Sulfur Stable Isotope Discrimination in Rice: A Sulfur Isotope Mass Balance Study

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The use of sulfur (S) stable isotopes to study S metabolism in plants is still limited by the relatively small number of studies. It is generally accepted that less S stable isotope discrimination occurs during sulfate (SO_4^{2-}) uptake. However, S metabolism and allocation are expected to produce separations of S stable isotopes among the different plant S pools and organs. In this study, we measured the S isotope composition of the main S pools of rice plants grown under different SO_4^{2-} availabilities in appropriate closed and open hydroponic-plant systems. The main results indicate that fractionation against ^{34}S occurred during SO_4^{2-} uptake. Fractionation was dependent on the amount of residual SO_4^{2-} in the solution, showing a biphasic behavior related to the relative expression of two SO_4^{2-} transporter genes (*OsSULTR1;1* and *OsSULTR1;2*) in the roots. S isotope separations among S pools and organs were also observed as the result of substantial S isotope fractionations and mixing effects occurring during SO_4^{2-} assimilation and plant S partitioning. Since the S stable isotope separations conserve the memory of the physiological and metabolic activities that determined them, we here underline the potential of the $^{32}S/^{34}S$ analysis for the detailed characterization of the metabolic and molecular processes involved in plant S nutrition and homeostasis.

Keywords: fractionation, *Oryza sativa* L., sulfate uptake, sulfur assimilation, sulfur stable isotopes

INTRODUCTION

Since 1865, sulfur (S) has been recognized as an essential element for plant growth (Sachs, 1865; Epstein, 2000). In plants, S is found in the amino acid cysteine and methionine, short peptides, vitamins and cofactors, and secondary compounds (Takahashi et al., 2011).

Plants mainly utilize sulfate (SO_4^{2-}), an inorganic form of oxidized S present in the soil solution, to support their growth. SO_4^{2-} is taken up by roots and allocated to various sink tissues, where it is stored in the cell vacuoles or assimilated into S organic (S_{org}) compounds (Saito, 2004; Takahashi et al., 2011). To accomplish the assimilation of S into biomolecules, SO_4^{2-} is first activated by ATP sulfurylase to adenosine-5′-phosphosulfate (APS), which is then channeled toward reduction or sulfation (Leustek et al., 2000). Most of the APS enters the reductive pathway along which sulfite and, subsequently, sulfide are produced through two sequential reactions catalyzed by APS reductase and sulfite reductase, respectively. Sulfide is finally incorporated into *O*-acetylserine

(OAS) to form cysteine in a reaction catalyzed by OAS(thiol)lyase (Takahashi et al., 2011). In the sulfation pathway, the APS is first phosphorylated by APS kinase to form 3′-phosphoadenosine-5′-phosphosulfate, the donor of SO_4^{2-} groups for a variety of sulfation reactions catalyzed by sulfotransferases (Günal et al., 2019).

Sulfur has four stable isotopes, namely, ^{32}S, ^{33}S, ^{34}S, and ^{36}S; their relative abundances are 0.9499, 0.0075, 0.0425, and 0.0001 atom fraction, respectively (De Laeter et al., 2003). Mass differences between the S isotopes result in small but significant variations in their chemical and physical properties, which may produce considerable separation of the S isotopes during chemical reactions. The most abundant isotopes, i.e., ^{32}S and ^{34}S, are now commonly measured using elemental analyzers coupled with Isotope Ratio Mass Spectrometers (IRMS), and S isotope abundance is generally reported in δ notation ($δ^{34}S$) as parts per thousand (‰) deviation relative to the Vienna-Cañon Diablo Troilite (VCDT; Coplen and Krouse, 1998) standard as follows:

$$δ^{34}S \ (‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \cdot 1,000$$

where R_{sample} and $R_{standard}$ are the isotope ratios ($^{34}S/^{32}S$) of the sample and standard, respectively.

Unlike what has happened with carbon and nitrogen, the natural abundance S stable isotope analysis techniques have so far scarcely been employed to study S allocation and metabolism in plants (Trust and Fry, 1992; Tcherkez and Tea, 2013), mainly due to the lack of knowledge about the $^{32}S/^{34}S$ isotope effects occurring during S metabolism and partitioning among the different organs. Most of the irreversible reactions involving S discriminate between ^{32}S and ^{34}S by favoring the lighter ^{32}S isotope, thus enriching in ^{34}S the residual substrate molecules left behind. That is to say that irreversible reactions that do not consume all the substrate may likely produce a detectable separation of the S stable isotopes, i.e., a fractionation, at natural abundance, providing crucial insights into the understanding of S metabolic fluxes inside the plants, without the need for costly labeling experiments with radioactive (^{35}S) or stable (^{34}S) isotopes (Tcherkez and Tea, 2013).

Sulfate uptake and allocation in plants involve a family of SO_4^{2-} transporter proteins whose activities are tightly regulated and coordinated with those of the assimilation pathways to control plant S homeostasis (Buchner et al., 2004; Gigolashvili and Kopriva, 2014; Sacchi and Nocito, 2019; Takahashi, 2019). A few pioneering studies indicated that a less S isotope discrimination occurs during SO_4^{2-} uptake since the isotope composition measured for plant total S (S_{tot}) is typically depleted in ^{34}S by 1–2‰ with respect to that measured for the SO_4^{2-} source feeding the plants (Mekhtiyeva, 1971; Krouse et al., 1991). In contrast, less is known about the S isotope composition of the SO_4^{2-} ions in the plant tissues, which should reflect the metabolic activities in which SO_4^{2-} is involved as a substrate. Although the isotope effects linked to SO_4^{2-} metabolism largely remain to be investigated in plants, it is possible to suppose that reductive SO_4^{2-} assimilation fractionates against ^{34}S, since it involves changes in the covalent bonding of the S atoms (Rees, 1973). Significant isotope effects have been reported for bacterial SO_4^{2-} reduction, which enriches both the sulfide produced in the lighter ^{32}S isotope and the remaining SO_4^{2-} in the heavier ^{34}S isotope (Thode et al., 1949; Kemp and Thode, 1968).

This study presents a detailed study on the dynamics of S stable isotopes occurring in appropriate closed or steady-state hydroponic-plant systems to dissect the $^{32}S/^{34}S$ isotope effects associated with SO_4^{2-} uptake, allocation, and metabolism in rice plants. In this study, we also provided the first complete S isotope mass balance in rice which considers organic and inorganic S pools in roots and shoots.

MATERIALS AND METHODS

Plant Material and Pre-growing Conditions

Rice (*Oryza sativa* L. cv. Vialone Nano) caryopses were surface sterilized with 70% (v:v) ethanol for 1 min, washed three times with sterile deionized water, and finally sown on filter paper saturated with deionized water to be incubated in the dark at 26°C. After 7 days, seedlings selected for uniform growth were transferred into 3-L plastic tanks (18 seedlings per tank), containing the following complete nutrient solution: 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 100 μM $MgSO_4$, 250 μM $NH_4H_2PO_4$, 25 μM Fe-EDTA, 46 μM H_3BO_3, 9 μM $MnCl_2$, 1 μM $ZnCl_2$, 0.3 μM $CuCl_2$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, and 30 μM Na_2O_3Si (pH 6.5). Seedlings were kept for a 14-days pre-growing period in a growth chamber maintained at 26°C and 80% relative humidity during the 16-h light period and at 22°C and 70% relative humidity during the 8-h dark period. The photosynthetic photon flux density was 400 μmol m^{-2} s^{-1}. Nutrient solutions were renewed two times a week to minimize nutrient depletion. At the end of the pre-growing period, roots were gently washed for 30 min in 3 L of deionized water (>18.2 MΩ cm). Plants were then transferred into fresh solutions and used in two distinct experimental setups (A and B). The parts of the plants were sampled, frozen in liquid N_2, and stored at −80°C for further analysis.

Experimental Setup and Tissue Sampling

In experimental setup A, pre-grown rice plants were transferred into fresh complete nutrient solutions and then grown further, under the same conditions described before, for 3–11 days, not renewing the growing media. Both plants and nutrient solutions were sampled at the beginning of the experiment and every day (starting from the third day).

In experimental setup B, pre-grown rice plants were transferred into fresh complete nutrient solutions ($+SO_4^{2-}$) or fresh minus sulfate nutrient solutions ($-SO_4^{2-}$), in which an equimolar amount of $MgCl_2$ replaced $MgSO_4$. Plants were grown under these conditions for 48 or 72 h by renewing the growing media every day.

In both the experimental setups, before sampling, plant roots were washed for 30 min in 3 L of deionized water to remove SO_4^{2-}, which was not absorbed, from the root apoplast. After

washing, plants were gently blotted with paper towels, shoots were separated from roots, and then, both were frozen in liquid N_2 and stored at $-80°C$ for further analysis.

Xylem Sap Sampling

In each sampling period (experimental setup B in the presence of SO_4^{2-}), the shoots of four rice plants were cut at 1 cm above the roots with a microtome blade to collect, with a micropipette, the xylem sap exuded from the lower cut surface during a 90-min period (Maghrebi et al., 2021).

Preparation of Samples for Sulfur Isotope Analysis and Quantitative Determination of the Sulfur Pools

Frozen samples were ground to a fine powder using mortar and pestle in liquid N_2 and stored frozen in a cryogenic tank.

For S_{tot} analysis, powder samples of 5 g [fresh weight (FW)] were digested at $150°C$ in 10 ml 2:1 (v:v) nitric:perchloric acid mixture, in order to quantitatively convert all the S forms into SO_4^{2-} (Blair and Till, 2003). Samples were then added with 1 ml of concentrated HCl and finally evaporated to dryness at $200°C$ to release any HNO_3 still present. The mineralized material was dissolved in 50 ml of deionized water and then brought to pH 2.0 with a tiny volume of 6 N HCl.

Sulfate was extracted from roots and shoots by homogenizing powder samples of 5 g (FW) in 50 ml of deionized water. After heating at $80°C$ for 40 min, the extracts were filtered and then brought to pH 2.0 with a tiny volume of 6 N HCl.

Residual nutrient solutions were boiled to evaporate water until their volumes were reduced to 50 ml. Samples were then filtered and brought to pH 2.0 with 6 N HCl.

Xylem sap samples were diluted with deionized water to a final volume of 50 ml, filtered, and then brought to pH 2.0 with a tiny volume of 6 N HCl.

Aliquots of 2 ml were collected from each diluted sample for the quantitative determination of SO_4^{2-}, using the turbidimetric method described by Tabatabai and Bremner (1970). The SO_4^{2-} ions of each sample were precipitated overnight as $BaSO_4$ by adding 2.5 ml of a 0.5 M $BaCl_2$ solution. $BaSO_4$ was then collected by centrifugation, washed two times in 2 ml of deionized water, dried at $80°C$, ground to a fine powder, and finally used for the S isotope analyses.

The amount of the S_{org} pool of both root and shoot extracts was estimated as follows:

$$S_{org} = S_{tot} - SO_4^{2-}$$

Sulfur Isotope Analysis

The $\delta^{34}S$ values of samples were measured using a Flash 2000 HT elemental analyzer coupled, *via* a ConFLo IV Interface, with a Delta V Advantage IRMS and interconnected to the software Isodat 3.0 (Thermo). The reaction tube, packed with tungstic oxide and copper wires separated by Quartz wool, was maintained at $1{,}020°C$. The He carrier gas flow was 150 ml min^{-1}. The O_2 purge for flash combustion was 3 s at a flow rate of 250 ml min^{-1} per sample. The temperature of the gas chromatography separation column was $90°C$. The SO_2 reference gas pulse was introduced three times (20 s each) at the beginning of each run.

Samples ($BaSO_4$ precipitates and reference materials) were weighed in tin capsules. Capsules were carefully closed by folding them with cleaned tweezers and then transferred to the autosampler. The run time of the analysis was approximately 500 s for a single run. The analysis of each sample was performed five times. Calibration was performed using three secondary reference materials provided by the International Atomic Energy Agency (IAEA): IAEA-S-1 ($\delta^{34}S = -0.30 \pm 0.03‰$); IAEA-S-2 ($\delta^{34}S = 22.62 \pm 0.08‰$); IAEA-S-3 ($\delta^{34}S = -32.49 \pm 0.08‰$). Two in-house standards were used for normalization and analytical quality assurance.

The data are reported in $\delta^{34}S$ notation, which is standardized to the VCDT international reference scale as follows:

$$\delta^{34}S\ (‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \cdot 1{,}000$$

The mass spectrometric uncertainty (1 σ) on the individual $\delta^{34}S$ measurements was better than $0.05‰$.

The $\delta^{34}S$ values of the S_{org} pool were estimated by imposing the following mass balance:

$$\delta^{34}S\_S_{tot} \cdot S_{tot} = (\delta^{34}S\_SO_4^{2-} \cdot SO_4^{2-}) + (\delta^{34}S\_S_{org} \cdot S_{org})$$

where SO_4^{2-} and S_{tot} are the amount of SO_4^{2-} and S_{tot}, respectively, measured in the same sample.

Fractionation factors ($\Delta_{L/H}$), i.e., in positive per mil (‰) units, were calculated by fitting an approximation of the Rayleigh equation to the data obtained by measuring $\delta^{34}S$ values of the residual SO_4^{2-} in the hydroponic solution ($\delta^{34}S\_SO_4^{2-}{}_{res}$), according to Fry (2006). For these purposes, the following equation was used:

$$\delta^{34}S\_SO_4^{2-}{}_{res} = \delta^{34}S\_SO_4^{2-}{}_{source} - \Delta_{L/H} \cdot \ln(f)$$

where f is the fraction of SO_4^{2-} remaining in the hydroponic solution, and $\delta^{34}S\_SO_4^{2-}{}_{source}$ is the initial S isotope composition of the S source.

Finally, the trajectories of the $\delta^{34}S$ values of the instantaneous product (S_{ist}) that forms, inside the plants, instant by instant in time were calculated using the following equation:

$$\delta^{34}S\_S_{ist} = \delta^{34}S\_SO_4^{2-}{}_{source} - \Delta_{(L/H)} \cdot [1 + \ln(f)]$$

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted from rice roots using TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA, United States) and then purified using PureLink® RNA Mini Kit (Life Technologies Corporation, Carlsbad, CA, United States), according to the manufacturer's instructions. Contaminant DNA was removed on-column using PureLink® DNase (Life Technologies Corporation, Carlsbad, CA, United States). The first-strand cDNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis SuperMix for quantitative real-time PCR (qRT-PCR; Life Technologies

Corporation, Carlsbad, CA, United States), according to the manufacturer's instructions.

The qRT-PCR analysis of *OsSULTR1;1* (LOC_Os03g09970) and *OsSULTR1;2* was performed on the first-strand cDNA in a 20 µl reaction mixture containing GoTaq® qPCR Master Mix (Promega) and the specific primers, using an ABI 7300 Real-Time PCR system (Applied Biosystems). The relative transcript level of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method using the expression of the *OsS16* (LOC_Os11g03400) gene as reference. Primers for qRT-PCR are listed in **Supplementary Table 1**.

Statistical Analysis

Quantitative values are presented as mean ± SEM of three independent experiments run in duplicate ($n = 3$). Two distinct 3-L tanks were used for each condition analyzed in each independent experiment. ANOVA was carried out using SigmaPlot for Windows version 11.0 (SYSTAT Software, Inc., San Jose, CA, United States). The significant values were adjusted for multiple comparisons using the Bonferroni correction. The Student's *t*-test was used to assess the significance of the observed differences between the values measured in root and shoot.

RESULTS

Sulfur Isotope Mass Balance in a Closed Hydroponic-Rice System (Experimental Setup A)

Potential $^{32}S/^{34}S$ isotope effects occurring during SO_4^{2-} uptake were investigated by setting up a closed hydroponic-rice system (**Figure 1A**) in which a finite amount of substrate (i.e., the SO_4^{2-} in the nutrient solution) was continuously removed from the solution, by the activity of the SO_4^{2-} transporters of the roots, and converted into a final product (i.e., S_{tot}). Using this system, we performed serial sacrifice experiments in which plant growth was terminated every 24 h (starting from the third day) for the S isotope analyses of both substrates and products.

During the experimental period (264 h), (i) plants continuosly grown (**Figure 1B**) and removed 98% of the SO_4^{2-} initially present in the nutrient solution (**Figure 1C**), (ii) SO_4^{2-} absorbed was quantitatively recovered in the plants as S_{tot} (**Figures 1D,E**), and (iii) no significant losses of S occurred during the growth (**Figure 1E**). The S_{tot} concentration of the plants ranged from 121.2 (at the beginning of the experiment) to 98.6 µmol g^{-1} dry weight (DW; at the end of the experiment), while the SO_4^{2-} concentration in the nutrient solution ranged from 100 to 6.5 µM, indicating that the regulation of plant S homeostasis occurred during SO_4^{2-} absorption (**Figure 1F**).

Figure 2A reports $\delta^{34}S$ data as a function of the fraction of SO_4^{2-} remaining in the hydroponic solution (f). The $\delta^{34}S$ of residual SO_4^{2-} in both the hydroponic solution and plant S_{tot} changed over time, tending toward higher values as f decreased. The $\delta^{34}S$ values of the residual SO_4^{2-} ($\delta^{34}S\_SO_4^{2-}$) increased from a minimum of $-1.92‰$ (at the beginning of the experiment) to a maximum of $-0.21‰$ (at the final sampling). In contrast, the $\delta^{34}S\_S_{tot}$ of the plants was always lower than the $\delta^{34}S\_SO_4^{2-}$ of the S source ($-1.92 \pm 0.02‰$) and increased from $-3.32‰$

(the starting isotope composition of total plant biomass) to $-2.30‰$ at the final sampling, indicating that SO_4^{2-} uptake significantly enriches plant S_{tot} in the lighter ^{32}S isotope. It is worth noting that, due to mass balance in the closed system, the $\delta^{34}S\_S_{tot}$ of the rice plants tended to the $\delta^{34}S\_SO_4^{2-}$ of the initial S source as SO_4^{2-} concentration in the external medium approached zero, indicating that (i) SO_4^{2-} ions in the nutrient solution were the only S source used by plants and (ii) no significant loses/fractionations of S isotopes occurred during the experiments due to H_2S gaseous emission (Wilson et al., 1978; Winner et al., 1981).

The isotope effects that occurred in the closed system were analyzed using an approximation of the Rayleigh equation describing isotope partitioning between two reservoirs as one of them decreases in size (Fry, 2006). The S isotope profile of the residual SO_4^{2-} in the nutrient solution (**Figure 2A**) showed a marked deviation from a typical Rayleigh enrichment ($R^2 = 0.79$; black dashed line) due to an unexpected data point distribution at the final steps of the experiment ($f \leq 0.21$). Considering the Rayleigh fractionation model, it was possible to calculate a single fractionation factor, $\Delta_{L/H} = 0.48 \pm 0.09‰$, which describes an average of the net fractionation along the overall trajectory (profile). However, data distribution could be more appropriately described by assuming that a dual-phase Rayleigh fractionation occurred during SO_4^{2-} uptake. In the first phase ($1 \leq f \leq 0.31$), a significant isotope fractionation against $^{34}S\_SO_4^{2-}$ took place ($\Delta_{1(L/H)} = 1.09‰$), while in the second phase ($f \leq 0.21$), a less pronounced isotope effect [$\Delta_{2(L/H)} = 0.16‰$] was associated with SO_4^{2-} uptake. **Figure 2A** also reports the calculated trajectories of the $\delta^{34}S$ values of S_{ist} that forms, inside the plants, instant by instant in time from the external SO_4^{2-} due to SO_4^{2-} uptake; such a product is always offset in the isotope composition of the substrate ($\delta^{34}S\_SO_4^{2-}$) by the fractionation factor $\Delta_{L/H}$ (Fry, 2006). In each phase (I and II), S isotope fractionation ($\delta^{34}S\_SO_4^{2-}-\delta^{34}S\_S_{ist}$) was practically independent of f, as can be easily observed by comparing the isotope signatures of the substrate and cumulative product for each data point.

Aiming to decompose the two phases into their physiological and molecular components, we performed a transcriptional analysis of *OsSULTR1;1* and *OsSULTR1;2*, i.e., the main rice genes reasonably involved in SO_4^{2-} uptake (Godwin et al., 2003; Kumar et al., 2011; **Figure 2B**). Results revealed that the transition from the two phases was associated with significant changes in the ratio between the transcript levels of the two genes: the *OsSULTR1;2* transcript was always independent of f and was more abundant than the *OsSULTR1;1* transcript during the first phase ($1 \leq f \leq 0.31$), while the *OsSULTR1;1* transcript level rapidly increased in the second phase ($f \leq 0.21$), when the SO_4^{2-} concentration in the nutrient solution became limiting for plant growth [$(SO_4^{2-}) \leq 37$ µM; **Figure 2B**].

Sulfur Isotope Mass Balance in a Whole Plant: Steady-State vs. Sulfur Starvation (Experimental Setup B)

The possible $^{32}S/^{34}S$ isotope effects associated with both S partitioning among plant organs and cell metabolism were investigated by comparing plants pre-grown in complete nutrient

FIGURE 1 | Time course of S partitioning in a closed hydroponic-rice system. **(A)** Experimental setup: A finite amount of sulfate (SO_4^{2-}) is continuously removed from the hydroponic solution and converted into the total S of the plant (S_{tot}). **(B)** Plant total biomass accumulation. **(C)** Residual SO_4^{2-} in the hydroponic solution over time. **(D)** S_{tot} accumulation over time. **(E)** Residual SO_4^{2-} in the hydroponic solution vs. plant S_{tot} accumulation. **(F)** Plant S_{tot} concentration vs. SO_4^{2-} concentration in the hydroponic solution. Data are means ± SE of three independent experiments run in duplicate ($n = 3$).

solutions and then continuously maintained on media containing SO_4^{2-} or deprived of SO_4^{2-} for 72 h (experimental setup B). Nutrient solutions were changed every day to minimize the changes in the isotope signature of the S source ($-1.92 \pm 0.02‰$) due to fractionation associated with SO_4^{2-} uptake.

Results showed that the S isotope composition of the whole plants did not significantly change over time since similar $\delta^{34}S\_S_{tot}$ values were measured at each time period (0, 48, and 72 h) in both of the growing conditions (**Figure 3**). At the beginning of the experiment (0 h), the S_{tot} of the whole plants was significantly depleted in ^{34}S by $-1.40 \pm 0.08‰$ relative to the S source (**Figure 3**).

Plants maintained in hydroponic solutions containing SO_4^{2-} grew linearly in the observation period (**Figure 4A**). As expected, the concentrations of SO_4^{2-}, S_{tot}, and S_{org} did not significantly change in both root and shoot over time (**Figures 4B,C**). The invariance of each S pool was associated with the invariance of their isotope signatures, indicating that plants reached metabolic and isotope steady-states (**Figure 5**). The S_{tot} of root and shoot was significantly depleted in ^{34}S by $-1.94 \pm 0.08‰$ and $-1.09 \pm 0.09‰$, respectively, relative to the S source (**Figure 5A**);

moreover, $\delta^{34}S\_S_{tot}$ values were significantly lower in the root than in the shoot in all the conditions analyzed (**Figure 5A**). In the root, the SO_4^{2-} pools were significantly ($P < 0.001$) ^{34}S-depleted relative to the S source, while in the shoot, they were significantly ($P < 0.001$) ^{34}S-enriched relative to the same S source (**Figure 5B**). The S_{org} pool of both root and shoot were significantly ^{34}S-depleted with respect to the S source; interestingly, both the S_{org} pools were also significantly ^{34}S-depleted with respect to their relative SO_4^{2-} pools of the cells (**Figure 5C**). Finally, no differences were found in comparing the $\delta^{34}S\_SO_4^{2-}$ values of the SO_4^{2-} pools in the xylem sap and in the whole root system (**Figure 5D**).

In contrast, SO_4^{2-}-deprived plants dynamically allocated S previously absorbed during the preliminary growth phase, preserving both the overall S isotope signature (**Figure 3**) and the total amount of S_{tot} over time (**Figure 4**). However, due to both continuous growth (**Figure 4D**) and S allocation processes, the S_{tot} concentration in rice organs changed over time, decreasing linearly in both root ($R^2 = 0.993$; **Figure 4E**) and shoot ($R^2 = 0.999$; **Figure 4F**). The SO_4^{2-} concentration sharply decreased over time in both root and shoot due to SO_4^{2-}

FIGURE 2 | Sulfur isotope dynamic in the closed system and transcriptional analysis of *OsSULTR1;1* and *OsSULTR1;2*. **(A)** S isotope dynamic as a function of the fraction (*f*) of SO_4^{2-} remaining in the hydroponic solution. Black dashed line is the Rayleigh curve calculated over the entire *f* interval, while the black continuous lines are the Rayleigh curves calculated over the two phases: Phase I ($1 \leq f \leq 0.31$) and Phase II ($f \leq 0.21$). Red continuous lines describe the trajectory of the calculated instantaneous product (S_{ist}) over the two phases. The dashed green line indicates the $\delta^{34}S\_SO_4^{2-}$ value of the initial S source. **(B)** Changes in the relative transcript levels of *OsSULTR1;1* and *OsSULTR1;2* in the roots. The numbers in brackets refer to the concentration of residual SO_4^{2-} (expressed as μM) in the hydroponic solution corresponding to each *f* value. Data are means ± SE of three independent experiments run in duplicate (*n* = 3).

assimilation (**Figures 4E,F**). In fact, the concentration of the S_{org} in the root slightly decreased over time from 7.26 ± 0.41 to 4.36 ± 0.14 μmol g^{-1} FW, while in the shoot, it remained relatively constant. The S_{tot} isotope composition of both root and shoot did not change over time (**Figure 6A**) and was significantly ^{34}S-depleted relative to the S source. As previously observed, the $\delta^{34}S\_S_{tot}$ values were significantly lower in the root than in the shoot (**Figure 6A**). Differently, the SO_4^{2-} pools of both root and shoot became progressively enriched in ^{34}S over time. It is worth noting that the most pronounced changes in the SO_4^{2-} isotope composition were observed in the shoot: the maximum variations observed at 72 h were $2.70 \pm 0.05\%$ and $6.71 \pm 0.19\%$ for root and shoot, respectively (**Figure 6B**). The S_{org} pools of both roots and shoot were significantly ^{34}S-depleted compared to the S source; their $\delta^{34}S\_S_{org}$ values changed differently over time since, in the root, they increased moving from 0 to 48 h and then remained constant at 72 h, while in the shoot, a significant increase was observed when moving from 48 to 72 h (**Figure 6C**).

DISCUSSION

It is generally assumed that terrestrial plants assimilate S from the soil (SO_4^{2-}) and from the atmosphere (SO_2) with less S isotope fractionation since the foliar $\delta^{34}S$ values are generally intermediate between those of the soil and the atmosphere or near to one extreme (Kennedy and Krouse, 1990; Krouse et al., 1991). However, the correct evaluation of the isotope effects due to S acquisition needs a direct comparison between the isotope compositions of the S_{tot} of a whole plant and the S source used by the same plant, since the $\delta^{34}S\_S_{tot}$ value of a single

plant organ may result from fractionations and mixing effects occurring during SO_4^{2-} uptake, assimilation, and partitioning.

To address the S isotope effects during S acquisition, we performed an S isotope mass balance in a closed system, in which the accumulation of S_{tot} in the plants is considered as the result of the continuous consumption of a unique and finite S source (SO_4^{2-}) initially present in a hydroponic solution (**Figure 1A**). In such a model system, if fractionation occurs, the enrichment in a given isotope in one part of the system results in its depletion in the other, so that isotopic mass balance is always maintained (Fry, 2006).

Our data indicate that isotope discrimination against ^{34}S occurred during SO_4^{2-} uptake, which resulted in transient lighter S isotope compositions of the plants and concomitant ^{34}S enrichments of the residual SO_4^{2-} in the hydroponic solution (**Figure 2A**). Interestingly, fractionation exhibited two phases characterized by distinct fractionation factors [$\Delta_{1(L/H)}$ and $\Delta_{2(L/H)}$] that can be considered as "isotope phenotypes" reflecting plant physiological adaptation to the SO_4^{2-} concentrations in the nutrient solution, which changed during the experiment (**Figures 1F, 2B**). The maximum fractionation observed was associated with *f* values ranging from 1 to 0.31 (corresponding to external SO_4^{2-} concentrations ranging from 100 to 50 μM), while the minimum isotope effect was associated with the smallest *f* values, when the concentration of SO_4^{2-} in the nutrient solution became critical (≤ 37 μM) and was potentially able to induce an array of S-deficiency physiological responses (Maruyama-Nakashita et al., 2003), including changes in the expression of the root high-affinity SO_4^{2-} transports, *OsSULTR1;1* and *OsSULTR1;2*, involved in SO_4^{2-} uptake (**Figure 2B**). Although a certain degree of

FIGURE 3 | Experimental setup and $^{32}S/^{34}S$ isotope effects associated with S partitioning and metabolism in rice plants. Plants were pre-grown in complete nutrient solutions and then continuously maintained on media containing SO_4^{2-} or deprived of SO_4^{2-} for 72 h (experimental setup B). S_{tot}, total S amount in a whole plant; $\delta^{34}S\_S_{tot}$, S isotope composition of the whole plant; Δ, ^{34}S depletion relative to the S source ($\delta^{34}S\_SO_4^{2-}{}_{source} = -1.92 \pm 0.02‰$). Data are means $\pm$ SE of three independent experiments run in duplicate ($n = 3$).

functional redundancy may exist, *OsSULTR1;2* is considered the major gene involved in SO_4^{2-} uptake under normal conditions, while *OsSULTR1;1* is a more specialized gene that is strongly induced under S limitation (Kumar et al., 2011). The prevalence of *OsSULTR1;1* or *OsSULTR1;2* under different environmental conditions could explain the two isotope phenotypes observed during plant SO_4^{2-} acquisition, assuming that the different isotope effects may be associated with the activity of the two SO_4^{2-} transporters. Thus, the plasticity of the isotope phenotype could reflect gene expression in response to changes in both environmental conditions and plant S-nutritional status.

Less information is currently available about the $^{32}S/^{34}S$ isotope effects occurring during S partitioning and metabolism in plants since the cycling of the S pools in a whole plant may attenuate the isotope differences between organs potentially caused by S reduction and assimilation. Most of the SO_4^{2-} ions that are taken up by root are translocated to the shoot, where they are assimilated into organic compounds (Takahashi et al., 2011). However, part of SO_4^{2-} is also assimilated into the root, and the continuous exchanges of SO_4^{2-} and S_{org} compounds occur in a shoot-to-root direction in order to ensure the S homeostasis of

the root (Cooper and Clarkson, 1989; Bell et al., 1995; Yoshimoto et al., 2003; Larsson et al., 2006).

To analyze the isotope effects occurring during S partitioning and metabolism, we carried out experiments aimed at the following: (i) preventing possible perturbations due to the continuous changes of the $\delta^{34}S\_SO_4^{2-}$ values of the external solution and (ii) obtaining rice plants with the same overall S isotope composition (**Figure 3**). In these experiments, plants can be considered systems continuously supplied by an S source that does not change in concentration and isotope composition.

As previously described, plants continuously grown in the presence of SO_4^{2-} reached metabolic and S isotope steady-states characterized by the invariance of the concentration and the isotope signature of each S pool. It should be noted that the S isotope distribution between root and shoot observed in this study strongly differs from the pattern described by Tcherkez and Tea (2013), concerning the S natural isotope composition in different organs (roots, leaf, stem, glumes, and grains) of mature wheat. Such a discrepancy might depend on the growth conditions (closed hydroponic-plant system vs. field) or, more likely, on the different S nutritional status and/or growth stage

FIGURE 4 | Changes in the concentration of the S pools (S_{tot}, SO_4^{2-}, and S_{org}) in root and shoot of rice plants grown in the presence or in the absence of SO_4^{2-} in the hydroponic solution. **(A)** Root and shoot fresh weight (FW) in the presence of SO_4^{2-}. **(B)** S_{tot}, SO_4^{2-}, and S_{org} in the root of plants grown in the presence of SO_4^{2-}. **(C)** S_{tot}, SO_4^{2-}, and S_{org} in the shoot of plants grown in the presence of SO_4^{2-}. **(D)** Root and shoot FW in the absence of SO_4^{2-}. **(E)** S_{tot}, SO_4^{2-}, and S_{org} in the root of plants grown in the absence of SO_4^{2-}. **(F)** S_{tot}, SO_4^{2-}, and S_{org} in the shoot of plants grown in the absence of SO_4^{2-}. Data are means ± SE of three independent experiments run in duplicate ($n = 3$). Different letters indicate significant differences between the samples at different times ($P < 0.05$).

of the plants considered in the two studies since S stable isotope separations conserve the memory of the physiological and metabolic activities that determined them. Finally, differences may also originate from the different distribution of the S assimilation enzymes in the leaf, since rice assimilates SO_4^{2-} mainly in the bundle sheaths, while other species, also in the mesophyll (Hua et al., 2021).

The isotope composition of the SO_4^{2-} pools of the root was lighter relative to the S source but heavier with respect to the expected composition calculated according to the isotope discrimination occurring during SO_4^{2-} uptake at high external concentrations [i.e., $\delta^{34}S\_SO_4^{2-} > \delta^{34}S\_SO_4^{2-}{}_{source} - \Delta_{1(L/H)}$]. Interestingly, SO_4^{2-} translocation from root to shoot did not discriminate the S isotopes since no differences were found when comparing the isotope signatures of the SO_4^{2-} ions in root and xylem sap (**Figure 5**). However, the SO_4^{2-} pools of the shoot were significantly ^{34}S-enriched with respect to the SO_4^{2-} pools of both root and xylem sap. This was likely due to SO_4^{2-} assimilation that, favoring the lighter ^{32}S isotope, causes a ^{34}S enrichment of the residual SO_4^{2-} ions left behind. The occurrence of an S isotope separation during SO_4^{2-} assimilation is consistent with the observation that the S_{org} pools of the shoot were significantly depleted in ^{34}S relative to both the SO_4^{2-} pools of the shoot and the S source. Since the aerial portion of the plant is fed by the SO_4^{2-} ions continuously translocated from root to shoot and

the S_{tot} of the shoot was lighter relative to the SO_4^{2-} coming from the root, we can reasonably suppose that a non-negligible portion of the ^{34}S-enriched SO_4^{2-} of the shoot is translocated to the root. Thus, the isotope signature of the SO_4^{2-} pool of the root could be the result of mixing effects due to the overall S isotope circulation, SO_4^{2-} uptake, and local S assimilation. Assuming that during the S isotope steady-state, (i) the $\delta^{34}S\_SO_4^{2-}$ values measured in the root are mainly influenced by root SO_4^{2-} uptake and SO_4^{2-} translocation from shoot to root, and (ii) the S isotope composition of the instantaneous SO_4^{2-} that continuously enters the root cells should theoretically differ from the S source by the fractionation factor $\Delta_{1(L/H)}$, so that

$$\delta^{34}S\_SO_{4\ ist}^{2-} = \delta^{34}S\_SO_{4\ source}^{2-} - \Delta_{1(L/H)}$$

$$= -1.92\ \permil - 1.09\permil = -3.01\permil$$

We can estimate the maximum amount of SO_4^{2-} that, coming from the shoot, is translocated and accumulated into the root (defined as $SO_4^{2-}{}_{StoR}$) by imposing the following mass balance:

$$\delta^{34}S\_SO_{4\ root}^{2-} \cdot SO_{4\ root}^{2-} = \delta^{34}S\_SO_{4\ shoot}^{2-} \cdot SO_{4\ StoR}^{2-}$$

$$+ \delta^{34}S\_SO_{4\ ist}^{2-} \cdot (SO_{4\ root}^{2-} - SO_{4\ StoR}^{2-})$$

where $\delta^{34}S\_SO_4^{2-}{}_{root}$ is the steady-state isotope composition of the SO_4^{2-} pool of the root, $SO_4^{2-}{}_{root}$ is the total amount of the

FIGURE 5 | Sulfur isotope composition of the main S pools in root and shoot and of SO_4^{2-} in the xylem sap of rice plants grown in the presence of SO_4^{2-} in the hydroponic solution. **(A)** S isotope composition of S_{tot} in root and shoot. **(B)** S isotope composition of SO_4^{2-} in root and shoot. **(C)** S isotope composition of S_{org} in root and shoot. **(D)** S isotope composition of SO_4^{2-} in xylem sap. Dotted lines indicate the $\delta^{34}S$ value of the S source used in the experiment ($\delta^{34}S\_SO_4^{2-}{}_{source} = -1.92 \pm 0.02‰$). Data are means $\pm$ SE of three independent experiments run in duplicate ($n = 3$). Asterisks indicate significant differences (Student's t-test; *$0.001 \leq P < 0.05$; **$P < 0.001$) between root and shoot of plants sampled at the same time. Different letters indicate significant differences between the samples (root, shoot, or xylem sap) at different times ($P < 0.05$).

SO_4^{2-} measured in the root, and $\delta^{34}S\_SO_4^{2-}{}_{shoot}$ is the isotope composition of the SO_4^{2-} ions coming from the shoot. Solving the equation for the unknown $SO_4^{2-}{}_{StoR}$ reveals that, in our conditions, 21.7% of the steady-state SO_4^{2-} pool of the rice root is inherited from the shoot.

Although less information is currently available on the long-distance transport of SO_4^{2-} from shoot to root, we can reasonably suppose that such an activity may involve the phloem and specific isoforms of SO_4^{2-} transporters mediating the loading of SO_4^{2-} into the sieve tubes (Takahashi, 2019). Feeding experiments with $^{35}SO_4^{2-}$ performed on *Arabidopsis* (Yoshimoto et al., 2003) support our finding, indicating the retranslocation of SO_4^{2-} as an important activity in controlling root SO_4^{2-} homeostasis and S isotope composition.

In contrast, during the growing period in the absence of SO_4^{2-}, rice plants can be considered closed systems assimilating the SO_4^{2-} ions previously absorbed during the preliminary

growth phase and allocating the S_{org} pools to optimize the distribution of the limited S resources between root and shoot. It is worth noting that in these conditions, the invariance of the S_{tot} isotope composition of both root and shoot was associated with dramatic changes in the isotope composition of the relative SO_4^{2-} and S_{org} pools (**Figure 6**), mainly caused by the $^{32}S/^{34}S$ isotope effects occurring during SO_4^{2-} assimilation. During the observation period, plants rapidly consumed the available SO_4^{2-} pools: at the end of the experiment, the overall S_{org} pool was about 94% of the S_{tot}. The S isotope mass balance that was carried out considering the overall SO_4^{2-} and S_{org} pools of the plants (i.e., root + shoot; **Table 1**) revealed that continuous S assimilation progressively enriched both the overall S_{org} pool in the lighter ^{32}S isotope and the residual SO_4^{2-} in the heavier ^{34}S isotope, producing an apparent isotope separation that was closely dependent on the severity of the imposed S starvation, as indicated by calculated Δ values ($\Delta = \delta^{34}S\_S_{org} - \delta^{34}S\_SO_4^{2-}$)

FIGURE 6 | Sulfur isotope composition of the main S pools in root and shoot of rice plants grown in the absence of SO_4^{2-} in the hydroponic solution. **(A)** S isotope composition of S_{tot} in root and shoot. **(B)** S isotope composition of SO_4^{2-} in root and shoot. **(C)** S isotope composition of S_{org} in root and shoot. Data are means ± SE of three independent experiments run in duplicate ($n = 3$). Asterisks indicate significant differences (Student's t-test; *$0.001 \leq P < 0.05$; ** $P < 0.001$) between root and shoot of plants sampled at the same time. Different letters indicate significant differences between the samples (root and shoot) at different times ($P < 0.05$).

TABLE 1 | Amount and sulfur (S) isotope composition of the overall SO_4^{2-} and S organic (Sorg) pools of rice plants grown in the absence of SO_4^{2-} in the hydroponic solution.

	Time (h)					
	0		**48**		**72**	
	Amount (μmol)	**δ^{34}S (‰)**	**Amount (μmol)**	**δ^{34}S (‰)**	**Amount (μmol)**	**δ^{34}S (‰)**
SO_4^{2-}	7.48 ± 0.13[a]	−1.47 ± 0.05[c]	2.80 ± 0.05[b]	2.07 ± 0.08[b]	0.90 ± 0.02[c]	4.06 ± 0.18[a]
S_{org}	9.65 ± 0.38[c]	−4.76 ± 0.28[a]	14.32 ± 0.31[b]	−4.73 ± 0.17[a]	16.22 ± 0.18[a]	−3.73 ± 0.17[b]

Data are means ± SE of three independent experiments run in duplicate (n = 3). Different letters indicate significant differences between the samples at different times (P < 0.05).

that ranged from −3.29 ± 0.40 (at the beginning of the experiment) to −7.80 ± 0.18‰ (at 72 h). As expected, the most pronounced isotope separations were observed in the shoot, confirming the prominent role of the rice aerial portion in SO_4^{2-} assimilation and S allocation (Takahashi et al., 2011).

CONCLUSION AND PERSPECTIVES

Our results provide an overview of the ^{32}S/^{34}S isotope effects occurring during SO_4^{2-} uptake, partitioning, and metabolism in rice. The main results clearly show that SO_4^{2-} uptake discriminates against ^{34}S, enriching plant total biomass in the lighter ^{32}S isotope relative to the S source. The S isotope discrimination observed during SO_4^{2-} acquisition closely depends on the amount of SO_4^{2-} in the growing medium, as well as on the plants' molecular and physiological responses aimed at optimizing S nutrition under different environmental conditions. Although further experiments will be necessary to directly measure the isotope effect associated with the activity of a single SO_4^{2-} transporter, we can reasonably conclude that *OsSULTR1;1* and *OsSULTR1;2* differently discriminate against ^{34}S, producing S isotope phenotypes closely dependent on their relative expression.

Results also indicate that the steady-state S isotope composition of the different S pools of both root and shoot mainly results from the substantial S isotope fractionations occurring during SO_4^{2-} assimilation and mixing effects due to the overall isotope circulation inside the whole plant. Finally, the extreme variability of the S isotope phenotypes observed under various S conditions underlines the potential of the δ^{34}S analysis to provide information for further detailed characterization of the metabolic and molecular processes involved in plant S homeostasis, as well as of the plant S systemic fluxes occurring in different nutritional and environmental conditions, since the S stable isotope separations conserve the memory of the activities that determined them.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

VC, MC, GS, and FN designed the experiments. VC, MM, and MC performed the experiments. FN analyzed the data and wrote the manuscript. All authors revised the manuscript draft and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.837517/full#supplementary-material

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