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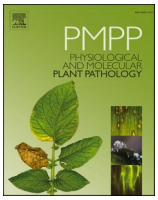

Initial characterization of a bacterial leaf streak susceptibility gene suggests it encodes a membrane transporter that influences seed nutrition and germination

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ABSTRACT

The type III secreted transcription activator-like effector Tal2g of the rice bacterial leaf streak (BLS) pathogen *Xanthomonas oryzae* pv. oryzicola promotes lesion development and bacterial exudation through stomata by binding to and upregulating a putative sulfate transporter gene in rice, *OsSULTR3;6*. To understand how OsSULTR3;6 contributes to disease development, we are characterizing its transport mechanics, subcellular localization, and potential substrates, and phenotyping *OsSULTR3;6* knockout lines generated by genome editing. Following a brief introduction to the plant SULTR gene family, this chapter summarizes our findings so far and presents speculative functional models for the role of OsSULTR3;6 in BLS.

Bacteria in the genus *Xanthomonas* cause disease in many crop species. During infection, many *Xanthomonas* species inject transcription activator-like effectors (TALEs) into plant cells, where these proteins directly activate specific, corresponding 'susceptibility' (*S*) genes important for disease development [1]. *Xanthomonas oryzae* pv. oryzicola (Xoc) causes bacterial leaf streak (BLS) in rice (*Oryza sativa*). Xoc enters the rice leaf through stomata or wounds and colonizes the mesophyll apoplast. The disease manifests as water-soaking at the site of entry, which then progresses into interveinal streaks. Late disease is characterized by tissue necrosis. As the disease progresses, bacteria ooze out of stomata onto the leaf surface, forming yellow droplets. Virulence depends on type III secretion of numerous effectors into host cells, including upwards of thirty distinct TALEs. Cernadas and coworkers identified one of these, Tal2g, as a major virulence factor [2]. A *tal2g* mutant caused shorter lesions and exuded less onto the leaf surface than the wild-type bacterium. Consistent with its importance as a virulence factor, Tal2g is conserved across a global collection of Xoc strains studied [3]. Tal2g exerts its effect by transcriptionally activating the *S* gene *OsSULTR3;6*, which encodes a putative sulfate transporter. The processes downstream of *OsSULTR3;6* induction that promote lesion expansion and bacterial exudation have not yet been elucidated. An important step toward understanding how OsSULTR3;6 contributes to disease development is to determine its native function in the absence of the pathogen. We are addressing this unknown by characterizing the transport mechanics, subcellular localization, and potential substrates of OsSULTR3;6. We are also investigating the function of OsSULTR3;6 by phenotyping knockout rice lines generated by genome editing. Following a brief introduction to the SULTR gene family in plants, this chapter summarizes our findings to date and presents speculative models, based on these findings, for how OsSULTR3;6 might promote

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lesion expansion and bacterial exudation from the leaf when its expression is induced by Tal2g.

1. The sulfate transporter (SULTR) gene family

In plants, members of the Sulfate Transporter (SULTR) gene family are hypothesized to encode integral membrane proteins that function as secondary active transporters. Such proteins coordinate the movement of protons (H^+) along a favorable electrochemical gradient to power the movement of anions against an unfavorable electrochemical gradient [4,5]. The SULTR family name derives from the role of some gene members in the uptake of sulfate from the soil and its movement internally within the plant [6,7]. Subsequent work has shown that some SULTR isoforms facilitate molybdate and phosphate transport, while others contribute to the abscisic acid (ABA) biosynthetic pathway, seed phytate accumulation, and pathogen susceptibility [8–12].

The SULTRs are grouped into four main subfamilies based on amino acid sequence similarity, which largely correlate with their differing functions *in planta*. The functional differences arise from their unique localization patterns and substrate affinity. The plasma membrane-localized members of the SULTR1 and SULTR2 groups demonstrate high and low-affinity transport of SO_4^{2-}, respectively. SULTR1 members are expressed in the root epidermis and cortex, while SULTR2 members are localized to the root endodermis and root and shoot xylem and phloem parenchyma, where they facilitate transport between the vasculature and surrounding tissues [6,13,14]. SULTR3 is the largest sub-group of the gene family and has several members that display functions in phosphate transport and seed phytate accumulation [9,10,15–17]. Individual SULTR3 isoforms localize to the plasma membrane, endoplasmic reticulum, and chloroplast membrane. SULTR4 members localize to the tonoplast and facilitate SO_4^{2-} export from the vacuole [18].

Much of the research on the function of the proteins encoded by SULTR genes has been done in the model plant *Arabidopsis thaliana,* and literature on the SULTR gene family in rice (*Oryza sativa*) is limited. While both Arabidopsis and rice each have 12 SULTR genes, group 3 in rice has 6 rather than 5 gene members, and OsSULTR3;6 is the one without an Arabidopsis ortholog [9,19,20]. It is most similar in sequence to OsSULTR3;5 but contains an additional, *C*-terminal domain, COG2252 [21]. Inversely, there is only a single SULTR4 gene in rice while Arabidopsis contains 2 functional members. The functional relevance of these discrepancies in the OsSULTR gene family, as compared to AtSULTR, is not yet clear. Since the functionality, localization, and any substrate(s) of OsSULTR3;6 are unknown, investigation of these properties is an important first step to understand the role of the protein in BLS and plant nutrition and may inform our understanding of the evolution of this gene family.

2. Transport function of OsSULTR3;6

To understand the native role of OsSULTR3;6 and its contributions to BLS susceptibility, we first wanted to determine whether the protein is a functional membrane transporter. The assessment of transport function was carried out in *Xenopus laevis* oocytes—unfertilized eggs from the African Claw Frog—using the two-electrode voltage clamp (TEVC) method [22].

We microinjected oocytes with RNA comprising a YFP::OsSULTR3;6 coding sequence (CDS). Confocal microscopy of these oocytes showed YFP expression at the periphery of the cell, suggesting localization to the oocyte plasma membrane (Fig. 1a). Next, we microinjected oocytes with untagged OsSULTR3;6 CDS or with sterile water as a negative control. After several days of incubation to allow for protein expression, oocytes

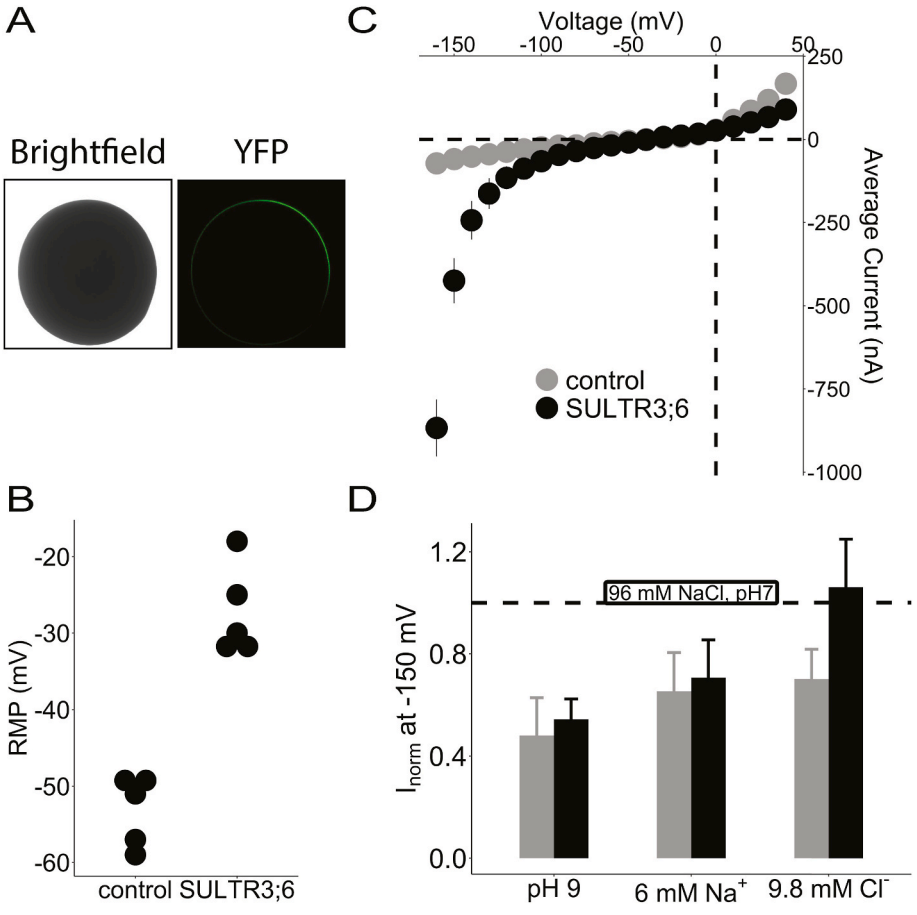

Fig. 1. OsSULTR3;6 is a membrane transporter. A) Microinjection of RNA encoding YFP-tagged OsSULTR3;6 shows YFP at the periphery of the oocyte. The left image displays a brightfield view of the oocyte and the right confocal imaging for the YFP signal (false-colored green). B) Resting membrane potential (RMP) of oocytes injected with water or RNA encoding OsSULTR3;6. Each point represents one cell. C) Current-voltage relationship displaying the average current measured in cells injected with water or RNA encoding OsSULTR3;6. D) Effect of bath solution changes on observed current in OsSULTR3;6-injected and control cells, relative to the standard bath solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, pH 7). Labels on the X-axis refer to the change of a single ionic constituent of the bath solution.

were impaled with a microelectrode to measure their resting membrane potential (RMP), which reflects the net ion balance across the cell's membrane. When compared to the water-injected controls, OsSULTR3;6-injected oocytes maintained less negative resting potentials, indicating that the expression of OsSULTR3;6 resulted in a change in the net ion balance of the cell (Fig. 1b).

Impalement of the oocytes with a second microelectrode allowed us to carry out TEVC and measure the amount of current flowing (i.e., ions transported) across the membrane at a constant, "clamped" voltage across the membrane. This technique helps to discern when the transporter is activated and how much transport occurs. When compared to water-injected controls, OsSULTR3;6-injected cells yielded large, negative currents at the various negative clamped voltages tested, indicating robust transport activity (Fig. 1c).

The bath solution in which the oocytes reside can be altered to determine how different ionic conditions affect transport. Therefore, we altered the pH, Na^+, or Cl^- levels to observe their effects on OsSULTR3;6 function. A reduction in either the bath concentration of H^+ or Na^+ led to significant reductions in currents for OsSULTR3;6-injected cells, while removal of Cl^- had no effect (Fig. 1d); this behavior contrasted with that of control cells, for which the low currents present were affected by reduction of any of the three ions (Fig. 1d). These data suggest that OsSULTR3;6 responds to extracellular cation concentrations, and they agree with the broader observations of SULTRs behaving as proton-driven co-transporters. Altogether, these observations indicate that OsSULTR3;6 is a functional transporter and that, similar to other SULTR members, its activity appears to be influenced by voltage and pH.

Looking ahead, we aim to define the substrate, or range of substrates, that OsSULTR3;6 is capable of co-transporting through further work with heterologous systems. For this, the oocyte and TEVC system will continue to be useful, as we can easily alter extracellular bath solutions and determine whether these affect the output current under different voltages. Additionally, expression of OsSULTR3;6 in *Saccharomyces cerevisiae* will be useful for screening nutrient transport via growth assays. *S. cerevisiae* mutant strains exist for both the sulfate (YSD1) and phosphate (PAM2) uptake pathways, allowing for growth complementation assays on limiting media [23,24]. Further hints from the literature and our plant nutrition experiments (discussed below) point toward either phosphate or sulfate as substrates of OsSULTR3;6, making these yeast strains an attractive starting point.

3. OsSULTR3;6 localization

In planta subcellular localization, combined with the functional data from the *X. laevis* and *S. cerevisiae* system, will provide insight into the physiological impact of OsSULTR3;6 activity by revealing the compartments between which transport occurs. In contrast to the other SULTR sub-groups, which each maintain consistent subcellular localization within a group, the SULTR3 members have been reported to localize to the plasma membrane, endoplasmic reticulum, or chloroplast [9,10,25].

We have undertaken initial efforts to localize OsSULTR3;6 by transient expression of a YFP::OsSULTR3;6 fusion protein in *Nicotiana benthamiana* epidermal cells. Confocal microscopy shows good expression in the epidermal cells with clear localization to the periphery (Fig. 2). Furthermore, co-expression alongside plasma membrane- or tonoplast-mCHERRY membrane markers shows large areas of overlap of the fusion protein and marker signals (Fig. 2). In the images we obtained, however, due to the large volume of the vacuole of the *N. benthamiana* epidermal cells, we were unable to unequivocally distinguish the two membranes. We will be expressing OsSULTR3;6 in both *Oryza sativa* and *Arabidopsis thaliana* mesophyll protoplasts to address this challenge.

4. Editing of *OsSULTR3;6*

Gene editing technology has been used to modify *S* genes to reduce disease susceptibility, and to knockout genes to study their function [26–28]. We previously developed two sets of edited rice lines as 1) proof of principle for resistance to BLS through loss of susceptibility and 2) as a resource to elucidate the function of OsSULTR3;6 (our unpublished results). The first set of lines are edited at the Tal2g effector binding element (EBE) in the promoter of *OsSULTR3;6*. The second are edited to remove the CDS of *OsSULTR3;6*. In representative lines from each set, the wild-type bacterium showed no difference in virulence from the *tal2g* mutant, indicating loss of the susceptibility function of *OsSULTR3;6* in these lines (Fig. 3). Further, based on preliminary phenotyping the lines had no apparent growth defects compared to unedited plants under normal, controlled conditions (Fig. 3).

Both sets of lines yielded seeds. We do not know yet whether the EBE edit affects endogenous expression patterns of *OsSULTR3;6*, but if not, it appears that this edit could indeed be deployed as a tractable form of quantitative resistance to BLS that might slow disease progression and protect yields while imposing less selection on the pathogen to evolve to

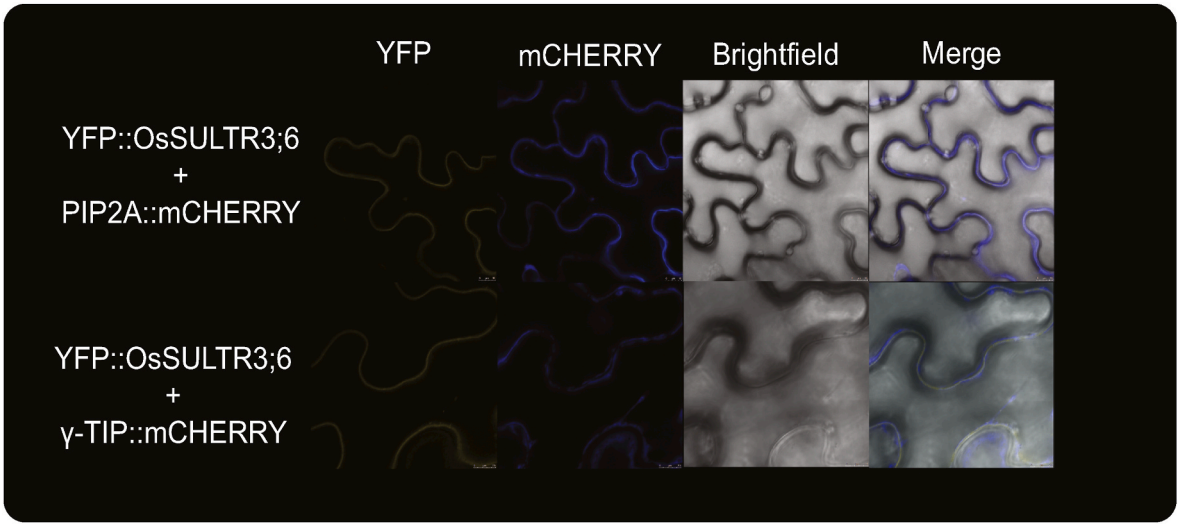

Fig. 2. Expression of a YFP-OSSULTR3;6 fusion protein in *Nicotiana benthamiana* epidermal cells: A YFP::OsSULTR3;6 fusion protein was transiently co-expressed with a plasma or tonoplast membrane marker, consisting of a PIP2A or γ-TIP fusion to mCHERRY, respectively. Panels left to right show YFP, mCHERRY (false-colored cyan), brightfield, and a merge of all channels.

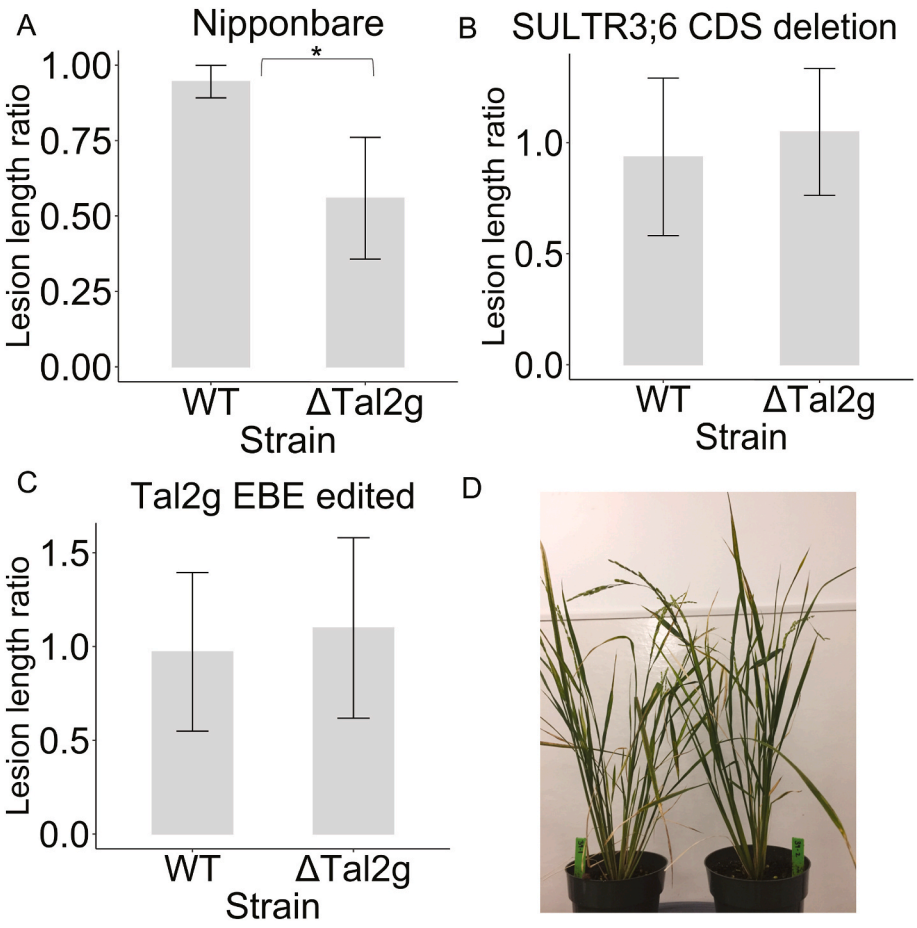

Fig. 3. Disruption of the Ta2g effector binding element (EBE) in the *OsSULTR3;6* promoter or deletion of the coding sequence of *OsSULTR3;6* reduces disease development without obvious developmental defects. Results of inoculation of A) wild-type Nipponbare plants, B) plants of a *SULTR3;6* CDS deletion line, and C) plants of a Tal2g EBE deletion line, with Xoc strain BLS256 (WT) and a *tal2g* mutant (M27). To account for leaf-to-leaf and position-to-position variability [2], both the *tal2g* mutant and the WT bacterium were inoculated side-by-side across the midrib with another WT inoculation, and the ratio of the resulting respective lesion lengths within each side-by-side pair was recorded. Shown are the mean ratios for 10 or more paired inoculations each (*$p < 0.05$). D) Growth habit of representative plants of an *OsSULTR3;6* CDS deletion line (left) and an unedited line (right).

overcome it. This approach contrasts with major, dominant resistance genes, which impose strong selection. The CDS deletion lines (*Δsultr3;6*), being fertile, are a useful resource for functional characterization of OsSULTR3;6, some of which we have begun and describe below.

5. Contributions of OsSULTR3;6 to seed physiology and nutrition

Public transcript expression data reveal that *OsSULTR3;6* is

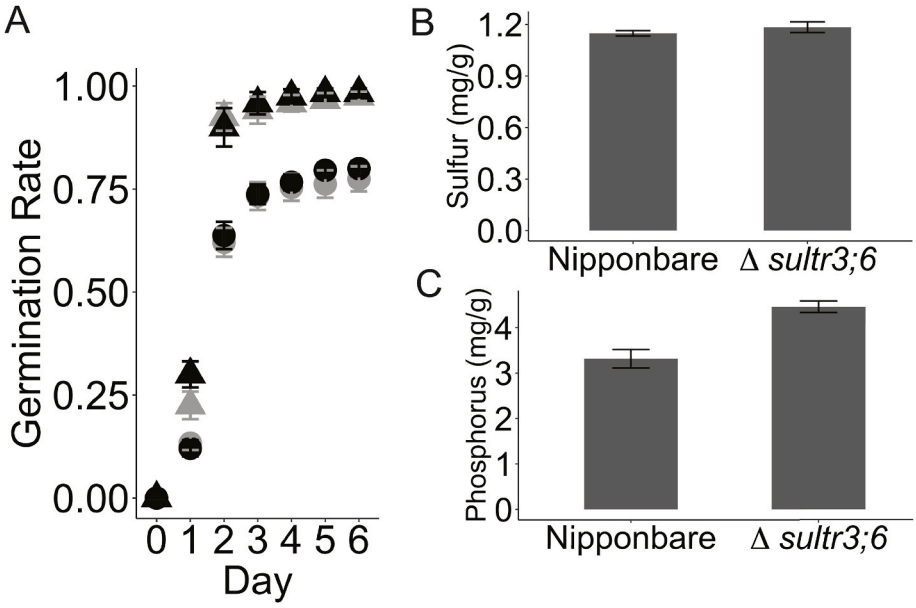

Fig. 4. OsSULTR3;6 influences rice seed function and nutrition. A) Proportion of total wild-type Nipponbare vs. *Δsultr3;6* seeds germinated over a six-day period, with or without GA_3 supplementation. Triangles, Nipponbare; circles, *Δsultr3;6*. Black fill, with GA_3; grey fill, without. B) Total phosphorus concentration measured by ICP-OES in Nipponbare and *Δsultr3;6* seeds. C) Total sulfur concentration measured by ICP-OES in Nipponbare and *Δsultr3;6* seeds.

upregulated in the rice inflorescence, specifically within the developing embryo (https://ricexpro.dna.affrc.go.jp; Locus ID Os01g0719300). We noticed in working with seeds of *OsSULTR3;6* CDS deletion (*Δsultr3;6*) plants that they were slow to germinate. We quantified this phenomenon in germination assays, and found a 25% reduction in germination compared to the wild type (Fig. 4a). This observation led us to hypothesize that the loss of OsSULTR3;6 transport causes nutritional changes in the seed directly, or indirectly via effects on plant metabolism.

Given that the plant hormone abscisic acid (ABA) is associated with repressing seed germination, we investigated the connection between OsSULTR3;6 and ABA [29]. Cysteine, a product of the sulfate assimilation pathway, is a sulfur donor required for the maturation of a molybdenum co-factor (MoCo) used by abscisic aldehyde oxidase 3 (AAO3) to catalyze the final oxidation step of the pathway to generate abscisic acid from abscisic aldehyde [30]. We hypothesized that OsSULTR3;6 activity influences sulfate levels in the reproductive tissue and subsequently the ABA concentration of the seed.

As a first step to test this proposed connection between OsSULTR3;6 and ABA, we repeated the same germination assays, but with or without gibberellic acid (GA_3) added to the medium. GA_3 had no effect on the germination rate of the of the *Δsultr3;6* seeds or of the wild-type seeds (Fig. 4a). These results do not exclude the possibility of a connection between OsSULTR3;6 and ABA, but prompted us to next investigate the possibility of direct nutritional effects. We used inductively coupled plasma-optical emission spectroscopy (ICP-OES) to measure the concentration of elements within *Δsultr3;6* vs. wild-type seeds, with a focus on sulfur and phosphorus. Seeds from *Δsultr3;6* plants had similar levels of sulfur to those measured in wild-type seeds, but contained more phosphorus (Fig. 4b and c).

The above data suggest two attractive hypotheses surrounding the role of OsSULTR3;6 in seed germination: 1) OsSULTR3;6 transports sulfate, affecting sulfur metabolism, and consequently, levels of ABA biosynthesis and accumulation in seeds; 2) OsSULTR3;6 is a phosphate transporter that controls P content in seeds. The first hypothesis is plausible even though total sulfur in *Δsultr3;6* seeds was similar to that in wild-type seeds. Plant tissues may have similar overall nutrient contents, yet different subcellular partitioning of ions and downstream metabolites. Further, SULTR3s are involved in sulfate loading into the chloroplast and its transport function is positively correlated to a higher concentration of cysteine and ABA in *Arabidopsis thaliana* [25]. We plan to further interrogate the first hypothesis through direct measurement of ABA levels in the seeds and reproductive organs of wild-type vs. *Δsultr3;6* plants.

The second hypothesis is supported by the increased levels of total phosphorus observed in *Δsultr3;6* seeds compared to the wild type. Despite the gene family name, several SULTR homologs in dicots and monocots have been reported as functional phosphate transporters [9,15,31]. Outside of the SULTR family, increased seed phosphorus mediated by the OsPHO1;2 transporter was identified as the cause of defective grain-filling phenotypes observed in forward genetic screens [32]. This excess of P prevented adequate grain filling due to a negative feedback loop between inorganic phosphate (P_i) and ADP-glucose pyrophosphorylase (AGPase), limiting starch biosynthesis. Investigation of total starch levels and starch granule morphology in *Δsultr3;6* seed relative to wild-type seed will be a useful first step in testing the phosphate transport hypothesis for OsSULTR3;6 function.

Aside from direct effects on the seed itself, the germination defects we observed could be due to effects in the reproductive tissues in the parent plant. This notion is supported in the literature for both S and P nutrition. For example, mutation of a rice transporter involved in facilitating ABA movement between the flag leaf to the nascent embryos on the panicle led to decreased grain filling and seed viability [33]. This finding supports the idea that reduced sulfate accumulation in the flag leaf, leading to reduced ABA, could result in less successful grain filling and development. In Arabidopsis, loss of a vacuolar P transporter resulted in excess P in the pistil, causing a physiological defect that restricted pollen tube growth [34]. These considerations necessitate an investigation of differences in *Δsultr3;6* plants, relative to wild-type plants, with respect to mineral nutrition in the reproductive organs, throughout floral and seed development.

6. How OsSULTR3;6 might contribute to BLS

Macro- and micronutrients regulate various physiological processes relevant to disease such as stomatal closing, callose deposition, and defense hormone signaling [35]. The substrate specificity and directionality of OsSULTR3;6-mediated transport will provide clues regarding the downstream processes that might be contributing to its role in BLS. Based on what we have learned so far, one possibility is that pathogen induction of OsSULTR3;6 in leaf tissue contributes to disease development by regulating abscisic acid signaling via a sulfate transport function [11,36]. Perturbations in ABA in guard cells could be expected to dysregulate stomatal opening and closing. Depending on the nature and timing of the dysregulation, this could contribute to water accumulation in the leaf apoplast (water-soaking) and/or facilitate bacterial exudation to the leaf surface, phenotypes both attributed to OsSULTR3;6 induction [2]. A second possibility is that OsSULTR3;6 mediates nutrient efflux from cells, creating a hypertonic apoplast, and this draws water out of cells via osmosis, resulting in greater water-soaking, lesion expansion, and exudation. A third way OsSULTR3;6 might contribute to disease development is by dampening the plant defense-associated oxidative burst by driving biosynthesis of sulfur-containing scavengers of reactive oxygen species such as glutathione [37]. Considering the temporally dynamic nature of plant-pathogen interactions, it is not unreasonable to speculate that more than one of these (or other) mechanisms underlie the contribution of OsSULTR3;6 to BLS. Ongoing elucidation of the fundamental properties of the transporter and its impact on plant physiology will help determine and refine the models to be tested.

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Declaration of competing interest

The authors declare no competing interests for this work.

Data availability

Data will be made available on request.

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