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The half-size ABC transporters STR1 and STR2 are indispensable for mycorrhizal arbuscule formation in rice

Caroline Gutjahr[1], Dragica Radovanovic[1], Jessika Geoffroy[1], Quan Zhang[2,†], Heike Siegler[1], Marco Chiapello[1], Leonardo Casieri[1,†], Kyungsook An[3], Gynheung An[3], Emanuel Guiderdoni[4], Santhosh K. Chellian[5,†], Venkatesan Sundaresan[5], Maria J. Harrison[2] and Uta Paszkowski[1,*]

[1]*Department of Plant Molecular Biology, University of Lausanne, Biophore Building, 1015 Lausanne, Switzerland,*
[2]*Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY 14853, USA,*
[3]*Crop Biotech Institute and Department of Plant Molecular Systems Biotechnology, Kyung Hee University, Yongin 446-701, Korea,*
[4]*CIRAD, UMR AGAP 1096, TA 108/03 Avenue Agropolis, 34398 Montpellier Cedex 5, France, and*
[5]*Department of Plant Biology, University of California Davis, Davis, CA 95616, USA*

Received 13 October 2011; accepted 27 October 2011.
[*]For correspondence (fax +41 21 692 4195; e-mail uta.paszkowski@unil.ch).
[†]Present address: LC; UMR INRA 1088/CNRS 5184/U. Bourgogne, Plant-Microbe-Environment, INRA-CMSE, BP 86510, 21065 Dijon cedex, France. SKC; Bayer Crop Science, Breeding and Trait Development, 108 Pasir Panjang Rd, # 05-14, Singapore 118535. QZ; Department of Biology, Campus Box 1137, Washington University in St Louis, St Louis, MO 63130, USA.

SUMMARY

The central structure of the symbiotic association between plants and arbuscular mycorrhizal (AM) fungi is the fungal arbuscule that delivers minerals to the plant. Our earlier transcriptome analyses identified two half-size ABCG transporters that displayed enhanced mRNA levels in mycorrhizal roots. We now show specific transcript accumulation in arbusculated cells of both genes during symbiosis. Presently, arbuscule-relevant factors from monocotyledons have not been reported. Mutation of either of the *Oryza sativa* (rice) ABCG transporters blocked arbuscule growth of different AM fungi at a small and stunted stage, recapitulating the phenotype of *Medicago truncatula stunted arbuscule 1* and *2* (*str1* and *str2*) mutants that are deficient in homologous ABCG genes. This phenotypic resemblance and phylogenetic analysis suggest functional conservation of STR1 and STR2 across the angiosperms. Malnutrition of the fungus underlying limited arbuscular growth was excluded by the absence of complementation of the *str1* phenotype by wild-type nurse plants. Furthermore, plant AM signaling was found to be intact, as arbuscule-induced marker transcript accumulation was not affected in *str1* mutants. Strigolactones have previously been hypothesized to operate as intracellular hyphal branching signals and possible substrates of STR1 and STR2. However, full arbuscule development in the strigolactone biosynthesis mutants *d10* and *d17* suggested strigolactones to be unlikely substrates of STR1/STR2. Interestingly, rice STR1 is associated with a *cis*-natural antisense transcript (*antiSTR1*). Analogous to *STR1* and *STR2*, at the root cortex level, the *antiSTR1* transcript is specifically detected in arbusculated cells, suggesting unexpected modes of STR1 regulation in rice.

Keywords: symbiosis, ABC transporter, arbuscules, *Gigaspora rosea*, *Glomus intraradices*, *Oryza sativa*.

INTRODUCTION

Arbuscular mycorrhizal (AM) symbioses are intimate associations between most terrestrial plants and fungi of the *Glomeromycota* (Schüßler *et al.*, 2001 and citations therein). The symbioses are based on reciprocal nutrient exchange between the symbiotic partners. The fungus depends on a supply of carbon from the plant, and in turn delivers mineral nutrients, especially phosphate and nitrogen, to its host (Smith and Read, 2008).

A pre-symbiotic molecular dialogue represents the start of the interaction. Plant-released strigolactones and fungal Myc factors induce symbiotic responses in the interacting counterpart (Akiyama *et al.*, 2005; Maillet *et al.*, 2011). Upon contact with the root surface the fungal hypha differentiates into an attachment structure, called the hyphopodium, from where the fungus passes the rhizodermis (Genre *et al.*, 2005) and subsequently proliferates inter- and intracellularly

within the cortex. A network of eight plant signaling proteins is essential for rhizodermis penetration by the fungus, and is conserved across the two angiosperm classes (Banba *et al.*, 2008; Gutjahr *et al.*, 2008; Parniske, 2008; Groth *et al.*, 2010). Inside cortical cells the fungus branches dichotomously and differentiates into a tree-shaped haustorium: the arbuscule that expands until it reaches the physical limits of the host cell. Development of an arbuscule is a highly complex process that involves dramatic architectural reorganization of the colonized cell. This includes invagination of both the plasma membrane and the tonoplast (Pumplin and Harrison, 2009, and citations therein), dramatic plasma membrane proliferation and, in consequence, the formation of a large surface area for signal and nutrient exchange. In parallel, the cytoskeleton is rearranged, and possibly guides membrane deposition and the accumulation of Golgi, endoplasmic reticulum (ER), mitochondria and plastids, as well as peroxisomes, around arbuscule branches (Lohse *et al.*, 2005; Genre *et al.*, 2008; Pumplin and Harrison, 2009). The newly formed periarbuscular membrane (PAM) is continuous with the plasma membrane of the cortex cell. However, immunolocalization and life-cell imaging revealed that it represents a distinct membrane domain that is further subdivided into discrete 'trunk' and 'branch' subdomains (Pumplin and Harrison, 2009). Therefore arbuscule formation induces a polarization of the plant cortex cell. Consistent with a specific function in symbiotic phosphate uptake, the branch domain of the arbuscule harbours highly specific phosphate transporters that mediate symbiotic phosphate acquisition (Harrison *et al.*, 2002; Javot *et al.*, 2007a). Mutation of the Medicago arbuscule-specific phosphate transporter *Mt*PT4 leads to higher arbuscule turnover, indicating that phosphate might not only act as a nutrient but also a signal (Javot *et al.*, 2007b; Yang and Paszkowski, 2011). It is likely that arbuscules are also predominantly involved in fungal nutrient acquisition. Support for this view is lent by the recent observation that the knock-down of symbiosis-induced *Medicago truncatula* sucrose synthase *MtSUC1* causes an arbuscule phenotype (Baier *et al.*, 2010).

Arbuscules represent an intimate and extreme form of compatibility between two organisms that must be the result of a precisely orchestrated molecular dialogue. Prior to arbuscule formation the cortex cell is reshaped and forms a tunnel-like structure, the so-called pre-penetration apparatus (PPA), to guide the anticipated fungal development within the cell (Genre *et al.*, 2008). This indicates that arbuscule development is initiated by the plant rather than the AM fungus. The search for plant proteins involved in arbuscule development is therefore a promising start in understanding this process. The first proteins required for arbuscule formation have recently been identified. VAPYRIN, a protein that consists of a major sperm protein domain and an ankyrin domain, is indispensable for intercellular accommodation of AM fungi and for arbuscule formation (Feddermann *et al.*, 2010; Pumplin *et al.*, 2010). A Medicago steroid-binding protein is also required for proper arbuscule formation, possibly by regulating sterol homeostasis in the root (Kuhn *et al.*, 2010). Two *M. truncatula* half-size ABCG transporters called STR1 and STR2 (for stunted arbuscule) interact in the periarbuscular membrane. As mutation or expression perturbation of one of these proteins results in small and stunted arbuscules, the dimer is probably necessary for the export of an essential but yet unknown compound into the peri-arbuscular space (Zhang *et al.*, 2010).

To identify genes necessary for AM development in monocotyledons we combined whole-genome transcriptome profiling of mycorrhizal *Oryza sativa* (rice) roots (Güimil *et al.*, 2005) with a reverse genetics screen (Hirochika *et al.*, 2004) for altered mycorrhizal phenotypes. Two genes strongly induced by AM colonization corresponded to two half-size ABCG transporters with homology to *M. truncatula STR1* and *STR2* (Güimil *et al.*, 2005; Zhang *et al.*, 2010). Here we characterized their temporal and spatial expression and found transcript accumulation to be associated with arbuscules. Interestingly, *STR1* and *STR2* expression was accompanied by a natural antisense transcript (*cis*-NAT) of *STR1*, the expression pattern of which suggests an unexpected involvement in the regulation of *STR1*. Importantly, we show that rice *STR1* and *STR2* are indispensable for arbuscule formation, demonstrating that their function is evolutionarily conserved between di- and monocotyledons.

RESULTS

Transcripts of *STR1*, *STR2* and *antiSTR1* accumulate in arbusculated cells

To detect mycorrhiza-regulated genes with a possible function in AM development in monocotyledons, we previously performed whole-genome microarray analysis of mycorrhizal rice roots (Güimil *et al.*, 2005). One strongly AM-induced gene encoded an ABCG transporter homologous to Medicago STR1 (Zhang *et al.*, 2010). Different versions of the *STR1* transcript (TIGR ID, LOC_Os09g23640; RAP ID, Os09g0401100) were found in rice genome databases (http://www.orygenesdb.cirad.fr, http://www.rice.plantbiology.msu.edu and http://www.rapdb.dna.affrc.go.jp). Sequencing of the cDNA from mycorrhizal roots revealed that the *STR1* gene consists of four exons that correspond to LOC_Os09g23640.1 (Figure 1a; http://www.orygenesdb.cirad.fr and http://www.rice.plantbiology.msu.edu). BLASTP searches identified a close homologue of STR1 in the rice genome that shared homology with *M. truncatula* STR2 (Zhang *et al.*, 2010). A whole-genome transcriptomics study using an updated version of the Affymetrix gene chip also revealed *STR2* to be induced by AM colonization (C. Gutjahr, R. J. H. Sawers, H. Angliker, T. Roloff, E. Oakeley, U. Paszkowski, unpublished data). The *STR2* transcript contained

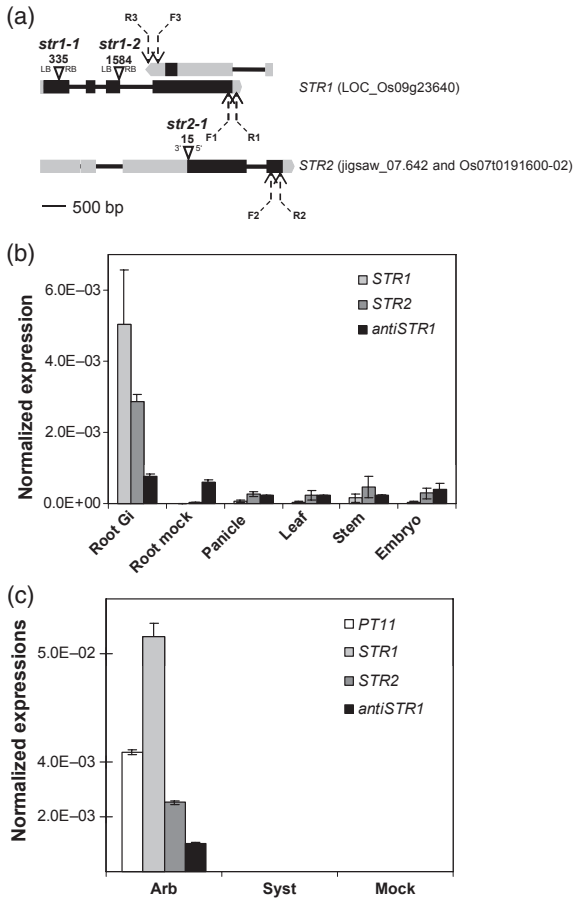

Figure 1. Gene structure and tissue- and cell-specific expression of *STR1*, *STR2* and *antiSTR1*.

(a) Gene structure and position of insertions of *STR1*, *STR2* and *antiSTR1*, drawn to scale. Black boxes indicate exons separated by introns (solid lines). Grey boxes indicate untranslated regions (UTRs). The mutants *str1-1* and *str1-2* carry T-DNA insertions (LB, left border; RB, right border) and *str2-1*, a dSpm insertion. The position of the insertion with respect to the A of ATG is displayed. Dashed arrows indicate primers used for real-time RT-PCR.

(b) Tissue-specific expression of *STR1*, *STR2* and *antiSTR1*, as determined by real-time RT-PCR. Error bars represent SDs of three technical replicates. The experiment was repeated twice with similar results.

(c) Real-time RT-PCR based expression of *PT11*, *STR1*, *STR2* and *antiSTR1* in rice cortex cells obtained by laser microdissection: arb, arbusculated; syst, systemic (from colonized roots, but not containing arbuscules); mock, from mock-inoculated roots. Expression is shown relative to the constitutively expressed gene *GAPDH*. Error bars show SDs of three technical replicates. The experiment was repeated three times with similar results.

two exons, confirming the computational prediction model, jigsaw_7.642, available on the rice genome browser (http://www.rapdb.dna.affrc.go.jp).

Database analyses indicated that *STR1* was accompanied by a natural antisense transcript of 1842 nt in length (*cis*-NAT; RAP ID, Os09t0401200-02), termed *antiSTR1* hereafter. According to a full-length cDNA clone (AK106846), *antiSTR1* contains two exons and is complementary with *STR1* in a tail-to-tail orientation: the second exon of *antiSTR1* overlaps with the third intron of *STR1* by 80 nt, and with its fourth exon by 819 nt (Figure 1a). The first exon of *antiSTR1* matches 131 nt of another gene adjacent to *STR1* (RAP ID, Os09t0401200-01; data not shown) in sense orientation. *AntiSTR1* is predicted (http://www.rapdb.dna.affrc.go.jp) to encode a protein of 94 amino acids. However, no corresponding protein sequence has been reported from any organism, as determined by BLAST searches. Therefore, *antiSTR1* is not likely to encode for a protein but instead represents a bona fide *cis*-NAT. Computational searches for similar *cis*-NAT sequences from other plant species did not yield any match. The presence of *antiSTR1* was confirmed by real-time RT-PCR, with primers specifically targeting *antiSTR1* and with cDNA synthesized by an *antiSTR1*-specific primer (Tables S1 and S3).

Natural antisense transcripts have been proposed to regulate complementary mRNA in many different ways, such as transcriptional interference, RNA masking, chromatin remodelling, RNA editing and RNA interference (Lapidot and Pilpel, 2006). For RNA interference, small interfering (si) RNAs of mostly 21–25 nt in length are produced from the antisense transcript, and lead to Argonaut-dependent degradation of mRNAs (Okamura and Lai, 2008). We considered the possibility that siRNAs might be produced from *antiSTR1* and could also target other genes in *trans*. Applying a cut-off of 21-nt sequence complementarity revealed a matching sequence of 121 nt in length contained within the first exon of the *STR2* open reading frame, indicating the possibility that *STR2* could be targeted by *antiSTR1* in *trans*. However, as antiSTR1 originates from the same locus as STR1 we kept the name *antiSTR1*. Small complementary stretches of 23–34 nucleotides were also found in three other genes located on chromosomes 4 and 7, and encoding an inorganic pyrophosphatase, a glycosyltransferase and a hypothetical protein (Table S2). However, these genes are not regulated by AM colonization (Güimil *et al.*, 2005; C. Gutjahr, R. J. H. Sawers, H. Angliker, T. Roloff, E. Oakeley, U. Paszkowski unpublished data).

Next, to confirm the AM-responsive induction of *STR1*, and to assess AM induction of *STR2* and *antiSTR1*, real-time RT-PCR analysis was performed on cDNA from non-colonized and *Glomus intraradices*-colonized roots. High levels of *STR1* and *STR2* mRNA were detected in mycorrhizal roots, thereby confirming previous microarray data, whereas expression was at the background level in non-colonized roots (Güimil *et al.*, 2005; C. Gutjahr, R. J. H. Sawers, H. Angliker, T. Roloff, E. Oakekly, U. Paszkowski, unpublished data). *antiSTR1* mRNA also accumulated in roots, but the level did not change upon AM colonization (Figure 1b). Examination of the transcript levels of *STR1*, *STR2* and *antiSTR1* in panicle, leaf, stem and embryo revealed only background levels of expression in these organs for all three genes.

As *STR1* and *STR2* were induced in mycorrhizal roots we next examined their expression in arbusculated cells

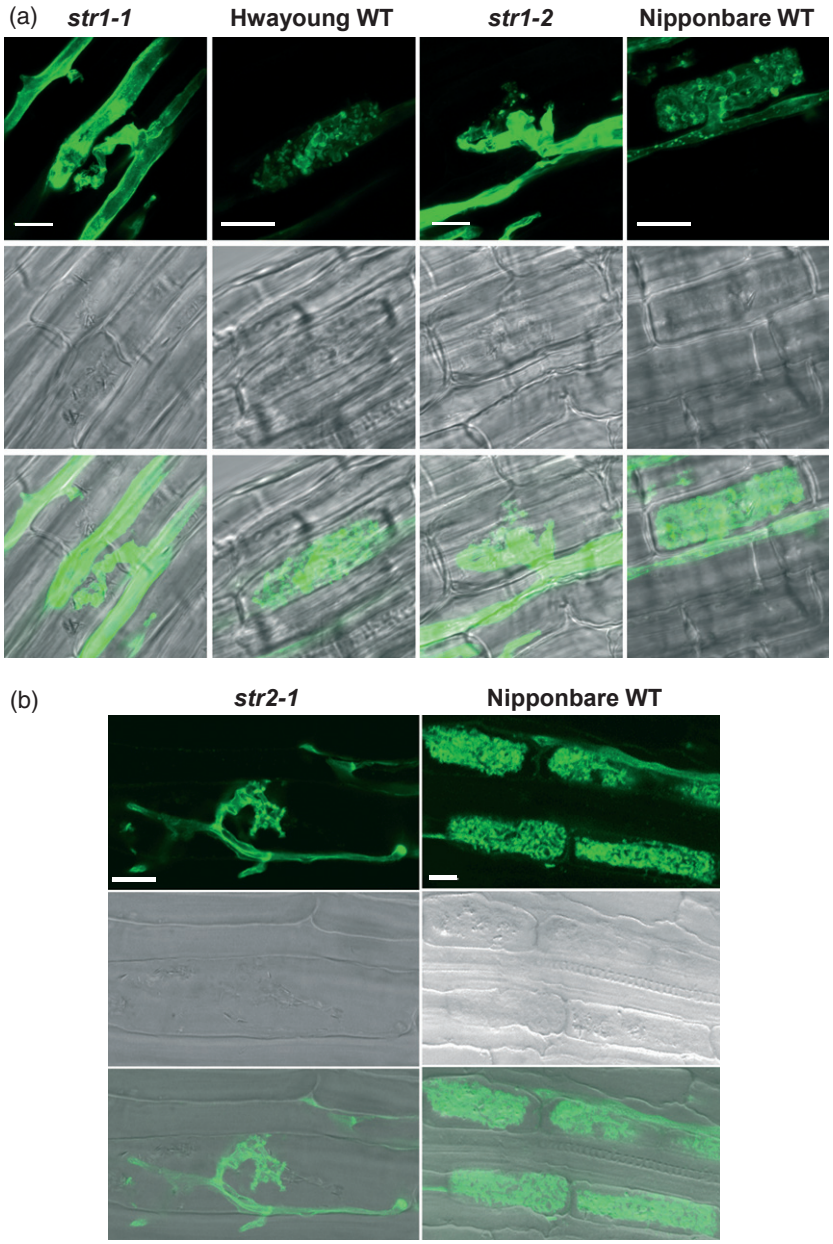

Figure 2. Phenotypes of rice *str1* and *str2* mutants colonized by *Glomus intraradices*.
Confocal images of *G. intraradices* arbuscules stained with wheatgerm agglutinin (WGA) conjugated with Alexa Fluor 488 in cortex cells of (a) *str1-1* and *str1-2* and (b) *str2-1*, their corresponding wild type. Bright-field images and overlays show the outline of cortical cells. Whereas the arbuscules in the cortex cells of the wild type are well developed, they are small, stunted and clumped in *str1-1*, *str1-2* and *str2-1*. Scale bars: 10 μm.

collected by laser microdissection (Figure 1c). Transcript levels were compared between arbusculated and non-arbusculated cortex cells from mycorrhizal roots (hereafter called systemic cortex cells) and cortex cells from mock-inoculated roots. To ensure the specificity of the samples, we first recorded the expression of the arbuscule marker *PT11*. *PT11* was specifically expressed in arbusculated cells, but not in systemic or mock control cells, as reported earlier (Gutjahr *et al.*, 2008). Similarly, *STR1* and *STR2* were specifically expressed in arbusculated cortex cells. Arbuscule-specific expression was also observed for *antiSTR1*. This cell-specific expression pattern was unexpected as the expression level of *antiSTR1* was similar between mock and *Glomus intraradices*-inoculated root systems (Figure 1b). *antiSTR1* transcript accumulation might therefore differ spatially between colonized and non-colonized roots, but the total level of *antiSTR1* transcripts in the root system might not differ between the two treatments. In summary,

all three transcripts accumulate in arbusculated cells, suggesting they play a role in arbuscule development, maintenance or function.

STR1 and *STR2* are conserved across grass species

The G subfamily of the half-size ABC transporters is the largest subfamily of ABC transporters in plants (Verrier *et al.*, 2008), and encompasses STR1 and STR2. An earlier phylogenetic analysis of STR1 and STR2 proteins revealed that they form a distinct clade with respect to the *M. truncatula* and *Arabidopsis thaliana* subfamily of ABCG transporters (Zhang *et al.*, 2010). To determine the conservation of rice STR1 and STR2 across the *Poaceae* we performed BLAST searches against the available grass genomes representing the subfamilies *Pooideae*, *Paniceae* and *Anthopogoneae*, and found putative orthologs for both genes in *Brachypodium dystachion*, *Setaria italica*, *Sorghum bicolor* and *Zea mays* (maize). Surprisingly, among the plants with duplicate genomes, such as *Populus* sp. (poplar), *Glycine max* (soybean) and maize, only the latter contains a single putative copy of STR1 and STR2, whereas in the others, at least one of the two genes has been duplicated. (Zhang *et al.*, 2010).

A phylogenetic tree was constructed including the full complement of rice and Arabidopsis ABCG protein sequences, and additionally sequences of STR1 and STR2 from dicotyledons and *Poaceae*, with sequenced genomes (Figure S1). STR1 and STR2 formed distinct clusters with respect to the rice and Arabidopsis ABCG transporter subfamily, confirming earlier observations (Zhang *et al.*, 2010). Within the STR1 and STR2 clusters the putative monocotyledon and dicotyledon orthologs formed distinct subclusters, where the monocotyledon clusters recapitulated the phylogenetic relationships of the grass species that were included in the analysis (Vicentini *et al.*, 2008). In summary, STR1 and STR2 are conserved across grass subfamilies. Furthermore, the distinct clustering of the STR1 and STR2 sister clades, both containing proteins from di- and monocotyledons, indicates that orthologs in these two major angiosperm lineages originated from a common ancestor.

STR1 and *STR2* are indispensable for arbuscule formation

To assess the functional relevance of *STR1* and *STR2* for rice interaction with AM fungi, we searched public databases (http://www.orygenesdb.cirad.fr; http://www.signal.salk.edu) for lines carrying insertions in the *STR* genes. We chose two insertion lines with T-DNA insertions in the first and third exon of *STR1* (1C-04850 and CL522472) and a line carrying a dSpm transposon insertion within the first exon of *STR2* (RdSpm 2654D). PCR analysis and sequencing of 5′ and 3′ boundaries of the insertion confirmed the presence of the insertion for all lines, which were named *str1-1*, *str1-2* and *str2-1*, respectively (Figure 1a). Homozygous *str1* and *str2* mutants and corresponding wild-type varieties were inoculated with *Glomus intraradices* and fungal structures were inspected at 7 weeks post inoculation (wpi). In the wild-type cultivars arbuscules were well developed and highly branched, completely filling cortical cells (Figure 2). In contrast, arbuscules appeared small, stunted and clumped in cortical cells of *str1-1*, *str1-2* and *str2-1* (Figure 2). The morphology of other mycorrhizal structures in *str1* and *str2* was comparable with those of the wild type (data not shown). We conclude that STR1 and STR2 are required for arbuscule development of *Glomus intraradices* in rice, consistent with previous observations made for *M. truncatula str1* and *str2* mutant roots (Zhang *et al.*, 2010). Therefore, both ABC transporters are functionally conserved between Medicago and rice, and probably in other angiosperms. Because of the phenotypic equivalence between *str1* and *str2* mutants, we concentrated our further studies on the *str1* alleles.

It has been observed that the phenotype of mycorrhizal plant mutants can vary depending on the species of the AM fungal partner (Manjarrez *et al.*, 2009). To determine the role of STR1 for arbuscule development of diverse AM fungi the two *str1* alleles were inoculated with *Gigaspora rosea*, which is distantly related to *Glomus intraradices* (Schüßler *et al.*, 2001). Although roots of both wild-type genotypes contained fully developed and highly branched *Gigaspora rosea* arbuscules in both *str1* mutants, the arbuscules appeared smaller, stunted and particularly clumped (Figure 3). Thus, STR1 is required for arbuscule formation in at least two genera of different and distantly related AM fungal species.

Arbuscule-related plant signaling is intact in *str1* mutants

It could be expected that abnormal arbuscule development affected the signaling pathways required for the wild-type induction of arbuscule-responsive genes. The transcript level of eight AM-specific marker genes was assessed. Previously these were classified as early (*AM1*, *AM2*, *AM3*, *AM11*) or late (AM10, AM14, AM15, PT11; Gutjahr *et al.*, 2008) with respect to induction profiles preceding or coinciding with arbuscule formation. To capture possible transience in marker gene expression, a time-course experiment was performed and *Glomus intraradices*-inoculated *str1-1*, *str1-2* and wild-type roots were sampled at 3, 5 and 7 wpi (Figure 4). Since colonization kinetics vary across biologically independent experiments, we display the results as separate replicates.

As expected, Hwayoung and Nipponbare wild-type root length colonization was low at 3 wpi, and rose at later time points (Figures 4a and S2a). Root length colonization of *str1-1* and *str1-2* also increased with time, but remained significantly lower than wild-type colonization. In both wild-type cultivars all eight marker genes were expressed at 3 wpi, reflecting the presence of all mycorrhizal structures (Figures 4b and S2b). As previously established, the transcript level of marker genes increased with colonization level

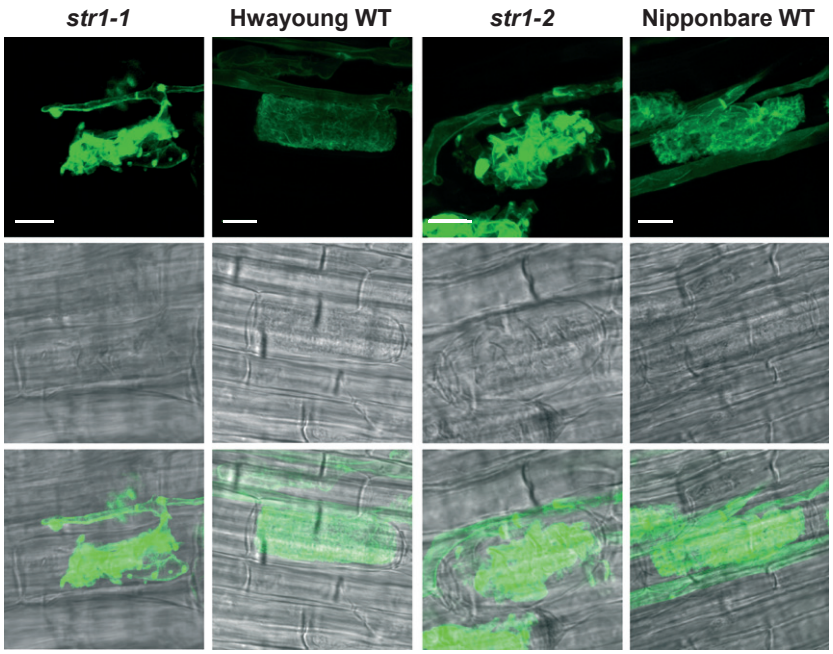

Figure 3. Phenotype of rice *str1* mutants colonized by *Gigaspora rosea*.
Confocal images of *Gigaspora rosea* arbuscules stained with wheatgerm agglutinin (WGA) conjugated with Alexa Fluor 488 in the cortex cells of *str1-1* and *str1-2*, and their corresponding wild type. Bright-field images and overlays show the outline of cortical cells. Whereas the arbuscules in the cortex cells of the wild type are well developed, they are small, stunted and clumped in *str1-1* and *str1-2*. Scale bars: 10 μm.

(Gutjahr *et al.*, 2008). In *str1-1* and *str1-2*, transcripts of the four early marker genes accumulated at 3 and 5 wpi, and increased with time and colonization level (Figure 4b). In contrast, mRNA of the late markers was generally not detected, despite the presence of stunted arbuscules at these time points. At 7 wpi, however, the four late marker genes, including *PT11*, were additionally expressed in both mutant alleles. The expression profile of the marker genes was equivalent between the two mutant alleles, and was additionally confirmed by an independent replicate experiment focusing on two early (*AM1* and *AM3*) and two late (*AM14* and *PT11*) marker genes (Figure S2b). Therefore, AM-specific signalling cues are not perturbed in *str1* roots containing stunted arbuscules, and the increased intraradical colonization, including an elevated number of stunted arbuscules, was sufficient to elicit detectable expression.

To confirm correlation between the morphological and the molecular *str1* phenotype across divergent AM fungal species, marker gene expression patterns in *str1* mutants colonized by *Gigaspora rosea* were determined (Figure 5). A reduced set of representative marker genes was employed, namely two early (*AM1* and *AM3*) and two late (*AM14* and *PT11*) marker genes. As observed for *Glomus intraradices*, colonization by *Gigaspora rosea* increased with time in wild-type cultivars, and remained significantly lower in the *str1* mutants (Figure 5a). The transcripts of the four markers were detected at high levels in both wild-type cultivars at all time points (Figure 5b). In contrast, in the two *str1* mutants only the mRNAs of the two early marker genes were detected. The distinct marker gene expression profile consistently observed in the mutant alleles was supported by an independent biological replicate (Figure S3b). Thus, despite the comparable abundance of arbusculated cells in plants inoculated with *Glomus intraradices* and *Gigaspora rosea*, the gene induction of the late markers *PT11* and *AM14* could not be detected in roots colonized by *Gigaspora rosea* (Figures 4, 5, S2 and S3). Morphological differences between the *Glomus intraradices* and *Gigaspora rosea* arbuscules observed in rice (Gutjahr *et al.*, 2008; Kobae and Hata, 2010) are a likely cause for variation in their capacity to elicit gene expression. Differences in the composition of signaling molecules released by the two AM fungal species offers an alternative explanation for variation in host gene expression. Nevertheless, the expression of late marker genes in *str1* mutants colonized by *Glomus intraradices* shows that the signaling pathway leading to arbuscule response marker gene expression is intact.

Within the same experiment we also examined the expression of *STR1*, *STR2* and *antiSTR*1 in the mutant background (Figure 6 and S4). In both wild-type cultivars *STR1* and *STR2* expression was induced by *Glomus intraradices* and *Gigaspora rosea* colonization, and correlated well with time and increasing levels of colonization. In both *str1* mutant alleles colonized with *Glomus intraradices* and *Gigaspora rosea*, transcripts of *STR1* and *STR2* did not accumulate beyond the background level of mock-inoculated roots, and remained close to the detection limit

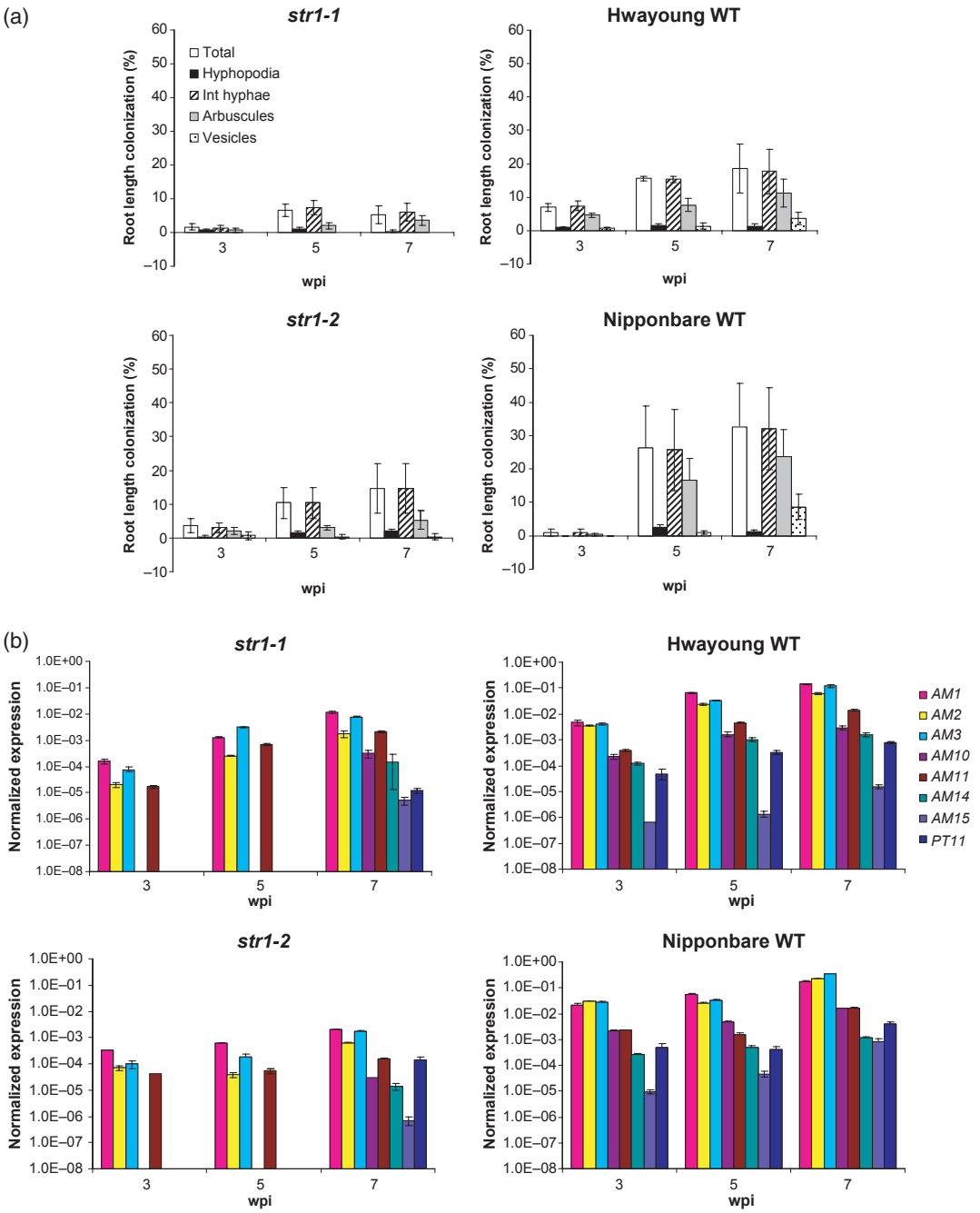

Figure 4. Molecular phenotype of *str1* mutants colonized by *Glomus intraradices*.
(a) *Glomus intraradices* colonization kinetics in *str1-1* and *str1-2*, as compared with their corresponding wild type. The percentage root length colonization was determined by the grid line intersect method. Means and SEs of three biological replicates each represented by duplicate samples are shown. Int hyphae: intraradical hyphae.
(b) Real-time RT-PCR-based expression kinetics of four early (*AM1, AM2, AM3, AM11*) and four late (*AM10, AM14, AM15, PT11*) rice arbuscular mycorrhizal (AM) marker genes in roots of *str1-1*, *str1-2* and the corresponding wild type in response to *G. intraradices* colonization. Error bars indicate SDs of three technical replicates. Each sample is a pool of three plants.

(Figures 6 and S4). Even at 7 wpi with *Glomus intraradices*, when late marker transcripts were detected in both *str1* mutants, the mRNA level of *STR1* and *STR2* in the mutant background did not increase consistently (Figures 6 and S4a). It could have been expected that the expression profile of the wild-type *STR2* gene in the *str1* mutant backgrounds was similar to late marker genes. The common absence of gene activity induction of both *STR* genes, however,

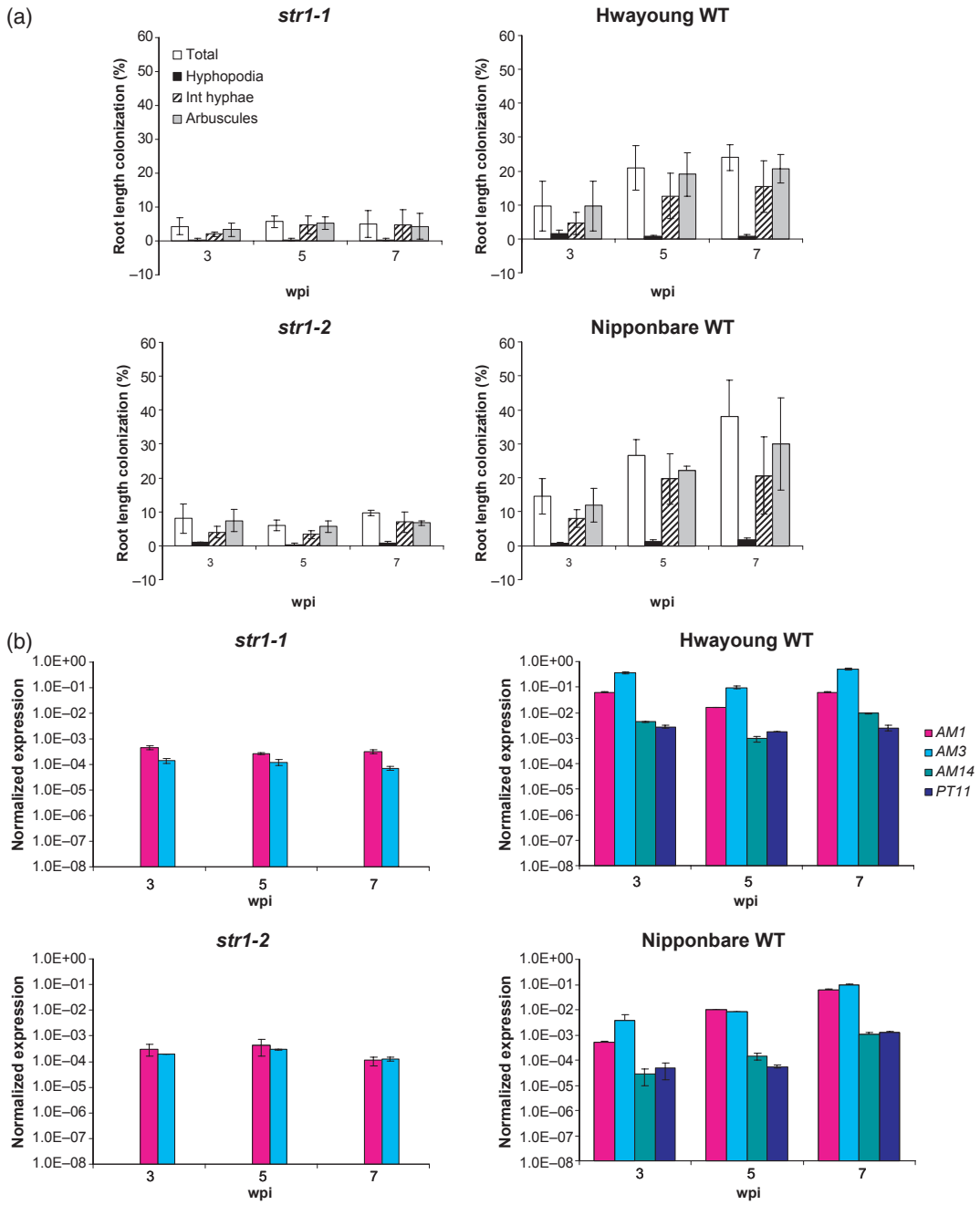

Figure 5. Molecular phenotypes of *str1* mutants colonized by *Gigaspora rosea*.
(a) *Gigaspora rosea* colonization kinetics of *str1-1* and *str1-2*, compared with their corresponding wild type. The percentage of root length colonization was determined by the grid line intersect method. Means and SEs of three biological replicates each represented by duplicate samples are shown. Int hyphae: intraradical hyphae. (b) Real-time RT-PCR-based expression kinetics of two early (*AM1, AM3*) and two late (*AM14, PT11*) rice arbuscular mycorrhizal (AM) marker genes in roots of *str1-1*, *str1-2* and the corresponding wild type in response to *G. rosea* colonization. Error bars indicate SDs of three technical replicates. Each sample is a pool of three plants.

suggests a positive feedback loop to co-regulate both genes. *AntiSTR1* was expressed in roots of all treatments at approximately the same level at all time points. In summary, the T-DNA insertions in *str1-1* and *str1-2* perturb *STR1* and *STR2* induction in response to AM colonization, but do not affect the expression of *antiSTR1*.

The stunted arbuscule phenotype of *str1* persists in nurse culture

In *M. truncatula*, STR1 and STR2 have been shown to reside and interact in the periarbuscular membrane, and with analogy to other half-size ABCG transporters are predicted

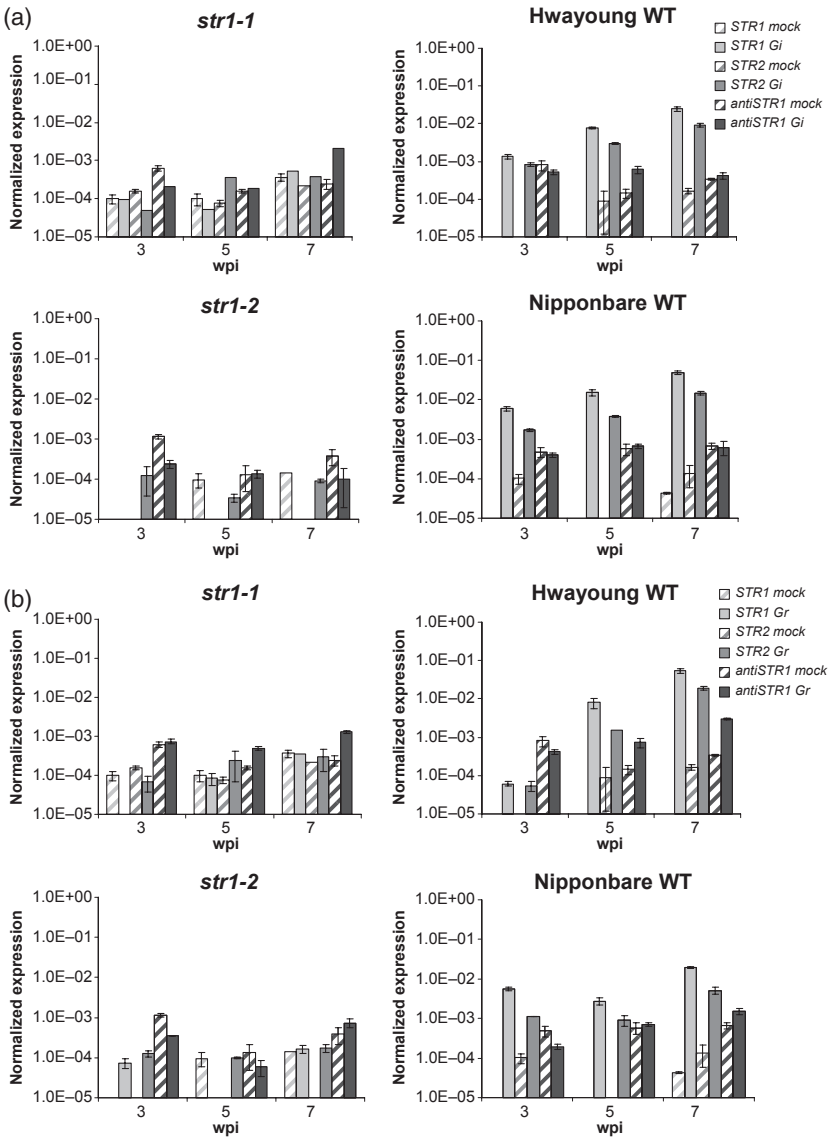

Figure 6. Expression kinetics of *STR1*, *STR2* and *antiSTR1* in *str1* mutants.
Three mock (hashed bars) and (a) *Glomus intraradices* (Gi, filled bars) or (b) *Gigaspora rosea* (Gr, filled) inoculated rice root systems were pooled per sample. Expression was determined by real-time RT-PCR. Means and standard deviations of three technical replicates are shown. As all plants were grown in parallel, the same data for mock-inoculated roots are shown in (a) and (b).

to be exporters (Zhang *et al.*, 2010). Consequently, the lack of the STR1/STR2 substrate in the periarbuscular interface might interfere with symbiosis signalling, and could ultimately lead to altered carbon flow and thus to energy deprivation of the fungus, reflected by stunted arbuscules. Fungal starvation as a cause for limited arbuscule development was examined by co-cultivating inoculated *str1-1* plants with wild-type nurse plants, which serve nutrients and energy to the fungus (Figure 7a). To obtain good resolution of possible quantitative differences, half-strength inoculum was used for this experiment. At 6 wpi monocultured wild-type plants had high colonization levels, whereas monocultured *str1-1* plants were barely colonized (Figure 7b). The presence of wild-type plants quantitatively enhanced the colonization of *str1-1* plants relative to the pure mutant culture (Figure 7b), yet the stunted arbuscule phenotype persisted (Figure 7c). Therefore, fungal fitness can be enhanced by community culture with wild-type nurse plants, but the morphological arbuscule defect is not complemented and thus not caused by a general lack of energy.

Strigolactones are unlikely substrates for STR1 and STR2

Root-released strigolactones induce branching of AM fungal hyphae prior to root colonization (Akiyama *et al.*, 2005). It

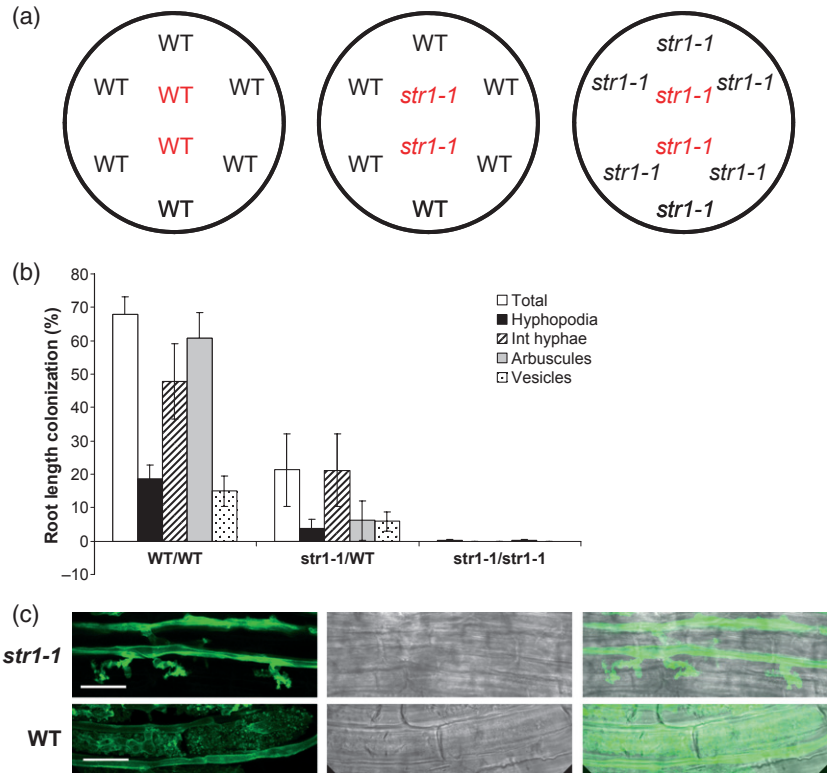

Figure 7. Enhanced colonization of *str1* by wild-type nurse plants.
(a) Experimental set-up: two *str1-1* mutant plants were surrounded by six wild-type plants and inoculated with *Glomus intraradices*. As a control, two plants of each genotype were surrounded by six plants of the same genotype.
(b) The percentage of root length colonization, as determined by the gridline intersect method, of the Hwayoung wild type surrounded by six wild-type plants (WT/WT), *str1-1* surrounded by wild type (*str1-1*/WT) and *str1-1* surrounded by *str1-1* (*str1-1/str1-1*) at 6 weeks post inoculation (wpi). Means and SEs of three biological replicates represented by duplicate samples are shown. Int hyphae: intraradical hyphae.
(c) Confocal images of *G. intraradices* arbuscules stained with wheatgerm agglutinin (WGA) conjugated with Alexa Fluor 488 in cortex cells of *str1-1* and Hwayoung wild type at 6 wpi. Bright-field images and overlays show the outline of the cortical cells. Although colonization in *str1-1* is enhanced by the presence of the wild type, the arbuscule phenotype is not complemented. Scale bars: 20 μm.

has been hypothesized that strigolactones might also be required as a signal for hyphal branching inside the root during arbuscule formation, and furthermore that the absence of wild-type arbuscule branching in *str1* and *str2* mutants might suggest an involvement of the STR1/STR2 exporters in strigolactone release (Zhang *et al.*, 2010). Therefore, mutants deficient in strigolactone biosynthesis should display a stunted arbuscule phenotype similar to *str1* and *str2*. Decreased colonization levels have been observed in strigolactone mutants of pea and tomato, but arbuscule morphology in roots of these mutants have not been reported (Gomez-Roldan *et al.*, 2008; Koltai *et al.*, 2010). We therefore tested this hypothesis by employing the *d17* and *d10* rice lines that carry mutations in CAROTEOID CLEAVAGE DIOXIGENASE 7 and 8 (CCD7 and CCD8), respectively, which are required for strigolactone biosynthesis (Umehara *et al.*, 2008), and for which the arbuscule phenotype is not known.

Similar to *str1* and *str2* mutants, colonization of *d10* and *d17* by *Glomus intraradices* was reduced at 6 wpi by more than half in both mutants with respect to the corresponding wild type (Figure 8a). However, a close inspection of arbuscule morphology revealed wild-type like highly branched arbuscules in roots of both strigolactone-deficient mutants (Figure 8b). Therefore, as strigolactone levels in *d10* and *d17* are below the detection limit (Umehara *et al.*, 2008), we conclude that strigolactones are unlikely to be required for arbuscule branching, and that the phenotype of *str1* and *str2* mutants is not caused by a lack of strigolactone transport.

DISCUSSION

Arbuscular mycorrhizal symbiosis has great potential for application in sustainable agricultural practices, reducing fertilizer input because of the nutritional benefit it confers to plants. To improve crop performance through the application of AM fungi it is crucial to understand the molecular components that are important for arbuscular mycorrhiza development and function, especially in cereals, which represent the world's most important staple crops. Here we analyzed the function of STR1 and STR2, two half-size ABCG

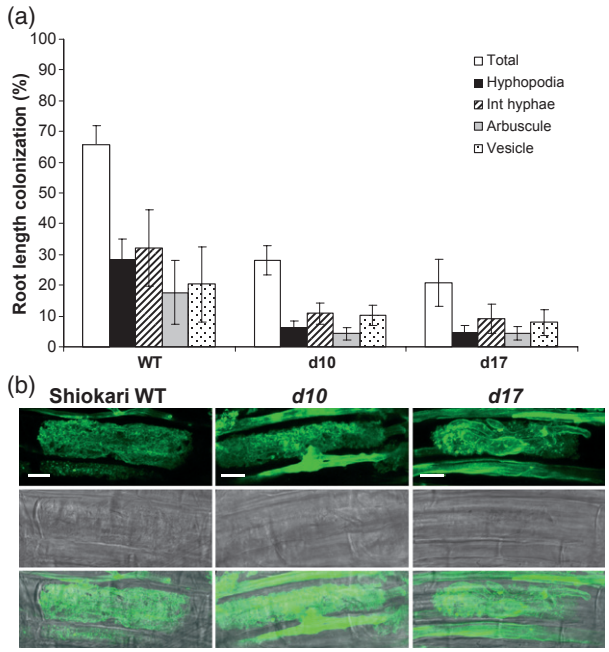

Figure 8. Colonization of strigolcatone biosynthesis mutants by *Glomus intraradices*.
(a) The percentage root length colonization of strigolactone biosynthesis mutants defective in CCD8 (*d10*) and CCD7 (*d17*), as determined by the grid line intersect method, at 6 wpi. Means and SEs of three independent biological replicates represented by duplicate samples are shown.
(b) The arbuscule morphology in *d10* and *d17* mutants is comparable with that of the Shiokari wild type. Bright-field images and overlays show the outline of cortical cells. Scale bars: 10 μm.

transporters in the major staple crop rice. We show that the mutation of rice *STR1* and *STR2* interferes with arbuscule formation, thus providing evidence for functional conservation of STR1 and STR2 between members of the di- and monocotyledons (Zhang *et al.*, 2010).

We found putative orthologs of STR1 and STR2 in the currently sequenced representatives of the *Pooideae*, *Paniceae* and *Anthopogoneae*. This evidence, together with the presence of putative orthologs in the genome of the lycophyte *Sellaginella moellendorfii* (Zhang *et al.*, 2010), suggests that *STR1* and *STR2* arose in lower plants prior to the divergence of the angiosperms, and that their function is broadly conserved among mycorrhizal land plants. Also, the common SYM pathway, required for fungal penetration into the host root, is functionally conserved across the major angiosperm classes (Chen *et al.*, 2007, 2008, 2009; Banba *et al.*, 2008; Gutjahr *et al.*, 2008), and orthologs of common SYM genes have been found in all plant lineages (Wang *et al.*, 2010). Conservation of STR1 and STR2 consolidates the hypothesis that arbuscular mycorrhizal symbiosis arose simultaneously with the colonization of land by plants (Remy *et al.*, 1994; Kistner and Parniske, 2002), and that the genetic repertoire required for the development of arbuscular mycorrhiza might have emerged in a common ancestor of land plants.

Although the knock-out of *STR1* interferes with arbuscule development, the signaling pathway leading to arbuscule-related gene induction is not perturbed, as late marker gene expression can be detected at higher levels of colonization with stunted *Glomus intraradices* arbuscules. This suggests that the expression level of arbuscule-related marker genes is a function of average arbuscule growth in the root system, such that an early arrest of arbuscule growth leads to lower levels of induction, and a certain minimum number of arbuscules is required to reach the threshold for transcript detection. Corroborating our findings in *M. truncatula*, the transcript of the specifically arbuscule-induced *OsPT11* orthologue *MtPT4* was detected at a relatively high level at 7 days after contact with *Glomus intraradices* spores (Zhang *et al.*, 2010). The earlier time point of *PT4* detection in *M. truncatula* is explained by the use of an inoculation system that allows synchronized burst colonization in a very short time (Zhang *et al.*, 2010).

Interestingly, the severity of the molecular phenotype of rice *str1* mutants depended on the AM fungus employed, as roots colonized by *Gigaspora rosea* showed no arbuscule-related gene induction. It has been reported earlier that different fungal species might differ in their influence on host transcriptional responses (Hohnjec *et al.*, 2005; Liu *et al.*, 2007), and this might result from variation in colonization dynamics or the cocktail of signaling molecules produced by different fungal species. More importantly, differences in arbuscule morphology in rice roots have been reported for *Glomus intraradices* and *Gigaspora rosea* (Gutjahr *et al.*, 2008; Kobae and Hata, 2010). *Glomus intraradices* only forms highly branched arbuscules, whereas *Gigaspora rosea* develops a mixture of branched arbuscules and thick arbuscular and hyphal coils. As the PT11 protein accumulates only in the membranes surrounding highly branched arbuscules, but not in those surrounding thick coiled hyphae (Kobae and Hata, 2010), it is possible that the number of arbuscules with inductive capacity in *Gigaspora rosea*-colonized *str1* mutant roots was not sufficient to elicit late marker genes. Alternatively, differences in the composition of signaling molecules released by two AM fungal species might lead to differences in host gene expression, which might in turn impact on arbuscule morphology.

The STR1/STR2 complex resides in the peri-arbuscular membrane, which represents the plant border of the apoplastic symbiotic interface. Based on the structure of STR1 and STR2, it was predicted that they are involved in the export of a molecule, which might be required locally at the periarbuscular space (Zhang *et al.*, 2010). ABC transporters can transport a large variety of substrates ranging from small peptides, oligosaccharides, secondary metabolites, hormone xenobiotics and lipids to mineral ions (Rea, 2007; Rees *et al.*, 2009; Woodward *et al.*, 2011). An appealing

hypothesis was that strigolactones could be a possible substrate of the STR1/STR2 dimer, and a signal for arbuscule branching (Zhang *et al.*, 2010), as they induce hyphal branching outside of the root prior to colonization (Akiyama *et al.*, 2005). To test this hypothesis we took advantage of the availability of the rice mutants *d10* and *d17*, which are defective in strigolactone biosynthesis (Umehara *et al.*, 2008), and report for the first time the AM phenotype of those mutants in rice. The colonization level of *d10* and *d17* was reduced, consistent with previous observations on strigolactone biosynthesis mutants in *Pisum sativum* (pea) and *Solanum lycopersicum* (tomato) (Gomez-Roldan *et al.*, 2008; Koltai *et al.*, 2010). However, the arbuscule morphology was equivalent to that of wild-type plants, indicating that strigolactones are not required for arbuscule development, and are probably not the substrate of the STR1/STR2 dimer. It has recently been shown that in addition to strigolactones, hydroxy fatty acids can induce branching in germination hyphae of AM fungi (Nagahashi and Douds, 2011), and it is possible that similar compounds are transported by STR1 and STR2 to induce arbuscule branching inside cortical cells.

A second hypothesis was that the STR1/STR2 dimer might transport a plant-derived nutrient required for arbuscule development (Zhang *et al.*, 2010). Thus, reduced arbuscule development might result from energy deprivation of the fungus. Inoculation of a *str1* mutant with wild-type plants in the same pot enhanced the level of colonization in the mutant, but did not complement the stunted arbuscule phenotype, showing that although the vigour of the fungus can be enhanced by external energy supply, arbuscule stunting is not caused by a general malnutrition of the fungus. However, it cannot be excluded that the STR1/STR2 dimer exports a specific nutritious compound, which is locally required at the arbuscule.

In plants, half-size ABC transporters of the G subfamily have been implicated in the export of lipid molecules from the leaf epidermis to the cuticle (Bird *et al.*, 2007; Luo *et al.*, 2007; Panikashvili *et al.*, 2007; Bessire *et al.*, 2011), and also other hydrophobic compounds such as sporopollenin precursors, which are required for pollen exine development and male fertility (Quilichini *et al.*, 2010; Bessire *et al.*, 2011; Choi *et al.*, 2011; Dou *et al.*, 2011). Animal ABCG family members transport cholesterol and other sterols or hydrophobic molecules (Borst *et al.*, 2000; Schmitz *et al.*, 2001; Wittenburg and Carey, 2002; Wang *et al.*, 2011). Sterols and sphingolipids are structural constituents of membranes, and are important for the construction of specific membrane domains, for example lipid rafts that are believed to unite proteins involved in signal transduction processes (Simons and Toomre, 2000). Whereas the STR1/STR2 substrate could be a signal or a locally required nutrient, it might alternatively be a precursor of or a plant-derived structural component that serves to build specific fungal membrane domains, and is not synthesized by the AM fungus itself.

We found that rice STR1 is associated with a *cis*-NAT, *antiSTR1*. cDNA sequencing showed that in rice 7% of transcripts are associated with *cis*-NATs (Osato *et al.*, 2003), but their function is not clear to date. In Arabidopsis, antisense transcipts have been reported to generate small interfering RNAs (siRNAs) that negatively regulate genes involved in diverse processes (Borsani *et al.*, 2005; Zubko and Meyer, 2007; Held *et al.*, 2008; Swiezewski *et al.*, 2009) or influence the location of the polyadenylation site of the sense transcript (Zubko *et al.*, 2011). However, *cis*-NATs might regulate their corresponding sense transcript in other ways, such as RNA masking, chromatin remodelling or RNA editing, and it has been proposed that the expression pattern of sense and antisense transcript might give clues on the regulatory outcome of their interaction (Lapidot and Pilpel, 2006). It is surprising that *antiSTR1* accumulates at equal levels in mycorrhizal and non-mycorrhizal roots, in contrast to *STR1* and *STR2* mRNAs, which are barely detectable in non-mycorrhizal roots and abundantly induced upon mycorrhizal colonization. However, at the level of the root cortex *antiSTR1* is specifically expressed in arbusculated cells, along with *STR1* and *STR2*. It is likely that in non-colonized roots *antiSTR1* is expressed in cell types other than cortex cells, for example in the vascular tissue, as shown for Medicago *STR1* and *STR2* (Zhang *et al.*, 2010). Furthermore, constitutive *antiSTR1* transcript accumulation might serve to control possible deleterious *Os*STR1 (and *Os*STR2) accumulation in the absence of arbuscular mycorrhiza. Intriguingly, it has recently been reported that rice *PHO1* genes are also coupled with *cis*-NATs (Secco *et al.*, 2010). Rice *PHO1;2* maintains phosphate homeostasis, especially in phosphate starvation conditions. Interestingly, upon phosphate starvation, *PHO1;2* transcript abundance remains constant, whereas that of its *cis*-NAT is increased, pointing to a positive impact of the antisense transcript on PHO1;2 protein availability. Analogously, expression of *antiSTR1* in arbusculated cells might promote the translation of *STR1* by stabilizing its mRNA. Further experiments involving transcript localization and (cell type-specific) expression perturbation of *antiSTR1* are required to test these hypotheses.

EXPERIMENTAL PROCEDURES

Plant material

For all experiments *O. sativa* ssp. *japonica* cv. Nipponbare was used, if not indicated otherwise. For the functional analysis of *STR1* (TIGR ID, LOC_Os09g23640; RAP ID, Os09g0401100), two T-DNA insertion mutants were identified: *str1-1* (1C-04850; FST-Postech; Jeon *et al.*, 2000) in the cv. Hwayoung background; and *str1-2* (CL522472; FST-Genoplante; Sallaud *et al.*, 2004) in the cv. Nipponbare background. For *STR2* (RAP ID, jigsaw07_642) a dSpm transposon insertion mutant *str2-1* (RdSpm 2654D; http://sundarlab.ucdavis.edu/rice/query_database.html) in the cv. Nipponbare

background was obtained. Homozygous mutant lines were identified and the exact position of the insertion was determined by re-sequencing the insertion flanks, as described previously (Gutjahr *et al.*, 2008), using the primers shown in Table S1. The strigolactone biosynthesis mutants *d10* and *d17* (Umehara *et al.*, 2008) and the corresponding wild-type cv. Shiokari were kindly provided by Shinjiro Yamaguchi (RIKEN Plant Science Center, http://www.psc.riken.jp).

Growth conditions and inoculation by AM fungi

Plants were inoculated with *Glomus intraradices* or *Gigaspora rosea*, as described previously (Gutjahr *et al.*, 2008). For the community experiment, plants were inoculated with *Glomus intraradices* and grown in pots where two *str1-1* mutant plants or two Nipponbare wild-type plants were surrounded by six *str1-1* mutant or six wild-type plants, as shown in Figure 7a. All plants were watered three times a week for the first 2 wpi. Thereafter they were watered once a week and fertilized twice a week with a mix of 0.005% (w/v) Hauert-Flory 2 type K (Hauert, http://www.hauert.com) and 0.01% Sequestren Rapid (Syngenta, http://www.syngenta.com).

Root staining, quantification of AM colonization and confocal microscopy

Quantification of AM colonization after Trypan blue staining was performed as described previously (Gutjahr *et al.*, 2008). For confocal microscopy, roots were stained with WGA-Alexafluor 488 and imaged with a Zeiss LSM 700 confocal microscope (http://www.zeiss.de).

Amplification of full-length cDNAs

The full-length cDNAs of STR1 and STR2 were segmentally amplified for sequencing with the primers listed in Table S3. The 5′ and 3′ untranslated regions (UTRs) were obtained with the Invitrogen GeneRacer® kit (Invitrogen, http://www.invitrogen.com) according to the manufacturer's instructions and using the gene-specific primers shown in Table S3. Full-length cDNA sequences were submitted to GenBank under the accession numbers JN608807 (STR1) and JN608806 (STR2).

Laser microdissection

A small number of roots inoculated with *Glomus intraradices* were collected at 7 wpi and cut into 0.5-cm-long pieces. These were acetone fixed and embedded employing a microwave-enhanced paraffin embedding protocol, as previously described (Tang *et al.*, 2006). Tissue blocks were immediately cut into 12-μm sections on a rotary microtome (Leitz, http://www.leica-microsystems.com) and mounted on UV-treated PEN 1-mm PALM membrane slides (PALM, http://www.zeiss.de). A total of 2000 cells per replicate were laser captured with a PALM microbeam system (PALM) from deparaffinized sections within 48 h of preparation.

RNA extraction, cDNA synthesis and real-time RT-PCR

RNA extraction, cDNA synthesis and real-time RT-PCR were performed as described previously (Gutjahr *et al.*, 2008). Primer sequences not mentioned in Gutjahr *et al.* (2008) are listed in Table S3. The specificity of the primers designed to amplify *antiSTR1* from oligo dT-primed cDNA was tested by comparison with with cDNA primed with *antiSTR1*-specific primers (Tables S1 and S3). If not indicated otherwise, all expression values are displayed relative to *CYCLOPHILIN2* expression.

Phylogenetic analysis

Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007) using the maximum parsimony method. The bootstrap consensus tree inferred from 1000 replicates was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000), with search level 7, in which the initial trees were obtained with the random addition of sequences (10 replicates). All alignment gaps were treated as missing data. There were a total of 2457 positions in the final data set, out of which 1557 were parsimony informative. Protein sequences were adopted from Verrier *et al.* (2008) and Zhang *et al.* (2010), except for: *Brachypodium distachyon*, *Bd*STR1 (Bradi4g29810.1) and *Bd*STR2 (Bradi2g26560.1); *Setaria italica*, *Si*STR1 (SiPROV028709m.g) and SiSTR2 (SiPROV033915m.g); and *Zea mays*, ZmSTR1 (GRMZM2G357034_P01) and ZmSTR2 (GRMZM2G035276_P01). which were obtained by searches in http://www.phytozome.com and http://www.maizesequence.org.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Phylogenetic tree of rice and Arabidopsis ABCG transporters.

Figure S2. Experiment II: molecular phenotype of *str1* mutants colonized by *Glomus intraradices*.

Figure S3. Experiment II: molecular phenotype of *str1* mutants colonized by *Gigaspora rosea*.

Figure S4. Experiment II: expression kinetics of *STR1*, *STR2* and *antiSTR1* in *str1* mutants.

Table S1. Specificity test for real-time RT-PCR primers targeting *antiSTR1*.

Table S2. Putative *antiSTR1* targets.

Table S3. Primers used in this study.

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