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SULFUR NUTRITION AND PARTITIONING IN RICE UNDER DIFFERENT STRESS CONDITIONS

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Summary

Abstract

Sulfur (S) is an essential macronutrient required by plants for their correct development. This element is fundamental for the biosynthesis of different compounds, such as the two amino acids, cysteine (Cys) and methionine (Met), vitamins (biotin and thiamine), peptides involved in the response to abiotic stresses (glutathione - GSH, and phytochelatines - PCs), lipids and co-factors. Sulfate (SO_4^{2-}) is the main S form taken up from soil by root system and then assimilated inside the cells during the sulfur reductive pathway. The uptake and the systemic movements of this anion are accomplished by the *SULfate TRansporter* (*SULTR*) gene family, which encode for H^+/SO_4^{2-} membrane co-transporters with different localization, amino acidic sequences, and affinity to sulfate. Since has been demonstrated that S has a key role in the response to different abiotic stresses (such as sulfur deficiency, heavy metal exposure or salt stress), the expression of these genes must be finely regulated, according to the different environmental conditions and requests for S reduced compounds.

The general aim of the present thesis is the description of S systemic fluxes in rice in different stress conditions, to obtain more information about the contribution of S in determining plant tolerance to abiotic stresses. To achieve the goal, we also took advantage of analysis performed with an elemental analyzer coupled with an isotope ratio mass spectrometer (EA-IRMS), a powerful instrument which utilizes stable isotopes of elements as tracers.

The entire research has been divided in three different parts.
In the first work, potential $^{32}S/^{34}S$ isotope effects occurring during SO_4^{2-} uptake were investigated in a closed hydroponic system in which a limited amount of substrate (SO_4^{2-} in the nutrient solution) was continuously removed from the solution by the activity of the sulfate transporters of the root and converted in a final product (total S of the plant). An isotope discrimination against ^{34}S occurred during SO_4^{2-} uptake: plants had a lighter S isotope composition, and the residual SO_4^{2-} in the hydroponic solution was enriched in heavy stable isotope. Fractionation during uptake showed two phases characterized by different fractionation factors, reflecting changes in the expression of the *OsSULTR* deputed to the root uptake which may explain the different isotope phenotypes

observed during plant sulfate acquisition. Moreover, the possible $^{32}S/^{34}S$ isotope effects associated to both S partitioning and metabolism were investigated by comparing plants pre-grown in complete nutrient solutions and then continuously maintained on media containing SO_4^{2-} (steady-state) or deprived of SO_4^{2-} for 72h. The SO_4^{2-} pool of the steady-state shoot was significantly ^{32}S depleted with respect to the SO_4^{2-} pools of root, while the organic S (S_{org}) pools were significantly depleted in ^{34}S compared to both the SO_4^{2-} pool of both the organs and the S source. These results suggested a higher S assimilation in the aerial part of plants which favor the lighter isotope. Under S starvation, S assimilation progressively enriched the S_{org} pools in the lighter ^{32}S isotope and the residual SO_4^{2-} in both the organs in the heavier ^{34}S isotope. Most pronounced isotope separations were again observed in the shoot, confirming the prominent role of this organ in SO_4^{2-} assimilation and S allocation. No fractionation due to translocation activity was observed.

In the second part of the work, to validate the results previously obtained, we performed a mass balance study in rice plants exposed for 72h to different Cd concentrations, to investigate possible changes in S stable isotope fractionation due to this stress: in fact, adaptation of S metabolism has a pivotal role in responses to heavy metal exposure. As expected, Cd treatment strongly enhanced SO_4^{2-} uptake and assimilation, as indicated by the analyses of the S pools (S_{tot}, SO_4^{2-}, and S_{org}). S isotope analyses performed on the whole plants revealed changes in the S metabolism associated to variations in the discrimination against ^{34}S, which was less evident as Cd concentration in the external medium increased. Transcriptional analysis suggested again that change of the ratio between relative transcripts of *OsSULTR1;1* and Os*SULTR1;2*, as observed for S starvation, may be responsible for the progressive decreased in ^{34}S isotope discrimination. The important role of shoot in S assimilation was confirmed: isotope fractionation associated to sulfate assimilation was higher in shoot than in root, and progressively increased as Cd concentration did.

The last part of work was focused on fully characterize, under hydroponics-controlled conditions in the absence or in the presence of salt stress (80 mM NaCl), the phenotypic behavior in the already available salt tolerant introgression line (IL) Onice 11 (O11), obtained by Marker-Assisted Back-Cross (MABC) selection starting from the cross

between the Italian *japonica* elite cultivars Onice (sensitive recurrent parent) and the *indica* variety IR64-*SalTol* (tolerant), donor of the major QTL *SalTol*. Moreover, S acquisition and metabolism of O11 and both the parental lines were evaluated to investigate their possible implication in determining the different tolerance to salt stress. Results showed the beneficial effect of the introgression of the *SalTol* QTL from the *indica* variety into selected *japonica* rice line, based on different characteristics of selected phenotypic-biochemical-physiological parameters. However, salt stress strongly affected S uptake and assimilation, and we can reasonably suppose that these features do not justify the different salt tolerance in the considered IL O11.

In conclusions, rice plants can discriminate against ^{34}S during SO_4^{2-} uptake and assimilation. Between plants organs, shoot represents the predominant one involved in S assimilation. Abiotic stresses, such as S starvation or Cd exposure, lead to changes in the ratio of relative transcripts between the *OsSULTRs* involved in the uptake of sulfate, and this may be the cause of the different isotope phenotypes observed. Finally, salt tolerance in the IL O11 appears to not be dependent on different S metabolism.

Riassunto

Lo zolfo (S) è un macronutriente essenziale per il corretto sviluppo delle piante. Questo elemento è fondamentale per la biosintesi di diversi composti come i due amminoacidi cisteina (Cys) e metionina (Met), vitamine (biotina e tiamina), peptidi coinvolti nella risposta agli stress abiotici (glutatione - GSH, e fitochelatine - PC), lipidi e cofattori. Il solfato (SO_4^{2-}) è la principale forma di zolfo assorbita dal suolo attraverso le radici e poi assimilata all'interno delle cellule durante il *pathway* di riduzione dello zolfo. L'assorbimento e i movimenti sistemici di questo ione dipendono dalla famiglia genica dei *SULfate TRansporter* (*SULTR)* che codifica per co-trasportatori di membrana H^+/SO_4^{2-} con diversa localizzazione, sequenza amminoacidica e affinità al solfato. Considerato che lo zolfo ha un ruolo chiave nella risposta a diversi stress abiotici (come la solfo-carenza, l'esposizione a metalli pesanti o lo stress salino), l'espressione di questi geni deve essere finemente regolata a seconda delle condizioni ambientali, per soddisfare la corretta richiesta di composti ridotti dello zolfo.

L'obiettivo generale di questa tesi è la descrizione dei flussi sistemici di zolfo nel riso in diverse condizioni di stress, per ottenere maggiori informazioni sul contributo di questo elemento nel determinare la tolleranza della pianta agli stress abiotici. Per raggiungere l'obiettivo, è stata sfruttata anche l'analisi eseguita con un analizzatore elementare accoppiato con uno spettrometro di massa a rapporto isotopico (EA-IRMS), un importante strumento che utilizza gli isotopi stabili degli elementi come *tracer*.

L'intero lavoro è stato suddiviso in tre parti.
Nella prima parte del lavoro sono stati studiati i potenziali effetti isotopici $^{32}S/^{34}S$ che si verificano durante l'assorbimento di SO_4^{2-} in un sistema idroponico chiuso, in cui una quantità limitata di substrato (SO_4^{2-} nella soluzione nutritiva) è continuamente rimossa dalla soluzione, a causa dall'attività dei trasportatori radicali del solfato, ed è convertita in un prodotto finale (S totale della pianta). È stata osservata una discriminazione isotopica verso ^{34}S durante l'assorbimento di SO_4^{2-}: le piante mostravano una composizione isotopica di S più leggera, mentre il SO_4^{2-} residuo nella soluzione idroponica si arricchiva in isotopo stabile pesante. Il frazionamento durante l'*uptake* ha evidenziato due fasi caratterizzate da diversi fattori di frazionamento, riflettendo anche

cambiamenti nell'espressione degli *OsSULTR* deputati all'assorbimento radicale, che potrebbero spiegare i diversi fenotipi isotopici osservati durante l'acquisizione del solfato. Inoltre, sono stati studiati i possibili effetti isotopici associati sia al partizionamento dello S, che al metabolismo cellulare, confrontando piante pre-coltivate in soluzioni nutritive complete e poi trasferite in soluzioni idroponiche contenenti SO_4^{2-} (*steady-state*) o prive di SO_4^{2-} per 72h. Il *pool* di SO_4^{2-} del germoglio in *steady-state* era significativamente impoverito di ^{32}S rispetto ai *pool* di SO_4^{2-} della radice, mentre quelli dello zolfo organico (S_{org}) erano significativamente impoveriti in ^{34}S rispetto sia al *pool* di SO_4^{2-} del germoglio, che alla sorgente. Questi risultati suggeriscono una maggiore assimilazione di S nella parte aerea delle piante che favoriscono l'isotopo più leggero. Nelle piante in solfo-carenza l'assimilazione di zolfo ha arricchito progressivamente in ^{32}S il *pool* di S_{org}, e in ^{34}S il SO_4^{2-} residuo nei diversi organi. Frazionamenti più evidenti sono stati nuovamente osservati nel germoglio, confermando il suo ruolo principale nell'assimilazione di SO_4^{2-} e nell'allocazione di S. Non è stato osservato alcun frazionamento causato dall'attività di traslocazione.

Per convalidare i risultati precedentemente ottenuti, nel secondo lavoro è stato eseguito un bilancio di massa in piante di riso trattate per 72 ore con diverse concentrazioni di Cd, per investigare il possibile diverso frazionamento tra gli isotopi stabili dello zolfo causato da questo stress: l'adattamento del metabolismo dello zolfo, infatti, ha un ruolo fondamentale nella risposta all'inquinamento da metalli pesanti. Come previsto il trattamento con Cd ha fortemente incrementato l'assorbimento e l'assimilazione di SO_4^{2-}, come indicato dalle analisi dei *pool* dello zolfo (S_{tot}, SO_4^{2-}, and S_{org}). L'analisi isotopica degli isotopi stabili dello zolfo eseguita sulle piante intere ha rilevato cambiamenti nel metabolismo associati a variazioni nella discriminazione nei confronti di ^{34}S, che diventava meno evidente all'aumentare della concentrazione di Cd nella soluzione nutritiva. L'analisi trascrizionale ha suggerito ancora una volta che i cambiamenti nei rapporti di trascritto relativo di *OsSULTR1;1* e *OsSULTR1;2*, come osservato per la solfo-carenza, potrebbero essere responsabili della progressiva diminuzione della discriminazione isotopica. Il prevalente ruolo del germoglio nell'assimilazione dello S è stato confermato: il frazionamento degli isotopi associato all'assimilazione del solfato era maggiore nel germoglio che nella radice, e progressivamente aumentava con la concentrazione di Cd.

L'ultimo lavoro si è concentrato sulla completa caratterizzazione in condizioni idroponiche in assenza o in presenza di stress salino (NaCl 80 mM) del fenotipo della già disponibile linea di introgressione (IL) tollerante al sale Onice 11 (O11), ottenuta tramite un approccio di *Marker-Assisted Back-Cross* (MABC) *selection*, a partire dall'incrocio tra la *cultivar japonica* élite Onice (parentale ricorrente sensibile) e la varietà *indica* IR64-*SalTol* (tollerante), donatrice del *major* QTL *SalTol*. Inoltre, sono stati valutati l'acquisizione e l'assimilazione del solfato di O11 e di entrambe le linee parentali, per indagare la loro possibile implicazione nel determinare una diversa tolleranza allo stress salino. I risultati hanno mostrato l'effetto benefico dell'introgressione del QTL *SalTol* dalla varietà *indica* nella linea di riso *japonica* selezionata, in base alle diverse caratteristiche dei diversi parametri fenotipici-biochimici-fisiologici selezionati. Tuttavia, lo stress salino ha fortemente influenzato negativamente l'assorbimento e l'assimilazione di S e possiamo ragionevolmente supporre che questi non giustifichino la diversa tolleranza al sale osservata nella IL O11.

In conclusione, le piante di riso sono in grado di discriminare l'isotopo stabile pesante ^{34}S durante l'assorbimento e l'assimilazione di SO_4^{2-}. Tra gli organi della pianta, il germoglio rappresenta quello maggiormente coinvolto nell'assimilazione di S. Gli stress abiotici come la solfo-carenza o l'esposizione al Cd determinano cambiamenti nei rapporti di trascritto relativo dei *SULTR* coinvolti nell'assorbimento radicale e questo potrebbe determinare i diversi fenotipi isotopici osservati. Infine, la tolleranza al sale della IL O11 non appare sia correlata ad un diverso metabolismo dello zolfo.

1. Introduction

1.1 Importance of sulfur in plants

With nitrogen (N), potassium (K) and phosphorus (P), sulfur (S) is one of the essential macronutrients required by plants which also has an important role in the response to abiotic stresses (Nazar *et al*. 2011).

S has numerous biological functions, even if it is present at approximately 0.1% of dry matter, and it generally has not structural propriety like carbon. This element is fundamental for the biosynthesis of different compounds, such as the two amino acids, cysteine (Cys) and methionine (Met), vitamins (biotin and thiamine), peptides involved in the response to abiotic stresses (glutathione - GSH, and phytochelatines - PCs), secondary products characteristic of *Brassicaceae* and *Alliaceae* (Leustek 2002; Nocito *et al*. 2007; Khan *et al*. 2014), and it is also important in regulating the activity of photosynthetic enzymes and chlorophyll content in leaves (Samanta *et al*. 2020).

Plants, differently from human and animals, can incorporate sulfate ion (SO_4^{2-}), the main source of S present in the soil solution, during the reductive assimilation pathway: the obtainment of Cys has a key role, because this amino acid is the precursor of most of the S compounds (Leustek *et al* 2000). After the absorption of SO_4^{2-} by the root system, this ion is translocated through the plants by xylem and phloem, reduced mostly in chloroplast, and then assimilated into Cys or stored in vacuoles as reserve (Davidian and Kopriva 2010; Takahashi *et al.* 2011).

The thiol group (-SH), reduced form of S found in Cys, is strongly nucleophilic and this propriety allows reactions with a broad of electrophilic compounds like metals, free radicals, and xenobiotics. Moreover, two molecules of Cys can be oxidized forming a stable covalent disulfide bond and this propriety has a pivotal role in the determination of proteins structure and function (Åslund and Beckwith 1999). This feature is also crucial in determine the redox status of an important thiol compound: GSH. Glutathione (γ-glutamylcysteinylglycine) is a tripeptide with structure Glu-Cys-Gly, which generally represents the 90% of intracellular non-protein thiols and which has a fundamental role as redox buffer. One molecule of GSH can react with another one to produce the

oxidized form (GSSG), maintaining the redox state of the cell. The reduced form can be restored by the activity of NADPH-glutathione reductase (Noctor *et al*. 2012). GSH has also other crucial functions in plants:

- it can defend and protect cells against the oxidative damage caused by biotic and abiotic stresses (Noctor 1998; Foyer and Noctor 2005; Rausch and Wachter 2005).
- it can enhance cell cycle and plants development, to adapt plant sessile life to different environmental constrains (May *et al*. 1998).
- it is involved in the detoxification of different xenobiotics, such as herbicide, mediated by the glutathione-S-transferases (Rea *et al* 1998).
- it is the precursor of phytochelatines (PCs), polypeptides with general structure (γ-Glu-Cys)$_n$-Gly, where n ranges from 2 to 11. These biomolecules have important role in heavy metals detoxification (Leustek *et al*. 2000).

Understanding more about S acquisition and assimilation in plants is essential both for its importance in human nutrition, as Met is an essential amino acid required by our diet, and because many S containing secondary metabolites and specialized peptides (such as GSH or PCs) are important in the response to biotic and abiotic stresses (Gigolashvili and Kopriva 2014).

1.2 Sulfate transporters

SO_4^{2-} in the rhizosphere is the most oxidized and stable form of S in nature. It is the main source of S taken up by the plant root system (Gruber *et al*. 2013), even if there are evidence of a small absorption of sulfur dioxide by the shoot through the stomata, generally in polluted environments (Saito 2004).

SO_4^{2-} absorption and its distribution through xylem and phloem are related to transporters codified by the family gene of *SULfate TRansportes* (*SULTR*s). *SULTR*s members have different localization, and their expression is controlled by different environmental conditions (Feldman-Salit *et al*. 2019). In higher plants SULTRs are H^+/SO_4^{2-} co-transporter integrated in membrane through 12 transmembrane regions and containing the carboxyl-terminal region named Sulfate Transporter/AntiSigma-factor (STAS), important in conferring correct activity, kinetic and interaction with other proteins (Shibagaki and Grossman 2004, 2006). Numerous protein sequences of SULTRs of different plants have been identified, for example in *Arabidopsis thaliana*, *Glycine max*, *Populustri chocarpa*, *Oryza sativa* and *Sorghum bicolor* (Takahashi *et al*. 2012). In *Arabidopsis*, *O. sativa* and *Popolus* 12, 11 and 13 genes have been identified, respectively, encoding for SULTRs (Kopriva *et al*. 2009; Gigolashvili and Kopriva 2014). Concerning how the transport of SO_4^{2-} from soil to root and inside the plants takes place, two different possibilities have been found:

- a probable $3H^+/SO_4^{2-}$ stoichiometry in *Lemna gibba*, producing an electrogenic transport across the plasma membrane (Lass and Ullrich-Eberius 1984);
- an H^+ gradient which drives the transport of SO_4^{2-} in yeasts expressing the *Stylosanthes hamata* sulfate transporter family (Smith *et al.* 1995).

In general, SULTRs can be divided in four different groups according to their amino acid sequences, localization, and affinity to SO_4^{2-} (Fig. 1):

- high affinity transporters of group 1 are the major responsible for the uptake of SO_4^{2-} in root system. In particular, SULTR1;1 and SULTR1;2 are mainly present in root hairs, root epidermal and cortical cells and they are involved in the uptake of SO_4^{2-} into roots (Takahashi *et al*. 2000; Yoshimoto *et al.* 2002; Takahashi 2019). To understand their correct function, several studies on *A. thaliana* single mutants lacking either *SULTR1;1* or *SULTR1;2* have been performed. These two genes have

functional redundancy, even if it is currently accepted that *SULTR1;2* is more expressed under normal S nutrition, while *SULTR1;1* is upregulated during S starvation and other abiotic stresses (Yoshimoto *et al*. 2002; Rouached *et al.* 2008; Ferri *et al.* 2017; Sacchi and Nocito 2019). The third isoform of the high-affinity sulfate transporter (SULTR1;3) is involved in the retrieval of SO_4^{2-} within the phloem: it is expressed in sieve elements and companion cells of the phloem and, according to this localization, an implication of this transporter in long distance SO_4^{2-} translocation processes has been postulated (Yoshimoto *et al.* 2003). Considering the importance of the gene expression of group 1, numerous studies have been dedicated to its transcriptional regulation. Among them, an interesting result was the identification of the transcription factor SULFUR LIMITATION1 (SLIM1)/ETHYLENE-INSENSITIVE3-LIKE3 (EIL3), obtained from the study of the S starvation response in *Arabidopsis* response-less mutants (Maruyama-Nakashita *et al*. 2006). This transcription factor induces gene expression of *SULTR1;1* and *SULTR1;2* in *A. thaliana* roots under S deficiency and it also plays a pivotal role in S metabolic network regulation, controlling SO_4^{2-} uptake and balancing the flux of S (Aarabi *et al.* 2016).

- Group 2 encodes for the low affinity SO_4^{2-} transporters, which regulate the translocation of SO_4^{2-} and its distribution to sink organs. The first transporter of this group, SULTR2;1, is mainly localized in pericycle and xylem parenchyma of the root and it seems to have a crucial role in loading the ion into the xylem (Sacchi and Nocito 2019). A particular regulatory control of this gene has been observed under S deficiency, activated by the transcription factor SLIM1/EIL3: mRNA is degraded by the miRNA-395, which accumulates in companion cells of phloem in root and shoot, controlling the transcript level. In this way the translocation of SO_4^{2-} to the leaves through xylem during S starvation is advantaged (Kawashima *et al*. 2011). The second transporter involved in translocation is SULTR2;2, which seems to regulate the source to sinks distribution via phloem. It is localized in leaves vasculature, palisade, and mesophyll, where the large part of SO_4^{2-} assimilation takes place (Takahashi *et al*. 2000).
- The third group is the most abundant. Recent studies performed on *A. thaliana* have pointed out that members of group 3 are redundantly expressed in

chloroplast membrane, suggesting a fundamental role in the assimilation of SO_4^{2-} (Cao *et al*. 2014; Chen *et al*. 2019). Despite these, there are different hypothesis regarding their function and localization, based on several observations. *SULTR3;5* seems to enhance the activity of *SULTR2;1* when they are heterologously expressed in yeast (Sacchi and Nocito 2019). In rice *SULTR3;4* is expressed in xylem region and its expression strongly affects phosphorous distribution: for this reason, it is indicated as a SULTR-like Phosphorus Distribution Transporter (SPDT) (Yamaji *et al.* 2017). At the same way *SULTR3;3* in rice has a double role in translocation of phosphate and sulfate since mutations of this gene affect the concentration of both P and SO_4^{2-} in plants. Studies on its expression have highlighted that it is localized in vascular bundles of leaves, shoot, flowers, and seeds but not in the root system (Zhao *et al.* 2016).

- The group 4 is the last one and it includes tonoplast transporter (SULTR4;1 and SULTR 4;2) involved in the efflux of sulfate from vacuoles. Proteins that can mediate the influx of SO_4^{2-} in vacuoles have not been identified yet (Kataoka *et al*. 2004).

The coordinated expression of all these genes leads to the obtainment of optimal management and balance of SO_4^{2-} uptake and translocation, considering also different regulations that occur under different environmental conditions (i.e., sulfur deficiency, salt stress or heavy metal pollution) (Kumar *et al*. 2011).

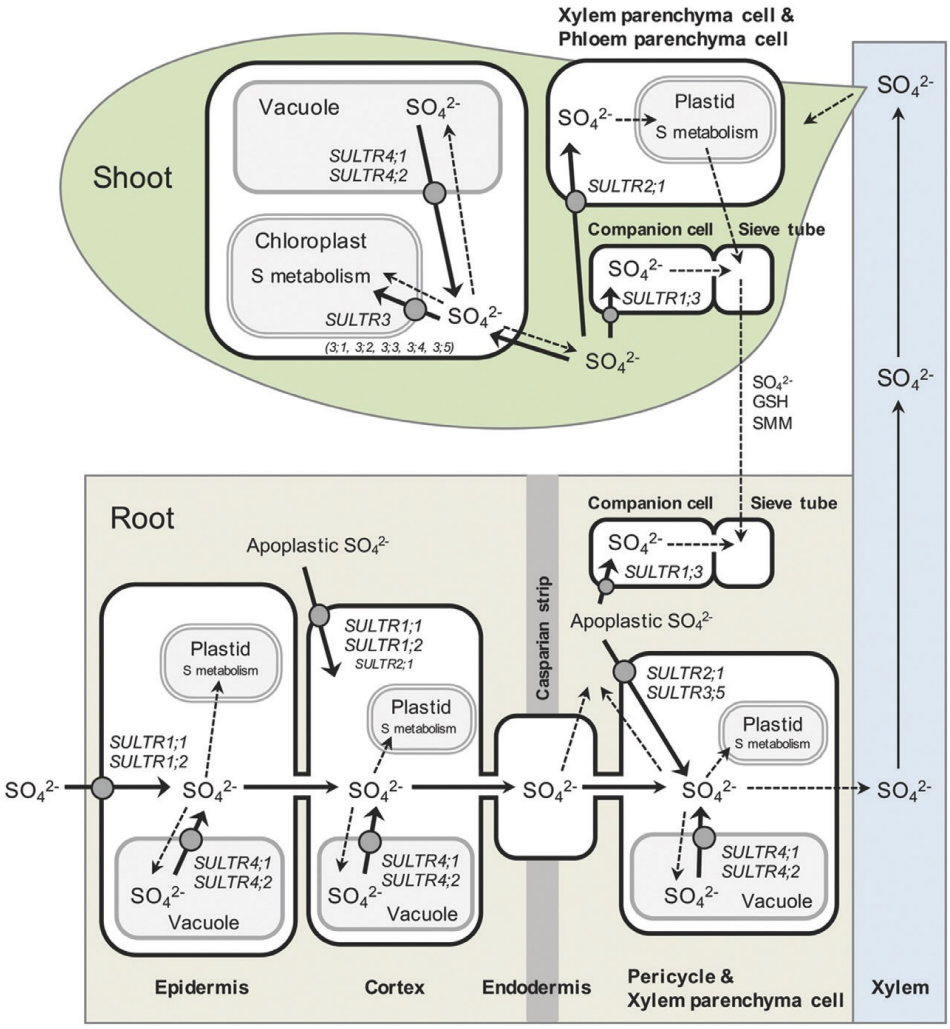

Figure 1. Schematic representation and localization of SULTR in *Arabidopsis thaliana* (Takahashi 2019).

1.3 Sulfate metabolism

Before entering the assimilatory pathway, SO_4^{2-} inside the cells must be activated by ATP sulfurylase in the following reaction:

$$SO_4^{2-} + ATP \rightarrow 5'adenylylsulfate\ (APS) + PPi$$

This enzyme is mostly accumulated in plastids, and it leads to the obtainment of APS, an energy rich compound which is a common intermediate in both reductive and non-reductive (sulfation) sulfate assimilatory pathway: the first pathway ends with the biosynthesis of Cys, while the second leads to the obtainment of 3'-phosphoadenosine 5'-phosphosulfate (PAPS), a donor of activated SO_4^{2-} for many sulfation reactions (Leustek *et al.* 2000).

1.3.1 Sulfation

The non-reductive assimilatory pathway in animal system has been long known, but different studies have pointed out its importance also for plant development and growth. Sulfation is catalyzed by sulfotransferases present in cytosol, which use PAPS as sulfuryl donor (Leustek *et al*. 2000). The biosynthesis of PAPS from APS and ATP is catalyzed by the enzyme APS kinase and in *Arabidopsis* at least three genes codifying for APS kinases have been identified, one of them with localization in chloroplast (Lee and Leustek 1998). There is a broad range of compounds sulfated by this pathway, such as jasmonates, flavonoids, peptides, brassinosteroides, gallic acid glucosides, extracellular polysaccharides and glucosinolates (Nocito *et al.* 2007).

1.3.2 Reductive assimilatory pathway: from APS to cysteine

In this pathway APS is reduced to sulfide, and then assimilated into Cys. Sulfate reduction is the main route for assimilation and all the enzymes involved are localized in plastid (Brunold and Suter 1989; Leustek *et al.* 2000). The first reaction is catalyzed by the APS reductase which transfers two electrons to APS, leading to the obtainment of sulfite. The electron donor is GSH (Bick *et al*. 1998; Kopriva and Koprivova 2004).

$$APS + 2\ GSH \rightarrow SO_3^{2-} + 2H^+ + GSSG + AMP$$

In the second step, sulfite is reduced to sulfide after a six-electron transfer from ferredoxin. This reaction is catalyzed by the sulfite reductase (Aketagawa and Tamura 1980; Krueger and Siegel 1982; Bork *et al*. 1998; Yonekura-Sakakibara *et al.* 1998; Leustek *et al*. 2000; Nocito *et al*. 2007).

$$SO_3^{2-} + 6\ ferredoxin_{red} \rightarrow S^{2-} + 6\ ferredoxin_{ox}$$

Two enzymes are involved in the last part of the reductive pathway: serine acetyltransferase (SAT), which catalyzes the formation of O-acetyl serine (OAS) starting from serine and acetyl-CoA, and OAS(thiol)-lyase (OAS-TL), which transfers sulfide into β-position of OAS leading to the final production of Cys. Both the enzymes are localized in cytosol, chloroplasts, and mitochondria. Studies on SAT mutant allowed to discover that OAS is predominantly synthetized in cytosol and mitochondria, while Cys in plastids. It is possible to speculate that in leaves sulfide is generated in chloroplast, OAS in

mitochondria, and in general the large part of the production of cysteine takes place in cytosol (Haas *et al.* 2008; Watanabe *et al.* 2008).

$$\text{Serine + acetyl-CoA} \rightarrow \text{OAS + CoA}$$

$$\text{OAS + } S^{2-} \rightarrow \text{Cys + acetate}$$

Cys represents not only the final product of the reductive pathway, but also a starting point for the synthesis of Met, GSH and several other S compounds (Noji *et al*. 1998; Saito 2000, 2004; Nocito *et al.* 2007).

1.3.3 Biosynthesis of GSH

GSH is a tripeptide, nonprotein thiol compound with several important roles in plant defense and protection. Even small changes in the intracellular concentration of GSH lead to important consequences for cells redox status, metabolic processes, and gene transcription (Noctor 1998; Rausch and Wachter 2005; Noctor *et al*. 2012). This molecule is an important redox buffer which acts in the scavenging of ROS that can accumulate during different biotic and abiotic stresses (Rausch *et al*. 2007; Astolfi and Zuchi 2013).

GSH biosynthesis takes place in plastids and cytosol from two sequential ATP-dependent reactions:

1. γ-EC synthetase catalyzes the bonding between the amine group of Cys and the γ-carboxyl group of glutamate (Glu), leading to the formation of the peptide γ-glutamylcysteine (γ-EC);

$$\text{Glu + Cys + ATP} \rightarrow \text{γ-EC + ADP +Pi}$$

2. in the last step, glycine (Gly) is added through the enzyme GSH synthetase to the C-terminal of γ-EC to obtain GSH (Nocito *et al*. 2007).

$$\text{γ-EC + Gly + ATP} \rightarrow \text{GSH + ADP +Pi}$$

The regulation of GSH biosynthesis is mainly controlled by the activity of γ-EC synthetase and the availability of Cys. Studies performed on *Arabidopsis* have shown that the antisense suppression of γ-EC synthetase caused a reduction of GSH in leaves. Vice versa, its overexpression increased the concentration of this tripeptide, without depleting the Cys pool: this result suggests that there is a strong and coordinate

regulation of Cys biosynthesis and GSH pathway (Xiang *et al*. 2001). It is also important to note that the activity of this biosynthetic pathway is finely regulated by its end product: in fact, GSH acts like negative feedback on γ-EC synthetase, and this is pivotal in controlling concentration and homeostasis of GSH (Hell and Bergman 1990; Noctor 1998; Noctor *et al*. 2002, 2012).

1.3.4 Sulfur metabolism is controlled by a demand-driven regulation

Cys is an important key-intermediate for several S metabolites and, for this reason, its biosynthesis is finely regulated to meet the total S demand of plants. This request depends on different factors, such as plant development and environmental conditions; in particular, several biotic and abiotic stresses cause an increase in the S assimilatory pathway to meet a higher request of S compounds (Rausch and Wachter 2005). For example, it is well and long known that during sulfur deficiency plant S request remains constant, creating different problems in the management of Cys biosynthesis and sulfur homeostasis related to SO_4^{2-} withdrawal in the growing media; this causes a dramatic decrease of the level of Cys, GSH and sulfate in tissues. To cope this situation, plants enhance transcript levels of different genes which codify for SULTR, ATP sulfurylase and APS reductase. However, normal situation can be easily restored by suppling sulfate or sulfur reduced containing compounds in the growth media (Lappartient and Touraine 1996; Smith *et al.* 1997; Bolchi *et al.* 1999; Lappartient *et al.* 1999).

Several experiments have also found out that different final products of sulfur reductive pathway can directly act as long distant repressor molecules, conferring to plants the ability to sense their nutritional status, and consequently optimize the biosynthesis of Cys (Lappartient and Touraine 1996; Lappartient *et al.* 1999). It has been discovered that GSH during its phloem translocation, and Cys in maize roots are possible repressors involved in the regulation of sulfur reductive pathway (Lappartient and Touraine 1996; Bolchi *et al.* 1999; Lappartient *et al*. 1999). It is possible to build an accepted model which can well describe S uptake and metabolism in plants. Adequate rates of SO_4^{2-}, Cys and GSH can down regulate the expression of key genes and enzymes involved in the S reductive pathway, acting in negative feedback that prevents an excessive use of energy; on the contrary, the reduction of the concentration of S containing compounds in plants

can up regulate the expression of these genes, enhancing SO_4^{2-} uptake and reduction. This fine regulation allows the survival of plants in a broad range of environmental conditions (Nocito *et al.* 2007).

OAS is another important key-molecule in the regulation of S reductive pathway. In fact, when availability of sulfur in plants is less than the ones of carbon and nitrogen, OAS can accumulate and it can act as a positive signal (Neuenschwander *et al.* 1991; Smith *et al.* 1997; Kim *et al.* 1999; Yamaguchi *et al*. 1999; Ohkama-ohtsu *et al.* 2004), partially overcoming the repression of important genes and favoring the biosynthesis of Cys (Hawkesford 2000; Hawkesford and Wray 2000).

Moreover, Cys biosynthesis is also controlled at post-translational level by the reversable formation of the complex between SAT and OAS-TL (Saito 2004; Wirtz and Hell 2006; Hell and Wirtz 2008). In normal condition, OAS-TL concentration is higher than the one of SAT in all cellular compartments and for this reason only a small part of OAS-TL can bind with SAT, to form enzymatic complexes constituted by an homotetrameric SAT and two homodimeric OAS-TL; their formation is promoted by sulfide, while the accumulation of OAS promotes the dissociation (Lunn *et al*. 1990; Rolland *et al*. 1993; Droux *et al.* 1998; Saito 2004). The kinetic proprieties of SAT are enhanced in this complex, on the contrary OAS-TL loses in efficiency. It is possible to speculate that a large amount of Cys is then synthetized by free OAS(thiol)-lyase (Droux *et al*. 1998). In the end, when sulfide limits the biosynthesis of Cys, OAS accumulates allowing the disruption of the enzymatic bond, thus preventing its excessive formation; when sulfide is not limiting, it sustains the formation of the complex and so the synthesis of OAS and the correct production of Cys. Moreover, Cys exerts negative feedback on SAT isoforms that can regulate OAS biosynthesis (Urano *et al*. 2000; Noji and Saito 2002; Wirtz and Hell 2003). This regulatory model is not only fundamental in controlling the homeostasis and biosynthesis of Cys, but also in coordinating OAS production from serine and sulfate reduction.

Despite these knowledges, lot of information about signal perception and transduction in maintaining S homeostasis remain unknown. It is possible that different molecules, such as auxin, methyl jasmonate, abscisic acid, cytokinins, and salicylate are involved in the transduction pathway (Hirai *et al*. 2003; Maruyama-Nakashita *et al*. 2003, 2004a;

Nikiforova *et al*. 2003; Rausch and Wachter 2005; Yakimova *et al.* 2006; Maksymiec 2011; Masood *et al*. 2012; Stroiński *et al*. 2013).

Speaking about the control at transcriptional level of different genes involved in S metabolism, several potential sulfur responsive elements (SUREs) in their promoter regions have been identified. For example, a study performed on *A. thaliana* and on the gene *SULTR1;1* has revealed that only 5 base pairs SURE was involved in promoting the expression of the gene under sulfur deficiency (Awazuhara *et al.* 2002; Kutz *et al*. 2002; Maruyama-Nakashita *et al*. 2004b).

1.4 Sulfur containing compounds and response to abiotic stresses

Abiotic stresses are in constant growing and are a major global problem that is limiting fertility and yield of crops. The rising of temperature is enhancing several abiotic stresses such as soil salinization, dehydration, extreme temperature, and intense sunlight, which are affecting the development of traditional cultivar (Capaldi *et al.* 2015). Plants respond in various and complex ways to cope with these difficulties, but the abilities of suppressing oxidative damage and ROS scavenging are of great importance (Das and Roychoudhury 2014). Moreover, different studies have well documented the pivotal role of different S containing compounds in the determination of plant tolerance to several abiotic stresses. Adaptation of SO_4^{2-} uptake and assimilation, related to a particular environmental condition, could be determinant for the correct development of plants (Nazar *et al*. 2011; Samanta *et al*. 2020).

- Cysteine plays different important roles. It enhances the activity of amylase, allowing the mobilization of crucial products to sustain growth and development during stress conditions (Genisel *et al.* 2015). This amino acid is also directly connected to ROS scavenging: even if this activity is more dependent to antioxidative enzymes (ascorbate peroxidase, superoxide dismutase, catalase, and glutathione reductase), evidence shows how in stressed condition in which high amounts of ROS are present, plants with high content of Cys have more resistive capacity to oxidative stress (Banerjee and Roychoudhury 2017). The induction of Cys biosynthesis was observed in canola plants during exposure to salt stress, due to a higher activity of SAT and OAS-TL (Ruiz and Blumwald 2002). Also, it has been

demonstrated in rice how in the salt tolerant *indica* variety Nona Bokra the accumulation of Cys was higher than in the susceptible variety M-1-48 and Gobindobhog (Roychoudhury *et al*. 2008).

- GSH acts as a redox buffer, and sufficient pool of this tripeptide can enhance antioxidant activity improving tolerance to different abiotic stresses. Cys and GSH together are also fundamental in the response to heavy metals pollution because they are the precursors of phytochelatines, secondary sulfur compounds and metal-binding peptides, relevant in determine detoxification of heavy metals. It has been documented how in transgenic *Arabidopsis* overexpressing OAS-TL, the availability of Cys and GSH increased and so also the Cd^{2+} tolerance of plants (Domínguez-Solís *et al.* 2004). Moreover, in salt stress condition, upregulation of Cys biosynthesis also determines an increase in GSH cellular concentration, with important benefits in reducing harmful effects of ROS (Queval et al. 2009).
- Thioredoxin is a disulfide reductase with low-molecular weight (12-14 kDa), fundamental in maintaining the reduction state of proteins and in lowering ROS level in different stage of plant development. It is possible to identify six types of thioredoxin, of which only three are present in eukaryotes, according to localization (cytosol, mitochondria, chloroplast) and amino acidic sequences (Gelhaye *et al.* 2005; Collet and Messens 2010). Abiotic stresses increase level of thioredoxin both at transcript and protein level (Chen et al. 2015). It was observed that during salt stress, mitochondrial thioredoxin increases its activity, enhancing the role of antioxidant enzymes and protecting mitochondria from ROS damages (Martí *et al*. 2011).

The ability of finely regulate S metabolism has a key role in the determination of plant tolerance against a plethora of stresses, both biotic and abiotic. Several studies have pointed out how different environmental conditions determine a behavior similar to the one observed during S starvation, to meet a higher demand of S reduced compounds to efficiently mitigate stresses and control the growth of plants (Noctor *et al*. 2002; Nocito *et al*. 2002, 2006, 2007; Hirai *et al*. 2003; Howarth *et al*. 2003b, a; Maruyama-Nakashita *et al.* 2003; Nikiforova *et al*. 2003; Rausch and Wachter 2005; Herbette *et al.* 2006; Khan *et al*. 2016).

1.4.1 Cadmium exposure and sulfur metabolism in higher plants

Heavy metals are a class of metals having a density higher than 5g/cm^3. They can be divided in two types:

1. essential for plants - like iron, copper, and zinc – which are required in small amounts for correct development.
2. not essential – like cadmium, mercury and lead – which are not necessary for plant growth, and which have deleterious effect (Elmsley 2001).

Among the not essential heavy metals, cadmium (Cd) is the most studied because considering its high mobility in soil and plant, it represents a serious toxic pollutant, dangerous not only for environment and plants, but also for all the living organisms. In soil it can be naturally present or added through anthropogenic activities, like depositions from mining activities, urban composts, and industrial sludges (Clemens 2006; Järup and Åkesson 2009; Gallego *et al*. 2012; Nazar *et al*. 20 12; Clemens *et al*. 2013; Choppala *et al.* 2014; Dalcorso *et al.* 2016).

Cd enters in root system through specific essential cations transporters (members of ZIP and Nramp families, or Ca^{2+} channels and transporters) (Pence et al. 2000; Perfus-Barbeoch et al. 2002; Baker et al. 2006) and it alters cellular functions, interfering with S and N atoms of amino acids, changing protein activity and structure. Moreover, this heavy metal interacts with enzyme catalysis, carbohydrates metabolism, photosynthetic pathway, nitrate metabolism and water balance (Khan *et al.* 2016). Cd also induces oxidative stress, enhancing the production of ROS (especially superoxide anion and hydrogen peroxide), because of its negative interaction with protein redox-active metals (Clemens *et al.* 2013; Choppala *et al*. 2014). Symptoms can be present in a variety of ways, such as chlorosis, growth reduction, nutrients deficiency and cell death (Mohanapuria *et al*. 2007; Ebbs and Uchil 2008; Khan *et al.* 2016).

Different mechanisms to prevent an excessive translocation of Cd to shoot (and then to seeds) have been developed by most of plant species, like a “firewall system” at root level that depends on different biochemical and physiological factors, for example capability of Cd chelation, translocation, absorption and compartimentalization (Nocito et al. 2011).

Concerning the importance of S compounds in Cd detoxification, phytochelatines (PCs) are molecules which have great relevance in the chelation and subcellular compartimentalization of this heavy metal. Their synthesis is enhanced by exposure to different metals and metalloids, among which Cd is one of the strongest inducers (Cobbett 2000; Merlos Rodrigo *et al.* 2016). Studies performed on *Arabidopsis* mutants have highlighted the crucial role of PCs in Cd detoxification: deficiency in their biosynthesis or in the formation of PC-Cd complexes determined a hypersensitivity to the heavy metal (Howden *et al.* 1995a, b). GSH is the precursor of PCs, and the biosynthesis is catalyzed by PC synthase (PCS) in a stepwise reaction: in the first step there is the transpeptidation of the γ-Glu-Cys unit of a GSH molecule into another GSH molecule to form PC2; consequently, other γ-Glu-Cys units arising from GSH are transferred to PC2 to form PC3 and so on (Rea *et al*. 2004; Rea 2012; Merlos Rodrigo *et al.* 2016) (Fig. 2).

There is a strong relationship between Cd exposure, SO_4^{2-} assimilation, GSH and PCs biosynthesis: in fact, Cd exposure immediately enhances PCs synthesis, creating an additional sink for S reduced compounds. This increases the request for Cys and GSH in a typical demand-driven coordinate transcriptional regulation of genes involved both in SO_4^{2-} assimilation and in GSH biosynthesis. This adaptation is essential for plant survival when exposed to Cd and it is a common feature in several species (Grill *et al.* 1989; Rauser 1990; Yadav 2010). Moreover, studies performed on *Zea mays* have pointed out how in seedlings exposed to Cd, there is an over expression of a gene root-expressed high-affinity sulfate transporter (*ZmST1;1*), like the one which can be observed in condition of S starvation. It is not clear yet, but it is possible that Cd accumulation inside cells can simulate a sort of S limiting condition, so also the regulation of SO_4^{2-} uptake and influx are determinant to reach the increased S demand for GSH and PCs biosynthesis, generated by Cd exposure (Nocito *et al*. 2002, 2006).

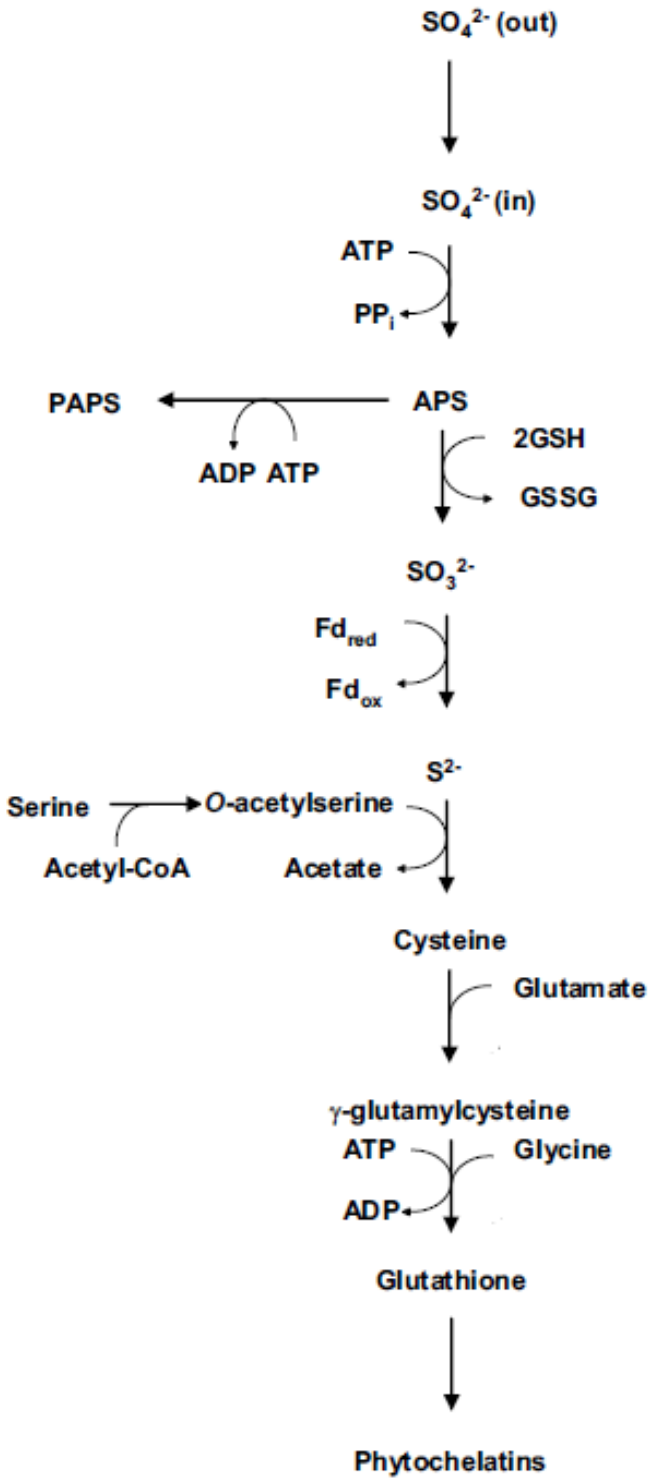

Figure 2. Sulfur assimilatory pathway, GSH and PCs biosynthesis (Nocito *et al*. 2007).

1.4.2 Sulfur metabolism and salt stress

One of the main problems caused by global warming is soil salinity: it is expected that by the end of our century, more of 50% of fertile land will be loss because of this abiotic stress (Manchanda and Garg 2008).

Salt stress-induced toxicity alters the cellular homeostasis of Na^+ and Cl^- ions and disturbs the uptake of major essential nutrients (Fatma *et al*. 2014). ROS over-production and extensive oxidative stress are other common and major symptoms caused by salinization: they can interfere with different aspects of plant life, causing damage in membrane and other cellular structures, metabolic disorders, and inhibition of photosynthesis (Bashir *et al.* 2021).

Plants have developed different mechanisms to cope with these problems, both enzymatic and non-enzymatic. It is evident that understand more about this topic will be useful in the identification of plants which can better face an important problem such as climate change and all its consequences. The enzymatic antioxidant system has been massively studied: the increased activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR), and the association to salt tolerance have been long reported (Nazar *et al*. 2011; Rossatto *et al.* 2017; Vighi *et al*. 2017); also the relationship between salinity and non-enzymatic antioxidants and osmoprotectans, such as GSH and proline, has been demonstrated (Hayat *et al.* 2012; Khan *et al.* 2012, 2014; Ashfaque 2014).

In the last years, involvement of S reduced compounds in the determination of an improved salt tolerance has been investigated and it has been demonstrated that they can enhance the tolerance by modulating physiological and molecular processes (Ashraf 2009; Türkan and Demiral 2009; Nazar *et al*. 2011; Ashfaque 2014; Khan *et al*. 2014). One of the mechanisms that plants have against salt stress adverse effect includes the upregulation of GSH synthesis, controlled by a fine regulation of the S assimilation enzymes and Cys biosynthesis (Mohd Asgher 2014). Accumulation of this tripeptide is enhanced by stress and consequently it determines an upregulation of SAT and APR (Queval *et al*. 2009). Several studies on different species have shown the importance of GSH in conferring different tolerance to salt stress. In *Vigna radiata* cultivars, tolerant ones had a major rate of GSH biosynthesis than sensitive ones, which shows also a greater oxidative damage (Nazar *et al*. 2011). In *Lycopersicum esculentum* (Shalata *et al.* 2002), *Oryza sativa* (Vaidyanathan et al. 2003) and *Archis hypogea* (Mittova et al. 2003), similar results were found, and sensitive plants showed lesser GSH content and redox state. Although the extensive number of studies available on GSH and salt stress

response, its mode of action is not clear yet. More integrated studies of physiology, biochemistry and molecular biology are needed to fully understand its involvement.

1.5 An overview on stable isotopes

Isotopes are elements that occupy the same position in the periodic table, and which have same number of protons and electrons in the nucleus but differ in the number of neutrons. They can be divided in unstable (radioactive) and stable species: nowadays 300 stable isotopes and 1200 unstable isotopes have been discovered (Hoefs 2009).

Stable isotopes are safe and abundant form of the same elements which do not decay and are not dangerous for human life (unlike the radioactive ones). All elements have at least two stable isotopes, one of them generally presents in grater abundance. The natural abundances of elements and their stable isotopes are constant in nature. They have been determined long time ago, at the beginning of the Universe, and for elements such as O, C, N, H and S the distribution of light stable isotopes (the one with low mass) represents more than the 95% (Tab. 1). In the last decades, their use as tracer has become a powerful tool in a broad of ecological studies (Peterson and Fry 1987; Fry 2006). Isotopes of any elements participate in the same way in several reaction, however rates and transport are dependent to the different mass and this difference causes deviations known as isotope effect (Hayes 2004). For example, in kinetic reactions, light stable isotopes react faster, while the heavy ones tend to concentrate where bonds are stronger. This phenomenon is called fractionation, and it causes different changes in isotope distribution during natural cycle of elements (Fry 2006).

Table 1. Isotope abundance of most common stable isotopes utilized in ecological studies (Fry 2006).

	Isotope Abundance (%)			
Element	**Low Mass**		**High Mass**	
Hydrogen	^{1}H	99.984	^{2}H	0.016
Carbon	^{12}C	98.89	^{13}C	1.11
Nitrogen	^{14}N	99.64	^{15}N	0.36
Oxygen	^{16}O	99.76	^{18}O	0.24
Sulfur	^{32}S	95.02	^{34}S	4.98

Stable isotopes are commonly measured through isotope ratio mass spectrometry (IRMS) in which organic and inorganic compound are transformed in gases (such as H_2, CO_2, N_2 and SO_2), permitting measurement of the isotope ratios of H, C, N, O and S. The instrument consists of four important components:

1. an inlet system containing capillary tubes, to avoid isotopes separation and so fractionation.
2. an ion source.
3. a mass analyzer.
4. an ion detector.

The pure gas is introduced at an inlet at one end of a flight tube and then it is ionized, accelerated, and deflected along the flight tube by a powerful magnet. Because of different mass-to-charge ratios, light and heavy isotopes will be deflected differently and sorted by the magnetic field. At the end, a series of collectors, which are positioned to capture the charged ions of different mass, convert the ionic impacts into a voltage, and then to a frequency. This voltage is then sent to a computer system that converts the signal to a ratio. It is important that the mass spectrometer has a good vacuum system to avoid external contamination (Michener and Lajtha 2008; Ehleringer and Osmond 2011).

1.5.1 Isotope measurement: the δ notation

The δ notation is the common value used to express isotope composition. It denotes a difference between the analysis on a sample and the isotope composition of an international standard, taken as reference (Tab. 2). δ value is calculated as follow:

$$\delta^{H}X = [(R_{SAMPLE}/R_{STANDARD} - 1)] * 1000$$

where "X" represents the element of interest (C, H, O, N or S), the superscript "H" refers to the heavy stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O, or ^{34}S) and "R" is the ratio between the heavy and light isotope ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, or $^{34}S/^{32}S$). The final multiplication by 1000 allow the amplification even of small differences and, for this reason, the δ value is expressed as "permil" (‰) (Hayes 2004).

Table 2. Isotope compositions of international reference standards .H and L indicate heavy and light isotope components, respectively (Fry 2006).

Standard	Ratio H/L	Value H/L	% H	% L
Standard Mean Ocean Water (SMOW)	$^{2}H/^{1}H$	0.0001558	0.015574	99.984426
	$^{17}O/^{16}O$	0.0003799	0.0379	99.76206
	$^{18}O/^{16}O$	0.0020052	0.20004	99.76206
PeeDee Belemnite (PDB) and Vienna PDV (VPDB)	$^{13}C/^{12}C$	0.01118	1.1056	98.8944
	$^{17}O/^{16}O$	0.0003859	0.0385	99.7553
	$^{18}O/^{16}O$	0.0020672	0.2062	99.7553
Air (AIR)	$^{15}N/^{14}N$	0.0036765	0.3663	99.6337
Canyon Diablo Troilite (CDB) and Vienna Canyon Diablo Troilite	$^{33}S/^{32}S$	0.0078772	0.74865	95.03957
	$^{34}S/^{32}S$	0.0441626	4.19719	95.03957
	$^{36}S/^{32}S$	0.0001533	0.01459	95.03957

After the IRMS analysis and computer calculation, it will be possible to obtain three different scenarios:

- A δ value of 0‰, which means that the sample has the same isotope composition of the international standard.

- A δ value greater than 0‰ signifies that the sample is more enriched in heavy stable isotopes (or more depleted in light stable isotopes) than the international standard.
- A δ value less than 0‰ signifies that the sample is more enriched in light stable isotopes (or more depleted in heavy stable isotopes) than the international standard (Hoefs 2009).

There are also other notations used in isotope studies, like fractional abundance (F), atom percentage (AP) or isotope ratio (R). However, the δ notation represents the most convenient because generates very small errors during measurement and calculation (Fry 2006).

1.6 Sulfur stable isotopes in plants

S has five natural isotopes: ^{32}S (≈ 95.02%), ^{33}S (≈ 0.75%), ^{34}S (≈ 4.21%) and ^{36}S (≈ 0.02%) are stable, while ^{35}S is present only in traces and it is radioactive. Since 1970, ^{35}S has been involved in labelling experiment to study S metabolism but nowadays there is a great interest in using stable isotopes as natural tracers (Tcherkez and Tea 2013). Given the higher natural amount, ^{32}S and ^{34}S are considered as the most useful, but it is necessary to underline how ^{33}S is mostly involved in NMR studies (Hobo *et al.* 2010).

S isotopes natural abundance is expressed as $\delta^{34}S$, and the value is relative to the international standard Vienna Canyon Diablo Troilite (VCDT). In EA-IRMS analysis, isotope ratio mass spectrometry coupled with an elemental analyzer, S organic compounds of a sample are converted in sulfur dioxide (SO_2) and then analyzed with a regard for the masses 64 ($^{32}S^{16}O_2$) and 66 ($^{34}S^{16}O_2$ or $^{32}S^{16}O^{18}O$) amu (Grassineau 1998). There is some disparity in $\delta^{34}S$ signature among Earth and its geological material: in fact, because of isotope effect, heavy stable isotopes tend to accumulate where bonds are more stable, and products are more oxidized than the substrate. For example, sedimentary sulfides and S in marine sediments are enriched in ^{32}S (≈ -50 and +10‰) because of the isotope effect caused by bacterial reduction. On the other side, to counterbalance this ^{34}S depletion, evaporites (≈ +5 and +40‰) and seawater sulfate (≈ +20‰) are enriched in heavy stable isotopes (Thode 1994). Chemical reactions which discriminate between light and heavy isotope occur also in plants: in fact, even if the

total biomass has generally a $\delta^{34}S$ signature similar to the one of the source, most of irreversible reactions during metabolism favor ^{32}S and this isotope fractionation can be useful to understand S systemic fluxes and differences among different S containing metabolites and plant organs (Tcherkez and Tea 2013). Nowadays there are scarce information about organ specific signature: a model study on *Triticum aestivum* (Fig. 3) have showed that SO_4^{2-} in roots and stems is more depleted in heavy isotopes, while the one in leaves and grains is more enriched in heavy isotopes than the source given at the beginning of the experiment. The abundance of ^{34}S in leaves is caused by a natural enrichment in S arriving at sink organs, and this can also explain the isotope effect occurred between roots and leaves (Tea et al. 2003).

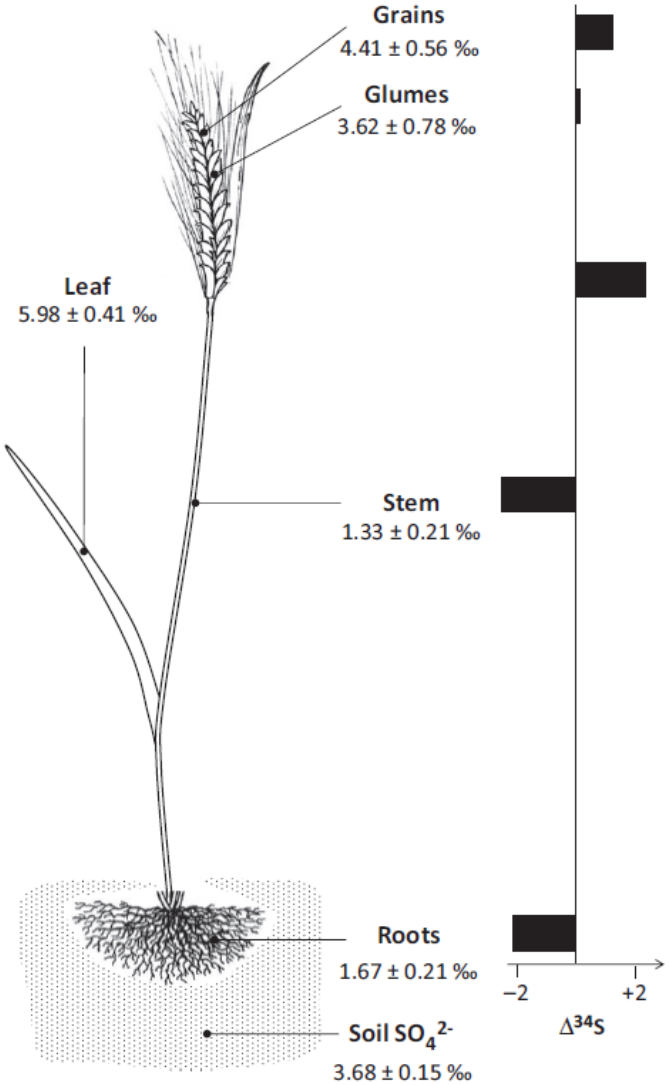

Figure 3. Sulfur natural isotope composition ($\delta^{34}S$) in *Triticum aestivum* (Tcherkez and Tea 2013).

Despite the small number of studies now available, it is possible to say that the $\delta^{34}S$ composition of plants does not reflect the one of the source, but it is dependent of fractionations that occur during absorption, translocation, and assimilation. These differences are useful to understand S metabolism and partitioning, and to obtain an integrated model of S incorporation in plants which will be important for studies on S use efficiency under different environmental condition.

2. Aim of the work

The general aim of the present PhD thesis is to describe S systemic fluxes in rice, to obtain more information about the contribute of S nutrition in determining plant tolerance under different environmental conditions. In particular, the work is articulated in three parts:

a. S isotope fractionation which occurs during sulfate acquisition in rice has been analyzed in a closed system (hydroponic condition) in which a certain amount of substrate (i.e., SO_4^{2-} in the solution) is progressively converted in a final product (i.e., total S in the plants). Moreover, isotope fractionation occurred during sulfate assimilation and partitioning was evaluated in plant organs (shoot and root) under different S nutrition (steady-state and sulfur starvation).

b. S isotope composition of rice plants exposed to different concentration of cadmium has been investigated, to validate the results previously obtained and to gain more information about a probable change in fractionation caused by the different S metabolism due to this abiotic stress.

c. The contribution of S acquisition and metabolism in determining different tolerance to salt stress in a *japonica* inbred lines (O11) have been analyzed.

3. Sulfur stable isotope discrimination in rice: a S isotope mass balance study

3.1 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 acids cysteine and methionine, short peptides, vitamins and co-factors, 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. Sulfate is taken up by roots and allocated to various sink tissues, where it is stored in the cell vacuoles or assimilated into S organic 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 is 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 sulfate groups for a variety of sulfation reactions catalyzed by sulfotransferases (Günal *et al*. 2019).

Sulfur has four stable isotopes: ^{32}S, ^{33}S, ^{34}S, and ^{36}S; their percentage abundances are 0.9499, 0.0075, 0.0425, and 0.0001 atom fraction, respectively (De Laeter *et al*. 2003). Mass differences among 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 – ^{32}S and ^{34}S – are now commonly measured using elemental analyzers coupled with isotope ratio mass spectrometers, and S isotope abundance is generally expressed in terms of $^{34}S/^{32}S$ abundance ratio, using the standard $\delta^{34}S$ notation:

$$\delta^{34}S = [(^{34}S/^{32}S)_{sample}/(^{34}S/^{32}S)_{VCDT} - 1] * 1000$$

which expresses the parts per thousand deviation of the isotope ratio $^{34}S/^{32}S$ of a sample relative to an international standard, the Vienna Canyon Diablo Troilite (VCDT; Coplen and Krouse 1998).

Unlike what has happened with carbon and nitrogen, 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 because of 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 – a fractionation – at natural abundance, providing crucial insights in the understanding of S metabolic fluxes inside the plants, without the need for costly labeling experiments with radioactive ^{35}S (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 little S isotope discrimination occurs during SO_4^{2-} uptake since the isotope composition measured for plant total S 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). On the other hand, little 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 remaining SO_4^{2-} in the heavier ^{34}S isotope (Thode *et al*. 1949; Kemp and Thode 1968).

This study aimed at dissecting the $^{32}S/^{34}S$ isotope effects occurring during SO_4^{2-} uptake, allocation, and metabolism in rice plants. For these purposes, dynamic changes in the S

isotope composition of different S pools were analyzed using mass balance approaches applied to various S exchanging systems.

3.2 Materials and methods

3.2.1 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 Milli-Q water, and finally sown on filter paper saturated with Milli-Q water to be incubated in the dark at 26 °C. Seven days later, 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 $(NH_4)_6Mo_7O_{24}$, 30 µM Na_2O_3Si (pH 6.5). Seedlings were kept for a 14-day pre-growing period in a growth chamber maintained at 26 °C and 80% relative humidity during the 16h light period and 22 °C and 70% relative humidity during the 8h dark period. Photosynthetic photon flux density was 400 µmol m^{-2} s^{-1}. Nutrient solutions were renewed twice 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 Milli-Q water. Plants were then transferred into fresh solutions and used in two distinct experimental setups (A and B). Part of the plants were sampled, frozen in liquid N_2 and stored at −80 °C for further analysis.

3.2.2 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 to 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 into 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 72h by renewing the growing media daily.

In both the experimental setups, before sampling, plant roots were washed for 30 min in 3 L of Milli-Q water to remove the not absorbed SO_4^{2-} 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.

3.2.3 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, in order to collect, with a micropipette, the xylem sap exuded from the lower cut during a 90 min period.

3.2.4 Preparation of samples for S isotope analysis and quantitative determination of the S pools

For total S analysis (S_{tot}), plant samples of 5 g (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-}. Samples were then clarified with 1 mL 33% (w/v) H_2O_2, and finally dried at 80 °C. The mineralized material was dissolved in 50 mL of water and then brought to pH 2.0 with 6 N HCl.

Sulfate was extracted from roots and shoots by homogenizing samples of 5 g (FW) in 50 mL of water. After heating at 80 °C for 40 min, the extracts were filtered, and then brought to pH 2.0 with 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 water to a final volume of 50 mL, filtered and then brought to pH 2.0 with 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 twice in 2 mL of water, dried at 80°C, and used for the S isotope analyses.

The amount of the S organic pool (S_{org}) of both root and shoot were estimated as follows:

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

3.2.5 S 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 Isotope Ratio Mass Spectrometer (IRMS), 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 1020 °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 GC separation column was 90 °C. The SO_2 reference gas pulse was introduced three times (20 s each) at the beginning of each run. 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 IAEA: IAEA-S-1 ($\delta^{34}S$ = -0.30 ± 0.03‰); IAEA-S-2 ($\delta^{34}S$ = 22.62 ± 0.08 ‰); IAEA-S-3 ($\delta^{34}S$ = -32.49 ± 0.08‰). Two in-house standards were used for normalization and quality assurance.

The data are reported in $\delta^{34}S$ notation, standardized to the Vienna-Canyon Diablo Troilite international reference scale (VCDT):

$$\delta^{34}S = [(^{34}S/^{32}S)_{sample}/(^{34}S/^{32}S)_{VCDT} - 1] * 1000$$

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

The isotope composition of the S_{org} ($\delta^{34}S$-S_{tot}) was estimated by imposing the following mass balance:

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

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

Fractionation factors ($\Delta_{L/H}$) – in positive permil (‰) 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, according to Fry (2006). For these purposes the following equation was used:

$$\delta^{34}S\text{-}SO_4^{2-}{}_{res} = \delta^{34}S\text{-}SO_4^{2-}{}_{source} - \Delta_{L/H} \ln(f)$$

where *f* is the fraction of SO_4^{2-} remaining in the hydroponic solution.

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:

$$\delta^{34}S\text{-}S_{ist} = \delta^{34}S\text{-}SO_4^{2-}{}_{sorce} - \Delta_{L/H} [1+ \ln(f)]$$

3.2.6 RNA extraction and qRT-PCR analysis

Total RNA was extracted from rice roots using Trizol Reagent (Invitrogen) and then purified using PureLink® RNA Mini Kit (Invitrogen), according to the manufacturer's instructions. Contaminant DNA was removed on-column using PureLink® Dnase (Invitrogen). First-strand cDNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen), according to the manufacturer's instructions.

qRT-PCR analysis of Os*SULTR1;1* (LOC_Os03g09970) and Os*SULTR1;2* (LOC_Os03g09980) 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 $2^{-\Delta Ct}$ method using the expression of the Os*S16* (LOC_Os11g03400) gene as reference. Primers for qRT-PCR are listed in Supplementary Table Tab. S1.

3.2.7 Statistical analysis

Quantitative values are presented as mean ± standard error of the mean (SE) of three independent experiments run in duplicate (n = 3). In each independent experiment, two distinct 3 L tanks were used for each condition analyzed. ANOVA was carried out using SigmaPlot for Windows version 11.0 (Systat Software, Inc., San Jose, CA, USA).

Significance values were adjusted for multiple comparisons using the Bonferroni correction.

Student's *t*-test was used to assess the significance of the observed differences between the values measured in root and shoot.

3.3 Results

3.3.1 S isotope mass balance in a closed system

Potential $^{32}S/^{34}S$ isotope effects occurring during SO_4^{2-} uptake were investigated by setting up a closed hydroponic system (Fig. 1a) in which a limited 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 in a final product (i.e., the total S of the plant; S_{tot}). Using this system, we performed serial sacrifice experiments in which plant growth was terminated every 24h (starting from the third day) for S isotope analyses of both substrates and products.

During the experimental period (264h): i) plants removed 98% of the SO_4^{2-} initially present in the nutrient solution (Fig. 1c); ii) SO_4^{2-} absorbed was quantitatively recovered in the plants as S_{tot} (Fig. 1d); iii) no significant losses of S occurred during the growth (Fig. 1e). The S_{tot} concentration of the plants ranged from 121.2 (at the beginning of the experiment) to 98.6 μmol g^{-1} DW (at the end of the experiment), whilst the SO_4^{2-} concentration in the nutrient solution ranged from 100 to 6.5 μM, indicating that regulation of plant S homeostasis occurred during SO_4^{2-} absorption (Fig. 1f).

Figure 2 reports isotope data as a function of the fraction of SO_4^{2-} remaining in the nutrient solution (*f*). The $\delta^{34}S$ of both residual SO_4^{2-} and plant S_{tot} changed over time, tending toward higher values as *f* decreased. The $\delta^{34}S$-SO_4^{2-} values increased from a minimum of -1.92‰ (at the beginning of the experiment) to a maximum of -0.21‰ (at the final sampling). On the other hand, 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 ± 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 – because of mass balance – 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; 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 which 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 SO_4^{2-} profile in the nutrient solution showed a marked deviation from a typical Rayleigh enrichment (R^2 = 0.79), because of an unexpected data point distribution at the final steps of the experiment (*f* ≤ 0.21). Considering the Rayleigh fractionation model, it was possible to calculate a single fractionation factor, $\Delta_{L/H}$ = 0.48 ± 0.09‰, which describes an average of the net fractionation along the overall trajectory. 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 ≤ *f* ≤ 0.31) a significant isotope fractionation against ^{34}S-SO_4^{2-} took place ($\Delta_{1(L/H)}$ = 1.09‰), whilst in the second phase (*f* ≤ 0.21) a less pronounced isotope effect ($\Delta_{2(L/H)}$ = 0.16‰) was associated to SO_4^{2-} uptake. Figure 2 also reports the calculated trajectories of the $\delta^{34}S$ values of the instantaneous product (S_{ist}) that forms – inside the plants – instant by instant in time from the external SO_4^{2-} because of SO_4^{2-} uptake; such a product is always offset in 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 was practically independent of *f*, as can be easily observed by comparing the isotope signatures of 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*, the main rice genes reasonably involved in SO_4^{-2} uptake (Godwin *et al*. 2003; Kumar *et al.* 2011). 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 ≤ *f* ≤ 0.31), whilst the *OsSULTR1;1* transcript level rapidly increased in the second phase (*f* ≤ 0.21), when the SO_4^{2-} concentration in the nutrient solution became limiting for plant growth ($[SO_4^{2-}]$ ≤ 37 µM; Fig. 2b).

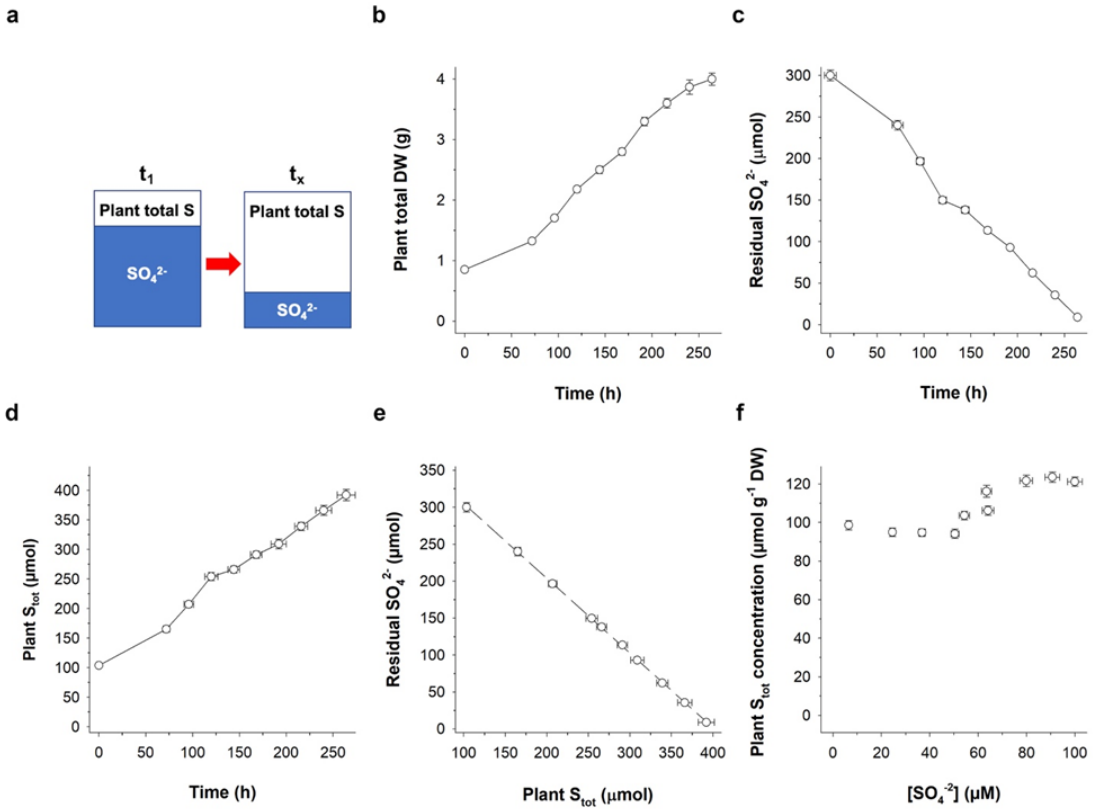

Figure 1. Time course of S partitioning in the closed hydroponic system. (**a**) Experimental setup: a limited amount of SO_4^{2-} is continuously removed from the hydroponic solution and converted into the total S of the plant. (**b**) Plant total biomass accumulation. (**c**) Residual SO_4^{2-} in the hydroponic solution over time. (**d**) Plant total S accumulation over time. (**e**) Residual SO_4^{2-} in the hydroponic solution vs. plant total S accumulation. (**f**) Plant S_{tot} concentration vs. SO_4^{2-} concentration in the hydroponic solution. Data are means ± SE of 3 independent experiments run in duplicate (*n* = 3).

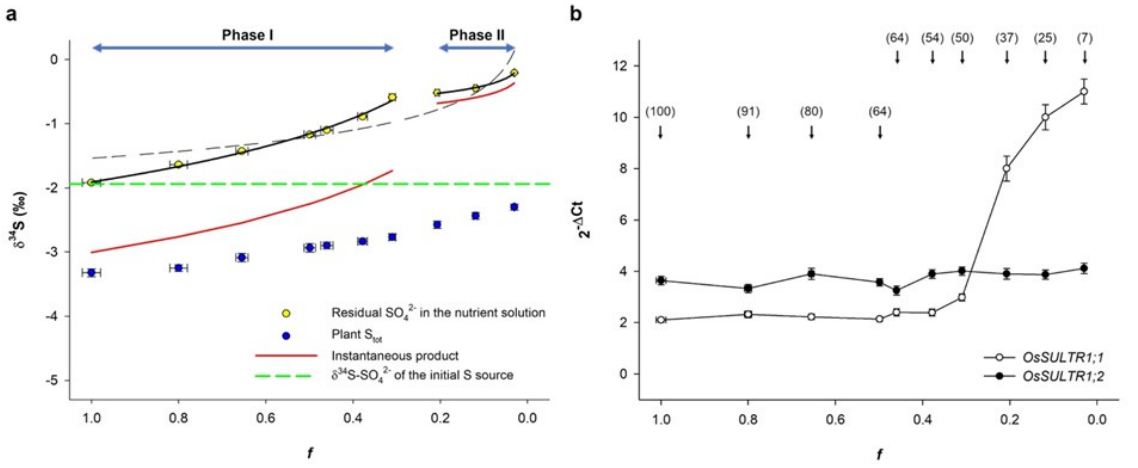

Figure 2. S isotope dynamic in the closed system and transcriptional analysis of *OsSULTR1;1* and *OsSULTR1;2*. (**a**) S isotope dynamic as a function of the fraction (*f*) of SO_4^{2-} remaining in the hydroponic solution. Black dashed line is Rayleigh curve calculated over the entire *f* interval, 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. 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 3 independent experiments run in duplicate (*n* = 3).

3.3.2 S isotope mass balance in a whole plant: steady state vs. S starvation

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 solutions and then continuously maintained on media containing SO_4^{2-} or deprived of SO_4^{2-} for 72h (experimental setup B). Nutrient solutions were changed daily to minimize changes in the isotope signature of the S source (-1,92 ± 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 72h) in both of the growing conditions (Fig. 3). At the beginning of the experiment (0h), the S_{tot} of the whole plants was significantly depleted in ^{34}S by -1.40 ± 0.08‰ relative to the S source (Fig. 3). Moreover, the analysis of organ-specific $\delta^{34}S$-S_{tot}

values indicated that the S_{tot} of both root and shoot was depleted in ^{34}S by -1.94 ± 0.08‰ and -1.09 ± 0.09‰, respectively, relative to the same S source (Tab. 1).

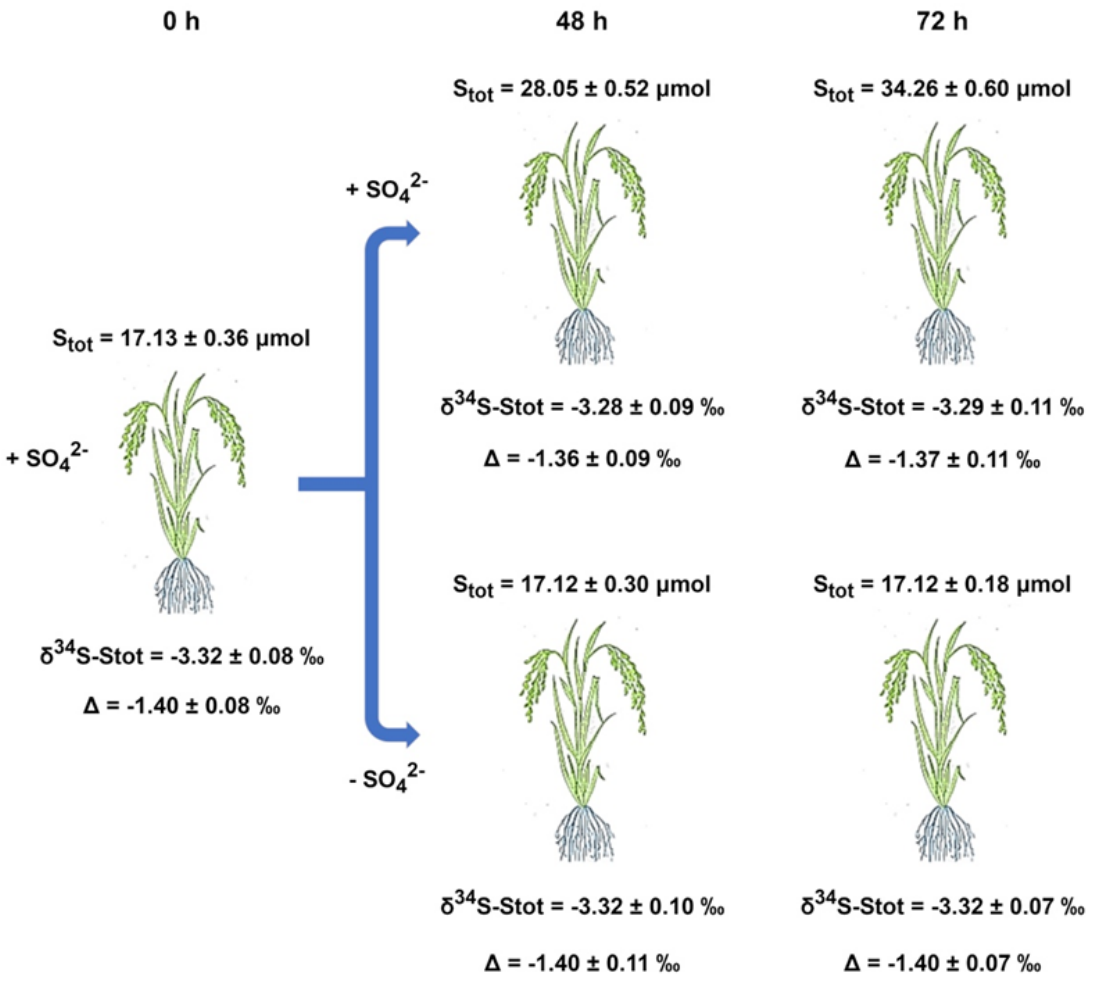

Figure 3. Illustration of the experimental setup used to study the $^{32}S/^{34}S$ isotope effects associated with S partitioning and metabolism in rice plants. Plants were pre-grown in complete nutrient solutions and then continuously maintained on media containing SO_4^{2-} or deprived of SO_4^{2-}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

Table 1. S isotope composition of the main S pools in root, shoot and xylem sap of rice plants grown in the presence of SO_4^{2-} in the hydroponic solution.

	Sample	Time (h)					
		0		48		72	
		$\delta^{34}S$ (‰)	Δ (‰)	$\delta^{34}S$ (‰)	Δ (‰)	$\delta^{34}S$ (‰)	Δ (‰)
S_{tot}	Root	-3.86 ± 0.08 ^{a}	-1.94 ± 0.08 ^{a}	-3.76 ± 0.09 ^{a}	-1.84 ± 0.09 ^{a}	-3.79 ± 0.08 ^{a}	-1.87 ± 0.08 ^{a}
	Shoot	-3.01 ± 0.09 ^{a*}	-1.09 ± 0.09 ^{a*}	-2.99 ± 0.07 ^{a*}	-1.07 ± 0.08 ^{a*}	-2.99 ± 0.06 ^{a*}	-1.07 ± 0.07 ^{a*}
SO_4^{2-}	Root	-2.51 ± 0.05 ^{a}	-0.59 ± 0.06 ^{a}	-2.49 ± 0.05 ^{a}	-0.57 ± 0.05 ^{a}	-2.48 ± 0.07 ^{a}	-0.56 ± 0.08 ^{a}
	Shoot	-0.71 ± 0.02 ^{a**}	1.21 ± 0.03 ^{a**}	-0.69 ± 0.02 ^{a**}	1.23 ± 0.03 ^{a**}	-0.68 ± 0.02 ^{a**}	1.24 ± 0.03 ^{a**}
S_{org}	Root	-5.20 ± 0.10 ^{a}	-3.28 ± 0.10 ^{a}	-5.19 ± 0.07 ^{a}	-3.27 ± 0.07 ^{a}	-5.15 ± 0.07 ^{a}	-3.23 ± 0.07 ^{a}
	Shoot	-4.55 ± 0.09 ^{a*}	-2.63 ± 0.09 ^{a*}	-4.53 ± 0.06 ^{a*}	-2.61 ± 0.06 ^{a*}	-4.46 ± 0.05 ^{a*}	-2.54 ± 0.06 ^{a**}
SO_4^{2-}	Xylem sap	-2.47 ± 0.09 ^{a}		-2.53 ± 0.07 ^{a}		-2.56 ± 0.06 ^{a}	

Δ indicates changes in ^{34}S relative to the S source ($\delta^{34}S\text{-}SO_4^{2-}{}_{source}$ = -1.92 ± 0.02‰). Data are means and SE of three independent experiments run in duplicate (*n* = 3). Asterisks indicate significant differences (Student's *t*-test; *0.001 ≤ *P* < 0.05; **P* < 0.001) between root and shoot of plants sampled at the same time. Different letters indicate significant differences among the samples at different times (*P* < 0.05).

Plants maintained in media containing SO_4^{2-} grew linearly in the observation period (Fig. 4a). As expected, the concentrations of SO_4^{2-}, S_{tot}, and S organic (S_{org}) did not significantly change in both root and shoot over time (Fig. 4b and c). The invariance of each S pool was associated with the invariance of their isotope signatures, indicating that plants reached a metabolic and isotope steady-state (Tab. 1). In particular: i) the S_{tot} of both root and shoot were significantly ^{34}S depleted with respect to the S source; ii) the $\delta^{34}S$-S_{tot} values were significantly lower in the root than in the shoot in all the conditions analyzed (Tab. 1). In the root, the SO_4^{2-} pools were significantly (*P* < 0.001) ^{34}S depleted relative to the S source, whilst in the shoot, they were significantly (*P* < 0.001) ^{34}S enriched relative to the same S source. 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. Finally, no differences were found on comparing the $\delta^{34}S\text{-}SO_4^{2-}$ values of the SO_4^{2-} pools in the xylem sap and in the root cells.

On the other hand, SO_4^{2-} deprived plants dynamically allocated S previously absorbed during the preliminary growth phase, preserving both the overall S isotope signature (Fig. 3) and the total amount of S_{tot} over time (Fig. 4). However, because of both continuous growth (Fig. 4d) and S allocation processes, the S_{tot} concentration in rice organs changed over time, decreasing linearly in both root (R^2 = 0.993; Fig. 4e) and shoot (R^2 = 0.999; Fig. 4f). The SO_4^{2-} concentration sharply decreased over time in both root and shoot due to SO_4^{2-} assimilation (Fig. 4e and f). In fact, the concentration of the S_{org} in the root slightly decreased over time from 7.26 $\pm$ 0.41 to 4.36 $\pm$ 0.14 μmol g^{-1} FW, whilst in the shoot it remained relatively constant. The S_{tot} isotope composition of both root and shoot did not change over time (Tab. 2) 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 (Tab. 2). 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 72h were 2.70 $\pm$ 0.05‰ and 6.71 $\pm$ 0.19‰ for root and shoot, respectively. The S_{org} pools of both roots and shoots 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 48h and then remained constant at 72h, whilst in the shoot a significant increase was observed when moving from 48 to 72h.

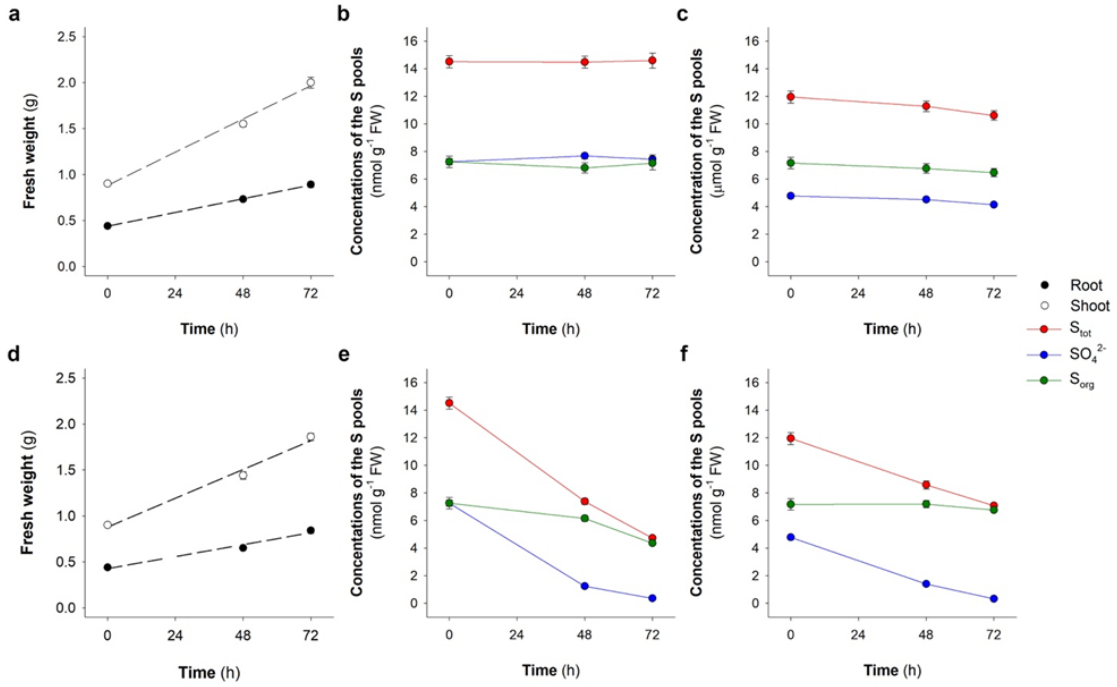

Figure 4. Changes in the concentration of the S pools (S_{tot}, SO_4^{2-} and S_{org}) in root and shoot of rice plants grown in the presence or in the absence of SO_4^{2-} in the hydroponic solution. (**a**) Root and shoot fresh weight in the presence of SO_4^{2-}. (**b**) S_{tot}, SO_4^{2-} and S_{org} in root of plants grown in the presence of SO_4^{2-}. (**c**) S_{tot}, SO_4^{2-} and S_{org} in shoot of plants grown in the presence of SO_4^{2-}. (**d**) Root and shoot fresh weight in the absence of SO_4^{2-}. (**e**) S_{tot}, SO_4^{2-} and S_{org} in root of plants grown in the absence of SO_4^{2-}. (**f**) S_{tot}, SO_4^{2-} and S_{org} in shoot of plants grown in the absence of SO_4^{2-}. Data are means ± SE of 3 independent experiments run in duplicate (*n* = 3).

Table 2. S isotope composition of the main S pools in root, shoot and xylem sap of rice plants grown in the absence of SO_4^{2-} in the hydroponic solution.

	Sample	Time (h)					
		0		48		72	
		$\delta^{34}S$ (‰)	Δ (‰)	$\delta^{34}S$ (‰)	Δ (‰)	$\delta^{34}S$ (‰)	Δ (‰)
S_{tot}	Root	-3.86 ± 0.08 ^{a}	-1.94 ± 0.08 ^{a}	-3.83 ± 0.08 ^{a}	-1.91 ± 0.08 ^{a}	-3.87 ± 0.09 ^{a}	-1.95 ± 0.09 ^{a}
	Shoot	-3.01 ± 0.09 ^{a*}	-1.09 ± 0.09 ^{a*}	-3.12 ± 0.07 ^{a*}	-1.20 ± 0.07 ^{a*}	-3.16 ± 0.07 ^{a*}	-1.24 ± 0.07 ^{a**}
SO_4^{2-}	Root	-2.51 ± 0.05 ^{c}	-0.59 ± 0.06 ^{c}	-1.50 ± 0.03 ^{b}	0.42 ± 0.04 ^{b}	0.19 ± 0.01 ^{a}	2.11 ± 0.03 ^{a}
	Shoot	-0.71 ± 0.02 ^{c**}	1.21 ± 0.03 ^{c**}	3.50 ± 0.07 ^{b**}	5.42 ± 0.08 ^{b**}	6.00 ± 0.19 ^{a**}	7.92 ± 0.19 ^{a**}
S_{org}	Root	-5.20 ± 0.10 ^{b}	-3.28 ± 0.10 ^{b}	-4.30 ± 0.14 ^{a}	-2.38 ± 0.14 ^{a}	-4.20 ± 0.2 ^{a}	-2.28 ± 0.21 ^{a}
	Shoot	-4.55 ± 0.09 ^{b*}	-2.63 ± 0.09 ^{b*}	-4.40 ± 0.15 ^{b}	-2.48 ± 0.15 ^{b}	-3.60 ± 0.21 ^{a}	-1.68 ± 0.21 ^{a}

Δ indicates changes in ^{34}S relative to the S source ($\delta^{34}S\text{-}SO_4^{2-}{}_{source}$ = -1.92 ± 0.02‰). Data are means and SE of three independent experiments run in duplicate (*n* = 3). Asterisks indicate

significant differences (Student's *t*-test; *0.001 ≤ *P* <0.05; **$P < 0.001$) between root and shoot of plants sampled at the same time. Different letters indicate significant differences among the samples at different times ($P < 0.05$).

3.4 Discussion

It is generally assumed that terrestrial plants assimilate S from the soil (SO_4^{2-}) and from the atmosphere (SO_2) with little 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 & 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 a 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 S source (SO_4^{2-}) initially present in a hydroponic solution (Fig. 1a). In such a model, 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 an isotope discrimination against ^{34}S occurred during SO_4^{2-} uptake, which resulted in transient lighter S isotope compositions of the plants and in concomitant ^{34}S enrichments of the residual SO_4^{2-} in the hydroponic solution (Fig. 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 (Fig. 1f and 2b). The maximum fractionation observed was associated with *f* values ranging from 1 to 0.31 (corresponding to external sulfate 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 (≤ 37µM) and was potentially able to induce an array of S-deficiency physiological responses (Maruyama-Nakashita *et al.* 2003), including changing in the expression of the root high-affinity SO_4^{2-} transports, *OsSULTR1;1* and *OsSULTR1;2*, involved in SO_4^{2-} uptake (Fig. 2b). Although a certain degree of functional redundance may exist, *OsSULTR1;2* is considered the major gene involved in SO_4^{2-}

uptake under normal conditions, whilst *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.

Little 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 among organs potentially caused by S reduction and assimilation. Most of the SO_4^{-2} ions 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 continuous exchanges of SO_4^{2-} and S organic compounds occur in a shoot-to-root direction, in order to ensure the S homeostasis of the root (Cooper & 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: i) preventing possible perturbations due to continuous changes of the $\delta^{34}S\text{-}SO_4^{2-}$ values of the external solution; ii) obtaining rice plants with the same overall S isotope composition (Fig. 3). In these experiment plants can be considered as systems continuously supplied by an S source which does not change in both concentration and isotope composition.

As previously described plants continuously grown in the presence of SO_4^{2-} reached metabolic and S isotope steady-states. 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 concentration (i.e., $\delta^{34}S\text{-}SO_4^{2-} > \delta^{34}S_{source} - \Delta_{1(L/H)}$). Interestingly, SO_4^{2-} translocation from root to shoot did not seem to discriminate the S isotopes, since no differences were found when comparing in the isotope signatures of the SO_4^{2-} ions in root and xylem sap. However, the SO_4^{2-} pools of the shoot were significantly ^{34}S depleted 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 heavier relative to the SO_4^{-2} coming from the root, we can reasonably suppose that a not negligible portion of both SO_4^{2-} and S_{org} of the shoot is translocated to the root. Thus, the isotope signature of the S pools of the root could be the result of mixing effects due to the overall isotope circulation and local S assimilation that account for the different S isotope composition of the S_{tot} in root and shoot (i.e., $\delta^{34}S\text{-}S_{tot}$ of the root < $\delta^{34}S\text{-}S_{tot}$ of the shoot).

Assuming that: i) the S isotope composition of the instantaneous SO_4^{2-} that enter the root cells should theoretically differ from the S source by the fractionation factor $\Delta_{1(L/H)}$; ii) the theoretical S isotope separation, Δ_{TR}, occurring during S assimilation in the root is the same as that observed in the shoot ($\delta^{34}S\text{-}SO_4^{2-}{}_S$ - $\delta^{34}S\text{-}S_{orgS}$ = -3.84‰), so that the theoretical isotope signature of the S_{org} produced during SO_4^{-2} assimilation in the root, $\delta^{34}S_{orgTR}$, differs by Δ_{TR} relative to the instantaneous SO_4^{-2}; iii) no fractionation occurs during S_{org} translocation from shoot to root, we can calculate the amount of the S organic compounds, $S_{orgStoR}$, coming from the shoot and accumulated into the root by imposing the following mass balance:

$$\delta^{34}S\text{-}S_{orgR}\ S_{orgR} = \delta^{34}S\text{-}S_{orgS}\ S_{orgStoR} + \delta^{34}S\text{-}S_{orgTR}\ (S_{orgR} - S_{orgStoR})$$

where, $\delta^{34}S\text{-}S_{orgR}$ is the steady state isotope composition of the S_{org} of the root, S_{orgR} is the total amount of the S_{org} measured in the root, $\delta^{34}S\text{-}S_{orgTR}$ is the theoretical isotope composition of the S_{org} fraction produced by the root. Solving the equation for the unknown $S_{orgStoR}$ reveals that under the S isotope steady-state about 71% of the S_{org} measured in the root is inherited from the shoot, indicating that only a small amount (about 29%) of the total S_{org} of the plant comes from the SO_4^{2-} ions directly assimilated into the root, according to the previous measurement performed in wheat by Larson *et al*. (2006), using a $^{35}S\text{-}SO_4^{2-}$ labelling approach. Finally, assuming that during the S isotope steady-state the $\delta^{34}S\text{-}SO_4^{2-}$ values measured in the root are mainly influenced by root SO_4^{2-} uptake and SO_4^{2-} translocation from shoot to root, we can also estimate the

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

$$\delta^{34}S\text{-}SO_4^{2-}{}_R \; SO_4^{2-}{}_R = \delta^{34}S\text{-}SO_4^{2-}{}_S \; SO_4^{2-}{}_{StoR} + \delta^{34}S\text{-}SO_4^{2-}{}_{TR} \; (SO_4^{2-}{}_R - SO_4^{2-}{}_{StoR})$$

where, $\delta^{34}S\text{-}SO_4^{2-}{}_R$ is the steady-state isotope composition of the SO_4^{2-} pool of the root, $SO_4^{2-}{}_R$ is the total amount of the SO_4^{2-} measured in the root, $\delta^{34}S\text{-}SO_4^{2-}{}_{TR}$ is the isotope composition of the SO_4^{2-} fraction coming from the shoot. Such an approach allows us to estimate that at least 79% of the steady-state SO_4^{2-} pool of the root come from SO_4^{2-} uptake, confirming shoot to root SO_4^{2-} translocation as an important activity in controlling root SO_4^{2-} homeostasis (Yoshimoto *et al*. 2003).

On the other hand, during the growing period in the absence of SO_4^{2-}, rice plants can be considered as a closed system 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 (Tab. 2), mainly caused by the $^{32}S/^{34}S$ isotope effects occurring during SO_4^{2-} assimilation. During the observation period plants rapidly consume 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 isotope mass balance carried out considering the overall SO_4^{2-} and S_{org} pools of the plants (i.e., root + shoot; Tab. 3), 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 which was closely dependent on the severity of the imposed S starvation, as indicated by calculated Δ values ($\Delta = \delta^{34}S\text{-}Sorg - \delta^{34}S\text{-}SO_4^{2-}$) that ranged from -3.29 $\pm$ 0.40 (at the beginning of the experiment) to -7.80 $\pm$ 0.18‰ (at 72h). 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.

Table 3. Amount and S isotope composition of the overall SO_4^{2-} and S_{org} pools of rice plants grown in the absence of SO_4^{2-} in the hydroponic solution.

	Time (h)					
	0		**48**		**72**	
	Amount (μmol)	**$\delta^{34}S$ (‰)**	**Amount (μmol)**	**$\delta^{34}S$ (‰)**	**Amount (μmol)**	**$\delta^{34}S$ (‰)**
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 and SE of three independent experiments run in duplicate (n = 3). Different letters indicate significant differences among the samples at different times ($P < 0.05$).

3.5 Conclusions 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 (i.e., SO_4^{2-} and S_{org}) of both root and shoot, mainly results from 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 $\delta^{34}S$ analysis to provide information for further detailed characterization of the metabolic and molecular processes involved in plant S homeostasis.

3.6 Supplementary material

Table S1. Sequences of primers (forward and reverse, 5'→3') of the housekeeping gene (*OsS16*) and of the *OsSULTR*s analyzed in the work. Primers were designed with the software Primer Express™ v. 3.0.1 (Thermo Fisher). Sequences of cDNA were found on "Rice Genome Annotation Project" website (http://rice.plantbiology.msu.edu/).

		Primer (5'-3')	
Gene	**Accession Number**	**Forward**	**Reverse**
OsS16	*LOC_Os11g03400*	ACGTCGACGAGGCATCCA	CGCGACCACCGAACTTCTT
OsSULTR1;1	*LOC_Os03g09970*	GGAGCATTCTTTGGCGTCAT	TCGCAACCGCAATTAGCA
OsSULTR1;2	*LOC_Os03g09980*	CGACCTTCTTTGCAGGAGTCA	TGAACCCTAGCCTGCAGAAAC

4. Sulfur stable isotope fractionation in rice under cadmium exposure

4.1 Introduction

More of 2.35 x 10^{12} m^2 of the worldwide arable lands are contaminated by heavy metals, among which cadmium (Cd) represents the one with higher mobility in plants and toxicity for human health (Bermudez *et al.* 2012; Song *et al*. 2015).

Rice (*Oryza sativa* L.) is one of the most important staple crops and its quality and yield are endangered by soil Cd contamination, thus also causing a major risk to human poisoning (Hu *et al*. 2009; Liu *et al*. 2016): this heavy metal is easily transferred from soil to plant, and it is accumulated in crop grains affecting food chain (Li *et al*. 2017).

Cd spreads in environment by anthropogenic activities. Its accumulation produces a wide range of phytotoxic effects on plants, such as nutrient imbalance, chlorosis, growth inhibition and excessive production of Reactive Oxygen Species (ROS), which causes peroxidation of membranes and damages to nucleic acids and proteins (Ebbs and Uchil 2008; Xu *et al*. 2012). Moreover, Cd toxicity also affects photosynthetic efficiencies, decreasing CO_2 assimilation and RuBisCO activity (Asgher *et al*. 2014). Cd can be easily absorbed by plant roots and translocated to different plant organs (stems, leaves and grains) through apoplastic and simplastic pathways (Lux *et al*. 2011). Several transporters mediate these processes such as ABC transporter, P-type ATPase, CAX family, LCT transporters, ZIP and CE family (Baig *et al*. 2019).

The first plant defenses against Cd injuries are based on exclusion or sequestration (Qureshi *et al*. 2010): 98% of total Cd in *Phaseolus vulgare* was retained in vacuoles and apoplast, while in *Arabidopsis thaliana* Cd sequestration was also observed in trichomes (Pielichowska and Wierzbicka 2004). It has been demonstrated in several species including rice, that Cd is mostly accumulated in root system: this initial “firewall” appeared to be of paramount importance in preserving the photosynthetic apparatus from Cd injury. Considering that xylem translocation is the main way determining Cd accumulation in grains, the storage capability of this heavy metal in roots determines the amount of free Cd available for translocation (Cakmak *et al*. 2000; Clarke *et al*. 2002; Grant *et al.* 2008; Uraguchi *et al.* 2009; Maghrebi *et al.* 2021).

During heavy metal exposure, phytochelatines (PCs) easily become the most abundant class of non-protein thiols in plants (Heiss *et al.* 1999; Zhu *et al*. 1999; Nocito *et al.* 2002, 2006, 2007). PCs are polypeptides synthesized starting from glutathione (GSH) and with general structure $(\gamma\text{-Glu-Cys})_n$-Gly, where n ranges from 2 to 11, mainly involved in Cd chelation in roots (Clemens 2006; Gill *et al*. 2011). Cd stress induces so the creation of reduced sulfur (S) additional sinks to sustains PCs biosynthesis, leading to a typical demand-driven coordinate upregulation of the genes involved in S-assimilation pathway, which allow to reach higher levels of Cysteine, precursor of GSH (Lee and Leustek 1998; Howarth *et al.* 2003a; Domínguez-Solís *et al.* 2004; Nocito *et al.* 2007). S is then an important element in this environmental condition and sulfate (SO_4^{2-}) uptake and translocation, accomplished by the *SULTR* gene family, must be finely modulated to sustain the increased activity of S reductive pathway during Cd detoxification (Takahashi *et al*. 2000; Nocito *et al*. 2006, 2007; Sarry *et al.* 2006; Akbudak *et al.* 2018).

As previously showed, S acquisition and metabolism in rice significantly discriminate against ^{34}S. That is to say that the exposure of rice plants to Cd could be a powerful tool to gain further information on the $^{32}S/^{34}S$ isotope effects occurring during S metabolism. So, the main aim of this work was to describe such hypothetical effects. To these purposes we performed a S stable isotope mass balance study in rice plant exposed for 72h to different Cd concentrations, considering the amount and the isotope composition of the main S pools of both root and shoot.

4.2 Materials and methods

4.2.1 Plant material and experimental set-up

Rice (*O. sativa* L. cv. Vialone Nano) caryopses were surfaced sterilized with 70% (v/v) ethanol for 30 s, washed three times with sterile distilled water and then sown on filter paper saturated with water to be incubated at dark for 7 days. Plantlets chosen for uniform growth were transferred into 3 L tanks (18 seedlings each) containing the following complete nutrient solution: 1.5 mM KNO_3, 500 µM $MgSO_4$, 250 µM $NH_4H_2PO_4$, 25 µM Fe tartrate, 46 µM H_3BO_3, 9 µM $MnCl_2$, 1 µM $ZnCl_2$, 0.3 µM $CuCl_2$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 30 µM Na_2Osi (pH 6.5). Plants pre-grown for 14 days in a controlled growth chamber maintained at a temperature of 26 °C and 80% relative humidity during the 16 hours of light, while 22 °C and 70% relative humidity during the 8 hours of dark. Photosynthetic photon flux density was 400 µmol m^{-2} s^{-1}. Nutrient solutions were renewed twice a week to minimize nutrient depletion. At the end of the pre-growing period, roots were washed for 30 min with 3 L of Milli-Q water and then plants were transferred to new complete nutrient solutions containing 0, 1, 10 and 25 µM $CdCl_2$. Plants growth under these condition for other 72h, renewing the nutrient solution every day. At the end of the three days, roots were washed for 30 min in 3 L of Milli-Q water containing 5 mM $CaCl_2$, to remove not absorbed sulfate from root apoplast and excess of Cd. After washing, plants were gently blotted with absorbent paper, roots were separated by shoots, and then frozen in liquid N_2 and stored at -80 °C to preserve them for further analysis.

4.2.2 Preparation of samples for quantitative determination of total sulfur, sulfate, Cd determination, and isotope analysis

For total S analysis (S_{tot}), plant 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 in SO_4^{2-}. Samples were then clarified with 1 mL 33% (w/v) H_2O_2, and finally dried at 80 °C. The mineralized material was dissolved in 50 mL of water and then brought to pH 2.0 with 6 N HCl.

Sulfate was extracted from roots and shoots by homogenizing samples of 5 g (FW) in 50 mL of water. After heating at 80 °C for 40 min, the extracts were filtered, and then brought to pH 2.0 with 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 residual SO_4^{2-} of each sample was 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 twice in 2 mL of distilled water, dried at 80 °C, and used for the S isotope analyses.

The amount of the S organic pool (S_{org}) of both root and shoot were estimated as follow:

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

For Cd determination 1 mL of every mineralized sample was diluted in 4 mL of 65% (v/v) HNO_3 and Cd content of roots and shoots was measured by inductively coupled plasma-mass spectrometry (ICP–MS; Bruker AURORA M90 ICP–MS).

4.2.3 S 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 Isotope Ratio Mass Spectrometer (IRMS), 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 1020 °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 GC separation column was 90 °C. The SO_2 reference gas pulse was introduced three times (20 s each) at the beginning of each run. 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 IAEA: IAEA-S-1 ($\delta^{34}S$ = -0.30 ± 0.03‰); IAEA-S-2 ($\delta^{34}S$ = 22.62 ± 0.08 ‰); IAEA-S-3 ($\delta^{34}S$ = -32.49 ± 0.08‰). Two in-house standards were used for normalization and quality assurance.

The data are reported in $\delta^{34}S$ notation, standardized to the Vienna-Canyon Diablo Troilite international reference scale (VCDT):

$$\delta^{34}S = [(^{34}S/^{32}S)_{sample}/(^{34}S/^{32}S)_{VCDT} -1] * 1000$$

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

The isotope composition of the S_{org} ($\delta^{34}S$-S_{tot}) was estimated by imposing the following mass balance:

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

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

4.2.4 RNA extraction and qRT-PCR analysis

Total RNA was extracted from 400 mg (fresh weight – FW) of rice roots and shoots using Trizol Reagent (Invitrogen) and then purified using PureLink® RNA Mini Kit (Invitrogen), according to the manufacturer's instructions. Contaminant DNA was removed on-column using PureLink® Dnase (Invitrogen). First-strand cDNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen), according to the manufacturer's instructions.

qRT-PCR analysis of *OsSULTR1;1* (LOC_Os3g09970), *OsSULTR1;2* (LOC_Os03g09980), *OsSULTR1;3* (LOC_Os08g31410), *OsSULTR2;1* (LOC_Os03g09930), and *OsSULTR2;2* (LOC_Os03g09930) 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 $2^{-\Delta Ct}$ method, using the expression of the *OsS16* (LOC_Os11g03400) gene as reference. Primers for qRT-PCR are listed in Supplementary table (Tab. S1).

4.2.5 Statistical analysis

Quantitative values are presented as mean ± standard error of the mean (SE) of three independent experiments run in duplicate (n = 3). In each independent experiment, two distinct 3 L tanks (18 plants each) were used for each condition analyzed. ANOVA was

carried out using SigmaPlot for Windows version 11.0 (Systat Software, Inc., San Jose, CA, USA). Significance values were adjusted for multiple comparisons using the Bonferroni correction. Student's *t*-test was used to assess the significance of the observed differences between the values measured in root and shoot.

4.3 Results

In this work we analyzed rice plants pre-grown in complete nutrient solutions and then exposed to different concentrations of Cd^{2+} (0, 1, 10 and 25 µM) for 72h. As described in the previous work, during the exposure period, nutrient solutions were changed daily to minimize the isotope effects due to S fractionation associated to SO_4^{2-} uptake.

4.3.1 Plants growth and Cd accumulation in root and shoot

Cd exposure significatively inhibited the growth of root and shoot as compared with the control (Fig. 1). The exposure to 1 and 10 µM Cd^{2+} resulted in a similar inhibition in both root (-16% and -27%, respectively) and shoot (-27% and -28%, respectively). Greatest decreases were observed in plants exposed to 25 µM Cd^{2+}: compared to the control, root was inhibited by -41%, while shoot by -42% (Fig. 1).

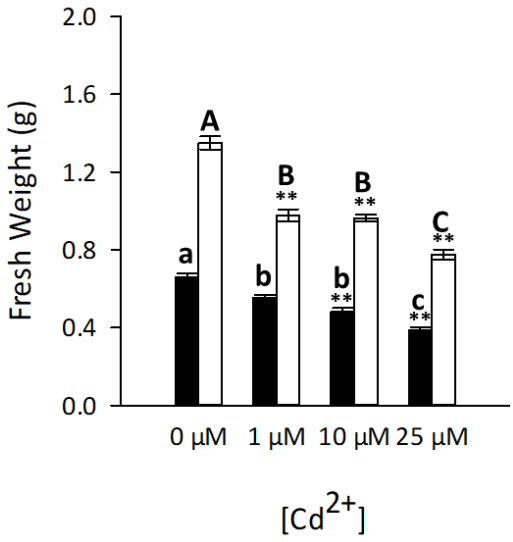

Figure 1. Growth of root (black columns) and shoot (white columns) of rice plants under exposure to different Cd concentration (0, 1, 10 and 25 µM) for 72h. Different letters indicate statistical differences among treatment ($P < 0.05$). Asterisks indicate statistical differences between treated and control condition with $P < 0.001$. Values are expressed as mean ± SE of 3 independent experiments run in duplicate ($n = 3$).

Cd concentration in root increased with the concentration in the growing media, ranging from 0.21 nmol g^{-1} FW (control) to 382.5 nmol g^{-1} FW (25 µM Cd^{2+}) (Fig. 2a). Conversely, Cd concentration in the shoot was lower than in the roots, representing the 9-13% of total Cd in plants, and, except for the control condition, it was not different among the other three different treatments (Fig. 2b).

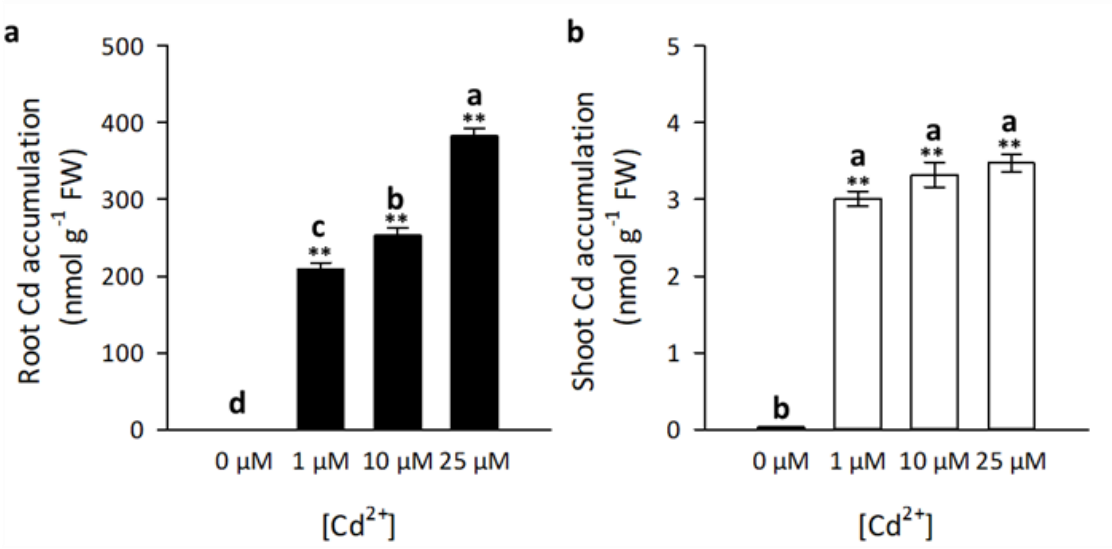

Figure 2. (**a**) Cd accumulation in root (black columns) and (**b**) Cd accumulation in shoot (white columns) of rice plants under exposure to different Cd concentration (0, 1, 10 and 25 µM) for 72h. Different letters indicate statistical differences among treatment ($P < 0.05$). Two asterisks indicate statistical differences between treated and control condition with a $P < 0.001$. Values are expressed as mean ± SE of 3 independent experiments run in duplicate ($n = 3$).

4.3.2 Effect of Cd on S isotope composition

Possible $^{32}S/^{34}S$ isotope effect due to Cd exposure and associated changes in S metabolism were evaluated, comparing the plants treated with different Cd^{2+} concentration (1, 10, 25 µM) for 72h with control non-treated plants (0 µM Cd^{2+}). During the entire experiment nutrient solutions were changed daily to minimize both nutrients depletion and changes in the isotope signature of the S source (-1.92 ± 0.02‰) caused by the fractionation which occurs during SO_4^{2-} uptake.

The isotope composition of the total S (S_{tot}) of the whole plants was significantly depleted in ^{34}S relative to the S source in all the conditions analyzed (Fig. 3). However, the depletion in ^{34}S significantly decreased as the Cd concentration in the growing medium increased. A strong effect of Cd on the amount of the S_{tot} was also observed (Fig. 3). Looking at the organ-specific isotope composition of S_{tot} (Tab. 1) it is possible to note that, while in root the $\delta^{34}S$-S_{tot} values remained constant under all the conditions, in shoot the ^{32}S enrichment progressively decreased as the concentration of the heavy metal increased, reaching values similar to the $\delta^{34}S$ of the S source.

Even if Cd treatments strongly inhibited plants growth, the concentration of SO_4^{2-}, S_{tot}, and organic S (S_{org}), increased in both root and shoot (Fig. 4a and b) as the concentration

of Cd in the growth media did. In particular, S_{org} increased linearly both in root and in shoot (R^2 of 0.973 and 0.976, respectively).

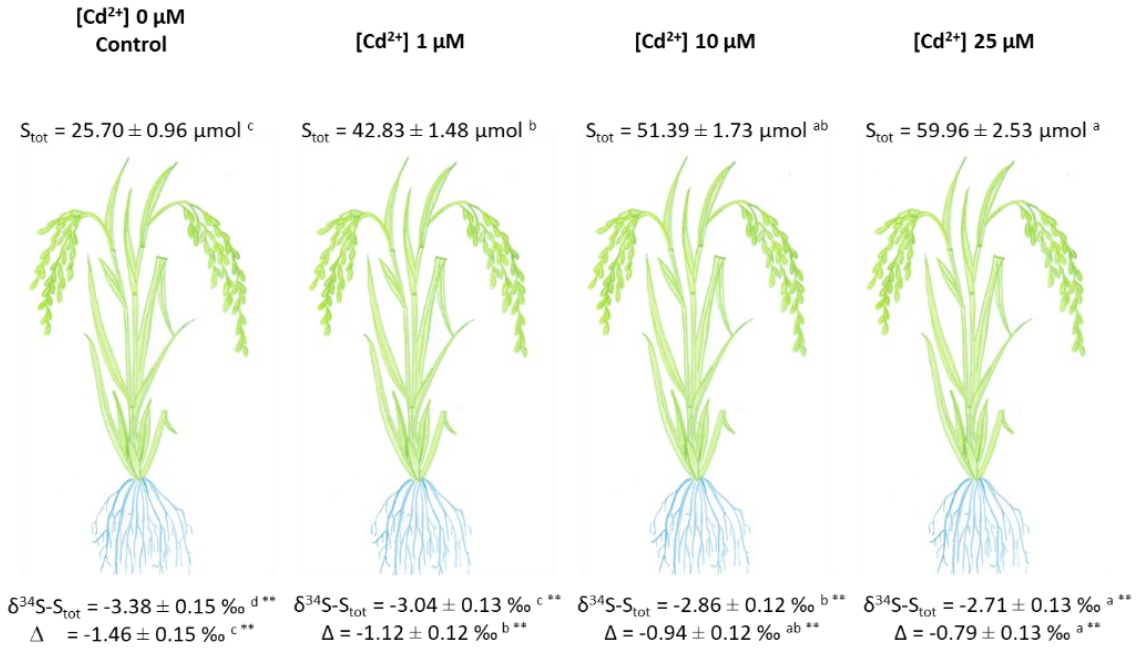

Figure 3. Illustration of the experimental setup used to study the $^{32}S/^{34}S$ isotope effects associated with different Cd treatments in rice plants. Plants were pre-grown in complete nutrient solutions and then exposed to different Cd concentration (0, 1, 10 and 25 µM) for 72h. S_{tot}, total S amount in a whole plant; $\delta^{34}S\text{-}S_{Stot}$, S isotope composition of the whole plant; Δ indicates changes in ^{34}S relative to the S source ($\delta^{34}S\text{-}SO_4^{2-}{}_{source}$ = -1.92 ± 0.02‰). Data are means and SE of 3 independent experiments run in duplicate (n = 3). Different letters indicate statistical differences among treatment (P < 0.05). Asterisks indicate significant differences (Student's t-test; *0.001 ≤ P <0.05; **P < 0.001) between plant and source signatures.

Concerning organ-specific isotope composition of the S pools (Tab. 1), it was possible to note that the sulfate pools in root were significantly depleted in ^{34}S compared to the shoot. $\delta^{34}S\text{-}SO_4^{2-}$ values of shoot were always significantly (P < 0.001) enriched in ^{34}S relative to the S source; in root system, $\delta^{34}S\text{-}SO_4^{2-}$ was depleted in ^{34}S compared to the same source only in control condition (Δ = -0.52 ± 0.06‰). In fact, Cd exposure resulted in a progressive ^{34}S enrichment of the SO_4^{2-} pool of the root with respect to the S source. Maximum of variations (respect to the S source) was always observed in shoot: Δ ranged between 1.24 ± 0.02‰, in control condition, to 3.12 ± 0.04‰, at highest concentration of Cd. The $\delta^{34}S\text{-}S_{org}$ values in root and shoot were always significantly (P < 0.001)

depleted in ^{34}S compared to the S source. At the same time, these values in shoot were always significantly enriched in heavy ^{34}S isotope compared to the root.

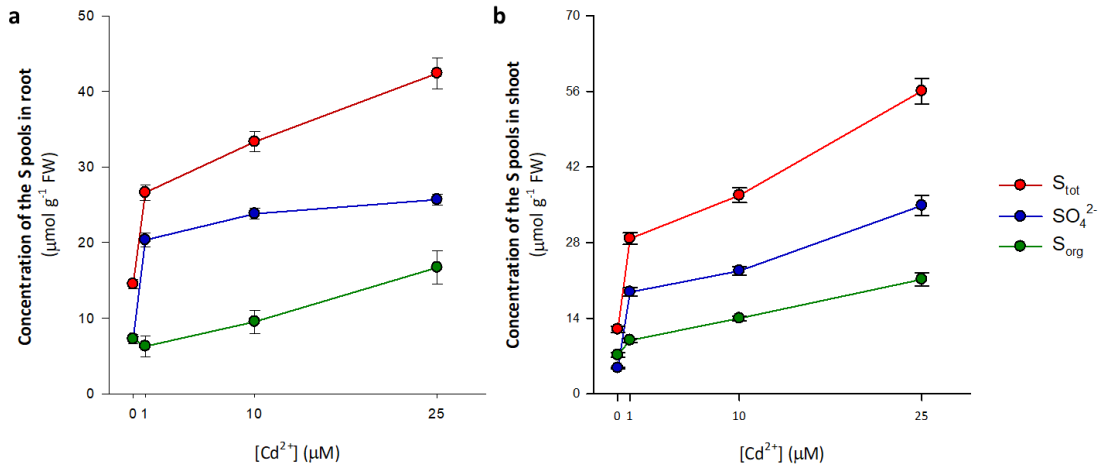

Figure 4. Changes in the concentration of the S pools (S_{tot}, SO_4^{2-} and S_{org}) in (**a**) roots and (**b**) shoots of rice plants under exposure to different Cd concentration (0, 1, 10 and 25 µM) for 72h. Data are means ± SE of 3 independent experiments run in duplicate (*n* = 3).

Table 1. S 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.

	Sample	Cadmium Concentration (µM)							
		0		1		10		25	
		$\delta^{34}S$ (‰)	Δ (‰)	$\delta^{34}S$ (‰)	Δ (‰)	$\delta^{34}S$ (‰)	Δ (‰)	$\delta^{34}S$ (‰)	Δ (‰)
S_{tot}	Root	-3.71 ± 0.08 ^{a}	-1.79 ± 0.08 ^{a}	-3.73 ± 0.06 ^{a}	-1.81 ± 0.06 ^{a}	-3.70 ± 0.07 ^{a}	-1.78 ± 0.07 ^{a}	-3.78 ± 0.06 ^{a}	-1.86 ± 0.06 ^{a}
	Shoot	-2.99 ± 0.08 ^{c*}	-1.07 ± 0.08 ^{c*}	-2.4 ± 0.07 ^{b**}	-0.48 ± 0.07 ^{b**}	-2.1 ± 0.05 ^{a**}	-0.18 ± 0.05 ^{ab**}	-1.9 ± 0.04 ^{a**}	0.02 ± 0.04 ^{a**}
SO_4^{2-}	Root	-2.44 ± 0.05 ^{c}	-0.52 ± 0.06 ^{c}	-2.30 ± 0.05 ^{a}	-0.38 ± 0.05 ^{c}	-2.00 ± 0.04 ^{b}	-0.08 ± 0.05 ^{b}	-1.30 ± 0.02 ^{a}	0.62 ± 0.03 ^{a}
	Shoot	-0.68 ± 0.01 ^{d**}	1.24 ± 0.02 ^{d**}	-0.01 ± 0.0002 ^{c**}	1.91 ± 0.02 ^{c**}	0.8 ± 0.02 ^{b**}	2.72 ± 0.03 ^{b**}	1.2 ± 0.03 ^{a**}	3.12 ± 0.04 ^{a**}
S_{org}	Root	-4.98 ± 0.10 ^{a}	-3.06 ± 0.10 ^{b}	-8.36 ± 0.08 ^{c}	-6.44 ± 0.08 ^{b}	-7.95 ± 0.08 ^{b}	-6.03 ± 0.08 ^{bc}	-7.59 ± 0.06 ^{b}	-5.67 ± 0.07 ^{b}
	Shoot	-4.53 ± 0.08 ^{a*}	-2.61 ± 0.08 ^{a*}	-6.94 ± 0.07 ^{b**}	-5.02 ± 0.07 ^{b**}	-6.82 ± 0.05 ^{b**}	-4.90 ± 0.05 ^{b**}	-7.00 ± 0.05 ^{b*}	-5.08 ± 0.05 ^{b*}

Δ indicates changes in ^{34}S relative to the S source ($\delta^{34}S\text{-}SO_4^{2-}{}_{source}$ = -1.92 ± 0.02‰). Data are means and SE of 3 independent experiments run in duplicate (*n* = 3). Different letters indicate significant differences among treatments ($P < 0.05$). Asterisks indicate significant differences (Student's *t*-test; *$0.001 \leq P < 0.05$; **$P < 0.001$) between root and shoot of plants with the same treatment.

4.3.3 Effect of Cd exposure on genes involved in sulfate uptake and translocation

Exposure to Cd leads to the coordinate upregulation of genes involved in SO_4^{2-} uptake and translocation, in order to sustain the higher demand for the biosynthesis of S reduced compounds. In the present work, the expression of the *SULTR* genes belonging to group 1 and 2 in different plant organs has been analyzed under different Cd concentrations (0, 1, 10 and 25 µM).

OsSULTR1;1 and *OsSULTR1;2* (Fig. 5) encode for high-affinity sulfate transporters expressed in root system and involved in SO_4^{2-} uptake (Takahashi *et al.* 2000; Yoshimoto *et al.* 2002; Takahashi 2019). These two genes in our results were both significantly upregulated by Cd. Nevertheless, while *OsSULTR1;2* was the main gene expressed in control plant, *OsSULTR1;1* became the prevalent one involved in SO_4^{2-} uptake in all the other treated conditions.

The expression of *OsSULTR1;3* (Fig. 6a) – transporter deputed at the phloematic retrieval of SO_4^{2-} from shoot to root (Yoshimoto *et al*. 2003) – showed significant differences only in the root under exposure to 25 µM Cd.

OsSULTR2;1 and OsSULTR2;2 are transporters involved in sulfate translocation from root to shoot (Sacchi and Nocito 2019; Takahashi 2019). In our results *OsSULTR2;1* (Fig. 6b) showed significant differences from the control only at 10 and 25 µM Cd^{2+}. *OsSULTR2;2* expression was always significantly upregulated compared to the control.

Concerning shoot system, *OsSULTR1;3* expression (Fig. 7a) increased as the concentration of the heavy metal in the nutrient solution did, reaching the highest value at 10 µM Cd^{2+}. At 25 µM Cd^{2+} the relative transcript level of *OsSULTR1;3* was higher than in control condition, but similar to the one observed at the concentration of 1 µM. Between the genes involved in root to shoot sulfate translocation, *OsSULTR2;1* (Fig. 7b) showed significant over-expression in all treated condition, compared to the control; *OsSULTR2;2* (Fig. 7c) showed significant differences from the control only at the Cd external concentration of 1 µM.

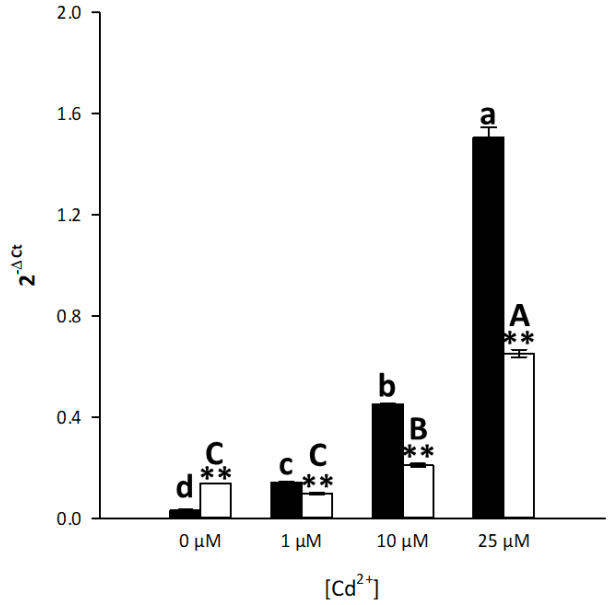

Figure 5. Expression of *OsSULTR1;1* (black columns) and *OsSULTR1;2* (white columns) in root of rice plants under exposure to different Cd^{2+} concentration (0, 1, 10 and 25 µM) for 72h. Different letters indicate statistical differences among treatment (P < 0.05). Two asterisks indicate statistical differences between the expression of the two genes with P < 0.001. Values are expressed as mean ± SE of 3 independent experiments run in duplicate (n = 3).

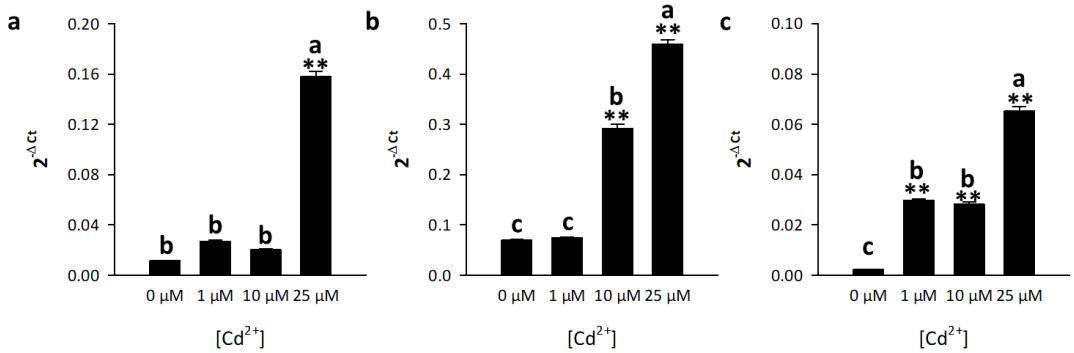

Figure 6. Expression of (**a**) *OsSULTR1;3*, (**b**) *OsSULTR2;1*, and (**c**) *OsSULTR 2;2* in root of rice plants under exposure to different Cd^{2+} concentration (0, 1, 10 and 25 µM) for 72h. Different letters indicate statistical differences among treatment (P < 0.05). Two asterisks indicate statistical differences between treated and control condition with P < 0.001. Values are expressed as mean ± SE of 3 independent experiments run in duplicate (n = 3).

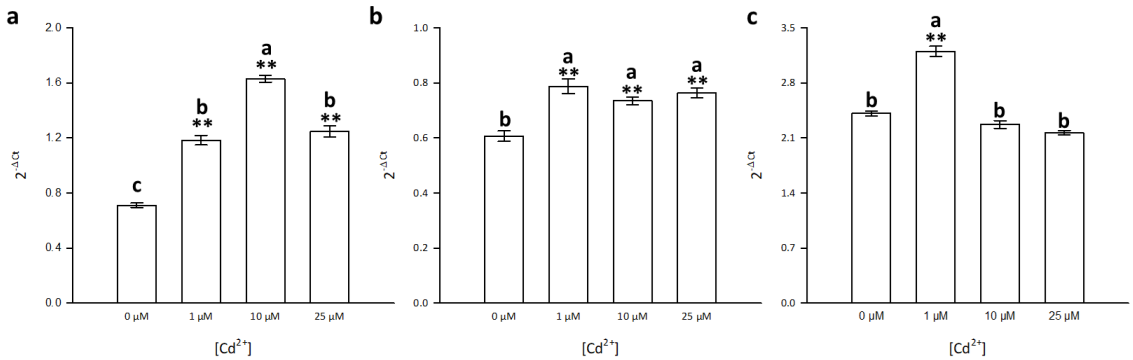

Figure 7. Expression of (**a**) *OsSULTR1;3*, (**b**) *OsSULTR2;1*, (**c**) and *OsSULTR 2;2* in shoot of rice plants under exposure to different Cd^{2+} concentration (0, 1, 10 and 25 µM) for 72h. Different letters indicate statistical differences among treatment ($P < 0.05$). Two asterisks indicate statistical differences between treated and control condition with a $P < 0.001$. Values are expressed as mean ± SE of 3 independent experiments run in duplicate ($n = 3$).

4.4 Discussion

Exposure to Cd activates different adaptive processes which increase the demand for S thought to be essential to sustain a higher synthesis of S reduced compounds, in particular GSH and PCs (Nocito *et al.* 2006, 2007). They are necessary not only for Cd chelation and detoxification, but also to mitigate the oxidative damage produced by free Cd^{2+} ions in cells (Noctor *et al*. 2012).

In our experiment, as expected, Cd treatment strongly induced SO_4^{2-} uptake and assimilation, as indicated by the analyses of the S pools (S_{tot}, SO_4^{2-}, and S_{org}) in both root and shoot. Most of the *OsSULTRs* of root and shoot system, involved in the uptake and translocation of sulfate, was induced under all the Cd conditions analyzed.

S isotope analyses clearly reveled that Cd-induced changes in the S metabolism and transport were associated to significant variations in the overall capability of the rice plants to discriminate against ^{34}S. In fact, such a feature progressively decreased as Cd concentration in the external medium increased, suggesting that changes in the relative expression of *OsSULTR1;1* and *OsSULTR1;2*, the main genes involved in sulfate uptake, could be involved in determining the different S isotope phenotypes observed under Cd stress. Thus, as previously reported, the higher expression of *OsSULTR1;1* observed in Cd exposed roots may account for the progressive decrease in ^{34}S isotope discrimination.

Data analyses also reveals that the isotope separation associated to sulfate assimilation was higher in shoot than in root, and progressively increased as Cd concentration did in both the organs. Such a finding confirms the prominent role of shoot in sulfate assimilation under Cd stress and suggests the possible interpretation of the S isotope separation as a diagnostic index of plant S nutritional status.

The higher expression of *OsSULTR2;1* and *OsSULTR2;2* observed under Cd stress, indicates that modulation in sulfate translocation from root to shoot are involved in controlling sulfate assimilation in the shoot. Moreover, since the expression of *OsSULTR1;3* increased under Cd exposure, it is possible to speculate that in this condition also phloematic retrieval of SO_4^{2-} increased. Thus, the isotope composition of

the SO_4^{2-} pools of the roots could be interpreted as the results of mixing and fractionation effects occurring during sulfate assimilation and cycling.

4.5 Supplementary material

Table S1. Sequences of primers (forward and reverse, 5'→3') of the housekeeping gene (*OsS16*) and of the *OsSULTR*s analyzed in the work. Primers were designed with the software Primer Express™ v. 3.0.1 (Thermo Fisher). Sequences of cDNA were found on "Rice Genome Annotation Project" website (http://rice.plantbiology.msu.edu/).

		Primer (5'-3')	
Gene	**Accession Number**	**Forward**	**Reverse**
OsS16	*LOC_Os11g03400*	ACGTCGACGAGGCATCCA	CGCGACCACCGAACTTCTT
OsSULTR1;1	*LOC_Os03g09970*	GGAGCATTCTTTGGCGTCAT	TCGCAACCGCAATTAGCA
OsSULTR1;2	*LOC_Os03g09980*	CGACCTTCTTTGCAGGAGTCA	TGAACCCTAGCCTGCAGAAAC
OsSULTR1;3	*LOC_Os08g31410*	GGAATATCTGCGCCTTGCTT	AGGGCTGCTTGAGTGATACCA
OsSULTR2;1	*LOC_Os03g09940*	CAAGGAGGCTCTCAGCATCTG	GCCGAGGCATGTGAGGAA
OsSULTR2;2	*LOC_Os03g09930*	GAATGATCTCACCGGCATGTT	GAAGCGCTTGTCTGCACCTT

5. Introgression of the *SalTol* QTL in a selected line of rice (*Oryza sativa* L.) ssp. *japonica*: effects on sulfur metabolism and phenotypic, physiological, and biochemical parameters

5.1 Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops on a worldwide basis, not only in Asian countries, where it represents 50%–80% of people's daily caloric intake, but also in Europe, where rice production represents almost 75% of the 4.2 Mt of milled rice consumed by European citizens (https://ricepedia.org/rice-around-the-world/europe, 2020). Rice production has major socio-cultural, economic, and ecological impacts in several countries of the Mediterranean area (Khush 2005). It has been estimated that the global demand for rice by the growing world's population will increase from the 480 million tons of 2013 to 715 million tons of milled rice in 2025 (FAO 2013); an adequate productive response is jeopardized by the climate change-related increase in temperatures and the rise in sea levels/salinization of the aquifers. In Europe, the issue is particularly relevant in Mediterranean areas of river deltas where an important fraction of rice production is located (Frouin *et al*. 2018).

Salinity is a soil condition characterized by a high concentration of soluble salts, in particular NaCl, of either natural or anthropic origin (Reddy *et al*. 2017). After drought, soil salinity is the second problem in rice-growing countries, and it poses a serious limitation to rice production (Gregorio 1997). Soils are classified as saline when their electrical conductivity (EC) is 4 dS m^{-1} (approximately 40 mM NaCl) or more (https://www.ars.usda.gov/pacific-west-area/riverside-ca/agricultural-water-efficiency-and-salinity-research-unit/docs/research-databases/) (Hoang *et al.* 2016). Salinity impairs plant growth and development, and ultimately yield, via water stress, cytotoxicity due to excessive uptake of ions like Na^+ and Cl^-, and nutritional imbalance.

Among the common cellular consequences of salinity there is the onset of secondary oxidative stress due to an excessive generation of reactive oxygen species (ROS), not efficiently counteracted by the antioxidant system. High level of ROS can severely affect the chemical stability of lipids, proteins, nucleic acids as well as that of cellular structures like membranes (Isayenkov and Maathuis 2019). Plants have developed different abilities to control their oxidative status and the increase in ROS production due to environmental stresses. The enzymatic antioxidant system has been massively studied, and the increased activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) have already been connected to salt

tolerance in different plant species (Gosset *et al*. 1994; Hernández *et al.* 1995; Lee *et al.* 2001). Moreover, non-enzymatic defense mechanisms are important in ROS scavenging, and, between them, sulfur (S) metabolism seems to play a crucial role. In fact, biosynthesis of different S containing compounds, and among them the tripeptide glutathione (GSH), is pivotal for ROS scavenging and for the determination of different degree of salt tolerance among crops and cultivars (Nazar *et al.* 2011; Mohd Asgher 2014; Samanta *et al.* 2020).

Rice sensitivity to salt depends on the genotype. In general, varieties belonging to the ssp. *japonica* are extremely sensitive even to mild salinity, especially during the seedling and reproductive stages (Moradi and Ismail 2007; Horie *et al*. 2012). In the ancient *indica* landrace Pokkali from Kerala (India), as well as in Nona Bokra, a major QTL (*SalTol*) associated with the shoot Na^+/K^+ ratio and salinity tolerance at seedling-stage has been identified on chromosome one. *SalTol* confers salt tolerance up to 150 mM NaCl (Bonilla *et al.* 2002; Karan *et al.* 2012). Marker-assisted selection (MAS) is a very effective method of transferring this desirable trait in rice (Thomson *et al*. 2010; Aliyu *et al.* 2011; Vu *et al*. 2012; Vinod *et al*. 2014; Singh and Singh 2015), and the *SalTol* QTL proved successful for this task (Thomson *et al*. 2010; Linh *et al.* 2012; Bimpong *et al.* 2016). At the International Rice Research Institute (IRRI), *SalTol* was introgressed from Pokkali into the salt sensitive IR29 line to obtain the FL478 genotype (IR 66946-3R-178-1-1), tolerant to salinity. In addition the FL478 genotype resulted insensitive to the photoperiod, and showed shorter life cycle and earlier flowering time than the original Pokkali landrace (Thomson *et al*. 2010; Chattopadhyay *et al*. 2014; Krishnamurthy *et al.* 2020). From FL478, *SalTol* was then transferred into IR64, a popular lowland variety released in 1985 and widely used as a representative *indica* variety in research studies and as a parent in breeding programs, allowing to obtain introgression lines (IR64-*SalTol*) with salinity tolerance significantly higher than the recurrent parent IR64 (Ho *et al.* 2016). The introgression of the *SalTol* region from FL478 into several *indica* rice varieties has successfully produced new salt tolerant genotypes (Huyen *et al*. 2012; Linh *et al*. 2012; Mondal *et al.* 2014) nevertheless, information for *SalTol* introgressions in temperate *japonica* rice accessions is not yet available. Currently, European farmers do not have at their disposal rice germplasm with sufficient salt tolerance to face salinity problems (Frouin *et al*. 2018). The gene *OsHKT1;5* that encodes for a xylem-expressed Na^+

transporter is suggested to be implicated in salt tolerance in Nona Bokra by decreasing Na^+ content in shoots and maintaining K^+ homeostasis. *OsHKT1;5* is one of the genes included in *Saltol* (Lin *et al.* 2004; Ren *et al.* 2005) and it represents the putative candidate gene regulating the salinity tolerance at seedling stage (Babu *et al*. 2017). Nevertheless, a genetic background effect of introgression of the *SalTol* QTL into sensitive rice varieties cannot be excluded, so that the physiological mechanisms conferring salt tolerance to the acceptor genotype might be more complex than those expected by the simple *OsHKT1;5* activity.

In the context of the Neurice H2020 project (http://neurice.eu/), an approach of Marker-Assisted Back-Cross (MABC) was followed to obtain new European *japonica* tolerant varieties carrying in their genome the *SalTol* QTL. After the initial cross between the Italian *japonica* elite cultivars Onice and Vialone Nano (both salt sensitive) and the *indica* donor parent IR64-*SalTol*, followed by three backcrosses to restore the elite genome and three self-pollination, 40 introgressed lines (ILs) were obtained (21 for Onice, and 19 for Vialone Nano) (Cavallaro 2018). After another self-pollination a selection of 16 ILs, which showed higher recovery of the genome of the recurrent parents, was evaluated in two different non-saline fields in Northern Italy, and in two different saline ones in the Po River delta. Between them, Onice 11 (O11), Vialone Nano 25 (VN25), and Vialone Nano 28 (VN28) were the ones with best performances under salt stress condition (Monaco, 2019, personal communication).

Aim of the present work was to study the phenotypic behavior under salt stress of the donor (IR64-*SalTol*) and the acceptor (Onice) genotypes, and one of the tolerant introgressed lines (O11) in hydroponic controlled condition. Morphological [growth inhibition and level of stress (Standard Evaluation System-SES)], physiological (stomatal conductance, membrane electrolytes leakage, shoot/root Na^+/K^+ ratio) and biochemical (malondialdehyde-MDA accumulation, and antioxidant enzymes specific activities in the leaves) parameters have been evaluated. A particular focus was dedicated to the effects of salt exposure on metabolites and activities belonging to the sulfur metabolism, known to participate in relieving oxidative stress conditions generated by primary salt stress. In particular, IR64-*SalTol*, Onice and O11, have been characterized for the amount of SO_4^{2-}, S total, non-protein thiols (NPTs), and the expression of the main *OsSULTR* of group 1 and 2 in different organs of the plant. The final aim was to verify a possible involvement

of S metabolism in determining salt stress tolerance in the line (O11) containing the *SalTol* QTL.

5.2 Materials and methods

5.2.1 Plant material and experimental set-up

Seeds of each genotype/line considered [Italian recurrent parent Onice (ssp. *japonica*), *SalTol* donor parent IR64-*SalTol* (ssp. *indica*), and the introgressed line IL O11 (ssp. *japonica*)] were sterilized as follows: 3 min in ethanol 70% (v/v); 30 min in sodium hypochlorite 30% (v/v) + 0.02% Tween (v/v); 5 washes of 3 min each with distilled water. Seeds germinated on filter paper saturated with distilled water, in dark, for a week at 26 °C. After 7 day (7d), 40 seedlings for each line were selected for uniform growth and transferred into tanks (26.5 x 36.7 x 17 cm) containing 10 L hydroponic solution (Fig. 1a), whose composition followed the protocol of Yoshida *et al*. (1976): 1.428 mM NH_4NO_3, 0.513 mM K_2SO_4, 0.849 mM KH_2PO_4, 0.123 mM K_2HPO_4, 0.754 mM $CaCl_2$, 1.644 mM $MgSO_4$, 20 mM Fe-EDTA, 9.5 µM $MnCl_2$, 0.075 µM $(NH_4)_6Mo_7O_{24}$, 18.89 µM H_3BO_3, 0.152 µM $ZnSO_4$, 0.156 µM $CuSO_4$. Final pH was adjusted to 6.0 and controlled every two days. The entire solution was renewed once a week.

The plantlets grew in controlled environment, at a temperature of 28 °C; 80% relative humidity; 16h of light, with 400 µmol m^{-2} s^{-1} of photosynthetic photon flux density, and 8h of dark. After 7d in the Yoshida solution, the salt treatment was started by adding 80 mM NaCl to reach an EC value of 8 dS m^{-1} (Field Scout, Direct Soil EC Meter, Spectrum Technologies Inc., Aurora, IL, USA). Plants of each line and at different growth conditions were sampled after 7d and 14d of treatment and used for the *in vivo* or *in vitro* experiments (Fig. 1b). For infrared thermography a precocious and additional sampling has been made after 48h of treatment (Fig. 1b). For each genotype/line six tanks were set up, three of which maintained in control condition (biological triplicate).

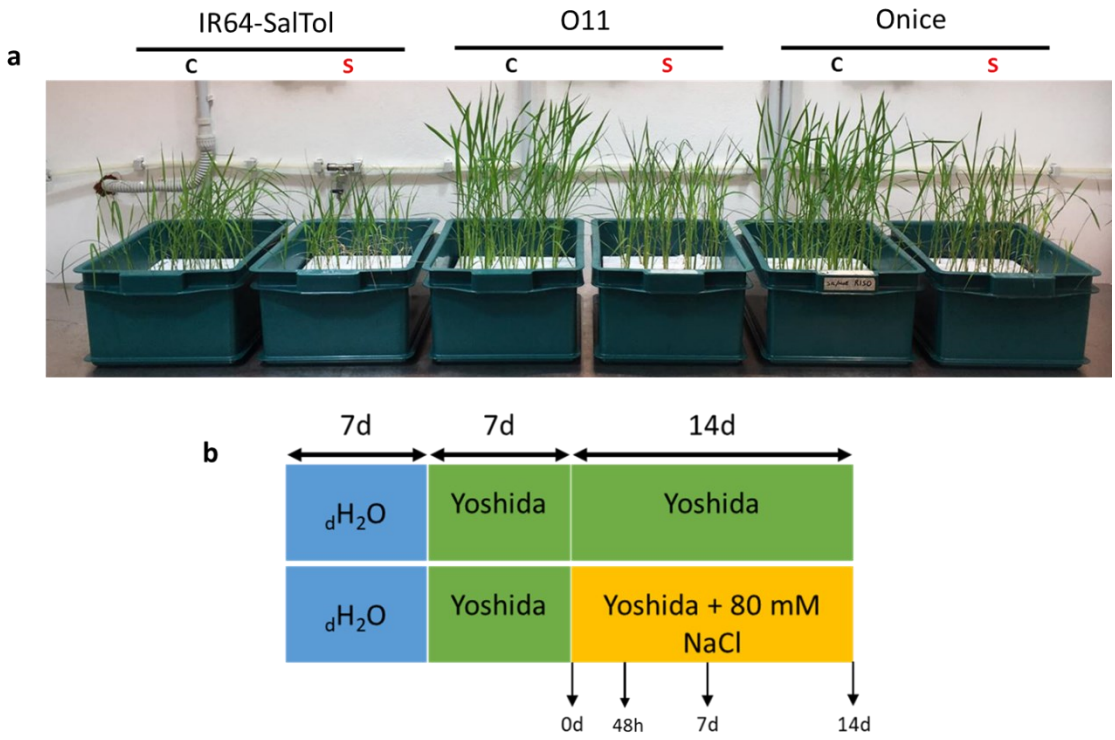

Figure 1. (**a**) Plant growth in hydroponic conditions. Tanks containing 40 plants of each single genotype/line in 10 L of different growth solution: C, control (Yoshida solution); S, salt (Yoshida solution + 80 mM NaCl). (**b**) Outline of the experimental hydroponic design: 7d of seed germination on filter paper saturated with distilled water; 7d of growth in hydroponic in Yoshida control solution; 14d growth in salt solution. 0d: addition of 80 mM NaCl to the control Yoshida solution; 48h: check of the salinity level; 7d and 14d: evaluation of the selected physiological parameters and tissue sampling.

5.2.2 Standard Evaluation System (SES)

The modified SES, issued and validated by IRRI, was used for the visual screening of the salt-induced symptoms at phenotypic level in the selected rice genotypes (Tab. 3). This scoring discriminates the susceptible genotypes from the tolerant and the moderately tolerant ones (IRRI 2002). SES score values were determined at 7d and 14d after salt stress treatment.

Table 1. Criteria for SES evaluation (IRRI, 2002).

Injury score		Description
1	Highly tolerant	Normal plant growth, only the old leaves show white tips with no symptoms in the younger leaves
3	Tolerant	Near normal plant growth, buy only leaf tips burn, a few older leaves become partially whitish and rolled
5	Moderately tolerant	Growth severely retarded, most old leaves severely injured, a few young leaves elongating
7	Sensitive	Complete cessation of growth, most leaves dried, only few young leaves still green, some plants dying
9	Highly sensitive	Almost all plants dead or dying

5.2.3 Plant fresh and dry weight determination

For the determination of fresh weight (FW), 7d after the administration of the salt stress plant material obtained from the different organs (young leaves, old leaves, sheath plus stems, roots, and shoots) was gently blotted with absorbent paper and then weighed. Dry weight (DW) was determined by drying in the oven (70 °C) the samples up to a constant weight.

5.2.4 Membrane lipid peroxidation: evaluation of membrane electrolyte leakage and malondialdehyde levels

Leaves of rice plants grown for 7d in hydroponics in the presence or in the absence of 80 mM NaCl were cut in strips (1 cm) and washed for 5 min with 15 mL of Milli-Q distilled water (Lutts *et al*. 1996). After this preliminary wash, samples were incubated in 15 mL of Milli-Q distilled water at room temperature (RT), and for 2h in gentle agitation, then the EC of the incubation medium was measured (EC1). Samples were then autoclaved at 120 °C for 20 min and, after cooling, the EC of the incubation medium was measured again (EC2). The value of electrolyte leakage (EL) was calculated as:

$$EC\ (\%) = EC1/EC2 * 100$$

High values of the ratio indicate severe membrane damage (Tantau and Dörffling 1991).

Malondialdehyde (MDA) extraction and assay was carried out on pooled leaf tissues of rice plants grown for 7d in control and salt conditions. The plant material was harvested, rapidly rinsed with bi-distilled water, gently blotted with towel paper, immediately weighed, and then frozen in liquid N_2. The frozen plant tissue was homogenized (mortar and pestle) to a fine powder in the presence of liquid N_2. Fifty milligrams of leaf powder were resuspended in a 2 mL Eppendorf® tube with 1 mL of ethanol 80% (v/v). Samples were vortexed and then centrifuged at 16,000*g* for 20 minutes at 4°C. Five-hundreds µL of the supernatant were transferred in new 2 mL tubes. After the addition of 500 µL of a solution composed by 20% (v/v) trichloroacetic acid (TCA) and 0.65% (w/v) thiobarbituric acid (TBA), the tubes were maintained at 85°C for 30 min and then centrifugated at 13,000*g* for 10 min, at 4 °C. After collecting the supernatants, the absorbances were measured at two different wavelengths: 532 and 600 nm (Secoman

UviLine 9400 spectrophotometer). Molar concentration of MDA was calculated as follow:

$$\mu mol\ MDA/g\ FW = ABS_{tot}/155/g\ FW$$

$$where\ ABS_{tot} = ABS_{532} - ABS_{600}$$

5.2.5 Measurement of antioxidant enzymes activity

Four-hundreds milligrams of leaf powder were weighed and homogenized in 4 mL extraction buffer composed by 100 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, and 10 mM ascorbic acid. The homogenate was centrifuged at 13,000*g* for 15 min at 4 °C, and the resulting supernatant was used to determine the activity of glutathione reductase (GR), catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD). The enzymatic assays were performed according to the methods reported by Vighi *et al*. (2017). The total protein content was evaluated according to Bradford (1976).

5.2.6 Measure of Na^+/K^+ ratio

After 7d of salt treatment, the plants were harvested and the aerial parts were washed with distilled water, while roots were washed twice with 50 mM Rb_2SO_4 for 10 min, at 4 °C to remove Na^+ and K^+ from the apoplast. Na^+ and K^+ contents and ratio were evaluated in the different parts of the shoots, sampled separately (i.e., young leaves, old leaves, steam and sheaths), and in the roots. On samples obtained from the plants of each line grown at different conditions FW and DW (after 3d in oven at 65 °C) were determined. Oven-dried samples were transferred in teflon tubes with 10 mL of 65% (v/v) HNO_3, mineralized by a microwave oven (Anton Paar MULTIWAVE-ECO), by linearly rising the temperature within 10 min up to 210 °C, and maintaining this condition for further 10 min. Mineralized samples were cooled for 20 minutes and then diluted forty times with distilled water. Concentration of Na^+ and K^+ ions were measured by inductively coupled plasma-mass spectrometry (ICP–MS; Bruker AURORA M90 ICP–MS).

5.2.7 Infrared thermography and acquisition of thermo-images

Thermo-images were obtained by the infra-red camera (FLIR T650S) on the plants grown at different conditions and harvested after 48h, 7d and 14d of salt stress imposition. The

thermo-images were shot in a controlled environment chamber, between 11:00 am and 2:00 pm since in this period plant stomatal conductance and transpiration tend to remain constant. The camera was placed at ca. 120 cm from the plants, and it was left in the growth chamber for about 2h before starting the measurements; this period allows the optics of the IR camera to reach thermal equilibrium with the air temperature. In every image, for each line, control and stressed plants were reproduced in comparison, trying to carefully avoid leaves overlay. Pictures were obtained in not more than 20 min, to prevent possible alterations of plant stomatal conductance due to the respiratory CO_2 produced by the operator (James and Sirault 2012).

The evaluation of shoot temperature was performed by using the software FLIR ResearcIR Max 4, considering 25 random points on young leaves for each biological replicate (Fig. 2).

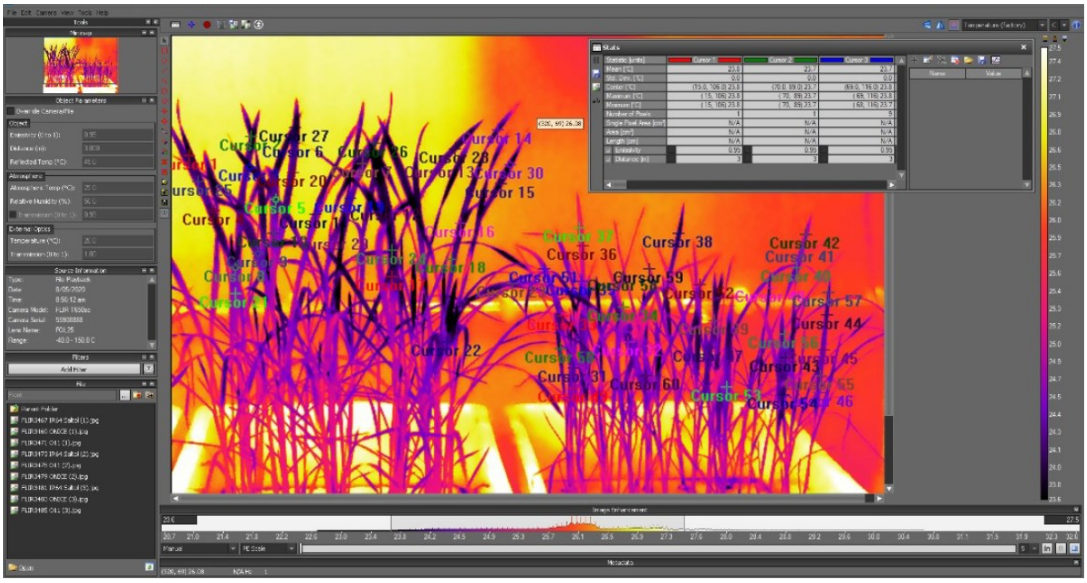

Figure 2. Example of analysis by the software FLIR ResarchIR Max 4. Control and treated condition were placed in the same photograph; in this picture IL O11 is showed (control on the left, treated on the right).

5.2.8 RNA extraction and qRT-PCR analyses

Onice, IR64-*SalTol* and O11 plants were sampled after 7d of growth in hydroponic conditions, in the presence or in the absence of 80 mM NaCl. Roots were washed with washed for 30 min in 3 L of Milli-Q water to remove the not absorbed SO_4^{2-} from the root apoplast, and then they were separated by shoots.

Samples, constituted by a pool of 40 plants per genotype and condition, were powdered using liquid N_2. Aliquots of 0.4 g (FW) of powder were transferred in a 2 mL sterilized tube and Trizol® reagent (Invitrogen) was added according to manufacturer's instructions for the nucleic acid extraction. The complete extraction of total RNA from shoots and roots was performed according PureLink® RNA Mini Kit (Invitrogen) protocol. Contaminant DNA was removed on-column using PureLink® DNase (Invitrogen). First-strand cDNAs of the different samples were obtained following the protocol SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen).

qRT-PCR analysis of *OsSULTR1;1* (LOC_Os3g09970), *OsSULTR1;2* (LOC_Os03g09980), *OsSULTR1;3* (LOC_Os08g31410), *OsSULTR2;1* (LOC_Os03g09930), and *OsSULTR2;2* (LOC_Os03g09930) was performed in the different organs of the plants by using an ABI 7300 Real-Time PCR system (Applied Biosystems). The first-strand cDNA was put in a 20 µL reaction mixture containing GoTaq® qPCR Master Mix (Promega) and the specific primers. The relative transcript level of each gene was calculated and expressed as $2^{-\Delta\Delta Ct}$, using the expression of the *OsS16* (LOC_Os11g03400) gene, coding for the 40S ribosomal protein S16, as reference. Primers for qRT-PCR are listed in Supplementary Table Tab. S1.

5.2.9 Quantitative determination of total sulfur, sulfate, and non-protein thiol content

For total S quantification, 0.5 g (FW) of the same pulverized utilized for RNA extraction were let dried overnight in oven at 80 °C and then DW was measured. Samples were digested at 150 °C in 3 mL 2:1 (v/v) nitric + perchloric acid mixture to convert all the S forms in sulfate. Samples were clarified with 1 mL 33% (w/v) H_2O_2 and dried at 80 °C. Mineralized materials of shoot and root were dissolved in 3 mL of distilled water, then brought to pH 2.0 with 6 N HCl.

For sulfate quantification 1 g (FW) of pulverized was let dried overnight in oven at 80 °C and then DW was measured. Samples were extracted by homogenizing them in 4 mL of distilled water. After heating at 80 °C for one hour, samples were filtered, then brought to pH 2.0 with 6 N HCl.

For quantitative determination of sulfate equivalent the turbidimetric method described by Tabatabai and Bremner (1970) was utilized.

Total non-protein thiols (NPTs) contents were determined as described by Fontanili *et al*. (2016).

5.2.10 Statistical analysis

Values are presented as mean ± standard error (SE). Statistical analyses were performed through the software SigmaPlot 11.0 (Sysat Software Inc., San Jose, California, USA). Student's *t*-test was used to assess the significance between control and treated plants of the same genotype, considering $P < 0.05$. Student's *t*-test applied on gene expression considered $P < 0.001$ and $0.001 \leq P < 0.05$. One-way ANOVA, adjusted for multiple comparisons using the Bonferroni method, was performed to assess significance between different genotypes and same treatment, considering $P < 0.05$.

5.3 Results

5.3.1 Effects of *SalTol* introgression on phenotypic parameters

The effects of salinity on Onice, IR64-*SalTol* and IL O11 growth, expressed as shoot and root fresh (FW) and dry weight (DW), were evaluated after 7d in plants grown in the absence or in the presence of 80 mM NaCl (Fig. 3). All lines, when grown in salt stress conditions, showed a significant reduction of the shoot and root biomass as compared to the controls with losses greater than 70% both for FW and DW. IR64-*SalTol* showed a growth significantly lower than that of the other two genotypes, both in control and in salt stress conditions.

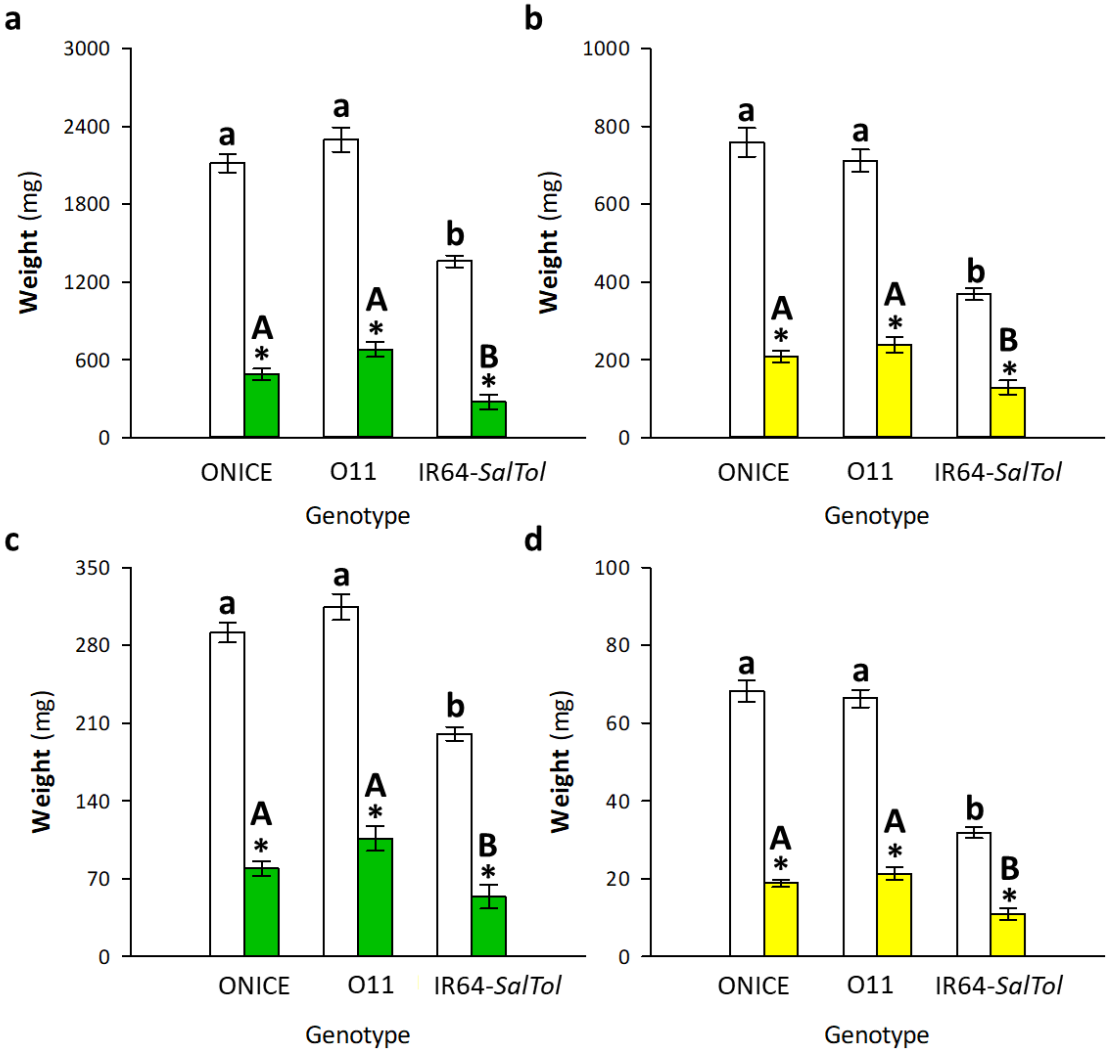

Figure 3. (**a**) Shoot and (**b**) root FW, (**c**) shoot and (**d**) root DW of the Italian recurrent parent Onice, the *SalTol* donor parent IR64-*SalTol*, and the introgressed line IL O11 after 7d of hydroponic growth in the absence (white columns) or in the presence (colored columns) of 80 mM NaCl. Values are the mean ± SE of data recorded in 10 plants (*n* = 10). Various letters

(lowercase and capital as referred to control and treated plants, respectively) indicate significant differences among genotypes after ANOVA ($P < 0.05$). Asterisks indicate significant differences between control and treated plants within each genotype after *t*-test ($P < 0.05$).

After 7d and 14d of growth in hydroponic culture in the presence of salt (80 mM NaCl), the injury score was assessed according to the SES by evaluating the suffering symptoms (leaf rolling, discoloration of leaf blades, burnt tips, chlorosis) on plants. A SES score value of 1 is associated to the absence of specific suffering symptoms and, in this context, it has been assigned to the plants of all genotypes grown in control conditions confirming the suitability of the adopted protocol for rice growing. Upon exposure to 7d of salt stress (Fig. 4a), in all genotypes the SES score value significantly increased in the treated plant with respect to the control ones, even if salinity differently affected the appearance of the suffering symptoms in the three lines considered. The highest value of SES (4.9) was assessed in the plants of the recurrent parent line Onice according to the salt susceptibility described for this genotype. IR64-*SalTol* had a SES score value of 3.9 while, interestingly, the IL O11 showed the lowest SES value (3). After 14d of salt treatment (Fig. 4b), in all genotypes the suffering symptoms became more severe as indicated by the further increase in the SES score that reached high and comparable values in Onice (7.3) and IR64-*SalTol* (6.5). Also, at this time of treatment O11 showed a lower SES score value (5.5) than the parental lines.

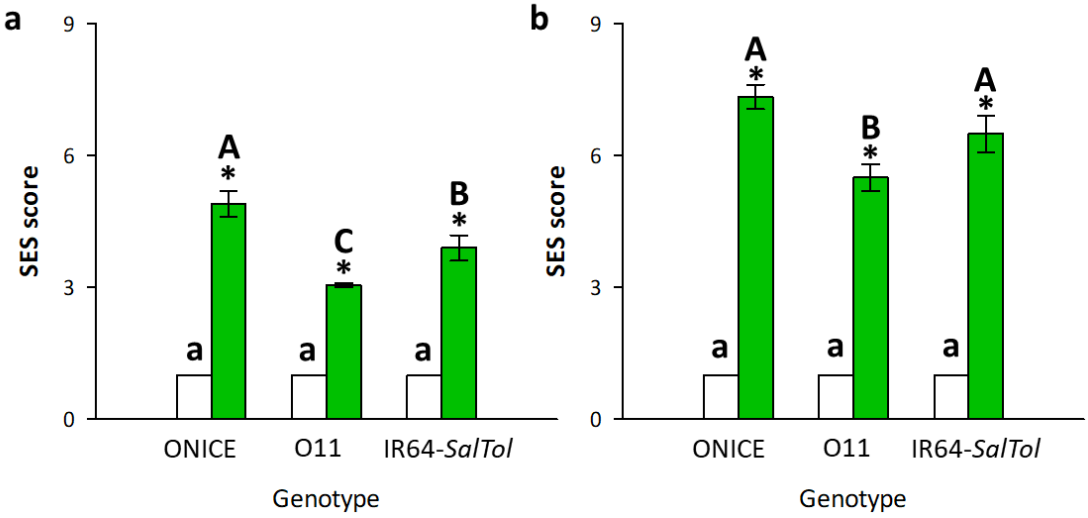

Figure 4. SES score of the Italian recurrent parent Onice, the *SalTol* donor parent IR64-*SalTol*, and the introgression line IL O11 after (**a**) 7d and (**b**) 14d of growth in the hydroponic solution in the absence (white bars) or in the presence (green bars) of 80 mM NaCl. Values are the mean ± SE of the data recorded in 40 plants at 7d and 12 plants at 14d. Various letters (lowercase and capital as referred to control and treated plants, respectively) indicate significant differences among genotypes after ANOVA ($P < 0.05$). Asterisks indicate significant differences between control and treated plants within each genotype after *t*-test ($P < 0.05$).

5.3.2 Effects of the *SalTol* introgression on MDA levels, membrane electrolytes leakage and activity of antioxidant enzymes

Under salinity, besides the osmotic stress and Na^+ toxicity, ROS (reactive oxygen species) overaccumulation is a secondary stress. A reduced stomatal conductance fosters ROS generation, due to electron outflow from the photosynthetic and respiratory electron transport chains, to oxygen, and to activities in peroxisomes and cell apoplast, determining oxidative damage of the biological membranes, that lose their physiological properties, and macromolecules. The malondialdehyde (MDA) level is a biochemical marker of membrane lipid peroxidation and the damage is indicated by the tissue electrolyte leakage (EL; Vighi *et al*. 2017).

The presence of 80 mM NaCl during the hydroponic growth induced, after 7d of treatment, a significant increase in the leaf MDA levels of the two parental lines, with a dramatic effect in the recurrent parent Onice and a lower effect in the *SalTol* donor IR64-*Saltol* (+129% and +58% as compared to the controls, respectively). In salt conditions the leaf MDA contents of IL O11 and IR64-*SalTol* were comparable and significantly lower to those observed in Onice. In IL O11 the leaf MDA levels did not significantly increase in treated plants respect to the control ones (Fig. 5a).

After 7d of salt treatment, in all genotypes considered the values of leaf EL were significantly higher than those recorded in the plants grown in control conditions (Fig. 5b). In particular, higher values (26%) of leaf EL were observed in the recurrent parent Onice than in the introgressed lines IR64-*SalTol* and O11 (about 12%), with an effect of salinity on this parameter that was consistent with what observed for leaf MDA levels.

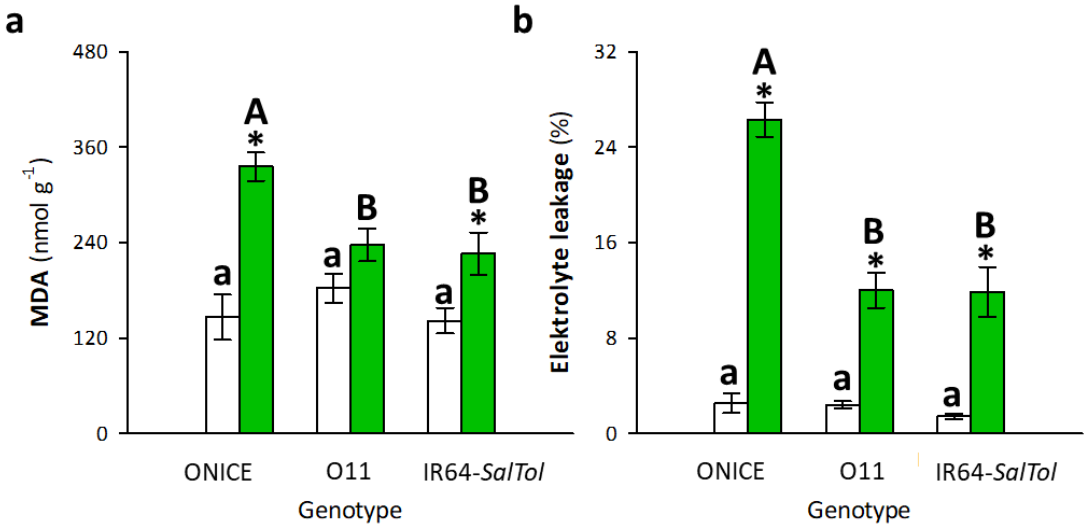

Figure 5. (**a**) Malondialdehyde contents and (**b**) electrolyte leakage values in the leaves of the Italian recurrent parent Onice, the *SalTol* donor parent IR64-*SalTol*, and the introgression line IL O11 after 7d of growth in the hydroponic solution in the absence (white bars) or in the presence (green bars) of 80 mM NaCl. MDA contents are expressed as nmol g^{-1} DW; EL was calculated as EL (%) = EC1/EC2*100. Values are expressed as the mean ± SE recorded in 4 plants of the same genotypes and same condition (*n* = 4). Various letters (lowercase and capital as referred to control and treated plants, respectively) indicate significant differences among genotypes after ANOVA ($P < 0.05$). Asterisks indicate significant differences between control and treated plants within each genotype after *t*-test ($P < 0.05$).

The excess of energy on photosynthetic systems, that occurs when stomatal conductance is reduced by effect of salt stress, is discharged on the molecular oxygen giving rise at first to the superoxide radical accumulation. This ROS is readily dismuted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 can be eliminated by catalase (CAT), preferably in peroxisomes, as well as by ascorbate peroxidase (APX), acting in different cell compartments, through ascorbate-glutathione cycle (Hossain and Dietz 2016).

Figure 6 shows the levels of the specific activities of SOD (Fig. 6a), CAT (Fig. 6b), APX (Fig. 6c) and glutathione reductase (GR; Fig. 6d) evaluated in the leaf tissue of the three genotypes grown for 7d in the absence or in the presence of 80 mM NaCl.

Salt stress induced in the three genotypes an increase in all the considered antioxidant activities with a lower effect in the donor parent IR64-*SalTol* than in the recurrent one Onice and in IL O11. IR64-*SalTol* showed the lowest levels of all the leaf antioxidant activities also in plants grown in control conditions.

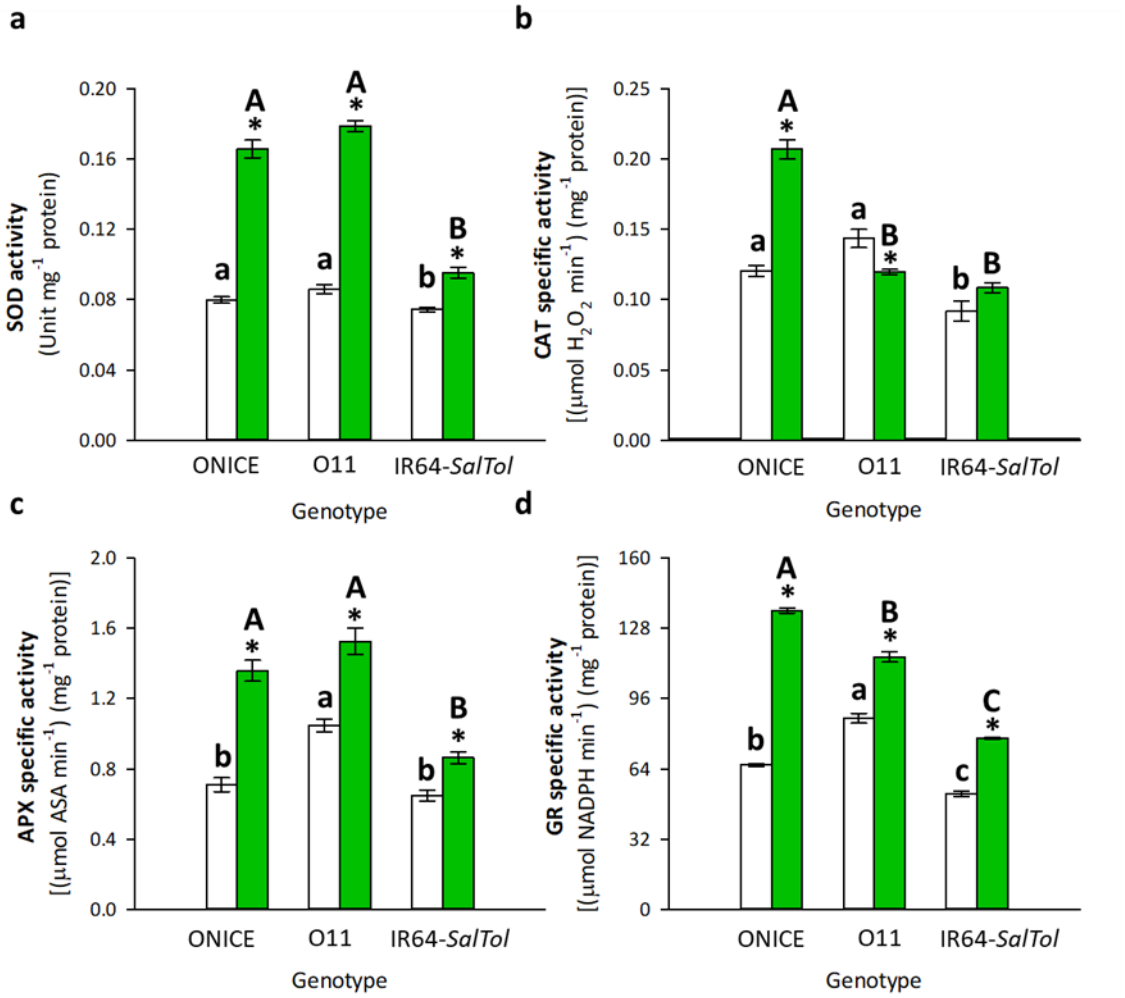

Figure 6. Specific activity of the antioxidant enzymes (**a**) SOD, (**b**) CAT, (**c**) APX, and (**d**) GR in the leaves of the Italian recurrent parent Onice, the *SalTol* donor parent IR64-*SalTol*, and the introgressed line IL O11 after 7d of growth in the hydroponic solution in the absence (white bars) or in the presence (green bars) of 80 mM NaCl. Values are expressed as mean ± SE of 4 different measurements of the same extract, constituted from pooled leaves of the same genotype and same condition (n = 4). Various letters (lowercase and capital as referred to control and treated plants, respectively) indicate significant differences among genotypes after ANOVA (P < 0.05). Asterisks indicate significant differences between control and treated plants within each genotype after t-test (P <0.05).

In particular, leaves SOD activity (Fig. 6a) that was already higher in control plants of the recurrent parent Onice and in IL O11, as compared to the donor parent IR64-*SalTol*, after salt treatment strongly increased (+108%) in these two genotypes, while it increased of only +28% in the donor parent IR64-*SalTol.*

Concerning leaf CAT activity (Fig. 6b), in the controls the differences among the three genotypes are like those observed for SOD activity in the plants when grown in the same conditions. Interestingly, salt stress induced a dramatic increase (+72%) in CAT activity only in the recurrent parent Onice. No changes or even a low but significant decrease (-

17%) in CAT activity, as compared to the controls, were observed in the treated plants of the donor parent IR64-*SalTol* and of IL O11, respectively.

APX activity (Fig. 6c), which in the leaves of plants grown in control conditions was significantly higher in IL O11 than in the parental lines, after salt treatment strongly increased in Onice (+91%) and to a lesser extent in the introgressed lines (+45% and +33% in IL O11 and in IR64-*SalTol*, respectively).

GR activity (Fig. 6d) was significantly different in the leaves of all three genotypes when plants were grown in control conditions, showing the highest value in IL O11, the lowest one in IR64-*SalTol* and an intermediate value in Onice. After salt treatment, a significant increase in GR activity occurred that was higher in Onice (+108%) and lower in IL O11 and IR64-*SalTol* (+32% and 48%, respectively).

5.3.3 Effects of *SalTol* introgression on Na^+/K^+ molar ratios

Plants of the different genotypes grown for 7d in presence of 80 mM NaCl were analyzed for Na^+ and K^+ contents in different organs: young leaves, old leaves, sheath plus stem, roots, and shoots (Tab. 2). Considering the contribution of all plant organs, significant differences in Na^+/K^+ molar ratio among the three genotypes were observed only in young leaves and in roots. In particular, in young leaves IL O11 showed value (0.28 ± 0.09) of Na^+/K^+ molar ratio that were significantly lower as compared to those observed in the recurrent parent Onice (2.25 ± 0.42), while the *SalTol* donor parent IR64-*SalTol* showed values (1.28 ± 0.39) of Na^+/K^+ molar ratio that were intermediate between those of Onice and IL O11. In roots, a significant higher value (5.32 ± 0.72) of Na^+/K^+ molar ratio was present in IR64-*SalTol* than in Onice (2.73 ± 0.42) and IL O11 (2.14 ± 0.17) that showed values statistically similar.

Table 2. Na^+/K^+ molar ratios in different parts of the Italian recurrent parent Onice, the *SalTol* donor parent IR64-*SalTol*, and the introgressed line IL O11 after 7d of growth in hydroponic solution in the presence of 80 mM NaCl.

	Na^+/K^+ molar ratio					
	Young leaves	**Old leaves**	**Leaf blades**	**Sheath + stem**	**Shoot**	**Root**
Onice	2.25 ± 0.42^a	2.51 ± 0.33^a	2.47 ± 0.36^a	3.95 ± 0.67^a	3.05 ± 0.35^a	2.73 ± 0.42^b
O11	0.28 ± 0.09^b	2.10 ± 0.24^a	1.59 ± 0.09^a	3.00 ± 0.57^a	2.19 ± 0.26^a	2.14 ± 0.17^b
IR64-*SalTol*	1.28 ± 0.39^{ab}	2.66± 0.54^a	2.25 ± 0.39^a	7.46 ± 2.40^a	3.19 ± 0.67^a	5.32 ± 0.72^a

Values are expressed as the means ± SE of value obtained considering 4 plants for each genotype (n = 4). Various letters indicate significant differences among lines and considered organs after ANOVA ($P < 0.05$).

The amounts of Na^+ in the whole plant, as well as its distribution between shoot and root, are reported in figure 7. In all three genotypes the greatest amount of the plant total Na^+ content was present in the shoot. No significant differences were found among the three genotypes in the plant material considered (Fig. 7).

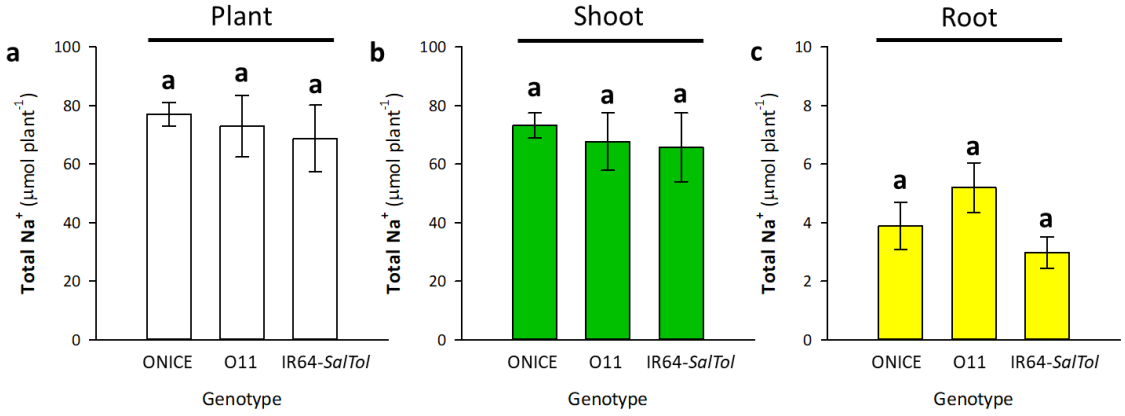

Figure 7. Total amount of Na^+ (**a**) in the whole plants (white bars), (**b**) in shoot (green bars), and (**c**) in root (yellow bars) of the Italian recurrent parent Onice, the *SalTol* donor parent IR64-*SalTol*, and the introgressed line IL O11 grown for 7d in the hydroponic solution in the presence of 80 mM NaCl. Values are expressed as means + SE of 3 plants (n = 3). After one-way ANOVA analysis, no significant differences were found.

5.3.4 Effects of *SalTol* introgression on leaves temperature

Figure 8 shows the effects of salinity on leaf temperature in the three genotypes considered, monitored at different times during the whole period of 14d of the salt treatment administration. Salt stress induced a slight but significant increase in leaf

temperature of the three genotypes with an effect that increased with the time of stress treatment.

After 48h of growth in salt stress conditions, significant increases of about +1.0 °C and +0.8 °C in leaf temperature were monitored in the plants of Onice and IR64-*SalTol*, respectively, as compared to the plants grown in control condition. At this short time of treatment, no changes of the leaf temperature were detected in IL O11.

After 7d of growth in salt conditions, the leaf temperatures in IR64-*SalTol* and Onice were higher of 1.5 °C and 1.7 °C when compared to those recorded in the control plants, and a lower but significant increase (+0.8 °C respect to the control) in leaf temperature occurred also in IL O11.

After 14d of salt treatment, the increases in leaf temperature were of 2.16 °C in Onice and 2.2 °C in IR64-*SalTol*, while in IL O11 the leaf temperature increased by 0.96 °C only.

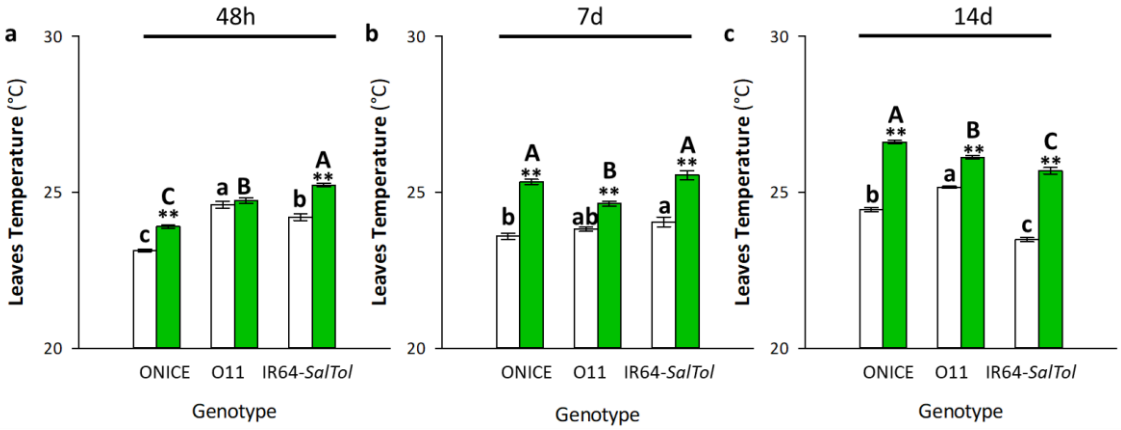

Figure 8. Temperature of the leaves of the Italian recurrent parent Onice, the *SalTol* donor parent IR64-*SalTol*, and the introgressed line IL O11 after (**a**) 48h, (**b**) 7d, and (**c**) 14d of growth in the hydroponic solution in the absence (white bars) or in the presence (green bars) of 80 mM NaCl. Values are expressed as the mean ± SE of the temperature recorded in all the spots considered for each genotype and condition (n = 75 at 48 h and 7d; n = 25 at 14d). Various letters (lowercase and capital as referred to control and treated plants, respectively) indicate significant differences among genotypes after ANOVA ($P < 0.05$). Asterisks indicate significant differences between control and treated plants within each genotype after t-test (P <0.05).

5.3.5 Effects of *SalTol* introgression on sulfur metabolism and *OsSULTR*s gene expression

In the present work sulfur (S) metabolism of the three lines has been investigated after 7d of salt treatment by measuring the amount of sulfate, total S (Fig. 9) and total non-protein thiols (NPTs; Fig. 10) in shoot and root systems. Moreover, the expression of

main *OsSULTR* genes of group 1 and 2 was investigated (Figs. 11 and 12). These genes encode for membrane co-transporters involved in the uptake and translocation of SO_4^{2-}, respectively, with an exception for *OsSULTR1;3* that, though belonging to group 1, is fundamental in the retrieval of SO_4^{2-} within the phloem (Takahashi *et al*. 2000; Yoshimoto *et al*. 2002; Yoshimoto *et al*. 2003; Takahashi 2019).

Concerning the root system, the total S amounts (Fig. 9a) were equal in all three genotypes when grown in control conditions. Salt treatment strongly reduced the total S content in all the lines considered (-54%, -57%, and -45% in Onice, IL O11, and IR64-*SalTol*, respectively), with an effect that was less severe in the parental *indica* line. The amount of sulfate (Fig. 9b) in the roots of control plants was high in the *indica* parental line, IR64-*Saltol*, low in the recurrent parent Onice and showed an intermediate value in IL O11. Under salinity, roots of the introgressed lines showed a very low (IL O11) or low (IR64-*SalTol*) decrease in total sulfate amount as compared to the recurrent parent Onice grown in the same stress conditions.

Considering the shoot system, the levels of total S (Fig. 9c) and sulfate (Fig. 9d) were higher than in roots. The total S amounts, which in control plants were higher in the *japonica* genotypes Onice and IL O11 than in the *indica* parental line IR64-*SalTol*, were dramatically reduced by salt stress in both introgressed lines IL O11 and the *SalTol* donor parental one (-70% and -47%, respectively), while resulted unchanged in the recurrent parent Onice. For what concerns the sulfate amounts, in control plants the trend of sulfate accumulation in the different genotypes was similar to that observed for total S. Salt stress significantly affected the sulfate amounts by inducing a strong increase in Onice and a strong decrease in IL O11, as compared to their controls. In particular, under salt conditions, the sulfate amounts in both the introgressed lines (IL O11 and IR64-*SalTol*) were low and similar, and essentially equal to those observed in control plants of IR64-*SalTol*.

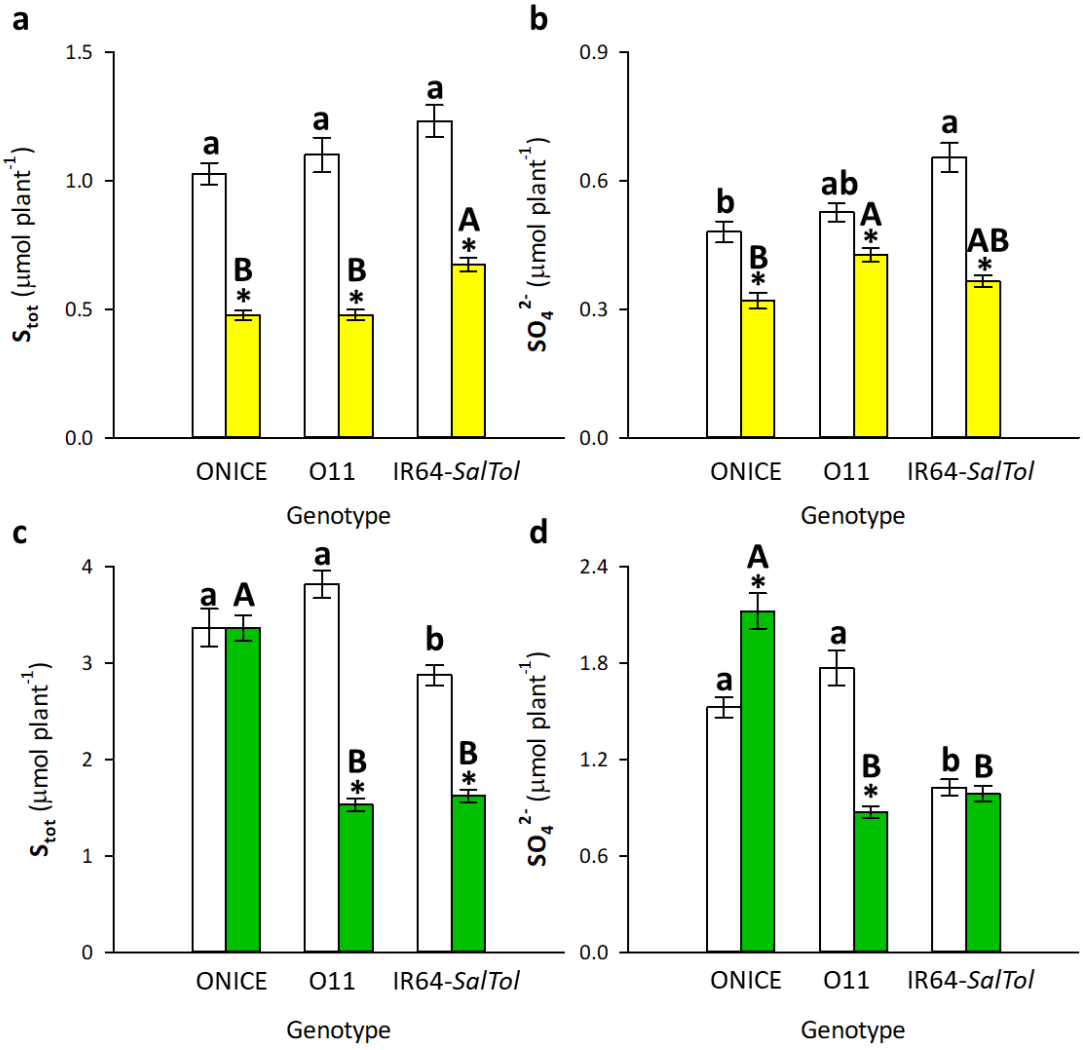

Figure 9. (**a**) Root total S and (**b**) sulfate amount, (**c**) shoot total S and (**d**) sulfate amount for plant of the Italian recurrent parent Onice, the *SalTol* donor parent IR64-*SalTol*, and the introgressed line IL O11 and after 7d of growth in the hydroponic solution in the absence (white bars) or in the presence (yellow and green bars for root and shoot, respectively) of 80 mM NaCl. Values are expressed as the mean ± SE of 3 different measurements performed on pooled roots and shoots composed from 40 plants of the same genotype and condition (n = 3) and are expressed on the DW basis. Various letters (lowercase and capital as referred to control and treated plants, respectively) indicate significant differences among genotypes after ANOVA (P < 0.05). Asterisks indicate significant differences between control and treated plants within each genotype after t-test (P < 0.05).

After salt treatment a strong decrease in the amounts of NPTs in all the lines could be observed, both in the root and in the shoot (Fig. 10a and b). In particular, the root amounts of NPTs were significantly lower in both control and treated plants of IR64-*SalTol* as compared to the other two genotypes.

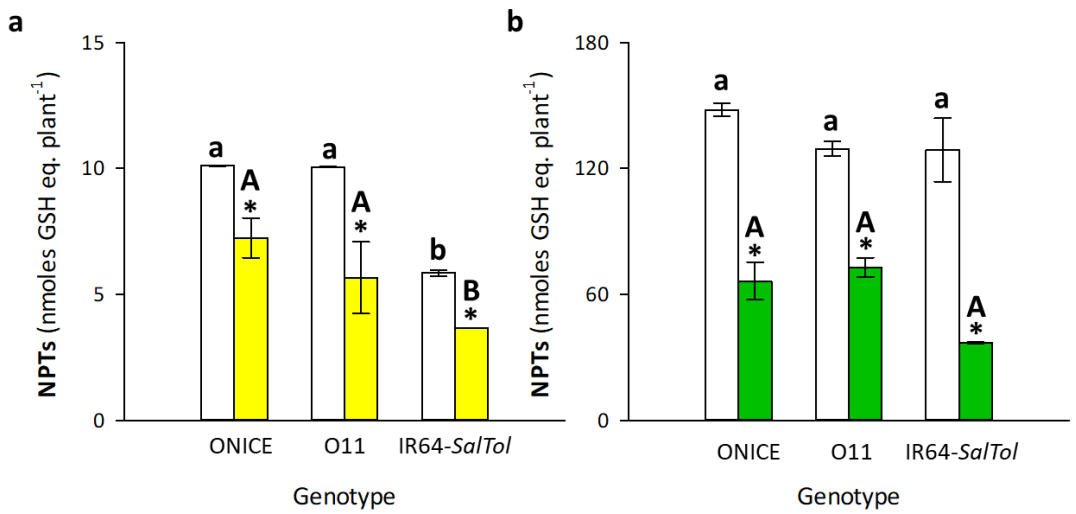

Figure 10. Amounts of non-protein thiols (NPTs) for plant in (**a**) root and (**b**) shoot of the Italian recurrent parent Onice, the introgressed line IL O11, and the *SalTol* donor parent IR64-*SalTol* after 7d of growth in the hydroponic solution in the absence (white bars) or in the presence (yellow and green bars for root and shoot, respectively) of 80 mM NaCl. Values, expressed as nmoles of glutathione (GSH) equivalents, are the mean ± SE of 3 different measurements performed on pooled roots and shoots composed from 40 plants of the same genotype and condition (n = 3) and are expressed on the DW basis. Various letters (lowercase and capital as referred to control and treated plants, respectively) indicate significant differences among genotypes after ANOVA (P < 0.05). Asterisks indicate significant differences between control and treated plants within each genotype after t-test (P <0.05).

The expression analysis of the main *OsSULTR* genes belonging to group 1 and 2 was performed in the roots [Fig. 11; *OsSULTR1;1* (a), *OsSULTR1;2* (b), *OsSULTR1;3* (c), *OsSULTR2;1* (d), and *OsSULTR2;2* (e)] and shoots [Fig. 12; *OsSULTR1;3* (a), *OsSULTR2;1* (b), and *OsSULTR2;2* (c)] of the three genotypes after 7d of plant growth in hydroponics, in the absence or in the presence of 80 mM NaCl .

All the *OsSULTRs* of root system, involved in sulfate uptake and translocation, were strongly downregulated by salt stress in all lines considered (Fig. 11).

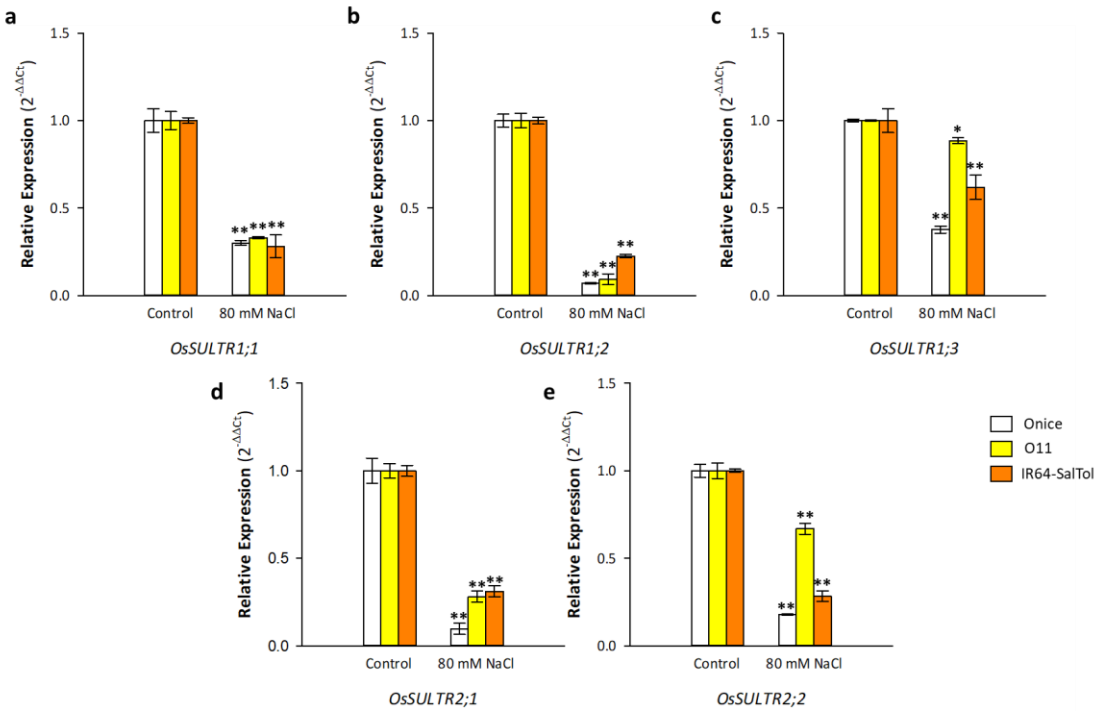

Figure 11. Transcript levels of the genes (**a**) *OsSULTR1;1*, (**b**) *OsSULTR1;2*, (**c**) *OsSULTR1;3*, (**d**) *OsSULTR2;1*, and (**e**) *OsSULTR2;2* in the roots of the Italian recurrent parent Onice (white bars), in the introgressed line IL O11 (yellow bars), and in the *SalTol* donor parent IR64-*SalTol* (orange bars) after 7d of growth in the hydroponic solution in the absence or in the presence of 80 mM NaCl. Values are expressed as $2^{-\Delta\Delta Ct}$ taking *OsS16* as gene reference. Values are expressed as the mean ± SE of 3 different measurements performed on pooled roots constituted by 40 plants (*n* = 3). Asterisks indicate significant differences between control and treated plants within each genotype after *t*-test at *P*-value $0.001 \leq P < 0.05$ (*) and at $P < 0.001$(**).

Concerning the shoot system, *OsSULTR1;3* (Fig. 12a) was upregulated by salt treatment in the tolerant donor parent IR64-*SalTol*, while its expression was not affected in the IL O11; on the contrary, salt stress caused a strong downregulation of this gene in the sensitive recurrent parent Onice. The expression of *OsSULTR2;1* (Fig. 12b) did not change in the IL O11, while it was downregulated in the parental genotypes, both the recurrent and the donor one. A strong downregulation of *OsSULTR2;2* (Fig. 12c) occurred in all the three lines after treatment with 80 mM NaCl.

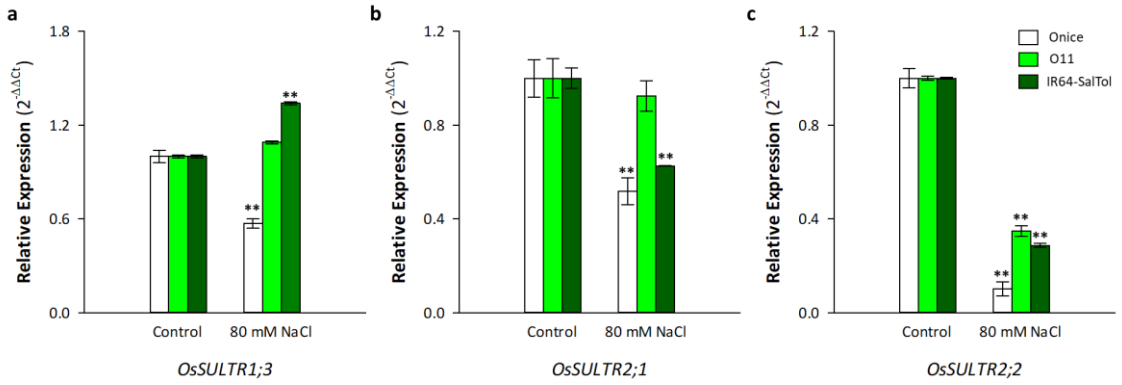

Figure 12. Gene expression of (**a**) *OsSULTR1;3*, (**b**) *OsSULTR2;1*, and (**c**) *OsSULTR2;2* in the shoots of the Italian recurrent parent Onice (white bars), the introgressed line IL O11 (light green bars), and the *SalTol* donor parent IR64-*SalTol* (dark green bars) after 7d of growth in the hydroponic solution in the absence or in the presence of 80 mM NaCl. Values are expressed as the mean ± SE of 3 different measurements performed on pooled shoots constituted by 40 plants (n = 3). Asterisks indicate significant differences between control and treated plants within each genotype after t-test at P-value $0.001 \leq P < 0.05$ (*) and at $P < 0.001$(**).

5.4 Discussion

5.4.1 Introgression of *SalTol* QTL in O11 and phenotypic behavior in salt stress

Climate changes and global rise in temperatures represent a serious ecological issue and induce several damages to agriculture, worsening abiotic stresses. Among these, salinization of soils and freshwater to date involve more than 20% of arable land on a worldwide basis. These aspects, together with the request for new arable territories to fulfill the food requirements of the increasing world's population, make it necessary to create and individuate new marketable rice varieties able to cope satisfactorily with the different stresses in relation to the territorial requirements.

Rice is a salt-sensitive crop (Munns and Tester 2008), with yield decreasing by 12% for every unit (dS m^{-1}) increase in EC above 3.0 dS m^{-1} (Maas and Grattan 2015). The development of salt stress-tolerant rice genotypes is therefore of utmost importance for the improvement of productivity of this crop in areas either salinized or at risk of salinization.

An in-depth characterization of the plant responses to specific stress factors is fundamental in the process of genetic improvement of stress tolerance in crop plants. In this context, breeding of new elite rice varieties needs to combine phenotypic, genotypic, transcriptomic, proteomic, and metabolomic analyses defined altogether as "Systems Biology" (Reddy *et al*. 2017). Amongst the different biotechnological techniques, the so-called Marker-Assisted Background Crossing (MABC) combines Marker Assisted Selection and conventional breeding and allows to introduce rapidly and efficiently into many cereals, and particularly so in rice, genes of interest involved in the response to several biotic and abiotic stresses, saving a few backcrosses compared with conventional breeding schemes.

In the present work, the major QTL *SalTol* from the donor parent IR64-*SalTol* was introgressed into the recurrent elite *japonica* line Onice yielding several ILs. The behavior of IL O11, one of the best performing in salinized fields, was confirmed in controlled conditions of hydroponic culture in the absence or in the presence of salt.

5.4.2 Performance of IL O11 in presence of salt: growth inhibition and SES

Growth inhibition is one of the general symptoms caused by salt stress, which leads to other injuries. Particularly, plants biomass reduction due to saline imposition can be caused by four different kinds of stresses: 1) water imbalance due to osmotic stress; 2) ion toxicity caused by high amount of Na^+ inside the cells; 3) nutrient imbalance due to high level of Na^+ and Cl^- which reduce the uptake of K^+, NO^-, PO_4^3; 4) higher production of ROS, which damages cells membrane and nucleic acids (Nawaz *et al.* 2010).
All the lines considered in this work were severely affected by salt treatment and they showed high decrease in biomass of shoot and root system. The significative differences of IR64-*SalTol* even in control condition can be explained by the natural different plant architecture which exists between the subspecies *japonica* and *indica* (Garris *et al.* 2005).

Visual examination of salt-induced leaf injury symptoms at vegetative stage, according to the widely accepted scale of IRRI, allows to classify rice cultivars from salt-tolerant (like *Pokkali* and FL478, SES score = 3 at 120 mM NaCl; Rahman *et al*. 2016) to moderately tolerant (SES score = 5), susceptible (SES score = 7), and highly salt-susceptible (SES score = 9) (Gregorio 1997) even if the phenotypic performance may slightly change in different conditions: at 80 mM NaCl, seedlings of salt-tolerant *Pokkali* show a SES score of about 4.5 (Yichie *et al*. 2018). IR64-*SalTol* is classified with a mean SES score, calculated on four different lines in the presence of 12 dS m^{-1} (approximately 100 mM NaCl) (Ho *et al.* 2016) of 4.31.
In our 80 mM NaCl conditions, with lighter salt stress, IR64-*SalTol* appeared to be only moderately tolerant (or slightly susceptible), with a recorded SES score of about 3.9 at 7d and 6.5 at 14d. In this context, it should be considered that unexpected differences in agronomic and physiological traits among IR64-*SalTol* lines have been described and explained invoking possible effects of undetected genes introgressed from the donor parent into chromosomal regions of the recurrent parent genome (Ho *et al.* 2016). Concerning the recurrent parent Onice, with a SES index of 4.9 at 7d and 7.3 at 14d, it was confirmed as salt-susceptible; introgression of the *SalTol* QTL ameliorated the salt effects in IL O11 (SES score at 7d = 3; 14d = 5.7).

5.4.3 Performance of IL O11 in presence of salt: thermal imaging

Osmotic stress imposed by saline condition causes a decrease in soil water potential, leading to a lesser capability of root system to extract water from soil (Epstein 1980; Munns 1993). Regarding specific plant tolerance to salt stress, it can be divided in 1) plants which tolerate high external salt concentration (osmotic tolerance); 2) plants which can control NaCl uptake; 3) plants which can tolerate high internal level of salt concentration (tissue tolerance). In particular, phenotyping for osmotic tolerance can be assessed by measuring stomatal conductance: in fact, it is very likely that the factors controlling this kind of tolerance are the same which control stomatal conductance (Rahnama et al. 2010). The infrared (IR) thermography is a powerful tool to measure stomatal conductance. This method can graphically show leaves temperature distribution, which is largely functional of stomatal conductance, by focusing the long-wave radiation emitted and reflected by plants (James and Sirault 2012). In 17 different genotypes of durum wheat, interesting results on stomatal conductance and leaves temperature were obtained through the IR thermography technique after treatment with 150 mM NaCl for 3d. This allowed the development of a performing screening method to understand more about salinity tolerance in plants (Sirault *et al.* 2009). Genotypes with a better tolerance to stress uptake water efficiently by maintaining higher stomatal conductance, and so they can be identified as plants with cooler leaves. A work performed by Siddiqui *et al.* (2014) on *O. sativa* cv. Donggin highlighted how 4d treatment with 225 mM NaCl statistically modified stomatal conductance, leading to higher leaves temperature in treated plants.
In our lines, thermo imaging results suggest a higher capability of IL O11 respect to Onice and IR64-*Saltol* to maintain an adequate stomatal conductance, so as to keep the hydration status of cells.

5.4.4 Performance of IL O11 in presence of salt: MDA levels, EL and antioxidant activity

Exposure of plants to salinity results in increased levels of ROS; the ROS-induced damage to cellular components particularly concerns biological membranes that lose their physiological properties (Vinod *et al*. 2014). Increased MDA levels are a biochemical

marker of membrane lipid peroxidation (Taïbi *et al.* 2016); under the physiological point of view, membrane damage is indicated by tissue electrolyte leakage (Xu *et al*. 2012).
In salt-stressed roots of the salt-susceptible IR28 and the salt-tolerant *Pokkali* rice cultivars, the levels of MDA increased in the former but remained constant in the latter, suggesting better protection against oxidative damage under this stress (Demiral and Türkan 2005). More recently, in the salinity-tolerant cultivar Giza 182 and the salinity-susceptible Sakha 105, consistent results have been described in leaves: the salt treatment induced an increase in MDA levels in both cultivars, albeit lesser in the tolerant cultivar than in the susceptible one (Abdelaziz *et al*. 2018).
Levels of MDA were similar in the IR64-*SalTol* donor line and IL O11 in the salt condition, while they were higher in the sensitive line Onice.

An investigation for salt tolerance performed on four rice genotypes (two salt-sensitive – Hitomebore and IR28 – and two salt tolerant – *Pokkali* and Bankat) showed that EL leakage under three different level of NaCl (0, 6 and 12 dS m^{-1}) remained unchanged in *Pokkali*, while in the other genotypes (even in the other salt-tolerant variety) EL increased as the salinity in the growth media did, and so did also MDA levels (Dionisio-Sese and Tobita 1998). In maize plants, treatment with 100 mM NaCl impaired membrane permeability by inducing increased electrolyte leakage in leaves (Kaya *et al.* 2013).
Considering EL values obtained by our investigation, on a percentage basis O11 performed better both than the recurrent parent and IR64-*Saltol*, showing a lesser damage of its membranes during salt stress. However, considering the absolute values reached after the 7d of salt treatment, the tolerant *SalTol* donor and the putative tolerant IL O11 lines showed equal EL value, lower than that of the salt sensitive Onice, whose membranes were more affected by ROS damages.

Plants have developed different mechanisms to cope with oxidative stress, and the connection between enzymatic one and salt tolerance is well and long known (Nazar *et al*. 2011; Rossatto *et al.* 2017; Vighi *et al.* 2017). Nevertheless, contrasting evidence are present in the literature concerning the effect of salt stress on the different antioxidant enzyme activities, so that it is currently still difficult to univocally associate the plant salt tolerance/sensitivity trait to a precise antioxidant pathway or to a candidate enzyme.

In the lines considered in this work, SOD activity was always significantly higher after salt treatment, even if to a lesser extent in IR64-*SalTol*. This agrees with the results of other studies in which sensitive and tolerant rice plants under salt stress condition showed an increase in SOD activity (Turan and Tripathy 2013; Khare *et al*. 2015; Vighi *et al.* 2017).

In the paper published by Vighi *et al.* (2017), no differences of CAT activity are reported between the sensitive genotype BRS Bojuru and the tolerant BRS Pampa. In their time course experiment (0, 6, 24, 48, and 72h after treatment with 150 mM NaCl), the two genotypes behaved similarly with the only difference at 48h when BRS Bojuru showed the highest level of CAT activity. Also, in the work performed by Rossatto *et al.* (2017) on the variety BRS AG no differences of CAT activity were found after 10, 15, and 20d of salt treatment (136 mM NaCl). Our results indicated that 7d treatment of plants with 80 mM NaCl did not affect, or even decrease, CAT activity in the tolerant introgressed lines IR64-*SalTol* and IL O11, while dramatically increased the enzyme activity in the sensitive recurrent parent Onice.

APX is of paramount importance in H_2O_2 scavenging through the ascorbate-glutathione cycle. The APX affinity for H_2O_2 is higher than that of CAT allowing the peroxidase to eliminate this ROS even at low concentration (Gill and Tuteja 2010). Some studies performed on rice and soybean indicated an increase in APX activity in salt tolerant genotypes respect to sensitive ones (Moradi and Ismail 2007; Hakeem *et al.* 2012; Mishra *et al*. 2013). On the contrary, other papers reported an increase in the activity of this enzyme only in sensitive rice genotypes (Turan and Tripathy 2013; Khare *et al.* 2015; Rossatto *et al.* 2017; Vighi *et al.* 2017). In our work, all the three lines showed an increment of APX activity after salt treatment. However, the increment measured in the sensitive parent Onice was double and triple respect to those measured in IL O11 and IR64-*SalTol*, respectively.

In different plants species like rice, wheat, tobacco, maize and peanut an increase in GR activity under salt stress was observed (Guo *et al.* 2006; Tang *et al.* 2010a, b; Tan *et al.* 2011). In our results, after stress administration Onice showed an increase in GR activity three times higher than the increase observed in the putative tolerant IL O11. In both

the tolerant lines the effect of salinity on the GR activity increase was lower than in the sensitive parent Onice.

Taken as a whole, the results on antioxidant activities are coherent with those on MDA levels and EL. In salt-stressed IL O11 and IR64-*SalTol* the lack of increase in CAT activity allows to hypothesize the presence of lower levels of H_2O_2 than in Onice, which appear coherent with and could possibly account for the better membrane stability observed in the introgressed lines respect to the recurrent parent Onice. Further studies are needed to confirm this hypothesis.

Also concerning the oxidative stress, the introgression of *SalTol* proved effective in ameliorating the plant response to salinity.

5.4.5 Performance of IL O11 in presence of salt: Na^+/K^+ ratios

Two components are involved in the plant's perception of salinity stress (Munns and Tester 2008), osmotic stress, perceived immediately upon plant exposure to highly saline conditions, and the subsequent saline stress due to the accumulation of high concentrations of toxic ions over a longer period (Long *et al*. 2013). Entry of both Na^+ and Cl^- into the cells causes severe ion imbalance with onset of biochemical-physiological disorders. Excess Na^+ inhibits uptake of K^+ with consequences on the activity of several enzymes for the antagonistic action of Na^+ against K^+ (Kronzucker *et al.* 2013), indicating the importance of maintaining a balanced concentration of the two cations (Zhu 2007). K^+ regulates the activity of several cytosolic enzymes, the values of transmembrane electric potential, while also acting as an osmotic in the regulation of cell turgor (Yamane *et al.* 2008). The Na^+/K^+ ratio is considered as an indicator of the plant response to high salinity (Ren *et al*. 2005; Shabala and Cuin 2008). In fact, plant tolerance to salt stress depends on the ability to maintain, through selective uptake mechanisms (Ismail *et al.* 2010; Platten *et al*. 2013), a proper ion balance, i.e., low Na^+ and high K^+ concentrations and lower Na^+/K^+. Salt-tolerant plant species and varieties accumulate in shoots lower Na^+ and higher K^+ as opposed to sensitive ones (Rahman *et al*. 2016). More particularly, reduced Na^+ delivery from root to shoot, Na^+ recirculation in the phloem, tissue and organ-specific compartmentation (including enhanced Na^+ loading into structures like senescing leaves, leaf sheaths or epidermis, of limited

physiological importance), as well as recirculation of Na^+ from the photosynthetic organs back to the roots, play a key physiological role in adaptation to salinity at the whole-plant level.

It has been suggested that sheath K^+ and Na^+/K^+, together with Na^+/Mg^{2+} may be useful indicators for genetic analyses of salt-tolerant rice varieties under salt-stress conditions (Thu *et al*. 2017). In roots of salt-tolerant rice cultivars grown in saline conditions the Na^+ contents are higher than in salt-susceptible ones due their ability to prevent the translocation of sodium from root to shoot (Rahman *et al*. 2016) with the beneficial consequence of limiting damage to the photosynthetic machinery of the plant (Ma *et al*. 2018). Therefore, roots of tolerant landraces present higher Na^+/K^+ ratios than susceptible ones, concurrent with lower shoot Na^+ concentrations and Na^+/K^+ ratios (Rahman *et al.* 2016).

In our study, we divided plants into different parts, roots, stem and sheath, young, and old leaves, to gain further insights into salt-tolerance mechanisms. In our material, IR64-*SalTol* did show extremely high Na^+/K^+ ratios in the root fractions, accompanied by relatively low Na^+/K^+ ratio in young leaves, which may be interpreted as a control of Na^+ accumulation in the aerial parts through the reduction of its translocation from roots and its accumulation in the roots. In the sensitive cultivar Onice a high amount of Na^+ was observed in young leaves, that was strongly diminished in the tolerant IL O11. A strong correlation has been described between Na^+ tolerance and leaf blade Na^+ concentration, and the major tolerance mechanisms found in *O. sativa* and *O. glaberrima* are those capable to limit Na^+ uptake and accumulation in active leaves (Platten *et al.* 2013). These observations are consistent with the reported action of the *SalTol* QTL (Lin *et al*. 2004; Ren *et al.* 2005; Thomson *et al.* 2010), whose action is referred to the Na^+ transporter SKC1 (*OsHKT1;5*, Platten *et al*. 2006). *OsHKT1;5* is involved, primarily in roots, in the unloading of Na^+ from the xylem (Ren *et al.* 2005) with the consequent reduction of its translocation to the leaves (Hauser and Horie 2010), and better control of the general Na^+/K^+ homeostasis under salt stress.

According to the role reported for *SalTol* QTL, O11 as well as the donor parent IR64-*SalTol* seem able to maintain a low Na^+/K^+ ratio in the photosynthetically active organs, like young leaves. This result fits well with the data obtained for the antioxidant activities. In fact, CAT, that is a peroxisomal enzyme and has a lower affinity for H_2O_2

than APX, does not increase its activity in the introgressed genotypes when exposed to salt condition suggesting that, under salinity, in these lines the photorespiratory process may have a low impact in the reduction of the photosynthetic yield. Further studies are needed to confirm this hypothesis.

5.4.6 Performance of IL O11 in presence of salt: effect on S metabolism

Change in SO_4^{2-} uptake and assimilation are related to the adaptation of plants to different environmental condition, such as exposure to salt stress, and are determinant for the correct development (Samanta *et al*. 2020). Uptake and translocation of sulfate under different abiotic stresses need to be finely regulated, however a gene expression study on the main *OsSULTR* of group 1 and 2 performed on the sensitive *indica* rice variety IR64 by Kumar *et al*. (2011) has pointed out how salt stress downregulates their expression. Other studies of gene expression on young seedling of *Medicago truncatula* and *Arabidopsis thaliana,* showed no significative differences in *A. thaliana* and an upregulation of *SULTR1;2* in *M. truncatula* after early treatment (6h after the application of the stress) (Gallardo et al. 2014). It is of paramount importance to take in account SO_4^{2-} uptake and translocation, as well as considering possible modifications in the content of total S and sulfate in tissues, which could occur after application of salt stress; a work performed on *Allium cepa* allowed to discover that a treatment with 200 mM NaCl has no effect on S composition of root system, but it reduced the levels of S_{tot} and SO_4^2 of shoots, probably due to the competition for the adsorption and translocation between SO_4^{2-} and Cl^-, which has a diffusion rate greater than sulfate (Aghajanzadeh *et al*. 2019). In Alfalfa plants, exposure to salt stress leaded to a decrease only in total S content (Bhattarai *et al*. 2020).

Even the assimilation of this macronutrient and the involvement of S reduced compounds in improving salt tolerance have been investigated in the last years; it has been demonstrated that the modulation of the related physiological and molecular processes are important in enhancing tolerance to salt stress (Ashraf 2009; Türkan and Demiral 2009; Nazar *et al*. 2011; Ashfaque 2014; Mohd Asgher 2014). In particular, different GSH synthesis, controlled by a fine regulation of S assimilation and Cys biosynthesis, has a key role in the scavenging of ROS which tends to accumulate during salt stress (Mohd Asgher 2014). In *Vigna radiata*, (Nazar *et al.* 2011), *Lycopersicum*

esculentum (Shalata *et al*. 2002), *Oryza sativa* (Vaidyanathan *et al.* 2003) and *Archis hypogea* (Mittova *et al*. 2003) cultivars, tolerant plants showed higher GSH content and minor level of ROS than sensitive ones.

In our lines, all *OsSULTR*s of group 1 and 2 in the root were strongly downregulated by salt stress, even in the tolerant lines. The amount of total S in the roots dramatically decreased after salt treatment: it is possible to speculate that this may due not only to the downregulation of the genes involved in S uptake, but also to the strong decrease in NPTs amount in treated roots of all the lines considered.

In shoot of Onice the amounts of S_{tot} and SO_4^{2-} were not affected by 7d of salt treatment. This could be due to the different relative transcript levels of *OsSULTR1;3* among the lines: salt treatment upregulated the expression of this gene in the tolerant line IR64-*SalTol*; in the tolerant IL O11 application of the abiotic stress had no effect on the expression of this gene; in Onice *OsSULTR1;3* was strongly downregulated in treated shoot. It is possible that because of the stress caused by salt imposition, while the two tolerant lines tried to maintain a partial but not sufficient SO_4^{2-} homeostasis, the sensitive recurrent genotype lose the capability of reallocating sulfate form shoot to root and so it tended to accumulate the ion in the aerial parts. However, NPTs amount in shoot of all the genotypes was dramatically reduced by salt stress, and so it is possible to say that this stress negatively affected correct S assimilation in all the genotypes.

5.5 Conclusions and perspectives

In the present investigation salinity tolerance in the *japonica* variety Onice (salt-sensitive) was achieved by introgression, via MABC breeding, of the *SalTol* QTL from the *indica* tolerant line IR64-*SalTol*. The IL considered in this study (O11) showed an enhanced salt tolerance at vegetative stage and it might be considered as a promising line to be grown in salt-affected soils. Taken as a whole, the data allow to better understand the beneficial effect on the agronomic behavior in salinized environmental conditions of the introgression of the *SalTol* based on different characteristics of selected biochemical-physiological parameters. The analysis of the sulfur metabolism parameters showed that they were generally strongly affected by salt stress in all lines, suggesting that sulfur metabolism might not be directly involved in determining salt stress tolerance in the *SalTol* introgressed line O11 considered.

For the future, analyzing S acquisition and assimilation in the other ILs obtained (VN25 and VN28) could allow to discover more about the possible relationship between S metabolism and different salt tolerance. Moreover, the possibility of performed EA-IRMS analysis could be a great tool to gain more information about S isotope composition of the genotypes considered, potentially permitting to obtain more knowledge about plants S nutritional status during application of salt stress.

5.6 Supplementary material

Table S1. Sequences of primers (forward and reverse, 5'→3') of the housekeeping gene (*OsS16*) and of the *OsSULTR*s analyzed in the work. Primers were designed with the software Primer Express™ v. 3.0.1 (Thermo Fisher). Sequences of cDNA were found on "Rice Genome Annotation Project" website (http://rice.plantbiology.msu.edu/).

		Primer (5'-3')	
Gene	**Accession Number**	**Forward**	**Reverse**
OsS16	*LOC_Os11g03400*	ACGTCGACGAGGCATCCA	CGCGACCACCGAACTTCTT
OsSULTR1;1	*LOC_Os03g09970*	GGAGCATTCTTTGGCGTCAT	TCGCAACCGCAATTAGCA
OsSULTR1;2	*LOC_Os03g09980*	CGACCTTCTTTGCAGGAGTCA	TGAACCCTAGCCTGCAGAAAC
OsSULTR1;3	*LOC_Os08g31410*	GGAATATCTGCGCCTTGCTT	AGGGCTGCTTGAGTGATACCA
OsSULTR2;1	*LOC_Os03g09940*	CAAGGAGGCTCTCAGCATCTG	GCCGAGGCATGTGAGGAA
OsSULTR2;2	*LOC_Os03g09930*	GAATGATCTCACCGGCATGTT	GAAGCGCTTGTCTGCACCTT

6. General Conclusions

Results obtained allowed to discover that:

- Rice plants can discriminate against ^{34}S during SO_4^{2-} acquisition and assimilation, and that shoot represents the predominant organ involved in S assimilation. However, different environmental conditions which are well known to have a strong relationship with S metabolism (i.e., sulfur starvation or Cd exposure), can modulate the expression of *SULTR* genes, and the observed changes in the prevalence of relative expression between *OsSULTR1;1* and *OsSULTR1;2* in root system may be determinant for different isotope fractionation during sulfate uptake, leading to a minor discrimination against the heavy ^{34}S stable isotope.
- Concerning salt stress and S metabolism, even if nowadays it is well known its involvement in determining different tolerance to this abiotic stress, this feature appears to not justify salt tolerance in the case of the IL O11.

In general, EA-IRMS represents a powerful tool to study sulfur systemic fluxes in plants and to potentially understand more about plant S nutritional status and its involvement in plant tolerance under different environmental conditions.

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