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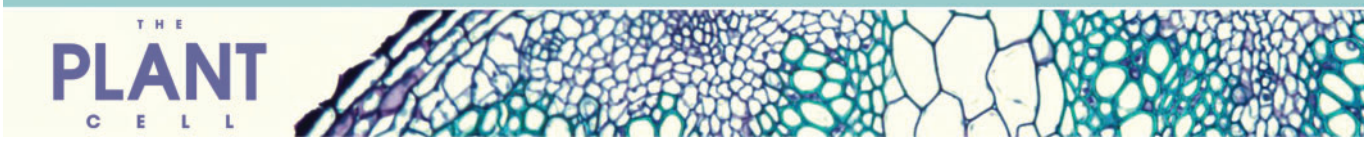

Research Article

# Sulfur transfer from the endophytic fungus *Serendipita indica* improves maize growth and requires the sulfate transporter SiSulT

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## Abstract

A deficiency of the essential macronutrient sulfur leads to stunted plant growth and yield loss; however, an association with a symbiotic fungus can greatly improve nutrient uptake by the host plant. Here, we identified and functionally characterized a high-affinity sulfate transporter from the endophytic fungus *Serendipita indica*. *Si*SulT fulfills all the criteria expected of a functional sulfate transporter responding to sulfur limitation: *SiSulT* expression was induced when *S. indica* was grown under low-sulfate conditions, and heterologous expression of *SiSulT* complemented a yeast mutant lacking sulfate transport. We generated a knockdown strain of *SiSulT* by RNA interference to investigate the consequences of the partial loss of this transporter for the fungus and the host plant (maize, *Zea mays*) during colonization. Wild-type (WT) *S. indica*, but not the knockdown strain (kd-*SiSulT*), largely compensated for low-sulfate availability and supported plant growth. Colonization by WT *S. indica* also allowed maize roots to allocate precious resources away from sulfate assimilation under low-sulfur conditions, as evidenced by the reduction in expression of most sulfate assimilation genes. Our study illustrates the utility of the endophyte *S. indica* in sulfur nutrition research and offers potential avenues for agronomically sound amelioration of plant growth in low-sulfate environments.

## Introduction

Sulfur is an essential macronutrient for plant growth and development and plays a fundamental role in metabolism. In addition to being a structural component of protein disulfide bonds, sulfur is present in amino acids (Cys and Met), vitamins (biotin and thiamin), cofactors (*S*-adenosyl-methionine), and the Fe-S groups of electron transport chains (Droux, 2004; Pilon-Smits and Pilon, 2007). Sulfur deficiency leads to a decrease in protein biosynthesis and chlorophyll content and, ultimately, a loss of crop yield (Sexton et al., 1997; Buchner et al., 2004; Lunde et al., 2008; Davidian and Kopriva, 2010). Although sulfur constitutes ~0.1% of the Earth's crust, it is mostly not present in a bioavailable form (Kertesz, 2000). Plants use sulfur primarily in its anionic form

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($SO_4^{2-}$), which is generally present in very small amounts in the soil. Furthermore, as sulfate is water soluble, it readily leaches out of the soil (Eriksen and Askegaard, 2000; Buchner et al., 2004; Davidian and Kopriva, 2010).

Plants are able to take up sulfate from soil over a wide range of concentrations through the use of high-affinity and low-affinity transporters (Leustek et al., 2000). These sulfate transporters belong to the major facilitator superfamily (MFS) group of membrane transport proteins. Members of this superfamily are integrated into membranes by 12–14 transmembrane regions and contain substantial similarity to the bacterial anti-sigma factor antagonist domain (STAS). Sulfate influx can be coupled to the cotransport of positively charged counterions such as protons ($H^+$) and sodium ($Na^+$), to the efflux of anions via a sulfate/anion ($A^-$) antiporter, or move directly across the membrane via an ABC-type complex transporter (Pao et al., 1998; Rouached et al., 2005; Piłsyk and Paszewski, 2009; Takahashi et al., 2012; Quistgaard et al., 2016).

In soils with low-sulfur availability, a symbiotic association between plants and an arbuscular mycorrhizal fungus (AMF) assists with sulfur acquisition from the soil: plants obtain nutrients from their fungal partner, which in return receives sugars from the plant (Parniske, 2008). In these associations, fungal and plant membrane transporters participate in nutrient transfer to the host plant. For instance, arbuscular mycorrhizal colonization of barrel clover (*Medicago truncatula*), spring onion (*Allium fistulosum*), carrot (*Daucus carota*), and trefoil (*Lotus japonicus*) substantially improves the sulfur status of the host plant especially in low-sulfate conditions (Guo et al., 2007; Allen and Shachar-Hill, 2009; Sieh et al., 2013; Giovannetti et al., 2014; Wu et al 2018).

The lack of a stable transformation system for AMFs precludes the genetic manipulation of AMF sulfate transporters to improve sulfur uptake in colonized plants and limits studies aimed at understanding mycorrhizal sulfur transfer to the host plant. However, the beneficial endophyte *S. indica* can be cultivated axenically under laboratory conditions and has a well-established transformation system. *S. indica* is a root endophytic fungus isolated from the roots of the plants *Prosopis juliflora* and *Zizyphus nummularia*, which grow in the Thar Desert in Rajasthan, India (Varma et al., 1999). Several studies have examined the functions of various genes in *S. indica* (Yadav et al., 2010; Akum et al., 2015; Jogawat et al., 2016). Colonization by *S. indica* improves plant's ability to acquire phosphorus, magnesium, and iron from a nutrient-deprived soil rhizosphere, due to the presence of dedicated nutrient transporters (Yadav et al., 2010; Prasad et al., 2019; Verma and Johri, 2019), providing benefits to the host plant such as improved growth and increased resistance to biotic and abiotic stresses (Waller et al., 2005; Kumar et al., 2009; Yadav et al., 2010; Johri et al., 2015; Jogawat et al., 2016; Narayan et al., 2017). Therefore, *S. indica* has been termed a plant probiotic (Aschheim et al., 2005).

In the present study, we identified, isolated, and functionally characterized a sulfur-specific transporter, *Si*SulT, from *S. indica*. We demonstrate that *Si*SulT is essential for sulfur transfer to the host plant and that it enhances plant growth and development in low-sulfate conditions. In addition, we report a thorough and complete analysis of the regulation of sulfur assimilation pathway genes in *S. indica* and maize (*Zea mays*) plants during colonization. Our results show that the biosynthetic steps are regulated at the level of the mRNAs of the encoding genes during adaptation to low-sulfate conditions. Characterization of the *Si*SulT sulfate transporter provides insight into the regulation of sulfate uptake during symbiosis. We propose that the use of *S. indica* would not only complement crop growth strategies but may also serve as a model system to study the molecular mechanisms underlying the indirect uptake of sulfur by plants via association with a beneficial fungus.

## Results

### *SiSulT* is a putative sulfate transporter from *S. indica*

We searched the *S. indica* genome (strain DSM11827) for putative sulfate transporters by BLAST analysis using *Saccharomyces cerevisiae* Sul1 and Sul2 as query sequences. We identified a single candidate on contig PIRI_contig_0011 (accession no CCA67103.1) that was annotated as a probable sulfate permease or related transporter (Zuccaro et al., 2011). We isolated a full-length cDNA with gene-specific primers for the transporter, which we named *Si*SulT. The gene encoding this putative *S. indica* transporter has an open reading frame (ORF) of 2,292 bp, and the encoded protein is 763 amino acids long with a predicted molecular weight of 83.2 kDa. *Si*SulT shares 42% and 49% sequence identity and highest query coverage of 90% and 73% with the yeast (*S. cerevisiae*) sulfate transporters Sul1 and Sul2, respectively. Sequence comparisons between genomic and cDNA showed that *SiSulT* consists of seven exons and six introns (Supplemental Figure 1). Our *in silico* analysis indicates that this gene could be a *S. indica* sulfate transporter.

### Phylogenetic and homology analysis

We subjected the predicted *Si*SulT protein sequence to a functional domain search. We identified several partially overlapping pfam domains with recognized roles in sulfate transport (Supplemental Table 1), which collectively point to *Si*SulT as a member of the MFS of inorganic ion transporters. The domain architecture of *Si*SulT is similar to that of the yeast sulfate permease/sulfate transporters Sul1 and Sul2 as well as to sulfate permeases in organisms across kingdoms, including human (*Homo sapiens*), Arabidopsis (*Arabidopsis thaliana*), and a number of AMFs. The tripeptide motif GLY (Gly-Leu-Tyr) is common to sulfate transporters and is present in *Si*SulT (Supplemental Figures 2 and 3).

We next constructed a phylogenetic tree of *Si*SulT-related proteins from bacteria, insects, mammals, plants, and fungi. *Si*SulT clusters with other sulfate permeases from Basidiomycota (filamentous fungi), as expected

(Supplemental Figure 4). *Si*SulT exhibits higher similarity to sulfate permeases from Basidiomycota (highest 75%; e.g. *Rhizoctonia solani*, *Acaulospora delicata*, *Gloeophyllum*, *Trabeum* sp., *Punctularia strigosozonata*, and *Dacryopinax primogenitus*) than from Ascomycota (sac fungi; highest 42%; Supplemental Tables 2 and 3). *Si*SulT shares 42%, 33%, 30%, 29%, and 27% amino acid sequence identity with sulfate transporters from fungus, human, fruit fly (*Drosophila melanogaster*), plants (Arabidopsis), and bacteria (*Escherichia coli*), respectively (Table 1). Thus, our phylogenetic analysis indicates that *Si*SulT falls into the group of sulfate transporters of fungi.

## *SiSulT* is upregulated under low-sulfate conditions in culture and in response to colonization

We set up *S. indica* liquid cultures in minimal nutrient medium (MNM) with sulfate concentrations ranging from 0 to 10 mM and collected samples after 1, 5, 10, and 15 days. Expression of *SiSulT* was induced in low-sulfate conditions ($\leqslant$100 μM) at all time points tested (1, 5, 10, and 15 days), consistent with its proposed function as a high-affinity sulfate transporter (Figure 1, A and B).

We next tested whether *SiSulT* expression was induced in response to colonization of maize roots. Under our experimental conditions, *S. indica* progressively colonized maize root cells to infect up to 70% of all root cells by 20-day postinoculation (dpi; Figure 1C). As the colonization of *S. indica* increased in the root, *SiSulT* transcript levels similarly rose over time (Figure 1D). In addition, colonization is associated with the developmental stage of the host tissue. *S. indica* showed stronger colonization with newly formed lateral roots than with tap roots. We observed pronounced intercellular colonization in the cortical tissue of the differentiation and elongation zones but a complete absence of colonization in the root tip meristem, including the root cap (Supplemental Figure 5A). To validate this observation, we determined the titer of *S. indica* in different root zones. We observed a much stronger signal intensity for the *S. indica* translation elongation factor gene *TEF* in the elongation and differentiation zone than in the meristematic zone, reflecting the relative colonization of these two tissues by *S. indica* (Supplemental Figure 5B). We conclude that *SiSulT* expression corresponds with progression of colonization to boost and maintain this mutualism.

## *Si*SulT complements a yeast sulfate transporter mutant

To test whether *SiSulT* encodes a functional sulfate transporter, we heterologously expressed this gene in a yeast mutant lacking the two high-affinity sulfate transporters Sul1 and Sul2 (mutant HK14; Kankipati et al., 2015). We cloned *SiSulT* into a yeast galactose-inducible expression vector, which we then introduced into the HK14 mutant. As a negative control, we transformed HK14 with the empty vector. HK14 transformed with *SiSulT* grew well under low-sulfate conditions (100 μM sulfate), whereas HK14 transformed with the empty vector did not (Figure 2A), suggesting that *SiSulT* indeed encodes a functional sulfate transporter.

We then extended our results with a growth curve analysis under various sulfate concentrations: both wild-type (WT) yeast (BY4742) and HK14 transformed with *SiSulT* sustained robust growth when the growth medium contained at least 100 μM sulfate, whereas they failed to grow at lower concentrations (Figure 2, B–D). The HK14 mutant transformed with the empty vector showed no growth, even at high concentrations of sulfate (Figure 2D). We conclude that *SiSulT* complemented the yeast HK14 mutant and encodes a functional sulfate transporter.

## *Si*SulT also transports chromate

The heavy metal chromate competes with sulfate for transport into yeast cells through the sulfate transporter (Roberts and Marzluf, 1971; Skeffington et al., 1976). To confirm the role of *Si*SulT in sulfate transport, we tested its ability to transport chromate. In the WT yeast strain BY4742, chromate toxicity is evident when the growth medium contains 20 μM chromate and 100 μM sulfate but is largely alleviated when sulfate concentrations are increased to 1 mM (Supplemental Figure 6). In contrast, the HK14 mutant, lacking high-affinity sulfate transporters, exhibited tolerance to high concentrations of chromate up to 60 μM. However, both the WT strain and the HK14 mutant transformed with *SiSulT* were highly sensitive to chromate (Supplemental Figure 7), further supporting the assigned function of *Si*SulT as a sulfate transporter.

## Biochemical characterization of *Si*SulT

To gain a more quantitative understanding of sulfate uptake by *Si*SulT heterologously expressed in yeast, we conducted uptake assays with radiolabeled $^{35}S$. We first grew the WT yeast strain BY4742 and the HK14 mutant transformed with either empty vector or with *SiSulT* in sulfate-replete

**Table 1** Summary of percentage identity between *S. indica* *Si*SulT and other fungal, plant, animal, insect, and prokaryotic sulfate transporters

| Organism | Sulfate transporter (number of amino acids) | GenBank accession No. | Identity with *SiSulT* (%) |
|---|---|---|---|
| *Serendipita indica* (Fungus) | *Si*SulT (763) | CCA67103.1 | 100 |
| *Saccharomyces cerevisiae* (Fungus) | Sul1 (859) | AJP84964.1 | 42 |
| *Homo sapiens* (Mammal) | SLC26A11 (606) | NP 001159819.1 | 33 |
| *Drosophila melanogaster* (Insect) | Esp (623) | AAD53951.1 | 30 |
| *Arabidopsis thaliana* (Plant) | SULTR1 (649) | OAO99615.1 | 29 |
| *Escherichia coli* (Bacteria) | YchM (550) | SCQ14120.1 | 27 |

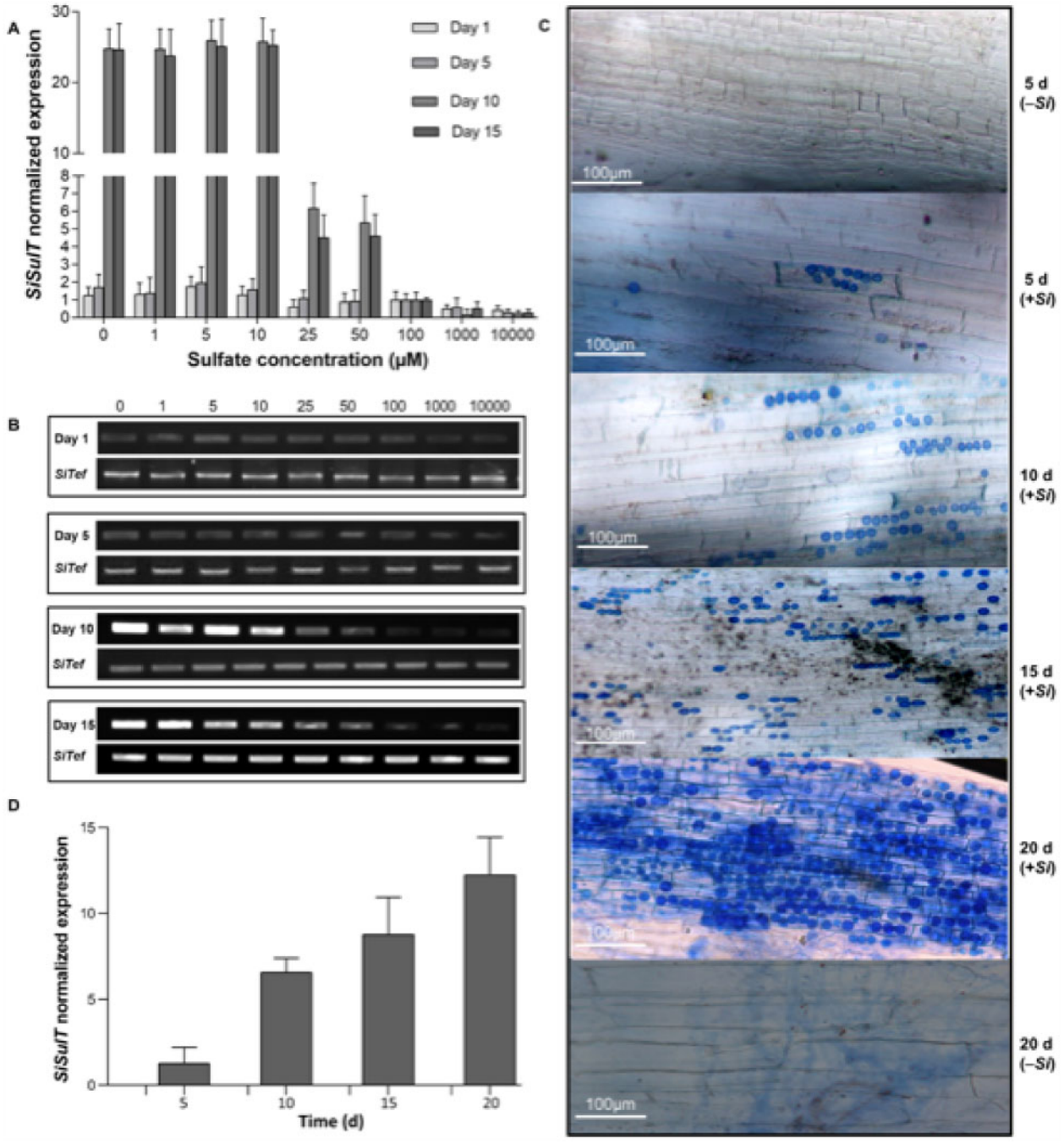


**Figure 1** Expression pattern of *SiSulT* in response to different concentrations of sulfate. A, *SiSulT* expression in *S. indica* grown in minimal medium containing the indicated sulfate concentrations for 1, 5, 10, and 15 days. *SiTEF* was used as a reference gene. Data are means (±SE), $n = 6$. B, PCR of *SiSulT* transcript levels in *S. indica* under the same sulfate concentrations as in A. C, Trypan blue staining of maize seedling roots showing the presence of intracellular *S. indica* chlamydospores in cortical cells after 5, 10, 15, and 20 days of colonization (+*Si*; spores are stained in blue). Maize roots grown without *S. indica* for 5 or 20 days (−*Si*) were used as a negative control. D, Transcript levels of *SiSulT* during colonization of maize seedling roots grown in the presence of 100 μM sulfate, as determined by RT-qPCR. *SiTEF* was used as a reference gene. Data are means (±SE), $n = 6$.

conditions before transferring them to sulfur starvation medium for 2 days. We then measured sulfur uptake by adding 10 μM $^{35}$S-labeled $Na_2SO_4$ for 4 min. Both BY4742 and HK14 transformed with *SiSulT* showed a strong accumulation of radioactive signal, with 296 pmol sulfate for BY4742 and 146 pmol for HK14 transformed with *SiSulT*. In contrast, the HK14 mutant transformed with the empty vector took up negligible amounts of sulfur, with a very low radioactivity count of 0.2 pmol sulfur equivalents (Figure 3A). These data suggest that *SiSulT* has the potential to complement the yeast HK14 mutant.

We then probed the uptake kinetics of Sul1 and Sul2 (in BY4742) and HK14 transformed with *SiSulT* under a range of $^{35}$S-labeled sulfate concentrations (from 1 to 100

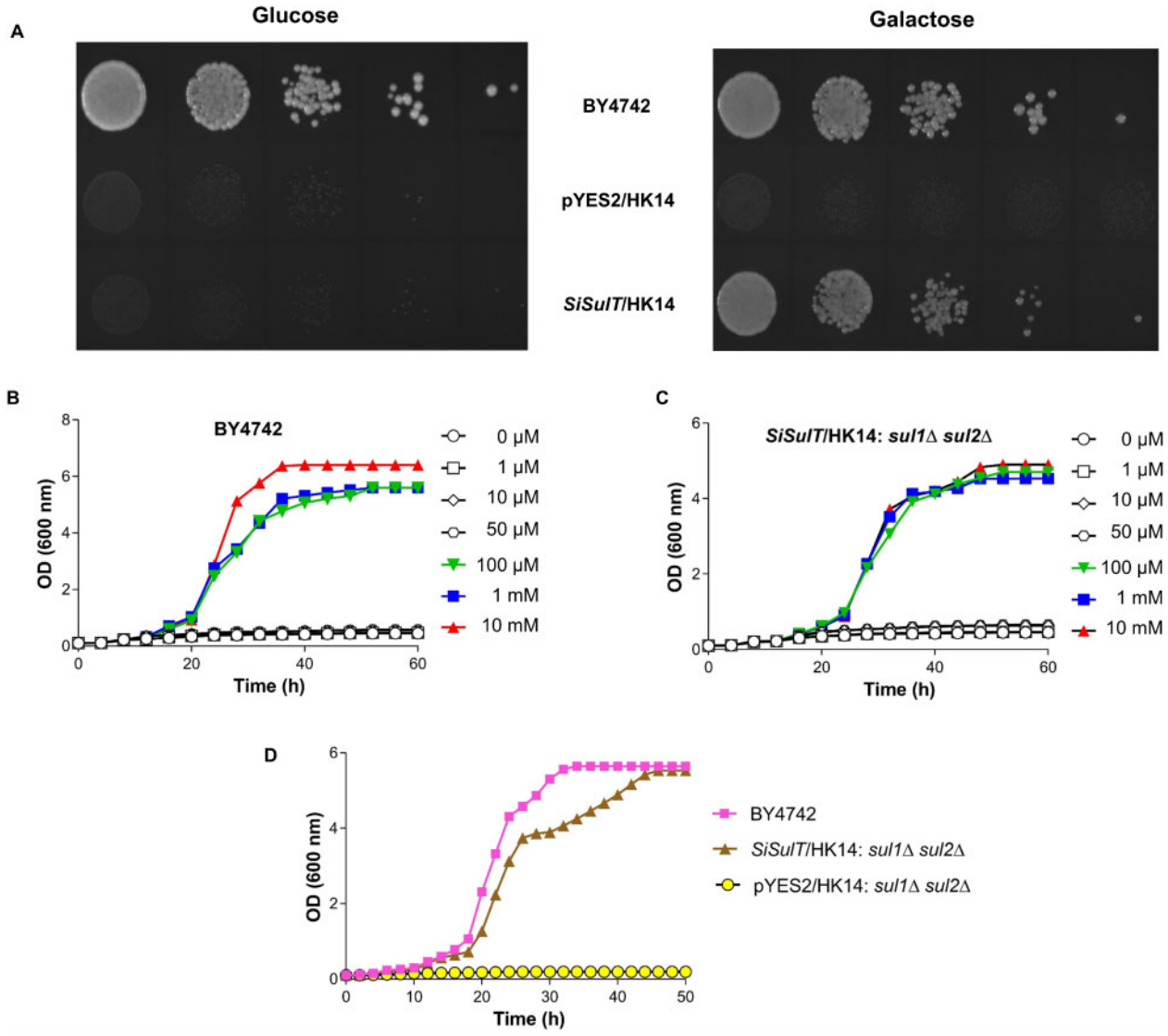


**Figure 2** Complementation of a yeast sulfate transporter mutant by *S. indica* *Si*SulT. A, Complementation of the HK14: *sul1*Δ *sul2*Δ yeast on plates. Yeast cells were grown at 30°C in liquid SD medium containing 0.1 mM of sulfate as a sole source of sulfur in the presence of 2% glucose (non-inducing condition) or 2% galactose (inducing condition). Cells were then washed, resuspended in sterile distilled water at a cell density of $OD_{600}$ = 0.1, followed by 1:10 serial dilutions. BY4742: wild type parental strain; pYES/HK14: *sul1*Δ *sul2*Δ mutant transformed with pYES2 empty vector; *SiSulT*/HK14: *sul1*Δ *sul2*Δ mutant transformed with *SiSulT*. B and C, Growth curve assays for BY4742 (B) and HK14: *sul1*Δ *sul2*Δ mutant expressing *SiSulT* (C), grown in the presence of various sulfate concentrations. Yeast cells were first starved for sulfur and then transferred to SD medium with the indicated concentrations of sulfur. D, Growth curves of the wild type BY4742, HK14: *sul1*Δ *sul2*Δ mutant expressing *SiSulT* and HK14: *sul1*Δ *sul2*Δ mutant carrying the pYES2 empty vector in SD medium + 3 mM sulfate, after sulfur starvation.

µM). Sulfate uptake in both strains (BY4742 and HK14 transformed with *SiSulT*) followed typical Michaelis-Menten kinetics, with an apparent $K_m$ of 15 ± 1.7 µM and $V_{max}$ value of 1.92 ± 0.06 pmol/min/$OD_{650}$ for HK14 transformed with *Si*SulT and a $K_m$ of 8.2 ± 1.4 µM and a $V_{max}$ of 3.20 ± 0.04 pmol/min/$OD_{650}$ for BY4742 (Figure 3B).

We also determined the pH optimum for sulfate transport by *SiSul*T, again based on $^{35}$S-labeled sulfate uptake assays between pH 2.0 and 8.0. Sulfate transport activity indeed exhibited a preferred pH, as the highest uptake was observed at pH 5.0 (Figure 3C). We conclude that *Si*SulT, when expressed in yeast, displays sulfate uptake kinetics that are quantitatively similar to those of the high-affinity yeast sulfate transporters Sul1 and Sul2.

## Generation of RNAi strains for *SiSulT*

To probe the role of *SiSul*T in sulfate transfer to the host plant, we generated knockdown strains of *SiSulT* in *S. indica* (kd-*SiSulT*) by RNA interference (RNAi). To this end, we used the pPiRNAi vector, which contains a multiple cloning site flanked by two converging *S. indica* promoters (*SiTEF-pro* and *SiGPD-pro* from glyceraldehyde-3-phosphate dehydrogenase, Supplemental Figure 8A) and selected a 452-bp fragment that is unique to *SiSulT* (Supplemental Figure 8B). We selected four colonies (named TC for transformed

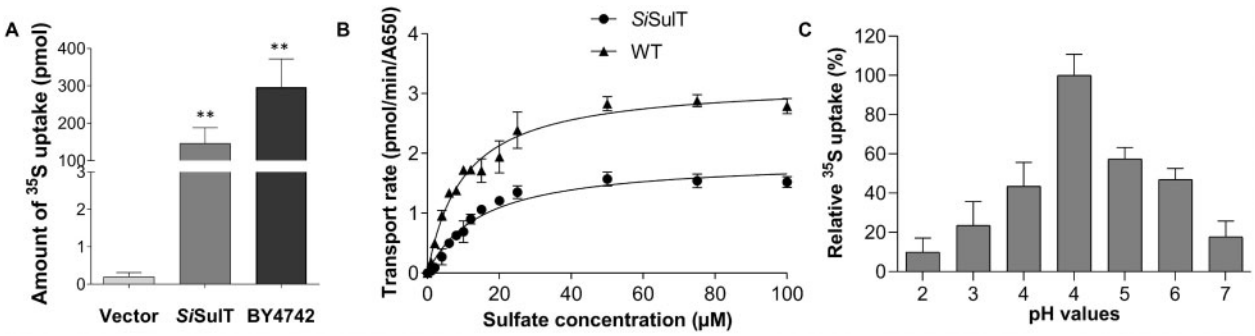


**Figure 3** Functional characterization of *Si*SulT expressed in the yeast HK14: *sul1*Δ *sul2*Δ sulfate transporter mutant. A, $^{35}S$ uptake assay in the wild–type yeast strain BY4742, the HK14: *sul1*Δ*sul2*Δ mutant expressing *SiSul*T and HK14: *sul1*Δ *sul2*Δ mutant carrying the pYES2 empty vector. Data are shown as means ± SEM ($n$ = 3 biological replicates and three technical replicates). B, Kinetics analysis of $^{35}S$ uptake in the WT yeast strain BY4742 and the HK14: *sul1*Δ *sul2*Δ mutant expressing *SiSulT*. Sulfate uptake for 4 min after transfer from sulfur-starvation medium to SD medium containing $^{35}S$-labeled $Na_2SO_4$ in the presence of galactose at pH 5.0 at the indicated concentrations. Data are shown as means ± SEM ($n$ = 3 biological replicates and three technical replicates). Statistical significance between BY4742 and the HK14: *sul1*Δ *sul2*Δ mutant expressing *SiSul*T is based on a two-sample $t$-test [$P$-value is 0.0065 at 95% CI (−1.431156, −0.260011)]. C, Determination of the pH optimum for sulfate uptake by *Si*SulT. Relative sulfate uptake was normalized to highest uptake values at pH 5.

colony) based on hygromycin resistance conferred by pPiRNAi (Supplemental Figure 9A). All colonies showed lower *SiSulT* transcript levels relative to untransformed *S. indica*, ranging from a 27% to 58% reduction depending on the colony tested (Supplemental Figure 9B). The presence of the RNAi construct was confirmed by genomic PCR (Supplemental Figure 9C), whereas the accumulation of small interfering RNAs was verified by RNA blot analysis (Supplemental Figure 9D). Because TC1 showed the strongest *SiSulT* silencing (Supplemental Figure 9B), we selected it for further experiments. Both WT *S. indica* and TC1 colonies grew at similar rates in KF medium without hygromycin (Supplemental Figure 10, A and D). Importantly, WT *S. indica* failed to grow in KF medium supplemented with hygromycin (Supplemental Figure 10, B and E), unlike TC1 (Supplemental Figure 10, C and F). The detection of siRNA suggests that RNAi machinery exists in *S. indica*. Further, it shows the successful use of RNAi and electroporation as a transformation system.

## *Si*SulT transports sulfate to host plants

We next assessed the contribution of *Si*SulT to sulfur transport from the fungus to the host plant (maize) by setting up bicompartment assays with WT and kd-*SiSulT S. indica* cultures (Supplemental Figure 11). We placed a small Petri dish containing *S. indica* inside a larger Petri dish in which maize seedlings were allowed to grow. We then facilitated colonization of maize seedling roots by *S. indica* by forming agar strip bridges between the two compartments. After hyphae were established, we refreshed the growth medium in the inner compartment and added 100 μM sulfate and 1 μM $^{35}S$-labeled $Na_2SO_4$. We determined the transfer of radiolabeled sulfur by autoradiography of maize seedlings after 35 days. In seedlings colonized with WT *S. indica*, autoradiography revealed extensive radiolabel in maize roots and leaves (Figure 4, A and B). In contrast, we detected a much weaker radioactive signal in maize seedlings colonized with the kd-*SiSulT* strain, consistent with a role for *Si*SulT in direct sulfate transport to the host plant (Figure 4, D and E). As expected, we observed no radioactivity transferred to maize seedlings in the absence of any colonization, indicating that $^{35}S$-labeled $Na_2SO_4$ did not cross compartments nor was it taken up by uninoculated maize roots (Figure 4, G and H).

Quantification of the radioactive signal in maize seedlings colonized with either WT *S. indica* or kd-*SiSulT* revealed that knocking down *SiSulT* expression in *S. indica* resulted in an over eight-fold decrease in sulfur uptake by the host plant [$362 \pm 25.3$ pmol sulfate transported by WT *S. indica* and $43 \pm 6.7$ pmol in the case of kd-*SiSulT* $\{P < 0.01\}$, Figure 4J]. However, both sets of maize seedlings (colonized with WT *S. indica* or kd-*SiSulT*) exhibited comparable colonization rates of ∼70% at 20 dpi (Figure 4, C and F). In the colonized state, *S. indica* forms both external (extracellular) and internal (intracellular) hyphae. External hyphae ramify out of the colonized root, whereas internal hyphae penetrate the root cortex including the root epidermis. To determine the expression of *SiSulT* in internal and external hyphae, we measured *SiSulT* transcript levels by reverse-transcription quantitative PCR (RT-qPCR). *SiSulT* was significantly more highly expressed (∼2.25-fold) in external hyphae relative to internal hyphae (Figure 4K). These observations support the hypothesis that *SiSulT* facilitates the transfer of sulfate from the fungus to the host plant and that external hyphae may constitute the initial uptake site.

## Plant responses to sulfur deficiency and the role of *Si*SulT in plant sulfate nutritional status

Based on the above results, we hypothesized that colonization of maize roots by *S. indica* would improve sulfur

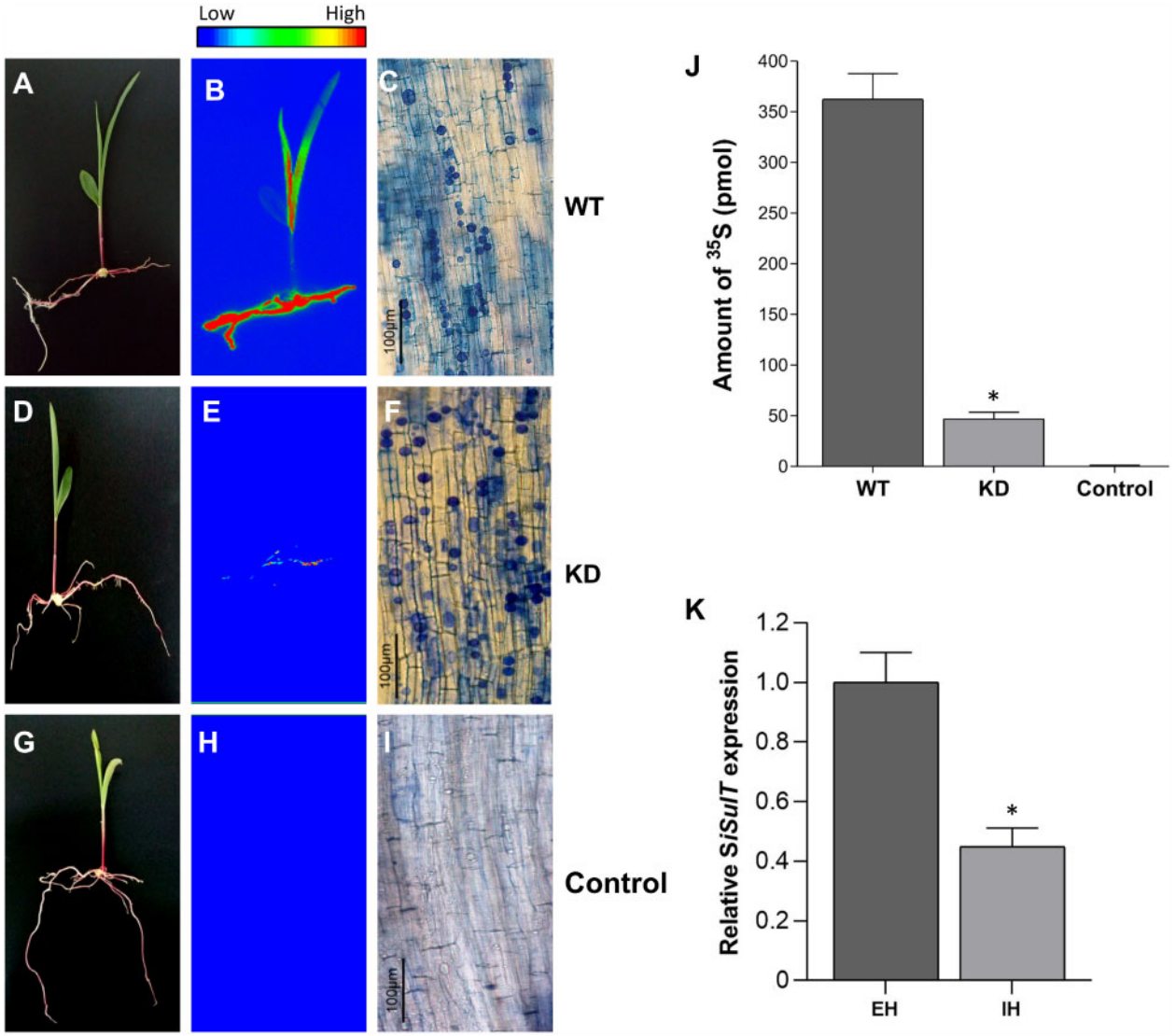


**Figure 4** Transport of sulfate to maize seedlings by *S. indica* by bi-compartment assays. Radioactivity incorporated into seedlings was visualized by autoradiography, and the intensity is shown as false colors. A–C, Maize seedlings colonized with WT *S. indica*, (D–F) Maize seedlings colonized with kd-*SiSulT S. indica*. G–I, Maize seedlings grown without *S. indica* (control). Whole maize seedlings were photographed (A, D, G) prior to autoradiography over 12 h (B, E, H). Root colonization by *S. indica* spores was determined by trypan blue staining (C, F, I). Scale bar: 100 μm. J, Amount of $^{35}$S transferred to maize seedlings by *S. indica*. Radioactivity was measured three times independently (the number of transformants used was $n = 3$). Data are shown as means ± SD ($n = 3$). Asterisks indicate a significant difference ($P < 0.01$) based on a *t*-test. K, Spatial expression of *SiSulT* during colonization. Expression of *SiSulT* in external (EH) and internal *S. indica* hyphae (IH) during colonization of maize seedling roots. Relative expression in EH was set to 1. The *SiTEF* gene was used as internal control. Data are shown as means ± SD ($n = 3$). Asterisks indicate significant difference from EH ($P < 0.01$) based on a *t*-test.

nutrition in low-sulfate conditions. To test this, we grew maize plants for 4 weeks on acid-washed sand soaked in a modified Hoagland solution with low sulfate (100 μM). Some plants were inoculated with WT *S. indica* and others with the kd-*SiSulT* strain, with noncolonized plants serving as negative controls. Maize plants colonized with WT *S. indica* were much larger and healthier than those in the other two groups (Figure 5A). Indeed, under similar growth conditions, maize plants colonized with WT *S. indica* accumulated 237% more fresh biomass and 230% more sulfur than noncolonized plants (Figure 5, B and C). Inoculation of maize plants with the kd-*SiSulT* strain resulted in a very limited improvement of plant sulfur nutritional status, as these plants only accumulated 130% more fresh biomass and 140% more sulfur than noncolonized plants (Figure 5, B and C).

The increase in plant biomass was largely dependent on available sulfate levels: maize plants colonized with WT *S. indica* produced 230% more biomass relative to noncolonized plants when grown in low-sulfur conditions, whereas they only accumulated 120% more biomass when grown in sulfur-replete conditions compared to noncolonized plants

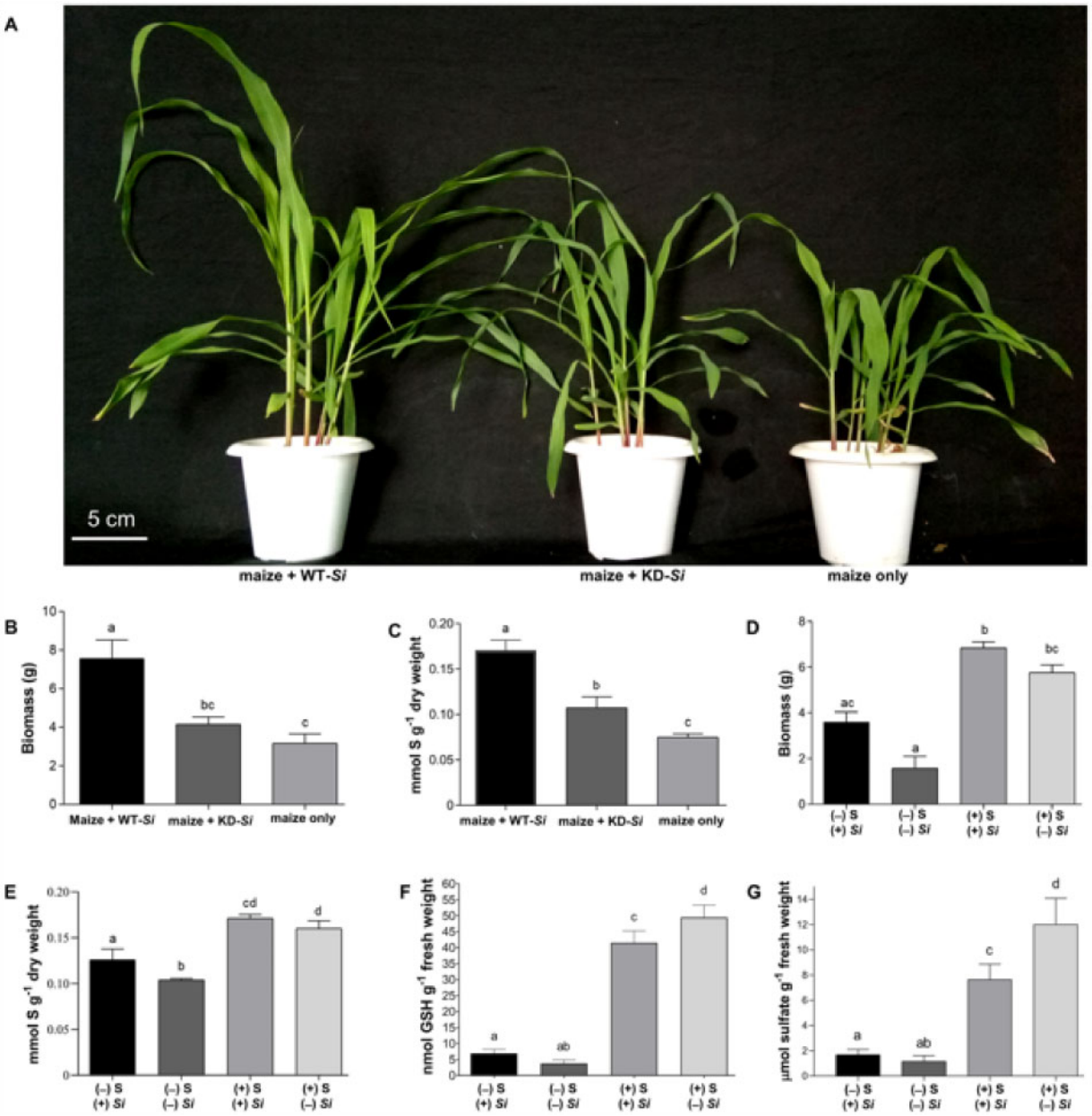


**Figure 5** Consequences of *SiSulT* silencing on maize plant health and development and sulfate nutritional status. A, Improved growth of maize plants colonized with WT *S. indica* relative to maize plants colonized with kd-*SiSulT S. indica* and noncolonized maize plants. Plants were photographed after 35 days. B and C, Determination of plant biomass (B) and total sulfur content (C) in maize plants colonized with WT *S. indica* or with kd-*SiSulT S. indica* and noninoculated maize plants. D–G, Consequences of inoculation with *S. indica* on maize plants grown in low (10 μM) or high (10 mM) sulfate concentrations. Total biomass (fresh weight) of maize plants grown under low (10 $\mu$M) and high (10 mM) sulfate conditions with or without *S. indica* colonization (D), total sulfur content (E), GSH content (F), and sulfate content (G). (−S, low sulfate; + S, high sulfate; + *Si*, plant colonized with *S. indica*; −*Si*, noncolonized plant). Different letters indicate significant differences in different treatments at $P \leqslant 0.05$ (Tukey's test). Data are shown as means ± SEM ($n$ = 3 biological replicates and three technical replicates). ANOVA results are given in Supplemental Data Set 1.

(Figure 5D). Total sulfur content mirrored the results obtained for fresh biomass: plants colonized with WT *S. indica* contained 130% more sulfur than noncolonized plants when grown in low-sulfur conditions, but this difference vanished when both sets of plants were grown in high-sulfur conditions (Figure 5E).

We also analyzed the content of metabolites such as glutathione (GSH) and sulfate ions in the host plant under low- and high-sulfate conditions as well as under colonized and noncolonized conditions. GSH content increased by 80% upon colonization in plants grown in low-sulfate conditions but decreased slightly (∼20%) in sulfur-replete

conditions. However, GSH content was 8- to 10-fold greater in both sets of plants between low- and high-sulfur conditions, as might be expected for the accumulation of a sulfur-containing metabolite (Figure 5F). Plant sulfate ion content exhibited a similar trend, with a 50% increase following colonization with WT *S. indica* under low-sulfur conditions and a 40% reduction in sulfur-replete conditions. Again, the sulfate ion content rose four- to six-fold between the two growth conditions, as expected (Figure 5G). Taken together, these observations suggest that *Si*SulT actively participates in transporting sulfur to plant roots during mutualism.

### Expression of sulfate assimilation pathway genes of *S. indica* grown axenically and during colonization with the host plant

To investigate the impact of partial silencing of the *SiSulT* on the relative expression of sulfate assimilation genes in *S. indica* during axenic growth and colonization of the host plant, we extracted total RNA from axenic *S. indica* cultures as well as from *S. indica* and maize roots during colonization in sulfate-limited and sulfate-replete conditions. We analyzed the relative expression of sulfate assimilation genes in *S. indica* by RT-qPCR. Out of 22 genes along the sulfate assimilation pathway, 11 were upregulated in low-sulfate conditions: the *S. indica* orthologs to the yeast methionine requiring (*MET*) genes *MET1*, *MET5*, *MET8*, *MET12*, *MET14*, *MET16*, *MET17*, and *MET22* as well as *SULFATE ADENYLYLTRANSFERASE* (*SiATPS*), *CYSTATHIONINE GAA-LYASE* (*SiCYS3*), and the sulfate transporter gene *SiSulT* itself (Supplemental Figure 12; Supplemental Table 4).

We repeated the experiment with the kd-*SiSulT* strain and observed fewer upregulated genes when the plants were grown in low-sulfate conditions: only *SiMET4*, *SiMET13*, *SiMET14*, *SiSulT*, and *SiCYS3* were upregulated. However, the relative expression of these genes reached higher levels than in sulfur-starved WT *S. indica*, with the exception of *SiSulT*, which is in agreement with the partial silencing of the transporter gene in this strain. The observation that these sulfur assimilation genes were more strongly induced in the kd-*SiSulT* strain than in the wild type under low-sulfate conditions is consistent with a sulfur deficiency phenotype associated with the partial loss of *Si*SulT function (Supplemental Figure 13; Supplemental Table 4).

We then quantified the relative transcript levels of the same genes during colonization of maize roots by the *S. indica* WT strain (Supplemental Figure 14) or the kd-*SiSulT* strain (Supplemental Figure 15). As observed previously, the *SiSulT* gene was strongly upregulated (~12-fold) in low-sulfate conditions in the WT *S. indica* background. Sulfur assimilation genes displayed very low expression levels, with the exception of *SiMET1* and *SiMET16* (Supplemental Figure 14; Supplemental Table 4). MET1 encodes *S*-adenosyl-L-methionine uroporphyrinogen III transmethylase, which catalyzes the synthesis of molecules essential for sulfate assimilation. A yeast mutant lacking MET1 has been reported to be compromised in sulfate assimilation, whereas MET16 encodes 3′-phosphoadenylsulfate reductase, which reduces 3′-phosphoadenylyl sulfate to adenosine-3′,5′-bisphosphate and free sulfite—a critical step in sulfate assimilation and methionine metabolism. It is possible that the fungus might be getting sulfur in the form of sulfur-containing amino acids, as the fungus also has plenty of amino acid transporters. In maize roots colonized with the kd-*SiSulT* strain, *SiMET10*, *SiMET12*, *SiMET13*, and *SiATPS* were induced four- to five-fold under low-sulfate conditions, and *SiMET1* was induced ~24-fold, much higher than that observed in WT *S. indica* under the same conditions (Supplemental Figure 15, Supplemental Table 4). As expected, *SiSulT* relative transcript levels in the kd-*SiSulT* strain dropped to ~30% of that measured in the WT strain, confirming the strong silencing of *SiSulT* in this background (Supplemental Figure 15; Supplemental Table 4).

### Expression of maize sulfate assimilation genes during *S. indica* colonization

To investigate the impact of partial silencing of *SiSulT* on the relative expression of sulfur assimilation genes in the host plant (maize) when grown in low-sulfate conditions, as evidenced by the high relative transcript levels of most sulfate assimilation genes, we analyzed the relative expression of sulfate assimilation genes in maize plants during colonization with WT and knockdown *S. indica* by RT-qPCR. We also extracted total RNA from maize roots grown in low- or high-sulfate conditions and colonized either with the WT *S. indica* strain or with the kd-*SiSulT* strain. We determined the relative expression of maize genes encoding components of the sulfur assimilation pathway by RT-qPCR. Out of our list of 31 selected genes, sulfate deficiency caused a two- to five-fold upregulation of only three genes: *methylthioadenosine nuclease* (*ZTN*), *serine acetyl-transferase2* (*ZmSAT2*), and *sulfo-transferase* (*ZmSOT*; Supplemental Figure 16; Supplemental Table 5). The number of genes whose expression was induced in low-sulfate conditions rose to 13 in maize roots colonized with the kd-*SiSulT* strain, with a fold-change ranging from 4 to 6 for most genes (Supplemental Figure 17; Supplemental Table 5). The expression of all three maize sulfate transporter genes, *ZmST1*, *ZmST3.4*, and *ZmST4.1*, was downregulated in maize roots colonized with the WT *S. indica* strain, consistent with an improvement of plant sulfur nutrition by the fungus. The same was true for maize roots colonized with the kd-*SiSulT* strain, although the effect was much less pronounced (Supplemental Figures 16 and 17; Supplemental Table 5). Taken together, our expression analyses indicate that plant sulfate assimilation machinery is activated during colonization, which was further found to be altered upon *SiSulT* knockdown.

## Discussion

The rhizosphere is a hotspot for microbial activity. Microbiota help nourish plants by mobilizing and cycling

nutrients. The surrounding soil is the main source of nutrients for plants, but many nutrients are not present in a bioavailable form, which can severely limit plant growth and crop yield. Sulfur is an essential macronutrient required for plant growth and development, with sulfur typically accounting for between 0.1% and 0.5% of a plant's total dry weight, below which plants are considered to be sulfur limited (Daigger and Fox, 1971; Kang and Osiname, 1976; Kamprath and Jones, 1986; Sakal et al., 2000; Marschner, 2011; Sutar et al., 2017). This translates to an optimum soil sulfate concentration range of 0.3%–1.0% of total soil dry weight (Little and Nair, 2009). A concentration of 3–5 p.p.m. of sulfate in the soil is adequate for the growth of many plant species, including maize (Sutar et al., 2017). However, over 95% of soil sulfur is in an organically bound form that is not directly available to plants and must therefore be broken down by bacteria and fungi before uptake (Fitzgerald, 1976; Tabatabai, 1986; Leustek, 1996; Scherer, 2001; Wilhelm Scherer, 2009; Gahan and Schmalenberger, 2014). In addition, the soil texture and precipitation are major factors determining the sulfur availability in the soil. Indeed, sandy and silty soil have less organic matter and low-sulfur content due to leaching caused by rainfall (Wilhelm Scherer, 2009).

To overcome sulfur deficiency, plants have developed intricate strategies that allow them to withstand nutrient-poor soil, including modulating the architecture of their root system and inducing the expression of dedicated transporters of various affinities for their substrates (Aibara and Miwa, 2014). For instance, plant sulfur deficiency causes the upregulation of sulfate transporters and related sulfur assimilation genes (Takahashi, 2019). Sulfur can be provided to the plant by the formation of a mutualistic symbiosis with a fungus. The endosymbiotic fungus *S. indica* has a well-established transformation method, which we capitalized on here for the characterization of a putative fungal sulfate transporter, *Si*SulT.

*Si*SulT possesses all the domains commonly seen in sulfate transporters (Sandal and Marcker, 1994; Smith et al., 1995a; Van De Kamp et al., 1999; Rouached et al., 2005; Yoshimoto et al., 2007). Transcript levels of a high-affinity transporter should reflect the nutritional status of its substrate, as seen for phosphate transporters in fungi and algae (Chung et al., 2003; Yadav et al., 2010; Kankipati et al., 2015). Indeed, *SiSulT* was expressed at high levels in low-sulfate conditions ($<100$ μM), which is consistent with a role in sulfate uptake (Figure 1). Heterologous expression of *Si*SulT in a yeast mutant lacking the two high-affinity transporters Sul1 and Sul2 rescued growth in low-sulfate conditions (Figure 2), as might be expected for a functional sulfate transporter. In addition, *Si*SulT conferred sensitivity to the heavy metal chromate when the fungus was grown in low-sulfate conditions (Supplemental Figures 6 and 7), which is another hallmark of a functional sulfate transporter (Ohta et al., 1971; Roberts and Marzluf, 1971; Campbell et al., 1981; Smith et al., 1995b; Cherest et al., 1997).

Sulfate uptake by *Si*SulT followed a typical Michaelis–Menten kinetics when expressed in yeast and resembled that observed for the endogenous yeast transporters Sul1 and Sul2 (Figure 3). With a measured apparent $K_m$ of 15 μM, *Si*SulT therefore falls within the range of values seen for high-affinity sulfate transporters, similar to the yeast transporters Sul1 and Sul2 (with $K_m$ 4–10 μM), or values that are much lower than those expected for low-affinity transporters (closer to 100 μM; Smith et al., 1995a, 1995b, 1997; Cherest et al., 1997; Takahashi et al., 2000; Vidmar et al., 2000; Yoshimoto et al., 2002; Howarth et al., 2003; Nocito et al., 2006). *Si*SulT transporters demonstrate a strong pH preference, with the highest uptake at pH 5.0. Sulfate uptake by *Si*SulT might be facilitated by a proton gradient across the plasma membrane, but this hypothesis needs further investigation.

As *S. indica* can be easily transformed, we were able to target *SiSulT* by RNAi (Supplemental Figure 8) and investigate its function in situ. WT *S. indica* facilitated sulfate transport into maize seedlings when grown in low-sulfate conditions, whereas the RNAi strain kd-*SiSulT* greatly decreased this uptake by 85% (Figure 4), although the extent of root colonization by either strain was comparable. *Si*SulT was more highly expressed in external hyphae, which are in direct contact with the transporter's substrate, compared to internal hyphae. We characterized the consequences of colonization of the host plant by *S. indica* in terms of overall growth, sulfur content, and secondary metabolites such as GSH and sulfate ions, which are all affected by low-sulfur availability (Figure 5; Nikiforova et al., 2005; Sieh et al., 2013). Colonization of maize plants by WT *S. indica* more than doubled plant fresh biomass and total sulfur content. Knocking down *SiSulT* expression to ∼40% of WT levels in the kd-*SiSulT* strain mostly abolished these gains, underscoring the importance of (1) colonization of maize roots by *S. indica* in plant fitness and (2) the critical role of the *Si*SulT sulfate transporter in this process. Importantly, the fitness advantage conferred by *S. indica* colonization was limited to low-sulfate conditions, as was observed in an AMF–barrelclover symbiosis (Sieh et al., 2013). These results parallel earlier findings with a phosphate transporter from *S. indica* during maize colonization (Yadav et al., 2010). Based on our collective data, we proposed a model suarizing the mechanisms of sulfate uptake by the host plant through a fungal mycelium in low-sulfate conditions (Figure 6).

Just as for other micro- and macronutrients, sulfur deficiency induces the expression of sulfur assimilation genes in both the fungus *S. indica* and the host plant (maize, in this case) when grown on their own (Droux, 2004; Casieri et al., 2012). Sulfate assimilation starts with the upregulation of a dedicated transporter or permease, which takes sulfur (as sulfate) from the environment and transports it to the sulfur cellular assimilation machinery, responsible for its ATP-dependent adenylation and reduction to sulfite and

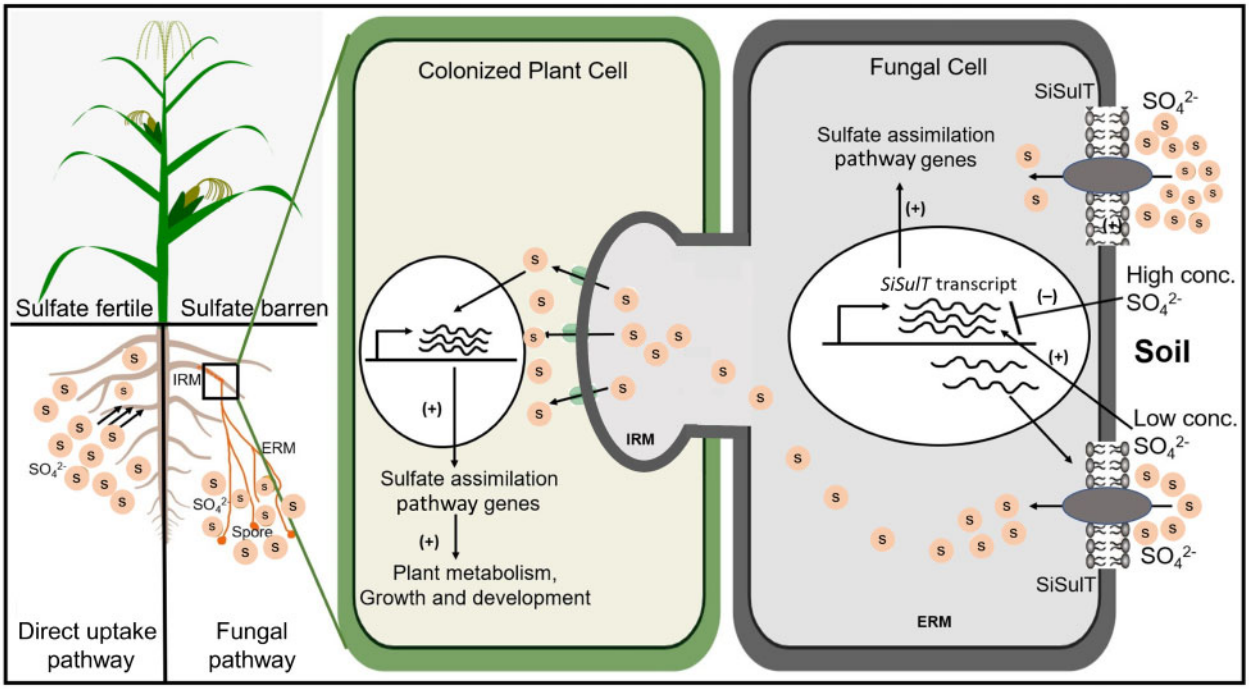


**Figure 6** Schematic diagram illustrating sulfur transfer to the host plant through *S. indica* endomycorrhizal symbiosis. Fungal mycelia import $SO_4^{2-}$ from external sources. The transfer of $SO_4^{2-}$ through *S. indica* symbiosis is regulated by the external concentration of sulfate. Under low external sulfate conditions, expression of high-affinity *SiSulT* of ERM results in uptake of $SO_4^{2-}$ and transfer to the colonized plant cell via IRM across the symbiotic interface (green dots with arrow). The mechanism might involve efflux of sulfate ions from the symbiotic fungus into the apoplast and uptake into the plant via plant sulfate transporters. ERM, extra radical mycelium; IRM, intra radical mycelium.

finally to sulfide. Genes involved in the biosynthesis of sulfur-containing amino acids such as Met and Cys, as well as *S*-adenosyl-methionine, were all highly upregulated in WT *S. indica* cultures exposed to low-sulfate conditions, likely as an attempt to compensate for the low pool of available sulfate for biosynthesis of these essential amino acids and sulfated compounds. In addition, partial silencing of the *S. indica* sulfate transporter *SiSulT* by RNAi imposed an even stronger sulfur deficiency in the kd-*SiSulT* background when grown in low-sulfate conditions, as evidenced by the very high relative transcript levels of most sulfate assimilation genes in kd-*SiSulT* relative to the WT.

A role for *Si*SulT in sulfate uptake during colonization of maize roots and in sharing resources with the host plant is clearly supported by the growth boost observed when colonized maize plants are grown in low-sulfate conditions relative to noncolonized plants (Figure 5, A and B). Although we might have expected to see strong upregulation of the plant sulfur assimilation machinery when grown in low-sulfate conditions, we were surprised to detect only three significantly upregulated genes in maize roots colonized with WT *S. indica* that responded to sulfur availability: *ADENOSINE 5′-PHOSPHOSULFATE REDUCTASE* (*APR*), *APS KINASE*, and *SERINE ACETYL-TRANSFERASE* (*SAT*), encoding enzymes that may constitute important regulatory points during sulfur assimilation (Hopkins et al., 2004). However, these observations are fully consistent with a beneficial interaction between the fungus and plant host whereby the fungus *S. indica* contributes to sulfur uptake and thus relieves the host plant from mounting a sulfur deficiency response. This hypothesis is consistent with the gene expression profile of maize sulfur assimilation genes in roots colonized with the kd-*SiSulT* strain. Indeed, overall expression for sulfur assimilation genes was generally higher in these roots, with 13 significantly upregulated genes compared to only 3 when colonization occurred with WT *S. indica*; Supplemental Figures 16 and 17). We hypothesize that kd-*SiSulT* provides less sulfate to the host plant during colonization, thus forcing fast-growing roots to induce their sulfur deficiency responses.

As they explore the soil, growing roots may encounter GSH originating from microbial activity or released by organic matter. This exogenous GSH pool constitutes a valuable source of reduced sulfur for the fungus but first requires conversion by the host plant. γ-Glutamyltransferase (GGT) catalyzed the hydrolysis of extracellular GSH, and its encoding gene *ZmGGT1* was highly induced during kd-*SiSulT*–maize symbiosis in low-sulfur conditions. It is possible that much of the plant GSH pool goes to the fungus during colonization. In the case of the WT *S. indica* strain, the net uptake of sulfur by the fungus more than makes up for the loss of GSH, which can be replenished by the plant. In contrast, maize plants colonized by kd-*SiSulT* may suffer from a net loss of GSH and therefore need to obtain alternate sources such as soil GSH. In agreement with this hypothesis, we also detected increased expression in the maize GSH *S*-transferase (*ZmGST*) gene, which has been proposed to play an

integral role in plant defenses against toxins (Leustek et al., 2000).

Our study offers a system with which to dissect beneficial plant–fungus interactions in the context of sulfate assimilation. The symbiotic relationship between *S. indica* and maize roots will further allow the thorough characterization of sulfur assimilation gene expression profiles, both in the fungus and in the host plant root, and provide insight into how they relate to the sulfur flux balance and sulfate demand for both organisms. Our results also suggest that the endophyte *S. indica* has potential agronomic uses in overcoming low-sulfate conditions and improving plant productivity.

## Methods

### Plant and fungal materials and bacterial strains

We used the maize (*Zea mays*) variety HQPM-5 and the fungus *S. indica* for all plant/fungus growth assays. The *E. coli* strains XL1-Blue and DH5α were used for cloning purposes (Sambrook et al., 1989). Maize seeds were surface sterilized for 2 min in ethanol, followed by 10 min in a NaClO solution (0.75% Cl), and finally washed six times with sterile water. Seeds were then treated with double-distilled $H_2O$ at 60°C for 5 min and germinated on water agar plates (0.8% Bacto Agar, Difco, Detroit, MI, USA) at 25°C in the dark for 3 days (Varma et al., 1999). Plants were grown under controlled conditions in a greenhouse with an 8-h-light:16-h-dark photoperiod (light intensity of 1,000 lux, using high-intensity fluorescent light) at a temperature cycle of 25°C/18°C with a relative humidity 60%–70%. Germinated maize seedlings were placed in pots filled with a mixture of sterile sand and soil in the ratio of 3:1 (garden soil from the Jawaharlal Nehru University campus and acid-washed riverbed sand). Plants were supplied with half-strength modified Hoagland solution (5 mM $KNO_3$, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 10 μM $KH_2PO_4$, 10 μM $MgCl_2$, 4 μM $ZnSO_4$, 1 μM $CaSO_4$, 1 μM $NaMoO_4$, and 50 μM $H_3BO_{3)}$ weekly.

### Colonization of *S. indica* and histochemical analysis

For colonization assays, we iersed the roots of 3-day-old maize seedlings in an aqueous solution containing $5 \times 10^5$/mL *S. indica* chlamydospores. Control seedlings were mock inoculated with sterile water and grown in sterile sand. Plants were given half-strength modified Hoagland solution weekly. Plant roots were harvested at different dpi (5, 10, 15, and 20 days) and were assessed for colonization by Trypan blue staining as follows. We selected 10 root samples randomly. Samples were softened in 10% KOH solution for 15 min and acidified with 1 N HCl for 10 min before staining with 0.02% Trypan Blue (Phillips and Hayman, 1970; Dickson et al., 1998; Kumar et al., 2009). After 2 h, samples were destained with 50% Lactophenol for 1–2 h before observation under a light microscope (Leica Microscope, Type 020-518.500, Germany and Nikon Eclipse Ti). The distribution of chlamydospores within the root was taken as colonization percentage, calculated as previously described (McGonigle et al., 1990; Kumar et al., 2009).

### Analysis of colonization pattern in maize roots

We selected equal amounts (by biomass) of maize roots that had been colonized by *S. indica* for 20 days from the root maturation and apical zones. Roots were harvested, frozen, and ground in liquid nitrogen, and then genomic DNA was isolated from 100 mg of root powder. We isolated total genomic DNA from two different root zones that contain both plant and fungal DNA as described (Huang and Schiefelbein, 2015). Further, this mixture of plant and fungal genomic DNA was resuspended in 100 μL nuclease-free water. We performed a PCR amplification of the *S. indica* translation elongation factor gene *TEF* and of the maize *Actin* gene (as a control). The sequences for the *TEF* and *Actin* primers are listed in Supplemental Table 7. PCRs were carried out in a final volume of 25 μL, containing 2 μL of DNA as template. The following PCR program was used: 98°C for 2 min (1 cycle), 98°C for 10 s, 60°C for 30 s, 72°C for 30 s (25 cycles), and 72°C for 5 min (1 cycle). PCR products were visualized on 1% agarose gels, stained with ethidium bromide, and quantified based on their band intensity. We also performed Trypan blue staining of representative maize roots for a visual estimation of *S. indica* spore density in different root zones.

### Isolation of *SiSulT* cDNA and cloning

We isolated total RNA from *S. indica* grown axenically in Aspergillus Minimal Medium (AMM; Hill and Kaefer, 2001) with low sulfate (10 μM) with TRIzol reagent (Invitrogen, USA). We synthesized first-strand cDNAs using a cDNA synthesis kit (AffinityScript cDNA Synthesis Kit Agilent). The 2,292-bp ORF for *SiSulT* was PCR amplified with gene-specific primers (Supplemental Table 7). For directional cloning, we added *Bam*HI and *Xba*I restriction sites to the gene-specific primers (Supplemental Table 7). We used *S. indica* first-strand cDNAs as template for PCR. 3 μg of RNA was used for cDNA synthesis, and 2 μL was taken from 100 times diluted cDNA sample as template for PCR in a final volume of 50 μL, containing 10 mM Tris–HCl (pH 8.3); 50 mMKCl; 1.5 mM $MgCl_2$; 200 μM dNTPs; 3 μM of each primer; 3 units of Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific); and 60–100 ng of cDNA as template. The PCR program used was as follows: 94°C for 2 min (1 cycle), 94°C for 45 s, 60°C for 1 min 15 s, 72°C for 1–2 min (35 cycles), and 72°C for 5 min (1 cycle). The PCR product was cloned into the pJET1.2 cloning vector (Promega) and further subcloned into the pYES2 yeast shuttle vector between *Bam*HI and *Xba*I sites.

### *SiSulT* expression analysis

We set up *S. indica* cultures in minimal media (0.4 mM NaCl, 2.0 mM $KH_2PO_4$, 0.3 mM $(NH_4)_2HPO_4$, 0.6 mM $CaCl_2$, 0.6 mM $MgSO_4$, 3.6 mM $FeCl_3$, 0.2 mM thiamine hydrochloride, 0.1% (w/v) trypticase peptone, 1% (w/v) glucose, 5% (w/v) malt extract, 2 mM KCl, 1 mM $H_3BO_3$, 0.22 mM

$MnSO_4.H_2O$, 0.08 mM $ZnSO_4$, and 0.021 mM $CuSO_4$, pH 5.8; Bécard and Fortin, 1988) containing different sulfate ($Na_2SO_4$) concentrations (1, 5, 10, 25, 50, 100 μM, 1, and 10 mM). We harvested *S. indica* after 1, 5, 10, and 15 days for RNA extraction with TRIzol reagent. We synthesized first-strand cDNAs with the Affinity Script cDNA Synthesis Kit (Agilent) from total RNA and used them as a template for RT-qPCR (Applied Biosystems 7500 Fast RT-qPCR System, Thermo Fisher Scientific) using SYBR green with gene-specific primers (Supplemental Table 7). The reaction mixture was heated at 95°C for 20 min and then subjected to 40 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 20 s. Heat dissociation curves confirmed that a single PCR product was amplified (*SiSulT* PCR product has a melting temperature 60°C–65°C). We used *S. indica TEF* as control (Yadav et al., 2010). The level of target mRNA, relative to the mean of the reference housekeeping gene, was calculated with the relative $\Delta\Delta C_t$ method as described by the manufacturer (Applied Biosystems 7500 Fast Real-Time PCR System, Thermo Fisher Scientific).

## Phylogenetic and homology analysis

We determined the functional sites in *SiSulT* and their pattern using the PROSITE database. We identified related sequences with the Basic Local Alignment Tool BLASTX algorithm (www.ncbi.nlm.nih.gov). Sequence alignments were generated with ClustalΩ and BLOSUM62 with a gap penalty of 10 for insertion and 5 for extension (Henikoff and Henikoff, 1992; Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses of *S. indica* putative *SiSulT* were conducted using MEGA X with the neighbor-joining analysis method and examined by bootstrap testing with 1,000 replications (Kumar et al., 2018).

## Yeast complementation assay

Yeast (*S. cerevisiae*) work was carried out in the WT strain BY4742 (*MATα his3Δ leu2Δ lys2Δ ura3Δ*) and the sulfate transporter double mutant HK14 (MATα *sul1::KanMX sul2::KanMX his3Δ leu2Δ lys2Δ ura3Δ*; Kankipati et al., 2015; Supplemental Table 6). Both strains have the same BY background. For complementation assays, we cloned *SiSulT* into the pYES2 yeast expression vector and introduced the resulting plasmid into HK14 mutant cells by the LiCl-PEG method (Bun-Ya et al., 1991; Riesmeier et al., 1992; Gietz et al., 1995; Akum et al., 2015; Jogawat et al., 2016). We used the parental strain BY4742 as positive control. As a negative control, we transformed HK14 cells with the empty vector pYES2. The pYES2 vector drives the galactose-inducible expression of the gene of interest. For growth tests, we grew yeast cells at 30°C on SD medium containing 0.1 mM of sulfate (as a sole source of sulfur) in the presence of 2% glucose (noninducing condition) or 2% galactose (inducing condition) as the sole carbon source. Cells were spun down and resuspended in sterile distilled water at a cell density adjusted to $OD_{600}$ = 0.1, as well as 1:10 serial dilutions. For each serial dilution and genotype, we plated 30 μL cell suspension on solidified agar plates containing glucose or galactose. We incubated plates for 2–3 days at 30°C before scoring growth by scanning the plates.

## Yeast growth analysis

We recorded the growth of yeast strains grown in various sulfate concentrations for 60 h. We first starved WT BY4742 and the HK14 mutant transformed with pYES2 or with *SiSulT* (cloned into pYES2) for sulfur by growing cells in sulfur starvation medium for 2 d (2% (w/v) galactose, 15 mM $NH_4Cl$, 6.6 mM $KH_2PO_4$, 0.5 mM $K_2HPO_4$, 1.7 mM NaCl, 0.7 mM $CaCl_2$, 2 mM $MgCl_2$, 0.2 mg/L biotin, 2 mg/L each of thiamine hydrochloride, pyridoxamine hydrochloride, and calcium pantothenate, 0.5 mg/L $H_3BO_4$, 0.04 mg/L $CuCl_2$, 0.1 mg/L KI, 0.05 mg/L $FeCl_3$, 0.2 mg/L $ZnCl_2$, and supplements of amino acids and nucleotides as per auxotrophic requirements of the strains). We then transferred all strains to growth medium containing different concentrations of sulfate (0, 1, 10, 50, 100 μM, 1, and 10 mM) as the sole sulfur source. The flasks were kept at 30°C; we measured $OD_{600}$ every 2 h for up to 60 h to check growth. Experiments were carried out in triplicates and repeated three times.

## Chromate toxicity

We grew WT BY4742 and the HK14 mutant transformed with *SiSulT* (cloned into pYES2) for 4 d at 30°C in yeast nitrogen base (YNB) containing 100 μM or 1 mM of sulfate as a sole source of sulfur and in the presence of 2% galactose as sole carbon source to $2 \times 10^8$ cells/mL. HK14 mutant transformed with pYES2 was supplemented with 100 μM homocysteine as an alternative source of sulfate to grow on YNB media. Cells were then spun down and resuspended in sterile distilled water at an adjusted cell density of $OD_{600}$ = 0.1, followed by 1:10 serial dilutions. We spotted 30 μL of cell suspension onto YNB plates containing 20 μM chromate and 100 μM or 1 mM sulfate, or 40 μM or 60 μM chromate and 100 μM sulfate. Plates were scanned after 4 days at 30°C. Chromate was omitted from control plates. The experiments were repeated three times.

## Sulfate uptake and kinetics assays

We grew WT BY4742 and the HK14 mutant transformed with pYES2 or with *SiSulT* (cloned into pYES2) for 4 days at 30°C in YNB liquid medium with 2% glucose and without uracil to $OD_{600}$ = 1.5–2 with shaking at 220 r.p.m. on a metabolic shaker (Infors, Switzerland). Exponentially growing cells were spun down and washed with sterile $ddH_2O$ before transfer to sulfur starvation medium as above, containing 2% galactose (pH 5, with 50 mM MES-KOH), and were allowed to grow at 30°C with shaking at 220 r.p.m. for 2 days. Cells were harvested and resuspended in fresh sulfur starvation medium at a cell density of 60 mg (wet weight)/mL. To start the sulfate uptake assay, we incubated 50 μL aliquots of cells for 10 min at 30°C and then added different concentrations of sulfate (1, 2, 4, 6, 10, 25, 75, and 100 μM) from a starting stock of 0.5 mM [$^{35}$S]sodium sulfate (specific activity of 2,000 cpm/nmol or 0.9 Ci/mol of sodium sulfate).

After 4 min, we stopped uptake by adding 5 mL of ice-cold sulfur starvation medium. Cells were recovered on a glass microfiber filter and washed three times with 5 mL of ice-cold sulfate starvation medium by centrifugation at 5,000*g* for 5 min at 4°C. For the controls, ice-cold sulfate starvation medium was added to cells before the addition of [$^{35}$S]sodium sulfate, and the cells were iediately filtered and washed. Filters were transferred into scintillation vials containing 5 mL of scintillation cocktail "O" (CDH), and the radioactivity was measured with a scintillation counter (Liquid Scintillation Analyzer TRI-CARB 2100TR; Packard). Uptake assays were performed at room temperature (25°C). Sulfate accumulation (in pmol) was measured by standard mathematical calculations to convert scintillation count to picomoles. We used a standard curve of radioactivity for the calculation. The amount of sulfate transported by the control (background) was used to normalize the data. Transport data at 10 μM concentration was used for plotting the uptake graph. The rate of sulfate uptake was expressed as nmol·min$^{-1}$ × (mg dry weight)$^{-1}$ or pmol/min/$OD_{650}$. GraphPad Prism 6 was used to plot nonlinear regression for sulfate uptake rate. Experiments were repeated three times, each time as technical triplicates.

## Development of the RNAi cassette and transformation

We selected a unique 452-bp *SiSulT* fragment (Supplemental Figure 8B) using the BLAST tool and analyzed it for uniqueness and RNA secondary structures. This fragment was PCR amplified using gene-specific primers (Supplemental Table 7) and cloned into the pGEM-T cloning vector before being subcloned into the pPiRNAi vector at the unique *Eco*RV site (Supplemental Figure 8A; Hilbert et al., 2012). The pPiRNAi vector bears two promoters, *SiTEF* and *SiGPD*, in a convergent configuration, such that each promoter expresses an RNA molecule corresponding to the sense or antisense strand of the gene of interest cloned at the *Eco*RV site. This construct was named as pRNAi-SiSulT. We transformed *S. indica* with the empty pRNAi and pRNAi-*SiSul*T vectors as described previously (Yadav et al., 2010). Briefly, chlamydospores were harvested from 14-day-old *S. indica* cultures and germinated on glucose-containing AMM (Yadav et al. 2010). Cell walls were disrupted with β-glucuronidase enzyme (Sigma: *Helix pomatia*). We introduced linearized pRNAi-SiSulT (1 μg) into *S. indica* by electroporation at 12.5 kV/cm, 25 μF capacitance, and 5 ms pulse length. We selected four transformants (TC1, TC2, TC3, and TC4) after primary and secondary selection using KF medium containing 100 μM (primary selection) and 200 μM (secondary selection) hygromycin (Supplemental Figure 9A). Successful transformation was confirmed by PCR using primers for hygromycin phosphotransferase (Supplemental Table 7; Supplemental Figure 9C). All transformants were also tested for expression of the silenced gene (*SiSulT*) by RT-qPCR as described (Jogawat et al., 2016). Transformants obtained were named kd-*SiSulT*.

## RNA blot analysis for siRNAs

We isolated total RNA from *S. indica* kd-*SiSulT* transformant TC1 (in duplicate) and WT *S. indica* with TRIzol reagent. Hybridization and autoradiography were performed as described (Yadav et al., 2010). RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water, heated to 65°C for 5 min, and then kept on ice. We then added polyethylene glycol (molecular weight of 8,000, Sigma) to a final concentration of 5% and NaCl to a final concentration of 0.5 M. After incubation on ice for 30 min, this mixture was centrifuged at 10,000*g* for 10 min. The supernatant was mixed with three volumes of ethanol. To precipitate the RNA, this mixture was incubated at −20°C for at 2 h before centrifugation for 10 min at 10,000*g*. The pellet was dissolved in DEPC-treated water and heated at 65°C for 5 min. We then added one-third volume of 4× loading solution [2× TBE (1× TBE is 0.09 M Tris–borate, pH 8.0, and 0.002 M EDTA), 40% sucrose, and 0.1% bromphenol blue] before loading onto a 15% urea–PAGE gel in 1× TBE (Tris–borate–EDTA). The RNA samples were electrophoresed at 2.5 V/cm and then blotted onto a Hybond N$^+$ membrane (Amersham Biosciences) and UV cross-linked. The membrane was prehybridized in 50% formamide, 7% SDS, 50 mM $NaHPO_4$/$NaH_2PO_4$, pH 7.0, 0.3 M NaCl, 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, and 0.02% bovine serum albumin), and 100 mg/mL sheared, denatured salmon sperm DNA at 37°C for at least 3 h. The probe was prepared by labeling the small fragment of the *SiSulT* gene (5′GTAATATCGACACGACCG) using [γ-$^{32}$P]ATP and polynucleotide kinase as per the manual (Molecular Labeling and Detection, Fermentas) and was added to the prehybridization solution. Hybridization was performed at 37°C overnight, and the membrane was subsequently washed at 37°C in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.2% SDS for 15 min twice. Final washing was given only with 2× SSC at room temperature for 10 min and autoradiography was done. DNA oligonucleotides of 16 and 22 nt were used as molecular size markers for siRNA analysis.

## Bicompartment assay

We placed a 6-cm-diameter Petri dish (compartment 2) inside a 15-cm-diameter Petri dish (compartment 1) to create a physical barrier between both compartments. We grew *S. indica* in compartment 2, whereas surface-sterilized maize seeds were placed in compartment 1. The leafy shoots protruded through a groove cut in the lid of each dish and were fixed in place by wrapping sterile nonabsorbent cotton wool around the portion of the subtending rhizome as it passed through the groove. We filled both compartments with MNM medium. We prepared three sets for the experiment: (1) maize seedlings colonized with WT *S. indica*; (2) maize seedlings colonized with kd-*SiSulT S. indica*; and (3) maize seedlings grown without *S. indica*. In all cases, MNM medium contained 10 μM sulfate in both compartments. For sets 1 and 2, we formed a connective bridge consisting of a 4 to 5 cm agar strip to allow colonization of maize

roots (in compartment 1) by *S. indica* (in compartment 2). For set 3, we placed a similar connecting bridge to control for any transfer of radioactive sulfate from compartments 2 to 1 due to diffusion. As the colonization develops, extraradical hyphae proliferate in the medium surrounding the roots in compartment 1 where they ramify and later sporulate. After colonization establishment, MNM medium in compartment 2 was replaced with fresh MNM medium containing 100 μM sulfate and 1 μM $^{35}S$ (specific activity, 200 mCi/mmol). We determined radioactivity by autoradiography iediately after harvesting the sample and measured the amount of $^{35}S$ incorporated on a liquid scintillation analyzer (Packard). The experiment was conducted three times independently.

## Spatial expression analysis of *SiSulT*

External hyphae projecting out from the surface of the colonized root were visualized under a binocular microscope and extracted using forceps (Harrison and van Burren, 1995; Allen and Shachar-Hill, 2009). Approximately 2 mg of hyphae was collected per sample. In the case of internal hyphal sample collection, external hyphae were first removed using forceps and/or brushed off with a paint brush. Small pieces (5–10) of colonized roots were collected. Colonization of the root pieces was confirmed as described previously (Narayan et al., 2017). We isolated total RNA from external and internal hyphal samples and synthesized first-strand cDNAs using the Superscript cDNA synthesis kit (Clontech). Diluted cDNAs were used as template for RT-qPCR with gene-specific primers for *SiSulT* and *SiTEF* (control) as above (Supplemental Table 7; Yadav et al., 2010).

## Analysis of the growth-promoting activity of *S. indica*

We prepared four sets of plants: (1) maize plants grown in low-sulfate conditions and treated with autoclaved macerated fungal mycelium (serving as control); (2) maize plants colonized with WT *S. indica* and grown in low-sulfate conditions; (3) maize plants grown in high-sulfate conditions and treated with macerated fungal mycelium; and (4) maize plants colonized with WT *S. indica* and grown in high-sulfate conditions. All four sets were grown in acid-washed sand fertilized with a modified half-strength Hoagland solution (Hoagland and Arnon, 1950) containing 100 μM or 10 mM sulfate. After 4 weeks, plants were harvested, and fresh weight was measured.

## Determination of sulfur and plant metabolite content

To determine the sulfur and plant metabolite contents, after 35 days we harvested and dried plants in an oven at 150°C and then crushed them into a fine powder, which was used for energy dispersive X-ray fluorescence (ED-XRF) on a PANalytical Epsilon 5 spectrometer. For sulfate ion measurements, we homogenized 50 mg of frozen plant material in 1 mL deionized water containing 20 mg of polyvinylpolypyrrolidone. Samples were incubated with constant shaking at 4°C for 2 h, at 95°C for 15 min, and finally centrifuged at 14,000*g* for 20 min. 200 μL of supernatant was used for high-performance liquid chromatography (HPLC; Agilent Technologies, Santa Clara, CA, USA, 1260 series) as described (Sieh et al., 2013). GSH was extracted from maize plant tissue by grinding 100 mg of frozen material in 1 mL of 0.1 M HCl. The extract was centrifuged at 20,000*g* for 10 min to remove cell debris. The supernatant was used to measure total GSH content after reduction with dithiothreitol and subjected to HPLC analysis using the monobromobimane derivatization method (Sieh et al., 2013).

## Expression analysis of sulfate assimilation pathway genes in *S. indica* and maize

We identified genes involved in sulfate uptake in the *S. indica* genome based on sequence similarity with related genes from budding yeast and quantified their relative transcript levels by RT-qPCR. We used the Saccharomyces Genome Database, which provides comprehensive integrated biological information for the budding yeast *S. cerevisiae*. We extracted total RNA from *S. indica* alone or after colonization of maize roots, grown in sulfate-limited and sulfate-rich conditions. *S. indica* mycelia were grown in AMM medium for 7 days in high-sulfate conditions (10 mM) and transferred to minimal growth medium containing low (LS = 10 μM) or high (HS = 10 mM ) concentrations of sulfate for an additional 7 days. In the case of *S. indica* alone, fungal mats were iediately harvested and frozen in liquid nitrogen and total RNA was isolated. In the case of colonization, maize plants were submerged in MNM medium supplemented with low (LS, 10 μM) or high (HS, 10 mM) concentrations of sulfate for 30 days along with a *S. indica* suspension of chlamydospores ($5 \times 10^5$/mL). Subsequently, the plant samples were frozen and total RNA was isolated. We explored genes related to sulfate assimilation in *S. indica* (Supplemental Table 4) and maize (Supplemental Table 5) by BLASTp search. We used a two-step RT-qPCR protocol in different conditions. RT-qPCR reactions were performed on an ABI 7500 Fast sequence detection system (Applied Biosystems, Life Technologies, USA) with the following PCR protocol: preincubation at 95°C for 5 min, denaturation 94°C for 10 s, annealing at 60°C for 10 s, extension at 72°C for 10 s, 40 cycles of amplification, and final extension at 72°C for 3 min. The Ct values were automatically calculated, and transcript levels were normalized to *SiTEF* expression in the case of *S. indica* (Kumar et al., 2009) and against *ZmGAPDH* in the case of maize. Fold-change was calculated relative to the nontreated control ($\Delta\Delta C^T$ represents the $\Delta C^T$ condition of the gene of interest—the $\Delta C^T$ condition of the control gene; Livak and Schmittgen, 2001).

## Statistical methods

The statistical analyses were performed with Microsoft Excel 2010 and GraphPad Prism 8. The significance of the study was calculated using *t*-test and one-way ANOVA. Statistical data are reported in Supplemental Data Set 1.

## Accession numbers

Sequence data of *S. indica* sulfate transporter (*SiSulT*) can be found in the GenBank Database (https://www.ncbi.nlm.nih.gov/genbank/). Accession numbers of genes mentioned in this study are listed in Supplemental Table 8.

## Supplemental data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Schematic representation of genomic orientation, ORF, and protein translation of putative *SiSulT*.

**Supplemental Figure 2.** Multiple sequence alignment analysis among sulfate permease of different kingdom.

**Supplemental Figure 3.** Multiple sequence alignment analysis among sulfate permease of different fungal species.

**Supplemental Figure 4.** Phylogenetic analysis of sulfate transporters.

**Supplemental Figure 5.** Colonization pattern of *S. indica* in maize roots.

**Supplemental Figure 6.** Chromate toxicity in the presence of different concentrations of sulfate.

**Supplemental Figure 7.** Effect of different concentrations of chromate on sulfate transport in the WT, HK14 (Δ*sul1*Δ*sul2*) mutant, and complemented strain.

**Supplemental Figure 8.** pRNAi yeast shuttle vector and RNAi insert map.

**Supplemental Figure 9.** Knockdown of *SiSulT*.

**Supplemental Figure 10.** Growth analysis of pRNAi-*SiSulT* transformed *S. indica*.

**Supplemental Figure 11.** Bi-compartment Petri dish culture system to study the transport of radiolabeled ($^{35}S$) sodium sulfate to maize plants via *S. indica*.

**Supplemental Figure 12.** Relative expression analysis of putative sulfate assimilation pathway genes of wild-type *S. indica* grown axenically under low and high sulfate conditions.

**Supplemental Figure 13.** Relative expression analysis of putative sulfate assimilation pathway genes of kd *S. indica* grown axenically under low- and high-sulfate conditions.

**Supplemental Figure 14.** Relative expression analysis of putative sulfate assimilation pathway genes of wild-type *S. indica* during colonized stage with maize plants under low- and high-sulfate conditions.

**Supplemental Figure 15.** Relative expression analysis of putative sulfate assimilation pathway genes of kd *S. indica* during colonized stage with maize plants under low- and high-sulfate conditions.

**Supplemental Figure 16.** Expression analysis of sulfate assimilation genes of maize plants during wild-type *S. indica* colonization under low- and high-sulfate conditions.

**Supplemental Figure 17.** Expression analysis of sulfate assimilation genes of maize plants during kd *S. indica* colonized stage under low- and high-sulfate conditions.

**Supplemental Table 1.** List of domain hits of all the important conserved domains of sulfate transporters.

**Supplemental Table 2.** Homology between highly similar fungal species with putative *Si*SulT.

**Supplemental Table 3.** Comparison of *Si*SulT sequence with members of the Basidiomycota.

**Supplemental Table 4.** Fold change of sulfur assimilation pathway genes of *S. indica*.

**Supplemental Table 5.** Fold change of sulfur assimilation pathway genes of the maize plant.

**Supplemental Table 6.** List of strains and plasmids used in this study.

**Supplemental Table 7.** List of oligonucleotides used in this study.

**Supplemental Table 8.** List of gene accession numbers used in this study.

**Supplemental Data set 1.** ANOVA data.

**Supplemental File 1.** Alignment corresponding to the phylogenetic tree in Supplemental Figure 4.

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