Identification and functional characterization of a sulfate transporter induced by both sulfur starvation and mycorrhiza formation inLotus japonicus

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Summary

Arbuscular mycorrhizas (AMs) are one of the most widespread symbioses in the world. They allow plants to receive mineral nutrients from the symbiotic fungus which in turn gets back up to 20% of plant carbon and completes its life cycle. Especially in low-nutrient conditions, AM fungi are capable of significantly improving plant phosphate and nitrogen acquisition, but fewer data are available about sulfur (S) nutrition.
We focused on S metabolism in *Lotus japonicus* upon mycorrhizal colonization under sulfur starvation or repletion. We investigated both tissue sulfate concentrations and S-related gene expression, at cell-type or whole-organ level.
Gene expression and sulfate tissue concentration showed that *Rhizophagus irregularis* colonization can improve plant S nutritional status under S starvation. A group 1 sulfate transporter, LjSultr1;2, induced by both S starvation and mycorrhiza formation, was identified. Its transcript was localized in arbuscule-containing cells, which was confirmed with a promoter-GUS assay, and its function was verified through phenotyping of TILLING mutants in nonmycorrhizal seedlings.
LjSultr1;2 thus appears to encode a key protein involved in plant sulfate uptake. In contrast to phosphate transporters, a single gene, LjSultr1;2, seems to mediate both direct and symbiotic pathways of S uptake in *L. japonicus*.

Introduction

Sulfur (S) is an essential macronutrient for plant growth, being a major component of the amino acids cysteine and methionine, and a large number of co-enzymes and prosthetic groups, as well as of many natural products of secondary metabolism (Takahashi et al., 2011a). Sulfur is often central to the biological function of many compounds because of its redox properties and the ability to create disulfide bonds between cysteine residues. Plants use inorganic sulfate as their major S source, but 95% of soil S is bound into organic compounds (Scherer, 2001) and therefore not directly available to plants (Leustek, 1996). In addition to sulfate, plants are able to supplement their S needs with reduced S forms absorbed from the atmosphere, such as sulfur dioxide and hydrogen sulfide (Leustek et al., 2000; Durenkamp & De Kok, 2004).

Sulfur deficiency has become an increasing problem for agricultural crops over the last few decades as a consequence of the combined effects of intensive farming methods, high-yield crop varieties, and declining atmospheric S deposition (McGrath et al., 2002). Available S in the soils of agricultural land now lies well below the recommended levels for crops. In Europe, S deficiency has already caused reduced crop yield, reduced nutritional value and increased disease susceptibility (reviewed by Lewandowska & Sirko, 2008).

It is well known that the formation of symbiosis with arbuscular mycorrhizal (AM) fungi leads to an improved nutritional status of the plant partner (Smith & Smith, 2012), especially under low nutrient availability. AM symbiosis allows the plant to access unexplored soil niches and absorb usually inaccessible nutrient sources: this dual nutritional advantage of AM symbiosis has long been known from both a physiological (Smith & Smith, 2011) and a molecular perspective (Harrison et al., 2002; Bucher, 2007). Extraradical AM hyphae are capable of absorbing minerals from the soil and transferring them to intraradical hyphae and through a specialized and highly branched structure – the arbuscule – to the plant.

The impact of mycorrhizal symbiosis on plant mineral nutrition has been extensively studied and, in particular, it has been demonstrated that plants possess a symbiotic phosphate uptake pathway (Smith & Smith, 2011). Also, ammonium transporters specifically expressed in arbusculated cells have been discovered (Gomez et al., 2009; Guether et al., 2009a; Kobae et al., 2010). Evidence for the involvement of AM symbiosis in the transfer of different mineral nutrients has been obtained in studies of different plant species (Paszkowski et al., 2002; Nagy et al., 2005; Guether et al., 2009b; Hogekamp et al., 2011).

Compared with the large number of reports on symbiotic phosphate and nitrogen (N) uptake, much less is known about the role of mycorrhizas in plant S acquisition. Sulfur nutrition has been investigated from the perspectives of both AM partners. On the fungal side, *Rhizophagus irregularis* was shown, in monoxenic conditions, to take up both sulfate- and S-containing amino acids and transfer them to the plant (Allen & Shachar-Hill, 2009). On the plant side, Casieri et al. (2012) investigated the transcriptomic response of *Medicago truncatula* to different sulfate concentrations in mycorrhizal and nonmycorrhizal plants, and showed that the regulation of M. truncatula sulfate transporters depends on S concentration and mycorrhizal colonization at both the root and shoot levels. In parallel, Sieh et al. (2013) shifted the focus to the interplay between the effects of phosphate and sulfate concentrations in the media and different mycorrhizal effects, and demonstrated the actual transfer of S from the fungus to the plant. The study also considered the impact of the AM symbiosis on the plant at the proteome and metabolome levels and the connections with S availability. However, notwithstanding the abovementioned recent data, the mechanisms and gene regulation underlying the transfer of S from the fungus to the plant are still unknown. With the general aim of shedding light on the effect of mycorrhiza formation on S metabolism, we identified and characterized a novel AM-dependent sulfate transporter, LjSultr1;2, that responds to S starvation. By the combination of different experimental approaches, we demonstrated that LjSultr1;2 is up-regulated in S starvation conditions and that in mycorrhizal roots its transcript accumulates specifically in arbusculated cells, which was also confirmed by promoter-GUS experiments. Through a reverse genetic approach, we showed the importance of this sulfate transporter in the uptake of sulfate in 4- and 10-d-old *L. japonicus* seedlings, even in the absence of the mycorrhizal partner. These results raise new, broader questions about plant sulfate uptake, where, in contrast to phosphate transport, a single gene, LjSultr1;2, seems to mediate both direct and symbiotic pathways in *L. japonicus*.

Materials and Methods

Plant material, growth conditions, and inoculation methods

Lotus japonicus (Regel) Larsen (ecotype 'Miyakojima' MG-20) seeds were chemically scarified with a 15-s sulfuric acid treatment, washed thoroughly with distilled water, surface-sterilized for 3 min in a 1% sodium hypochlorite and 1% Triton solution and washed again four times, for 10 min each, with sterile deionized water. Sterilized seeds were placed in 3% plant agar (w/v) in 15-cm Petri dishes at 25°C in the dark for 3 d to allow seed germination and in the light for another 3 d. Three or four seedlings were transplanted into 1-l pots filled with sand : vermiculite (50 : 50). Each plant was inoculated with 0.1 g of Rhizophagus irregularis inoculum (Symplanta; http://symplanta.com). Control plants were mock-inoculated with the same amount of double-autoclaved inoculum. Plants were grown in phytochambers with a day : night cycle of 16 h : 8 h and temperature 23 : 21°C, respectively, with illumination at 123 μmol s−1 m−2 and 26.7 W m−2. Plants were watered twice a week with a modified half-strength Long Ashton solution (2 × KNO3 and 0.25 mM phosphate; Hewitt, 1966) with the sulfate concentration depending on the treatment (−S: 10 μM; +S: 1 mM sulfate). This molarity of phosphate allowed a good level of mycorrhiza formation (Fiorilli et al., 2013) and there was no significant difference in plant phosphate and phosphorus (P) concentrations between mycorrhizal and control plants (Supporting Information Figs S1, S2). For the −S medium, all sulfate salts were exchanged by chlorides, except for MgSO4 which was replaced by Mg(NO3)2.

Four plants per treatment were harvested 8 wk after inoculation, allowing homogeneous mycorrhiza formation and S starvation. Mycorrhizal parameters were evaluated both using classical cotton blue coloration and microscopic evaluation (Trouvelot et al., 1986) and using the relative expression of plant and fungal marker genes (Lotus phosphate transporter and R. irregularis elongation factor, LjPT4 and GintEF, respectively) on the same material that was used for the other experiments (Fig. S3).

The generation of composite plants, for the promoter-GUS experiment, was performed using Agrobacterium rhizogenes 1193 (Stougaard et al., 1987) on L. japonicus wild-type seedlings. After the emergence of hairy roots from the root section, seedlings were grown on L medium (Stougaard et al., 1987) supplemented with cefotaxime (0.02% from a 300 mg l−1 stock solution) to eliminate A. rhizogenes. Plants with transformed roots were grown in pots and half of them were inoculated with Funneliformis mosseae. The selection of transformed roots was performed by analyzing the fluorescence of the reporter gene present in the plasmid employed (DsRED) using a stereomicroscope (Leica M205 FA; http://www.leica.com).

TILLING sultr1;2 mutants were generated by RevGen (Norwich, UK; http://revgenuk.jic.ac.uk/) (Perry et al., 2003) as shown in Fig. S7. The LjSultr1;2 genomic sequence was obtained from an L. japonicus genome database (http://www.kazusa.or.jp/lotus/) and four different mutants with altered amino acid sequences within one of the predicted transmembrane regions were selected for further analysis.

RNA isolation and expression analysis

Total RNA was extracted from L. japonicus shoot and root tissues by standard phenol/chlorophorm extraction and LiCl precipitation and analyzed as described by Kawashima et al. (2011). First-strand cDNA was synthesized from 600 ng of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Crawley, UK), which includes a DNAse step to remove possible DNA contamination. Quantitative real-time RT-PCR (qPCR) was performed using gene-specific primers (Table S1) and the fluorescent intercalating dye SYBR Green (Applied Biosystems, Warrington, UK) as described by Lee et al. (2011). All quantifications were normalized to the ubiquitin gene, LjUBQ10 (TC14054) gene. The RT-PCR reactions were performed in duplicate for each of the three independent biological samples.

Laser microdissection

Mycorrhizal and nonmycorrhizal root segments were fixed in freshly prepared methacarn (absolute methanol : chloroform : glacial acetic acid (6 : 3 : 1)) at 4°C overnight for paraffin embedding (Balestrini et al., 2007). A Leica AS laser microdissection system (Leica Microsystems Inc., Bensheim, Germany) was used to isolate cells from the prepared tissue sections as described by Balestrini et al. (2007). After collection, the RNA extraction buffer from the PicoPure kit (Arcturus Engineering, Mountain View, CA, USA) was added and samples were incubated at 42°C for 30 min, centrifuged at 800 g for 2 min, and stored at −80°C. Then, for the following RNA extraction steps, c. 1500 cells were pooled for each cell-type population in a single tube at a final volume of 50 ml. RNA extractions were performed using a slightly modified PicoPure kit protocol (Arcturus Engineering), as described by Balestrini et al. (2007). RNA quantification was obtained using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A one-step RT-PCR kit (Qiagen, Valencia, CA, USA) was used for the RT-PCR experiments that were conducted on the RNA extracted from several samples. Reactions were carried out as described in detail in Guether et al. (2009b). Amplification reactions with specific primers for LjSultr1;2 and the housekeeping gene LjUBQ10 (Guether et al., 2009b; annealing temperature 63 and 60°C, respectively) were used. The RT-PCR experiments were conducted on at least three independent biological replicates.

Histochemical analysis of root tissue

Seven hundred and thirty base pair upstream of LjSultr1;2 coding sequence were fused with the GUS gene in the vector pKGWFS7,0. The red fluorescent marker DsRED, under the control of the constitutive Arabidopsis Ubiquitin10 promoter (PUbq10) (Limpens et al., 2005), was inserted. Lotus japonicus composite plants carrying transformed roots were fertilized with 10 μM and 1 mM sulfate in the −S and +S treatments, respectively, and inoculated with Funneliformis mosseae BEG12 (http://www.mycagrolab.com/). Control plants were grown in the absence of the fungus. Root fragments, showing DsRED fluorescence and extraradical fungal structures, were selected under a stereomicroscope and excised. The root segments were covered with freshly prepared GUS buffer (0.1 m sodium phosphate buffer, pH 7, 0.5 mm K4Fe(CN)6, 5 mM K3Fe(CN)6, 0.3% Triton X, and 0.3% X–Glc). Samples

were incubated at 37°C for 16 h in the dark, washed with distilled water and observed under an optical microscope (Eclipse E400; Nikon; http://www.nikon.com).

Shoot and root metabolite measurements

For anion measurements, 20–30 mg of frozen ground material was homogenized in 1 ml of deionized water containing 20 mg of polyvinylpolypyrrolidone. Samples were shaken at 4°C for 1 h, left at 95°C for 15 min and then centrifuged at 14 000 g for 15 min. Then 200 µl of the supernatant was transferred to HPLC vials and analyzed as described by Loudet et al. (2003).

For P and S root quantification, c. 2 mg of dried material was digested in 1 ml of 6 M HNO3 for 1 h at 95°C. The digestion product was diluted to 6 ml with milliQ water and filtered. The P and S contents were determined in the final solution using inductively coupled plasma atomic emission spectrometry (ICP-AES) performed using a Liberty 100 Varian apparatus equipped with a V-Groove nebulizer and a Czerny-Turner monochromator (Varian, Palo Alto, CA, USA). The same treatments were applied to the control solution, which contained no sample. Quality control was based on the use of internal control samples and certified samples (Astasol-Mix from Analytica Ltd, Prague, Czech Republic).

The low-molecular-weight thiols cysteine and glutathione were analyzed as described by Koprivova et al. (2008). The extraction was performed in a 10-fold volume of 0.1 M HCl using 20–30 mg of ground leaf or root material. To remove cell debris, the extract was centrifuged at 16 000 g for 10 min, and 25 µl of the supernatant was neutralized with 25 µl of 0.1 M NaOH. To reduce disulfides, the neutralized extract was incubated for 15 min at 37°C with 1 µl of 100 mM dithiothreitol. Subsequently, 35 µl of water, 10 µl of 1 M Tris, pH 8.0, and 5 µl of 100 mM monobromobimane (Thiolyte® MB; Calbiochem, San Diego, CA, USA) were added and derivatization of thiols was allowed to proceed for 15 min at 37°C in the dark. The reaction was stopped and the conjugates stabilized by the addition of 100 µl of 9% acetic acid. Bimane conjugates were separated by HPLC (SpherisorbTM ODS2, 250 × 4.6 mm, 5 µm; Waters Corp., Milford, MA, USA) using 10% (v/v) methanol and 0.25% (v/v) acetic acid (pH 9.3) as solvent A and 90% (v/v) methanol and 0.25% (v/v) acetic acid (pH 9.3) as solvent B. The elution protocol employed a linear gradient from 96 to 82% A in B within 20 min, with a constant flow rate of 1 ml min−1. Bimane derivates were detected fluorimetrically (474 detector; Waters) with excitation at 390 nm and emission at 480 nm.

Determination of sulfate uptake

Sulfate uptake was measured using [35S]sulfate, essentially as described by Kawashima et al. (2011). Four- or 10-d-old seedlings of the wild-type and the sultr1;2 mutant were transferred into 24-well plates, with the roots submerged in 1 ml of nutrient solution adjusted to a sulfate concentration of 0.2 mM and supplemented with 6.7 µCi [35S]sulfate (Hartmann Analytic, Braunschweig, Germany) to a specific activity of 552 Bq (nmol sulfate)−1 and incubated in the light for 30 min. After incubation, seedlings were washed with nonradioactive nutrient solution and carefully blotted with paper tissue. Seedlings were weighed and transferred into scintillation vials, then 2 ml of tissue solubilizer Soluene (Perkin Elmer, Norwalk, CT, USA) was

added to dissolve the tissues. After 2–3 d, the 35S in plant material was quantified using scintillation counting.

Results

To better evaluate the impact of mycorrhiza formation on plant S nutrition and plant sulfate transport, first we had to control for Pi-dependent effects, as AM fungi enhance phosphate (Pi) uptake by their host (Smith & Smith, 2012) and cross-talk is known to take place between S and phosphate metabolisms (Rouached, 2011). To control for P-dependent effects, plants were grown at an intermediate phosphate concentration (250 μM), allowing both good mycorrhizal colonization (Fiorilli et al., 2013) and similar phosphate and P concentrations in control and mycorrhizal plants (Figs S1, S2).

Impact of AM symbiosis on the S status of *L. japonicus*: gene expression and sulfate, S and S metabolite concentrations

To investigate the effect of mycorrhiza formation on S starvation and S nutrition, half of the plants were inoculated with R. irregularis. −S and +S conditions showed similar levels of mycorrhizal colonization, on the basis of a quantitative evaluation of fungal presence (Trouvelot et al., 1986) and of mycorrhizal functionality indicated by LjPT4 transcript accumulation (Fig. S3), and similar Pi and P concentrations (Figs S1, S2). The effect of mycorrhiza formation on L. japonicus S nutrition was analyzed at two levels: gene expression, and tissue concentration of sulfate, S and S-containing metabolites.

First, we examined plant growth parameters; the only significant difference was found in +S mycorrhizal plants which showed a higher shoot biomass (Fig. S4). Then we focused on the expression of S starvation marker genes, orthologs of Arabidopsis sulfur deficiency-induced 1, Atsdi1 (Howarth et al., 2009), and a ChaC-like family protein. In Arabidopsis thaliana, transcript levels of these genes significantly increase during S starvation and are useful as genetic markers of the S nutritional status. We screened the current release of the L. japonicus genome v. 2.5 for their putative orthologs and similarity searches led to the identification of highly similar sequences (chr1.LjT43D18.180.r2.d and chr1.LjT36G06.150.r2.m, respectively, subsequently named LS1 and LS2, standing for Low Sulfur 1 and Low Sulfur 2). Both L. japonicus ortholog genes were dramatically up-regulated in −S nonmycorrhizal roots but not in −S mycorrhizal roots (Fig. 1), suggesting a different level of sulfate starvation as a result of the fungal presence. Consistently with this result, −S mycorrhizal plants showed a higher sulfate concentration than control plants at both root and shoot levels (Fig. 2); this was confirmed by total S contents at root level (Fig. S2). In contrast, +S plants did not show any difference in LS1 and LS2 mRNA levels or sulfate contents between mycorrhizal and nonmycorrhizal conditions (Figs 1, 2). As the first product of primary sulfate assimilation is cysteine (Mugford et al., 2011), we quantified its concentration in roots and shoots of mycorrhizal and nonmycorrhizal plants grown under both S conditions and found no differences between the different treatments (Fig. 3).

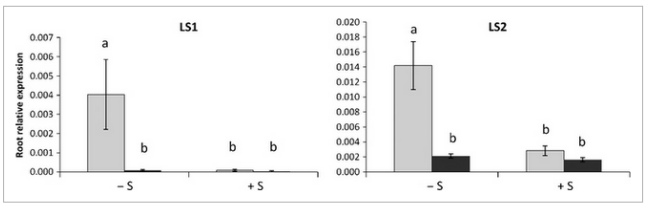

Figure 1. Relative expression of low-sulfur (S) gene markers LS1 and LS2 in Lotus japonicus. The expression levels of LS1 and LS2, Low Sulfur 1 and Low Sulfur 2, were measured by qRT-PCR and normalized against the reference gene, ubiquitin, LjUBQ10. Gray bars, control; black bars, mycorrhizal plants. Data shown are averages for three plants for each condition ± SD. Bars subtended by the same lowercase letter do not differ significantly at $P < 0.05$, according to Tukey's test. The only condition leading to overexpression of the two genes was sulfur starvation in nonmycorrhizal plants. By contrast, mycorrhizal plants did not show any gene induction.

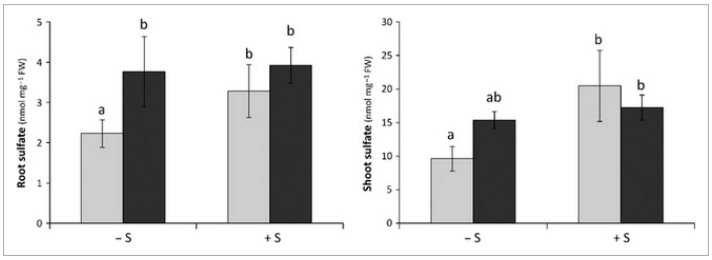

Figure 2. Sulfate concentration in root and shoot. Sulfate concentrations were determined in the roots and shoots of mycorrhizal and nonmycorrhizal Lotus japonicus plants grown under sulfur (S) starvation (−S) or repletion (+S) conditions. Sulfate content was measured by HPLC. Gray bars, control; black bars, mycorrhizal plants. Bars represent the mean of three biological replicates ± SD. Bars subtended by the same lowercase letter do not differ significantly at $P < 0.05$, according to Tukey's test.

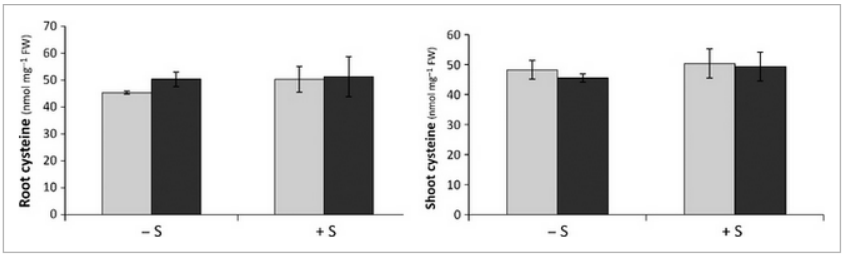

Figure 3. Cysteine concentration in root and shoot. Cysteine concentrations were determined in roots and shoots of mycorrhizal and nonmycorrhizal Lotus japonicus plants grown under sulfur (S) starvation (−S) or repletion (+S) conditions. Cysteine content was measured by HPLC. Gray bars, control; black bars, mycorrhizal plants. Bars represent the mean of three biological replicates ± SD. No significant differences were found between the treatments.

Focusing our attention on S starvation conditions in mycorrhizal plants, in a second set of plants where the expression of the mycorrhizal functional marker LjPT4 suggested variable colonization levels, we noted a correlation with the shoot sulfate concentration (Fig. S5). The more the root was colonized, the more sulfate was present until a plateau was reached.

In summary, gene expression studies revealed an attenuation of S starvation responses in AM plants, supported by higher tissue sulfate concentration, suggesting a positive

role for mycorrhizal fungi in plant S nutrition at low sulfate availability. This response was independent of the phosphate uptake. These results raise the question of whether specific sulfate transporters were activated in AM plants, allowing better S nutritional values.

Transcriptional analysis of *L. japonicus* sulfate transporters

A previous microarray study (Guether et al., 2009b) revealed a sulfate transporter that was up-regulated four-fold upon mycorrhiza formation, making it an interesting candidate for a detailed study. 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR was carried out to isolate the full-length cDNA of the corresponding LjSultr1;2 transporter. The resulting 1950-bp cDNA contained an open reading frame encoding a 71-kD polypeptide of 649 amino acid residues (LjSGA_011755.1). The sequence showed that LjSultr1;2 is closely related to MtSultr1;2 (CU6515893.1) and to XP_003526594.1, a sulfate transporter from Glycine max. The phylogenetic analysis confirmed that LjSultr1;2 belongs to group 1 of sulfate transporters and closely clusters with another L. japonicus sulfate transporter that we called LjSultr1;1 (chr6.CM0314.360.r2.m) (Fig. S6).

We also screened the current release of the *L. japonicus* genome v. 2.5 for putative sulfate transporters, finding other 10 members of this group, although sometimes with just a partial sequence.

In order to validate the microarray results (Guether et al., 2009b), the expression pattern of the LjSultr1;2 sulfate transporter was investigated under high and low S availability. Mycorrhizal and control roots were sampled 8 wk after fungal inoculation. Interestingly, quantitative RT-PCR showed that in −S conditions there was no significant difference between mycorrhizal and control roots, whereas in +S conditions mycorrhizal roots showed a strong up-regulation (Fig. 4). It is known that the expression of group 1 sulfate transporters is strongly induced by S starvation (Takahashi et al., 2011b), consistent with the different sulfate contents in the two conditions (Fig. 2). These results suggest that the expression of LjSultr1;2 depends on the one hand on the presence of the fungal symbiont and on the other hand on the S starvation conditions. If we exclusively focus on mycorrhizal conditions, we can conclude that LjSultr1;2 gene expression is not affected by S availability. In contrast, a closely related member of the group 1 sulfate transporters, LjSultr1;1, exclusively showed S starvation regulation and its expression was not correlated with fungal presence (Fig. 4).

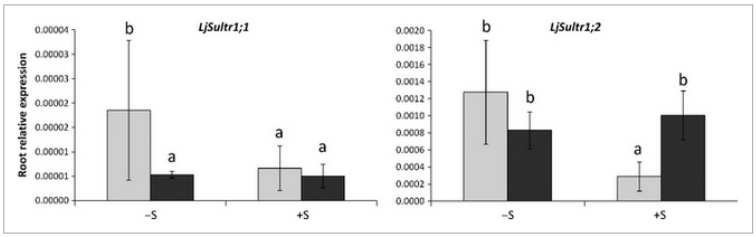

Figure 4. Relative expression of group 1 *Lotus japonicus* sulfate transporters (LjSultr1;1 and LjSultr1;2). The expression levels of LjSultr1;1 and LjSultr1;2 were measured by qRT-PCR and normalized against the reference gene ubiquitin, LjUBQ10. LjSultr1;1 showed regulation attributable exclusively to the sulfur (S) concentration; by contrast, LjSultr1;2 in +S showed a higher expression level in mycorrhizal than in control roots. Gray bars, control; black bars, mycorrhizal plants. Data shown are averages for three plants for each condition ± SD. Bars subtended by the same lowercase letter do not differ significantly at $P < 0.05$, according to Tukey's test.

To obtain a broader view of the regulation of sulfate uptake in L. japonicus, we performed a transcript analysis of the 11 putative sulfate transporter genes (Fig. S7). Consistently with regulation of sulfate transporters in Arabidopsis and group 1 expression, group 2 and 4 sulfate transporters also showed higher expression levels in −S control roots when compared with the other treatments. In addition, LjSultr3;1b was up-regulated in +S mycorrhizal roots, similar to LjSultr1;2, and could also be involved in the symbiotic pathway of sulfate metabolism. In contrast, transcripts of SST1, a group 3 sulfate transporter previously characterized in nodules, were absent in our samples (Krusell et al., 2005).

A laser microdissection approach using plants grown at high phosphate indicated a possible involvement of LjSultr1;2 in the uptake of S from the fungus to the plant, confirming accumulation of LjSultr1;2 transcript in arbusculated cells. As the nutrient exchange taking place between the plant and the fungus is probably located at the arbuscule level (Bonfante & Genre, 2010), cortical cells were chosen as a first target. The laser microdissection allowed the collection of three cell types: arbusculated cells (ARB), noncolonized cortical cells from mycorrhizal roots (MNM), and cortical cells from nonmycorrhizal roots (C). When the same primers used for LjSultr1;2 qPCR were used on RNA isolated from these three cell types, fragments of the expected size were specifically detected in arbuscule-containing cells (Fig. 5), indicating a specific location of the transcript in these cells and suggesting their involvement in the uptake of S from the fungus to the plant.

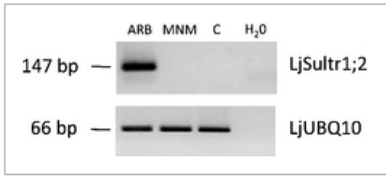

Figure 5. RT-PCR on laser-microdissected cells. RT-PCR analysis of the sulfate transporter LjSultr1;2 was carried out in Laser Microdissection (LMD) samples: LjSultr1;2 amplified fragments were only detected in arbusculated cells (ARB), and not in noncolonized cortical cells from mycorrhizal roots (MNM) or in cortical cells from nonmycorrhizal roots (C) of Lotus japonicus. No transcript was amplified from the blank sample (H2O). Data shown were obtained in three independent biological replicates.

To investigate the LjSultr1;2 expression pattern in detail and to understand the biological significance of the previous microarray study (Guether et al., 2009a) in the whole root system, the LjSultr1;2 promoter was fused to the reporter gene β-glucuronidase (GUS). This construct was introduced into L. japonicus roots by A. rhizogenes-mediated transformation (Stougaard et al., 1987). Composite plants were grown with or without AM fungal inoculation in half-strength Long Ashton solution. Transgenic hairy roots were generated and harvested after 8 wk. Roots of control plants showed a homogeneous blue coloration probably corresponding to epidermal and cortical cells, confirming our previous hypothesis (Fig. 6a) and consistent with data for other group 1 sulfate transporters (Takahashi et al., 2000; Buchner et al., 2010). In mycorrhizal roots, histochemical GUS reaction revealed a blue staining pattern in arbuscule-containing cells (Fig. 6b). Higher magnification showed that the GUS activity in arbusculated cells followed the arbuscule shape and did not accumulate in the space occupied by the fungal trunk (Fig. 6c). By contrast, epidermal, outer cortical and inner central cylinder cells of mycorrhizal roots did not show any GUS activity, consistent with the Laser Microdissection (LMD) results.

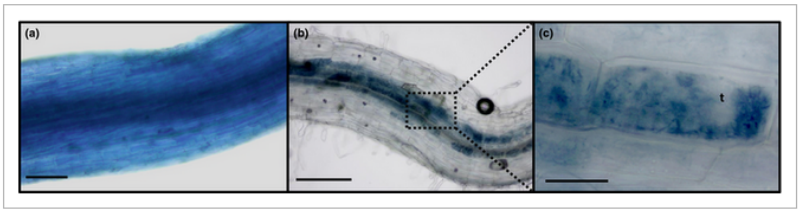

Figure 6. Histochemical GUS staining of Lotus japonicus roots expressing pLjST1.2:GUS in the presence and in the absence of Funneliformis mosseae. (a) Control roots revealed GUS activity homogeneously present in the whole root apparatus with a signal probably coming from epidermal cells. (b) In mycorrhizal roots, a vivid blue staining was detected exclusively in the arbusculated cells of the cortex. (c) A detailed picture of arbusculated cells shows how X-gluc accumulated in the cell with no signal coming from the space occupied by the arbuscular mycorrhizal (AM) fungal trunk (t). Bars: (a, b) 500 μm; (c) 50 μm.

LjSultr1;2 expression over colonization time

As under high sulfate conditions LjSultr1;2 expression in cortical cells was associated with the arbuscules, we followed the transcript levels of LjSultr1;2 from the fungal inoculation until the symbiosis had been fully achieved. The time course of LjSultr1;2 transcript levels was compared with that of LjPT4 to gather information on the relationship between LjSultr1;2 expression and arbuscule development (Giovannetti et al., 2012; Volpe et al., 2012) under +S conditions. Fig. 7 shows that LjSultr1;2 had a basal level of expression before the fungal colonization started (3 d post inoculation (dpi)), consistent with results presented in Fig. 4, but its transcript level increased in parallel with the time course of arbuscule formation. The experiment thus revealed two features of the LjSultr1;2 transporter: basal gene expression, presumably in the epidermis, to enable constitutive sulfate uptake into the roots, and an increase in transcript levels correlated with AM formation.

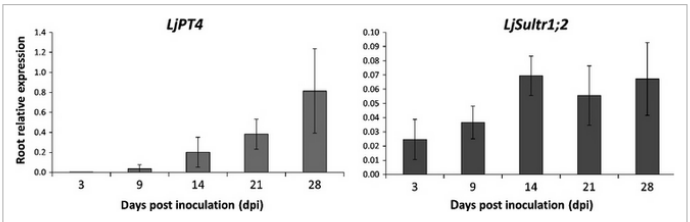

Figure 7. Time course of LjSultr1;2 expression. Relative expression of mycorrhiza-induced phosphate transporter, LjPT4, and the group 1 sulfate transporter, LjSultr1;2, over the colonization time was assessed by means of qRT-PCR at different time-points post inoculation of Lotus japonicus. The ct (threshold cycle) values of the samples were normalized against the ct values of the reference gene ubiquitin, LjUBQ10. The values are the means of three biological replicates and two technical replicates, ± SD. A positive Spearman's linear correlation was found ($r = 0.589$; $df = 19$; $P < 0.01$).

Characterization of *L. japonicus* sultr1;2 mutant

To validate the putative function of LjSultr1;2, different TILLING mutants were generated by RevGen (Perry et al., 2003). Four mutant genotypes were selected, carrying different missense mutations in the coding sequence, as shown in Fig. S8. As the first step in the mutant characterization, sulfate uptake into 4- and 10-d-old seedlings was analyzed. Homozygous mutants (sultr1;2Δ1 and sultr1;2Δ3) showed lower sulfate uptake in 4- and 10-d-old L. japonicus seedlings (Fig. 8). These two experiments confirm the function of LjSultr1;2 in S uptake, irrespective of the AM symbiosis, in agreement with the gene expression data. By contrast, heterozygous mutants (sultr1;2Δ4 and sultr1;2Δ5 lines) did not show any significant difference from the wild-type genotype, excluding the possibility that the different sulfate uptake capacity was attributable to any background mutations or different fitness of the mutant population (Figs S9, S10).

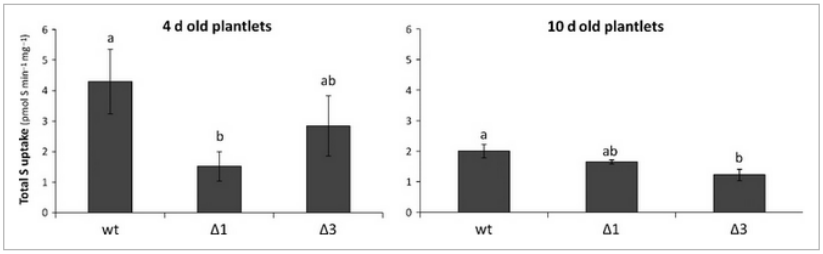

Figure 8. Total sulfate uptake in wild-type (wt) and sulfate transporter mutants sultr1;2 (delta1 and delta3). Lotus japonicus plants (4 or 10 d old) were fed with 35SO42− in the nutrient solution for 30 min. Radioactive sulfur in the whole plant was measured. Data shown are averages for four plants for each genotype ± SD. Bars subtended by the same lowercase letter do not differ significantly at $P < 0.05$, according to Tukey's test.

Discussion

Sulfate uptake: a direct and a symbiotic-dependent pathway

The connection between phosphate nutritional status and AM colonization has been extensively investigated, revealing that plants acquire Pi via two pathways: a direct AM-independent pathway and an AM-dependent pathway (Smith et al., 2003, 2004).

These pathways are active in most plants and involve a complex set of plant transporter-coding genes (Bucher, 2007), some of which are constitutive, while others are highly AM dependent. In this context, particular attention has been focused on plant systemic responses connected with P concentration (Breuillin et al., 2010; Balzergue et al., 2011), which led to the discovery of transcriptional regulators such as the microRNA miR399 (Branscheid et al., 2010) capable of moving from roots to leaves and acting as a signal of phosphate concentration. However, mycorrhiza formation is not exclusively connected to phosphate concentration: M. truncatula mtpt4 mutants are capable of forming arbuscules with the expected phenotype when grown at low concentrations of N, whereas this capability is disrupted at high N concentrations (Javot et al., 2011), revealing cross-talk between N and P metabolisms. Recently, another essential nutrient, S, has started to receive attention (Casieri et al., 2012; Sieh et al., 2013), as a consequence of its low availability in many soils (Schonhof et al., 2007; Mascagni et al., 2008). However, little is still known about S homeostasis in mycorrhizal plants, its molecular basis and the plant genes responsible for S symbiotic transfer.

The evidence presented here suggests that in L. japonicus sulfate is also acquired through two pathways, directly through plant roots and through an AM-specific pathway. These findings are consistent with the presence of five sulfate permease genes found to be expressed in the R. irregularis transcriptome (Tisserant et al., 2012). They also complement similar findings obtained with two different AM models, carrot (Daucus carota) roots and M. truncatula (Allen & Shachar-Hill, 2009; Casieri et al., 2012; Sieh et al., 2013), allowing a unified model of sulfate acquisition by mycorrhizal plants across legume and nonlegume plants. These dual modes allow the plant to access sulfate in soil surrounding the roots – through the direct pathway – but also to reach, through the AM extraradical hyphal network and the fungal-specific sulfate transporter (Tisserant et al., 2012, 2013), narrower soil pores and unexplored niches (Drew et al., 2003; Schnepf et al., 2011). Similar to phosphate nutrition, AM sulfate acquisition is particularly important in a low-S environment because, as shown previously for M. truncatula (Sieh et al., 2013), it alleviates the syndromes of sulfate deficiency. Mycorrhizal plants achieve higher sulfate concentrations in shoots and roots (Fig. 2), resulting in reduced transcript levels of S starvation marker genes (Fig. 1), group 1 LjSultr1;1 (Fig. 4) and group 4 sulfate transporters (Fig. S7).

Moreover, a correlation plot between root mycorrhizal rate and shoot sulfate concentration showed a positive dependence (Fig. S5) and typical saturated curve: with a finite amount of sulfate in the soil, the fungal component proves to be advantageous for the plant until the S is sufficient for both partners. In fact, the correlation curve reaches a plateau that probably corresponds to the maximum level of mycorrhiza formation providing a positive effect on the shoot sulfate concentration (Fig. S5).

Sulfate uptake into *L. japonicus* roots is facilitated by sulfate transporters. We identified 12 putative sulfate transporter genes in *L. japonicus*, which correspond well to the known classification into four groups in other species (Figs S6, S7; Takahashi et al., 2011b). For uptake from soil, the most relevant are transporters of group 1, which are transcriptionally induced upon sulfate deficiency in plants (Rouached et al., 2008). The pattern of lower transcript levels of putative group 1 sulfate transporter genes in roots of mycorrhizal *L. japonicus* plants as compared with roots of

nonmycorrhizal plants reflects reduced S starvation in mycorrhizal plants as a result of fungal uptake of S (Fig. 4). This is again consistent with regulation of sulfate transporters in *M. truncatula* (Casieri et al., 2012). However, one gene showed a different expression pattern: LjSultr1;2 was up-regulated in mycorrhizal roots grown in a high-sulfate regime (Fig. 4), in contrast to its closest paralog LjSultr1;1. LjSultr1;2 has a strong sequence homology with MtSultr1;2 (CU6515893.1) whose expression has been recently studied (Casieri et al., 2012; Sieh et al., 2013). Whereas LjSultr1;2 was induced by mycorrhiza formation in S-sufficient roots (Fig. 4), MtStultr1;2 was strongly up-regulated in mycorrhizal roots of S-starved but not S-replete plants. The different expression patterns may be attributable to different S concentrations in the soil or different mycorrhizal colonization, but might also reflect a specific function of this transporter in L. japonicus. Therefore, LjSultr1;2 is an excellent candidate for a detailed analysis of S metabolism in mycorrhizal plants.

LjSultr1;2: a dual role in *L. japonicus* S metabolism

The increased transcript levels of LjSultr1;2 in mycorrhizal roots suggested a possible specific role of this transporter in sulfate transport from fungi to plants, similar to AM-specific phosphate transporters. The accommodation process of the fungus leads to the biogenesis of a unique plant membrane, the periarbuscular membrane that surrounds the arbuscule (Bonfante & Genre, 2010), where many nutrient transporters are localized (Pumplin & Harrison, 2009; Zhang et al., 2010). Indeed, the analysis of RNA from laser-dissected cortex cells and the promoter GUS analysis showed a clear arbuscule-specific expression of LjSultr1;2, again similar to that of LjPT4 (Guether et al., 2009b). This not only supports the conclusions regarding AM-specific sulfate uptake, but identifies the plant transporter directly involved in this uptake pathway. Interestingly, however, in contrast to PT4, which has a unique role in mycorrhizal phosphate transport and is therefore mostly expressed in arbuscule cells (Harrison et al., 2002; Paszkowski et al., 2002), the LjSultr1;2 transcript is present also in nonmycorrhizal roots. This clearly indicates a dual function of the transporter: on the one hand, in direct root sulfate uptake and, on the other hand, in AM-specific transport in mycorrhizal roots. This dual function is also supported by the GUS experiment which showed that the LjSultr1;2 promoter was active exclusively inside the arbusculated cells of mycorrhizal roots (Fig. 6), whereas it was homogenously present in control roots, in agreement with the function of group 1 transporters in other plant species (Takahashi et al., 2011b). By contrast, LjSultr1;2 transcripts were absent in cortical cells of nonmycorrhizal roots on the basis of the LMD experiments. However, this discrepancy could be attributable to the different sensitivities of the techniques: nonquantitative RT-PCR on dissected cells is surely not comparable to the promoter GUS experiment. The most important finding of the LMD experiments was the detection of the transcript in arbuscule-containing cells, which was clearly confirmed by the GUS.

This assay clearly showed a possible shift from a direct to a symbiotic pathway (proposed model in Fig. 9), as happens with phosphate nutrition, where, by contrast, this shift requires different genes for the two pathways (Smith et al., 2011).

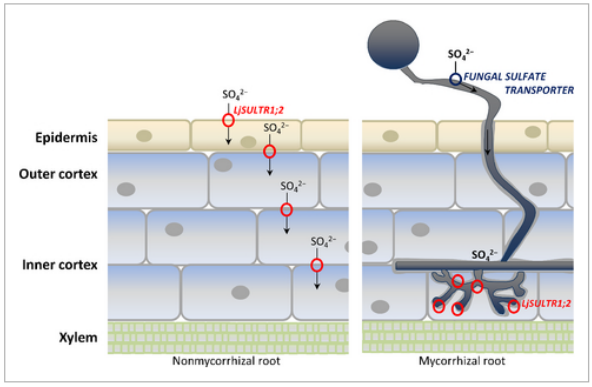

Figure 9. Proposed model of the role of group 1 sulfate transporter LjSultr1;2 in both the direct and symbiotic pathways. The two pathways of sulfate uptake in control and mycorrhizal roots probably involve the same sulfate transporter of Lotus japonicus. In the direct pathway, sulfate is absorbed by plant sulfate transporters, such as LjSultr1;2, expressed in the epidermis and cortex (red circles). In the mycorrhizal pathway, sulfate is taken up into arbuscular mycorrhizal (AM) fungal hyphae by fungal sulfate transporters (blue circles; Tisserant et al., 2012) and translocated to intracellular fungal structures in root cortical cells. The same transporter involved in the direct pathway, LjSultr1;2, is induced in the arbusculated cells (red circle), and is probably localized on the periarbuscular membrane, transferring sulfate from the interface to plant cortical cells.

The function of LjSultr1;2 in constitutive sulfate uptake is evident from the analysis of the TILLING mutants, which showed lower sulfate uptake capacity in sterile conditions (Fig. 8).

LjSultr1;2, however, was inducible by sulfate starvation in nonmycorrhizal roots (Fig. 4), as happens with its orthologs in other species (Takahashi et al., 2011b; Sieh et al., 2013). Thus, its expression in arbusculated cells might be triggered by local sulfate depletion. The transcripts of the sulfate starvation markers, however, were not detectable in arbusculated cells (Fig. S11). This result strongly suggests that the specific expression of LjSultr1;2 in arbuscule cells is linked to the fungal presence and not to S starvation. The dual localization and dual regulation by sulfate starvation and mycorrhiza formation thus explain the expression pattern of LjSultr1;2 (Fig. 4). Focusing on mycorrhizal plants, there were no significant differences between LjSultr1;2 expression levels under high- and low-S conditions, confirming that the AM symbiosis confers a stable sulfate concentration independently of S concentration in the soil. By contrast, in control plants there was a strong up-regulation caused by S starvation conditions. In S-sufficient cells, the control roots exhibited a low expression level, suggesting that the direct pathway was limited, whereas the high expression level in mycorrhizal roots could be attributable to the symbiosis pathway, consistent with the transcript accumulation and promoter activation in arbuscule-containing cells.

A dual biological role for a single gene within the AM symbiosis has already been shown for LjMAMI, an AM-induced MYB-family transcription factor strongly induced in arbuscule-containing cells but expressed also in root tips of nonmycorrhizal roots and responsible of a different root branching phenotype (Volpe et al., 2012).

In *A. thaliana*, during S deprivation, specific expression of AtSultr1;1 in the root epidermis and root hairs could aid in the search for limited amounts of sulfate at the

root/soil interface (Takahashi et al., 2000). Plants able to form a symbiosis could be using a similar mechanism by targeting gene expression to the plant/microbe interface. It has already been shown that a sulfate transporter is essential in N fixation in nodules (Krusell et al., 2005), but to date no sulfate transporter involved in AM symbiosis, specifically expressed in arbuscule-containing cells, has been found.

From an evolutionary point of view, it is interesting that genes involved in mineral nutrition have been recruited during AM establishment, in some cases leading to new paralogs, such as MtPT4, which are dominant in the arbusculated cells, or as in the case of LjSultr1;2 to the acquisition of new functions, leading to transporters with a dual role.

In conclusion, our findings complement those on other plant models and show improved sulfate concentration in mycorrhizal L. japonicus plants under S starvation conditions. Our results suggest that there are two sulfate acquisition pathways in L. japonicus and led to the identification of a sulfate transporter specifically involved in uptake from the arbuscules. The TILLING mutants, impaired in the capacity for sulfate uptake, will allow characterization and better understanding of the role of this protein within the symbiosis and how its deficiency could impact on sulfate homeostasis in AM plants. These mutant plants can be used for future integrated studies of the interplay between phosphate, N and S nutrition during the mycorrhizal interaction and investigations of the sulfate starvation and mycorrhizal signals controlling LjSultr1;2 expression.

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