transcription factors (Fukazawa et al. 2014, Yoshida et al. 2014, Hirano et al. 2017). GRAS proteins are prone to interact with each other, and several binary interactions of AM-relevant GRAS proteins have been revealed in heterologous systems (**Fig. 3**; Supplementary Table S1). It is possible that alternative GRAS protein complexes form with different DNA-binding transcription factors in a spatio-temporally controlled manner, to regulate subsets of genes needed for specific steps in AM development. The relevance of the observed physical interactions among GRAS proteins and of GRAS proteins with transcription factors from other families for AM development is still unknown. The interactions need to be determined in situ and correlated with specific stages of AM development using methods such as CoIP from mycorrhizal roots and in situ fluorescence resonance energy transfer-fluorescence lifetime imaging (FRET-FLIM) (Long et al. 2017). However, since transcription factor proteins usually occur in small amounts, and imaging AM symbiosis in deep root cortex layers of plants other than Arabidopsis is challenging, this will require increased efforts to improve techniques and accessibility of mycorrhizal tissue.

Ectopic expression of *MYB1* leading to accelerated arbuscule degeneration and reduced root colonization (Floss et al. 2017) indicates that an understanding of transcriptional regulators and networks will enable us to manipulate the symbiosis synthetically in the quest to understand the biological meaning of symbiotic traits such as arbuscule turnover. Furthermore, morphological and transcriptional phenotypic differences in *ram1* and *rad1* mutants of *Medicago* and *Lotus* and of *della* mutants in *Medicago* and rice suggest small regulatory variations and evolutionary differences even among closely and distantly related species. If the knowledge generated by our community should be used for breeding AM-optimized crops, which optimally benefit from the symbiosis upon low input of mineral fertilizer, we need to perform comparative studies including several plant species to uncover and understand species-specific differences. We predict that more of such differences will become apparent in the future.

Supplementary Data

Supplementary data are available at PCP online.

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The authors have no conflicts of interest to declare.

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