A Rice Glutamyl-tRNA Synthetase Modulates Early Anther Cell Division and Patterning

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D.Z. and W.L. conceived and designed the project; X.Y., G.L., Y.T., and Y.S. performed the experiments. X.Y., G.L., W.L., and D.Z. performed the analysis. W.L. and D.Z. provided supervision and were financially responsible for the work. X.Y., G.L., and D.Z. wrote the manuscript.
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Running title: OsERS1 modulates early anther development

One sentence summary:

OsERS1, a Glutamyl-tRNA Synthetase, modulates early rice anther development through affecting metabolic homeostasis and redox status.
ABSTRACT

Aminoacyl-tRNA synthetases (aaRSs) have housekeeping roles in protein synthesis, but little is known about how these aaRSs are involved in organ development. Here, we report that a rice (*Oryza sativa*) glutamyl-tRNA synthetase (OsERS1) maintains proper somatic cell organization and limits the over-proliferation of male germ cells during early anther development. The expression of *OsERS1* is specifically detectable in meristematic layer 2-derived (L2-d) cells of the early anther, and *osers1* anthers exhibit over-proliferation and disorganization of L2-d cells, producing fused lobes and extra germ cells in early anthers. The conserved biochemical function of OsERS1 in ligating glutamate to tRNA$^{\text{Glu}}$ is enhanced by its cofactor aminoacyl-tRNA synthetase co-factor (OsARC). Further, metabolomics profiling revealed that OsERS1 is an important node for multiple metabolic pathways, indicated by the accumulation of amino acids and tricarboxylic acid (TCA) cycle components in *osers1* anthers. Notably, the anther defects of *osers1* mutant are causally associated with the abnormal accumulation of hydrogen peroxide, which can reconstitute the *osers1* phenotype when applied to wild-type anthers. Collectively, these findings demonstrate how aaRSs affect male organ development in plants, likely through protein synthesis, metabolic homeostasis, and redox status.
INTRODUCTION

Reproductive cells in flowering plants link the dominant diploid sporophytic generation and the short haploid gametophytic generation. Male germ cells are produced within the sporophytic tissue called the anther (Ma, 2005). The anther primordium that forms from the floral meristem usually contains three layers: L1–L3. L1 and L3 cells subsequently differentiate into the epidermis and connective tissue, respectively, whereas the L2 layer forms germ cells in the center of anther lobes and three surrounding supportive somatic cell layers. The early stages of anther development (1-5) determine the formation of anther cell layers. At stage 2, the L2 layer undergoes rapid cell division and generates L2-derived (L2-d) cells. At stage 3, the central archesporial cells show a distinctive enlarged shape surrounded by the neighboring primary parietal cells (PPCs). At stage 4, PPCs form endothecium and secondary parietal cells (SPCs) via asymmetric cell division and archesporial cells differentiate into sporogenous cells. Following this, the sporogenous cells continue to divide for subsequent meiosis and the SPC layer symmetrically divides to form the middle layer and tapetum at stage 5 (Ma, 2005; Walbot and Egger, 2016; Wilson and Zhang, 2009; Zhang and Yang, 2014).

One critical event in pre-meiotic anther development is rapid periclinal cell division and strict cell lineage specification. However, the underlying mechanism controlling this process remains largely unknown. Previous studies revealed the critical functions of a few regulators – such as receptor-like protein
kinases, transcription factors, redox status, glycoprotein, and hormones — in
specifying cell lineage and the fate of germ and somatic cells in *Arabidopsis
thaliana*, rice (*Oryza sativa*), and maize (*Zea mays*) (Walbot and Egger, 2016;
Wilson and Zhang, 2009; Zhang and Yang, 2014).

Glutaredoxins (GRXs) are small oxidoreductases that modulate various
cellular events and responses to oxidative stress (Holmgren, 2000). Rice
MICROSPORELESS1 (MIL1) and maize Male Sterile Converted Anther 1
(MSCA1) are homologs to two Arabidopsis GRXs, ROXY1 and ROXY2. These
GRXs have been shown to regulate abaxial–adaxial anther lobe formation and
differentiation of microsporocytes, suggesting that glutaredoxins trigger
archesporial fate determination by controlling redox status. Further studies
show that GRXs interact with TGA (TGACGTCAG cis-element-binding protein)
transcription factors to transcriptionally regulate targets (Chaubal et al., 2003;
Hong et al., 2012b; Kelliher and Walbot, 2012; Xing and Zachgo, 2008; Yang et
al., 2016).

Recent studies have shown the function of cell-surface-localized leucine-rich
repeat receptor-like kinases (LRR-RLKs) and their ligands, such as rice
MULTIPLE SPOROCYTE 1 (MSP1) and TAPETAL DETERMINANT 1-like A
(OsTDL1A)/MICROSPORELESS 2 (MIL2), in determining early anther cell fate.
These are orthologous to Arabidopsis EXCESS MICROSPOROCYTES 1
(EMS1)/EXTRA SPOROGENOUS CELLS (EXS) and TAPETAL
DETERMINANT 1 (TPD1), respectively, and function in specifying anther cell
identity and controlling cell numbers of tapetum and microsporocytes (Canales et al., 2002; Hong et al., 2012a; Nonomura et al., 2003; Zhao et al., 2008; Zhao et al., 2002; Yang et al., 2003; Zhang & Yang 2014). MSP1 and OsTDL1A were suggested to exert their effects in early anther cell differentiation via modulating redox status in rice (Yang et al., 2016).

In maize, early-stage anthers also require hypoxia to maintain proper division and determine cell fate. The maize glutaredoxin MSCA1 may maintain growth-generated hypoxic conditions to induce archesporial cells. Also, archesporial cells might silence mitochondria to lower the concentration of reactive oxygen species (ROS) and maintain reducing conditions (Kelliher and Walbot, 2012).

Aminoacyl-tRNA synthetases (aaRSs) are found in all protein-synthesizing organisms, and can catalyze the ligation of amino acids and their cognate transfer RNA (tRNA), an essential step for translation and amino acid dynamics (Ibba and Söll, 2000; Yamakawa and Hakata, 2010). Studies in mammals showed non-canonical and surprising roles of aaRSs in amino acid signaling, transcriptional control, and anti-apoptosis (Gao et al., 2010). As housekeeping proteins, aaRSs are indispensable for plant development, but the detailed function in plant growth remains largely unclear. In Nicotiana benthamiana, silencing of Glutamyl-tRNA Synthetase (GluRS) and Seryl-tRNA Synthetase (SerRS) causes a severe yellowing phenotype in leaves due to significant reduction in the number of organelles (Kim et al., 2005). Mutation of a rice
ValRS results in a reduced level of chlorophyll and abnormal chloroplast ribosome biogenesis (Wang et al., 2016). Among the 45 aaRSs in Arabidopsis, 21 are required for ovule development and embryogenesis (Berg et al., 2005). In addition, an Arabidopsis Cysteinyl-tRNA Synthetase (CysRS) expressed in the central cell of the female gametophyte may specify the fate of adjacent cells (Kägi et al., 2010). Non-canonical and surprising roles of aaRSs have been uncovered, such as amino acid signaling, transcriptional control, and anti-apoptosis. Furthermore, eight members of aaRSs were found to exist in mammalian cytoplasm as a multi-tRNA synthetases complex (MSC) whose roles and mechanisms of formation are still unclear (Guo and Schimmel, 2013). However, the detailed role of aaRSs in plant organs and development is limited. Here, we report the critical function of the cytosol- and mitochondria-localized GluRS, OsERS1, in early anther cell proliferation and organization by affecting protein synthesis, amino-acid-derived metabolism, and cellular redox status.

RESULTS

OsERS1 Limits L2-d Cell Proliferation and Maintains Anther Cell Organization

To explore the mechanisms underlying anther development in the cereal model plant rice, we identified a new male sterile mutant, oryza sativa glutamyl-tRNA synthetase (osers1), because of the mutation in Glutamyl-tRNA Synthetase 1 (see below). The osers1 mutant displayed no obvious phenotypic changes from
wild type in vegetative growth, inflorescence architecture, female reproductive
organ, or seed shape (Supplemental Fig. S1A and S1B). However, during the
male reproductive stage, the mutant developed defective anthers that were pale
and had fused lobes, resulting in the production of ~20% viable pollen grains
and an ~10% seed setting rate (Supplemental Fig. S1C to S1F). The osers1
mutant showed a more tightly-woven texture in the anther wall, and the pollen
grains were shrunken and displayed rough, lumpy extine, in contrast to the wild
type, which had a well-arranged cuticle on the exterior of the anther wall, and
mature pollen grains with smooth extine (Supplemental Fig. S1G to S1N). All F1
plants of reciprocal crosses between osers1 and wild type were normal, and the
F2 offspring had an approximate 3:1 segregation of normal vs. abnormal
anthers (116: 42; $\chi^2 = 0.58$, $P>0.05$), suggesting that the osers1 mutants
contain a unifactorial and recessive mutation.

From stage 3 to 5 of anther development, ~40% of osers1 anthers had
adaxial fused lobes, which was distinct from the butterfly-shaped four lobes in
wild-type anthers (Fig. 1A and 1B; Supplemental Fig. S2A to S2F and S2I). At
stage 3, transverse-section analysis showed that each wild-type anther formed
~6.3 archesporial cells ($n=11$) enringed by a single layer of PPCs, while each
osers1 anther produced ~10 archesporial-like cells ($n=12$) enringed by PPCs
organized in an irregular mosaic pattern (Fig. 1C, 1D, 1I, and 1K). At stage 5,
the wild-type archesporial cells further divided into ~11 sporogenous cells ($n=15$)
surrounded by three circular inner layers derived from PPCs (Fig. 1E, 1G, 1I
and 1K). However, each osers1 anther produced ~20 sporogenous-like cells (n=13), and ~50% of the anthers had disordered wall layers with irregular boundaries (Fig. 1F, 1H, 1I and 1K; Supplemental Fig. S2I). Notably, the total number of somatic and germ cells derived from the L2 layer in the osers1 mutant was increased during stages 3–5 (Fig. 1J). Some sporogenous-like cells were ectopically embedded in subepidermal layers (Fig. 1I; Supplemental Fig. S2G and S2H).

The increased numbers of anther cells in the osers1 mutant prompted us to compare cell-division activity between wild type and the osers1 mutant. In situ hybridization showed stronger expression signals for HISTONE 4 (H4), a cell-division marker for the S-phase of mitosis, and CYCLIN-DEPENDENT KINASE B2-1 (CDKB2;1), a marker of the G2/M phase transition (Umeda et al., 1999), in both somatic and germ cells of osers1 lobes, compared with the wild type (Fig. 1L to 1Q). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis also showed increased expression of H4 and CDKB2;1 in osers1 anthers (Supplemental Fig. S3B). Due to the lack of marker genes for cell lineage during early anther development, we chose MSP1 to examine the relationship of the MSP1 pathway and OsERS1. In situ hybridization showed similar distribution patterns of MSP1 in both wild-type and osers1 anthers; that is, preferential accumulation on anther wall cells rather than in central sporogenous cells (Supplemental Fig. S3A). RT-qPCR showed no significant difference in the expression of MSP1, as well as in that of two
other genes involved in anther development, *MIL1* and *MIL2*, between wild-type and *osers1* anthers (Supplemental Fig. S3B). Unlike *osers1*, mutations of *MSP1, MIL2*, and *MIL1* led to misidentified anther wall cell layers and complete male sterility (Hong et al., 2012a, 2012b; Nonomura et al., 2003). These results indicate that the cell properties are still maintained in *osers1* anthers, and *OsERS1* is strongly implicated in cell proliferation and organization in the rice anther.

**OsERS1 Encodes a Glutamyl-tRNA Synthetase**

Using map-based cloning combined with the whole-genome sequencing-based MutMap method (Abe et al., 2012; Lü et al., 2015), we identified the mutated *OsERS1* gene as having an insertion of adenine (A) after A539 in the LOC_Os10g22380 coding region (also known as Os10g0369000, see Methods); this resulted in a premature stop codon in the open reading frame (Fig. 2A and 2B). The identity of *OsERS1* was confirmed by the complementary assay, in which the male sterile phenotype in the *osers1* mutant was rescued by expression of a genomic fragment that contained the wild-type *OsERS1* gene (Fig. 2C to 2E).

*OsERS1* encodes a putative glutamyl-tRNA synthetase (GluRS), which contains an N-terminal GST_C (C terminus of glutathione S-transferase) domain, a glutamyl-tRNA synthetase catalytic core, and a C-terminal tRNA-synt_C domain (Fig. 2B). RT-qPCR showed that *OsERS1* was expressed in the wild type at low levels in mature organs, but at high levels in young
tissues, including seedlings and young inflorescences (Fig. 3A). In the anther, *OsERS1* transcript levels were relatively high during stages 3–6 and decreased in later stages (Fig. 3B and 3C). *In situ* hybridization detected *OsERS1* mRNA in presumptive archesporial cells and neighboring L2-d cells before stage 3. During stages 3–5 when anther cells undergo rapid cell division, *OsERS1* mRNA was detected in PPC and archesporial cells of stage 3, the SPC and sporogenous cells of stage 4, and the tapetum, middle layer, and sporogenous cells of stage 5. The expression of *OsERS1* in germ cells decreased significantly after stage 5 (Fig. 3C; Supplemental Fig. S4A). The rice genome has two *OsERS1* homologs, LOC_Os01g16520 and LOC_Os02g02860, but these homologs had low expression in the anther and did not appear to have altered expression in the *osers1* mutant, suggesting that the phenotype of the *osers1* mutant was caused by aberrant *OsERS1* in the anther as opposed to the two homologs (Supplemental Fig. S4B). Considering that protein synthesis is essential in the cytosol, chloroplast, and mitochondria, we performed protein localization studies in rice protoplast cells and onion (*Allium cepa*) cells by co-expressing OsERS1-eGFP and a mitochondrial marker ScCOX4-mCherry (carried by vector *CD3-991*) (Nelson et al., 2007). Our results showed that OsERS1 is mainly localized in the cytoplasm and is partially shared by the mitochondria (Fig. 3D; Supplemental Fig. S4C).

Phylogenetic analysis of 40 OsERS1 homologs from 21 species from diverse kingdoms clearly divided these proteins into two clades: the eukaryote-specific...
GluRSs (Clade I) and the prokaryote-originated GluRSs (Clade II) (Fig. 3E; Supplemental Table S1). Intriguingly, most eukaryotes contain both types. Clade II GluRS contained only the catalytic domain, while Clade I GluRSs showed more complex structures. The progressive appearance of novel domains, such as GST_C, tRNA-synt_C, repeats of the WHEP domain (initially discovered in human tryptophanyl-tRNA synthetase [TrpRS] [W], histidyl-tRNA synthetase [H], GluRS-prolyl-tRNA synthetase [ProRS] [EP]), and chimeric GluRS-ProRS in Clade I, indicated multiple domain-recruiting events during evolution (Gao et al., 2010). In grass crops such as rice and maize, there seemed to be a duplication of GST_C containing GluRS, which we did not observe in other species (Fig. 3E), indicating that GluRS might have an expanded function for evolutionary adaption in higher organisms.

OsERS1 Functions as a Ligase between Glutamate and tRNA\textsuperscript{Glu}

The synthesis of glutamyl-tRNA is a two-step reaction: activation of glutamate by ATP and glutamylation of tRNA\textsuperscript{Glu}. In the catalytic domain, GluRS has a core active site (RFAPE in OsERS1) that binds glutamate and two key motifs called HIGH (amino acids of this domain) and KMSKS (LLSKR in OsERS1, amino acids of this domain) that are involved in ATP-binding and tRNA-binding, respectively (Freist et al., 1997; Ibba and Söll. 2000). Sequence alignment showed that these three motifs are highly conserved in the 40 GluRS homologs (Fig. 4A; Supplemental Fig. S5).

To confirm the function of OsERS1 in glutamyl-tRNA synthesis, we
performed an ATP-pyrophosphate (PPi) exchange assay, which showed that OsERS1 has PPi exchange activity and that its reaction rate is proportional to the concentration of tRNA$_{Glu}$ (Fig. 4B). An aminoacylation assay revealed that OsERS1 has the catalytic activity of aminoacylation of tRNA$_{Glu}$ with glutamate (Fig. 4C and 4D), yielding a Km value (tRNA$_{Glu}$ as substrate) of 2.20 μM, and a Kcat/Km value of 0.977 s$^{-1}$ μM$^{-1}$ (Fig. 4E). OsERS1’s affinity for tRNA$_{Glu}$ is similar to that of the *Thermus thermophilus* GluRS, but lower than that of *Rattus* liver and *Escherichia coli* (Freist et al., 1997). Also, OsERS1’s Km value is lower than that of wheat (*Triticum aestivum*) GluRSs, possibly because the latter functions as dimers (Thomes et al., 1983). However, it is still not clear whether GluRS can form dimers in rice.

Mutations of the three conserved motifs of OsERS1s – Arg209 and Glu213 in the active site region, His219 and His222 in the HIGH motif, and Ser442 and Lys443 in the KMSKS motif, respectively (Fig. 4A and Supplemental Fig. S5) – caused significantly reduced glutamylation activity and enzyme efficiency (Kcat/Km) (Fig. 4C to 4E). These results suggest that OsERS1 has catalytic activity in tRNA$_{Glu}$ glutamylation, and the RFAPE, HIGH, and KMSKS motifs are required for OsERS1’s enzymatic function.

**The Cofactor OsARC Stimulates the Catalytic Activity of OsERS1**

The GST_C domain of GluRS is non-catalytic because it lacks thioredoxin, but was shown to link the enzyme to protein complexes (Dixon et al., 2002). In yeast and mammals, GluRS/GluRS-ProRS interacts with the aaRS co-factor
Arc1p/p18 to form a complex and modulate enzyme activities (Galani et al., 2001; Gao et al., 2010; Graindorge et al., 2005). Rice has a single homolog of yeast Arc1p, OsARC (encoded by LOC_Os01g60660), which contains a heterogeneous GST_C domain (Fig. 5A). To examine whether OsERS1 can form a complex with OsARC, we conducted yeast two-hybrid and in vitro pull-down assays; this confirmed that OsERS1 can interact with OsARC and that the interaction requires the GST_C domains (Fig. 5B and 5C). Bimolecular fluorescent complementation (BiFC) showed that only the cells expressing full-length OsERS1-nYFP and OsARC-cYFP displayed yellow fluorescence signals (Fig. 5D), verifying this interaction in vivo. OsERS1-eGFP and OsARC-mCherry were highly co-localized when co-expressed in onion cells (Fig. 5E), supporting our hypothesis that OsERS1 and OsARC can form a complex. Moreover, compared with that of OsERS1 alone or OsERS1−ΔOsARC (truncated OsARC protein without the GST_C domain) (Fig. 5F and 5G), the OsERS1−OsARC complex had ~2.5 times the ATP-PPi exchange rate of glutamate and ~1.4 times the tRNA\textsubscript{Glu} aminoacylation efficiency in the plateau stage, indicating an enhancement effect promoted by OsARC. In addition, the GluRS and cofactor pairs are conserved components of MSCs in various organisms (Laporte et al., 2014). However, the structure and composition of plant MSC have not been dissected.

**Mutation of OsERS1 Leads to Metabolic Alterations in Anthers**

The substrate for OsERS1, glutamate, occupies a central position that links key
metabolic pathways (Newsholme et al., 2003). Loss of function of OsERS1 may cause an accumulation of free glutamate that is not used for protein synthesis in the anther. To test this possibility, we conducted non-targeted metabolomics profiling in wild-type and osers1 anthers. A total of 297 metabolites were identified, covering all major primary and several secondary metabolism pathways (Supplemental Table S2). Consistent with the deficiency in ligating glutamate with tRNA\(^{\text{Glu}}\), the osers1 mutant had 2.44 times the level of glutamate compared with the wild type. With the exception of glutathione in both reduced (GSH) and oxidized (GSSG) status, the levels of glutamate family members – including glutamine, \(\alpha\)-ketoglutarate (\(\alpha\)-KG), histidine, and arginine – were increased in the osers1 mutant. One glutamate derivative, \(\alpha\)-KG, is also an intermediate of the tricarboxylic acid (TCA) cycle, and links the metabolism of amino acids and carbohydrates (Jeong et al., 2013). As a result, most metabolites of the TCA cycle, such as oxaloacetate (OAA) and malate, were increased in the osers1 mutant (Fig. 6A; Supplemental Fig. S6B; Supplemental Table S2). This is also consistent with changes in the expression of genes involved in glutamate metabolism and the TCA cycle, such as genes encoding glutamine synthetases (GS2 and GS3), glutamate dehydrogenase (GDH1), and oxoglutarate dehydrogenase (ODH2) (Supplemental Table S3). Besides the glutamate family, the leucine, serine, and aspartate families derived from pyruvate, phosphoglycerate, and OAA were also increased in the osers1 mutant. We propose that increased levels of amino acids in the mutant may
have been caused by the accumulation of glutamate, OAA, and pyruvate, which
are important precursors for amino acid synthesis (Fig. 6; Supplemental Fig.
S6A; Supplemental Table S2).

In addition, we detected the expression level of 12 aaRS genes highly
expressed in reproductive organs. Most of the aaRS genes showed subtle
expression changes between the wild type and the osers1 mutant, except for
CysRS1 and TrpRS2 which had lower expression in the osers1 mutant
(Supplemental Fig. S7), implying that the amino acid changes in the mutant are
mainly caused by loss of function of OsERS1. By contrast, we observed
decreased levels of glycolysis intermediates, including glucose, glucose-6P,
and fructose-6P in the osers1 mutant (Fig. 6; Supplemental Fig. S6B); this
indicates higher consumption of these compounds in the osers1 mutant.
Decreased glucose supply and less lactate production suggests that pyruvate
was likely used by the TCA cycle and for amino acid biosynthesis. Increased
expression of pentose phosphate pathway (PPP) genes and accumulation of
purine and pyrimidine indicate that more intermediates of glycolysis were used
to support nucleotide biosynthesis in osers1 anthers (Supplemental Fig. S6C;
Supplemental Table S2; Supplemental Table S3). Metabolic analysis showed
abnormal fatty acid synthesis in the osers1 mutant, such as cutin and wax,
which is consistent with the scanning electron microscopic analysis of osers1
anther wall and pollen grain (Supplemental Fig. S1G to S1N and S6D;
Supplemental Table S2).
Overall, the *osers1* anthers show more active amino acid and carbohydrate metabolism with aerobic characteristics, such as an active TCA cycle and less lactate production. The increased glutamate family amino acids, as well as those derived from OAA and pyruvate, contribute to the significant changes in biosynthesis of the amino acids pathway in *osers1* anthers (Fig. 6B; Supplemental Fig. S6A; Supplemental Table S2). Notably, glutamate may provide mitochondrial anaplerosis to the TCA cycle to stimulate mitochondrial activities, which produces reactive oxygen species (ROS) as an inevitable byproduct. Consistent with this, decreased cellular antioxidants (GSH/GSSG, ascorbate) suggest a disturbed redox status in *osers1* anthers (Fig. 6B). Corresponding with activated cell division in *osers1* anthers, more carbohydrates are consumed in glycolysis to generate energy, and provide more intermediates for the PPP, which enhances the synthesis of nucleotides and macromolecules. These results reveal that the metabolic homeostasis is disturbed in *osers1* anthers, and that OsERS1 plays key roles in amino-acid-related metabolism.

**Altered Redox Status Affects Early Anther Development**

Global metabolomics profiling suggested that ROS might be produced by mitochondrial activities in the *osers1* mutant. To verify this, we first measured hydrogen peroxide (H$_2$O$_2$), a stable ROS molecule implicated in cell proliferation and differentiation (Møller et al., 2007; Tsukagoshi et al., 2010). Fluorometric quantitation assays showed that H$_2$O$_2$ content in the *osers1*
mutant at stages 4–5 was ~900 pmol/mg, twice that in wild-type anthers at the
same stage (Fig. 7A, left). Also, the precursor of H$_2$O$_2$, superoxide radical (O$_2$•-),
was slightly increased in the osers1 mutant (Fig. 7A, right), which contributed to
the redox status change in the osers1 mutant. In addition, 3,3'-diaminobenzidine (DAB) staining of wild-type anthers showed that the H$_2$O$_2$
signal was preferentially present in middle layer and endothecium cells until
stage 5 (Fig. 7B to 7E). However, osers1 anthers displayed detectable H$_2$O$_2$ in
the anther primordium before stage 3 (Fig. 7F), and strong H$_2$O$_2$ signals in
anther cells at stages 4–5 (Fig. 7G to 7I). In later stages, even though the
distribution of H$_2$O$_2$ was similar in both wild-type and osers1 anther wall layers,
stronger H$_2$O$_2$ signals were seen in osers1 anther wall layers (Supplemental Fig.
S8). Lower expression levels of genes encoding ascorbate peroxidases (APX)
in the mutant (Supplemental Table S3) may have contributed to the
accumulation of ROS.

To test whether the increased level of H$_2$O$_2$ in the osers1 mutant causes the
abnormal division and patterning of L2-d cells, we injected 100 µM H$_2$O$_2$ and 1
mM of the ROS-removal reagent potassium iodide (KI) into the young
inflorescences of the wild type and the osers1 mutant (Supplemental Fig. S9).
Transverse section observations revealed that each stage-4 wild-type anther
treated with water showed a slight increase in the number of sporogenous-like
cells, from 7.5 ($n$=16) to 9.2 ($n$=12), which may have been triggered by
wounding (Fig. 7J, 7K and 7S). Remarkably, the number of L2-d cells in each
wild-type anther treated with H$_2$O$_2$ increased from 109 ($n$=16) in untreated
anthers to 135.4 ($n$=18), and sporogenous-like cell numbers increased from 7.5
to 15.4. Therefore, treatment with 100 $\mu$M H$_2$O$_2$ in the wild type led to
over-proliferation of L2-d cells, ectopic sporogenous-like cells, and disordered
parietal cell layers, phenocopying the defects of the osers1 mutant (Fig. 7L, 7R,
and 7S). Wild-type anthers treated with KI also showed an increase in the
number of L2-d cells to 132.7 and sporogenous-like cells to 16.8 ($n$=17), while
parietal cells were organized differently compared to those treated with H$_2$O$_2$
(Fig. 7M, 7R and 7S). Compared with that of the untreated anther (139.3 L2-d
cells and 18.5 sporogenous-like cells, $n$=14), in the osers1 mutant, each anther
treated with water showed a slight decrease of L2-d cell numbers to 131.5 and
sporogenous-like cell numbers to 14.5 ($n$=13) (Fig. 7N, 7O, 7R, and 7S).
However, each osers1 anther treated with H$_2$O$_2$ and KI showed a reduced
number of L2-d cells and sporogenous-like cells (H$_2$O$_2$, 102.9 and 12.7, $n$=16;
KI, 110.5 and 14.5, respectively, $n$=15). Aside from changes in cell numbers,
we observed highly vacuolated cells in impaired osers1 anthers (Fig. 7P to 7S).
We reasoned that elevations of ROS by H$_2$O$_2$ injection and neutralization of
ROS by KI injection both disturbed ROS homeostasis, which may contribute to
repressed cell division, and that the osers1 anthers appear vulnerable to
changes in redox status. Together, these results suggest that changed redox
status disturbs early cell division in rice anthers.
DISCUSSION

Early anther development is a complex event characterized by rapid cell division and fate specification to support the development of functional anther layers that are properly organized within the anther (Walbot and Egger, 2016; Zhang and Yang, 2014). In this study, we revealed that OsERS1, a glutamyl-tRNA synthetase, functions in early anther cell division and patterning, likely through maintaining physiological homeostasis of amino acids and carbohydrate metabolism as well as redox status (Fig. 8).

Our genetic and cytological data demonstrate that loss of function of OsERS1 results in fused anther lobes with supernumerary L2-d cells and disarranged anther wall cell layers. In osers1 anthers, L2-d cell division was accelerated, but the L1 layer remained normal and formed a ‘rigid’ enclosure, which aggravated the disarranged cell layers and created abaxial fused lobes. In spite of its putative housekeeping function, expression of OsERS1 shows spatial-temporal characteristics rather than a constitutive pattern. In the anther, OsERS1 transcripts are initially expressed in cells of presumptive lobes at the four primordial corners. Then, OsERS1 mRNA is detected in both parietal and germ cells, preferentially in those undergoing periclinal division or newly formed cell layers (Fig. 3C). After stage 5, the germ cells develop into microspores via meiosis, and the abundance of OsERS1 mRNA in germ cells decreased to a very low level, indicating that OsERS1 mainly affects the early cell division and patterning. This expression pattern is in agreement with the phenotype of
increased L2-d cells and disarranged cell layers in the osers1 mutant.

Phenotypically, mutations of the LRR-RLK-ligand pair MSP1-MIL2 or glutaredoxins MIL1 lead to misidentified anther wall cell layers and complete male sterility (Hong et al., 2012a, 2012b; Nonomura et al., 2003; Zhao et al., 2008), while the osers1 anther still produces a low proportion of viable pollen grains (Supplemental Fig. S1). Anther defects in the osers1 mutant occur earlier than those in the reported rice mutants with abnormal early anther development such as gamyb-4, udt1 (undeveloped tapetum 1), and tip2 (tdr interacting protein 2) (Fu et al., 2014; Jung et al., 2005; Liu et al., 2010). We showed that there are no significant changes in the expression of MSP1, MIL2, and MIL1 in the osers1 mutant compared with the wild type, and previous studies showed that OsERS1 has no obviously altered expression in msp1, mil2, gamyb-4, udt1, or tip2 mutants (Aya et al., 2009; Fu et al., 2014; Jung et al., 2005; Liu et al., 2010; Yang et al., 2016;). Therefore, we propose that OsERS1 plays an independent role with known regulators in early anther development. Although loss of function of OsERS1 causes abnormal anther morphogenesis and decreased pollen vitality, the mutant still can form a small number of seeds with normal shape, suggesting that fertilization is not completely disturbed. However, we cannot exclude the possibility that female gametophyte development is also affected.

We confirmed the in vitro activity of OsERS1 in catalyzing the ligation between glutamate and tRNA\textsuperscript{Glu}. OsERS1 is dominantly localized in the cytosol
and partially shared by mitochondria. The product, glutamyl-tRNA, is an essential substrate for protein synthesis. Compared with the prokaryotic \((E. \text{coli})\) GluRS (Freist et al., 1997), OsERS1 possesses a relatively lower affinity to tRNA\(^{\text{Glu}}\). One explanation is that the additional GST\(_C\) domain may change space conformation and reduce substrate affinity. However, the interaction between OsERS1 and its cofactor OsARC overcomes this disadvantage by enhancing the activity of OsERS1 for synthesizing glutamyl-tRNA (Fig. 5). In the early anther, loss of function of \(OsERS1\) may impair translational processes in the cells that normally accumulate high \(OsERS1\). However, the \(osers1\) anthers maintain partial fertility and produce viable pollens, suggesting that other components may remedy the absence of OsERS1 to maintain protein synthesis in the anther.

Metabolic status is associated with tumorigenesis, stem cell renewal, pluripotency maintenance, and differentiation (Cairns et al., 2011; Ito and Ito, 2016; Schuster et al., 2014; Sperber et al., 2015;). In plants, glycolysis and mitochondrial metabolism activate the Arabidopsis root meristem via glucose-target of rapamycin (TOR) signaling (Xiong et al., 2013). In maize, the anther primordium is more likely glycolysis-dependent as the enclosed niche provides hypoxic conditions for archesporial cells (Kelliher and Walbot, 2012). We speculate that the hypoxia status specifying cell identity and cell division must be well controlled by the specific metabolic pathway within anther cells. In this study, we showed that as an important metabolic regulatory node, OsERS1 is
involved in intracellular glutamate dynamics, and glutamate could refuel the
TCA cycle that occupies a central position in multiple metabolic pathways.
Within this context, the Arabidopsis cytosolic GluRS is an important target of
14-3-3 proteins in carbon/nitrogen metabolism and sugar-sensing pathways.
GluRS is degraded when sugar starvation occurs in order to release glutamate
and support cell survival (Coruzzi et al., 2001; Cotelle et al., 2000). In this work,
we showed that the mutation of OsERS1 disturbs the homeostasis of the
metabolic network, as evidenced by increased amounts of amino acids, TCA
components, and active aerobic metabolism. We speculate that such a
metabolic shift acts as a signal to remove the limitation on cell proliferation in
the anther lobe. Consistent with this, some mutants of 21 Arabidopsis aaRSs
showed defective female gametogenesis and embryo development, but no
obvious defect during male gametogenesis except for in the ova9 mutant, which
had a mutation in Glutaminyl-tRNA Synthetase (GlnRS) (Berg et al., 2005).
However, how these specific aaRSs are involved in reproductive development
remains unclear. Although we observed no obvious change of other aaRS
genes in the osers1 mutant (Supplemental Fig. S7), we cannot rule out the
function of other aaRSs in male reproduction.

ROS exert dose-dependent impacts on cells. A lower or moderate basal level
of ROS is essential for cell proliferation and differentiation, but higher levels of
ROS is cytotoxic, causing cell damage and death (Mittler, 2017; Schieber and
Chandel, 2014; Sena and Chandel, 2012). In rice anthers, the ROS molecule
H$_2$O$_2$ is initially accumulated in the middle layer at stage 5; this may act as a signal that triggers cell differentiation (Yang et al., 2016; Zhang and Yang, 2014). In this study, we observed the production of H$_2$O$_2$ in osers1 anthers as early as the formation of archesporial cells, which may cause over-proliferation of anther cells and disorganized cell layers (Fig. 7). In support, disruption of the redox status in wild-type anthers by injecting low-concentrations of H$_2$O$_2$ resulted in a mimic of the phenotype of the osers1 mutant (Fig. 7). Similarly, in the shoot apical meristem of Arabidopsis, elevating H$_2$O$_2$ dramatically enlarges meristem size by increasing cell numbers in the peripheral zone (Zeng, et al., 2017). In maize, treatment with a high concentration of H$_2$O$_2$ (1 mM) on the anther inhibits cell division in both somatic and germ cells (Kelliher and Walbot, 2012), demonstrating multi-faceted regulatory aspects of ROS in cell division. The study in maize also indicates that the anther primordium may use glycolysis as a major energy source, rather than through glucose oxidation in mitochondria to avoid ROS pressure and maintain genomic integrity (Kelliher and Walbot, 2012). In this study, we demonstrated that increased ROS causes over-proliferation of early anther cells in the mutant, suggesting that reductive redox status is essential to maintain normal cell division and organization during rice anther development.

In summary, we discuss the role of a conserved glutamyl-tRNA synthetase, OsERS1, in limiting anther cell division and maintaining cell patterning (Fig. 8). The catalytic activity of OsERS1 in ligating glutamate to tRNA$^{\text{Glu}}$ can be
enhanced by its cofactor OsARC. Loss of function of OsERS1 causes changes in a variety of metabolites, such as the accumulation of amino acids and TCA cycle intermediates, and leads to aerobic metabolic features in the mutant. Together with the H₂O₂ assays, we concluded that OsERS1 maintains normal early anther cell division and patterning, likely through affecting protein synthesis, amino acid homeostasis, and redox status in rice. Our study reveals how the aaRSs control the cell division and organ development derived from meristematic cells in plants.

MATERIALS AND METHODS

Materials, Plant Growth, and Phenotypic Analysis

The osers1 mutant was isolated from a rice mutant library in the cultivar 9522 background (O. sativa L. ssp. japonica) (Chen et al., 2006). Rice plants were grown in the artificial atmospheric chambers on the Waite Campus, University of Adelaide, and in a paddy field at Shanghai Jiao Tong University. Plants and flowers were photographed with a Nikon E995 digital camera and a Motic K400 dissecting microscope. Photography of plant materials and observation of anthers were done as described by Li et al. (2006), and imaged with a Nikon Ni-E optical microscope and a Philips XL20 SEM. Cell counts were determined based on cross sections from >10 anthers and statistical analysis included all four lobes of whole anthers.
Map-based Cloning and Complementation of the Mutant

For fine mapping, we identified 158 mutants from an F2 population that was generated from a cross between the osers1 mutant (*japonica*) and 9311 (*indica*). To map the *OsERS1* locus, we used bulked-segregation analysis, and developed insertion–deletion (InDel) molecular markers based on the sequence differences between rice cultivars, described in the National Center for Biotechnology Information. The *OsERS1* gene was initially located to chromosome 10 between two InDel molecular markers, 10tys14 and 10tys2, and finer mapping narrowed down the gene to the region between RM25267 and 10tys28. Mapping combined with genome sequencing was performed using the previously published method (Abe et al., 2012; Lü et al., 2015).

For functional complementation, an 8.3-kb genomic DNA fragment containing the entire *OsERS1* coding region, a 3.2-kb upstream sequence, and a 1.7-kb downstream sequence was amplified from a BAC clone and subcloned through *EcoRI* and *BstEII* into the binary vector *pCAMBIA1301* (*p1301*), which carries a hygromycin resistance marker, to generate *p1301-OsERS1*. We induced calli from young panicles of the homogenous *osers1* mutant, and transformed the calli with *Agrobacterium tumefaciens* EHA105, which carried the *p1301-OsERS1* plasmid. At least 25 independent transgenic lines were obtained after PCR genotyping using primers listed in Supplemental Table S4.

RT-qPCR

We extracted total RNA from plants using the TRIZOL reagent (Invitrogen),
following the manufacturer’s instructions. cDNA was synthesized from 4 μg of total RNA using a reverse transcriptase kit (Bio-Rad). SYBR Green Mix (Kapa) was used for RT-qPCR on a QuantStudio Flex 6 (Life Technologies) machine. The level of ACTIN-2 (ACT) mRNA was used as the internal control. For expression analysis of OsERS1, aaRSs, metabolism-related genes, or anther developmental genes, we denatured the DNA at 95°C for 10 minutes (min), followed by 42–45 cycles at 95°C for 10 s, 56°C for 15 s, and 72°C for 15 s. The RT-qPCR results shown are from representatives of experiments repeated at least three times with similar results. Primer sequences are listed in Supplemental Table S4.

**RNA in situ Hybridization**

We amplified OsERS1-, MSP1-, H4-, and CDKB2;1-specific fragments of the exon region (OsERS1, 70–391 bp; MSP1, 3462–3841 bp; H4, full length; CDKB2;1, 415–739 bp) by PCR using specific primers fused with the T7 promoter (sequences are listed in Supplemental Table S4). Digoxigenin-labeled antisense and sense probes were transcribed using an *in vitro* transcription kit (Roche) according to the manufacturer’s instructions. *In situ* hybridization was performed according to a published protocol (Zeng et al., 2017).

**Subcellular Localization**

35Spro::OsERS1-eGFP (full-length OsERS1 cDNA fused with eGFP) was generated in pCAMBIA1301 and expressed in rice protoplasts and onion epidermal cells using PEG-mediated transfections and a particle delivery
system, respectively. The construction of the organelle marker (CD3-991) has been described by Nelson et al. (2007). For rice protoplast cells, stem tissues of 14-day-old seedlings were processed following the protocol described by Zhang et al. (2011). For onion epidermal cells, the onion was cut into 2 × 2 cm squares and placed in a plate for 4 h with Murashige and Skoog (MS) medium containing 40 g/L D-mannitol. For transformation, 5 μg of each plasmid DNA was coated with 1.0-μm gold particles (Bio-Rad) and delivered into onion epidermal cells using a PDS-1000/He particle delivery system (Bio-Rad). Fluorescence microscopy was performed using a Leica TCS SP5 laser scanning confocal microscope. Fluorescence signals for mCherry (excitation, 587 nm; emission, 580–630 nm; 15% power), eGFP (excitation, 483 nm; emission, 500–530 nm; 15% power), and chloroplasts (excitation, 504 nm; emission, 650–750; 15% power) were detected with 20× (dry) objective for onion cells, and 63× (oil) objective for protoplast cells, respectively.

**Phylogenetic Analysis and Motif Alignment**

We constructed a phylogenetic tree with aligned full-length sequences of homologs of OsERS1. MEGA (version 7.0) and the neighbor-joining (NJ) methods were used with a p-distance model and pairwise deletion and bootstrap (Gap Ktupel, 2; length penalty, 5; Window, 4; Diagonals, 4; 1000 replicates). The maximum parsimony method of MEGA was also used to support the NJ tree using the default parameter. Amino acid sequences from regions of 199-228 and 434-454 in OsERS1 were used for motif alignment by
MEGA.

**Recombinant Protein Purification**

Wild-type (WT) and mutant *OsERS1* were cloned into the *pET32(a)* vector for expression in the BL21 (DE3) *E. coli* strain. Expression of WT and its variants was induced in Luria-Bertani (LB) broth with 0.5 mM isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) for 4 h at 23°C. We purified the proteins with Ni-NTA affinity resin (Qiagen) according to standard procedures and dialyzed against storage buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM 2-mercaptoethanol, 3 mM MgCl\(_2\), and 50% (v/v) glycerol. The coding sequences of *OsARC* and \(\Delta OsARC\) (deletion of GST_C) were fused to the N terminus of maltose-binding protein (MBP) in the vector *pMAL2c-x*. We expressed the fusion proteins in *E. coli* (strain BL21) and purified them using amylose resin (NEB) according to the manufacturer’s instructions. Supplemental Table S4 lists the primer sequences for the constructs.

**tRNA Transcription and Purification**

The DNA fragment covering the *T7* promoter and tRNA\(^{\text{Glu}}\) gene (http://gtrnadb.ucsc.edu/#eukarya, chr1.trna78-GluTTC, sequence: 5’-GCCCTATCGTCTAGTGGTTCAGGACATCTCTTTCAAGG-3’) was synthesized as a double-stranded molecule by Invitrogen (Shanghai, China), phosphorylated, and ligated into *pUC19* (pre-cleaved by *EcoRI* and *BamHI*) to construct the plasmid *pUC19-tRNA\(^{\text{Glu}}\). *T7 in vitro* transcription was carried out at 37°C in a
reaction mixture containing 40 mM Tris–HCl (pH 8.0), 22 mM MgCl$_2$, 1 mM spermidine, 5 mM dithiothreitol (DTT), 0.5% (v/v) Triton X-100, 60–80 ng/μl tDNA template, 5 mM NTP (each), 0.8 U/μl ribonuclease inhibitor, 20 mM GMP, 500 U/μl T7 RNA polymerase, and 1 U/ml of pyrophosphatase for 3 h. Subsequently, 5 U/ml DNase I (RNase I free) was added and incubated for 1 h to digest the transcription template. We loaded the transcripts onto a 15% PAGE–8M Urea gel with 1-mm thickness and 40-cm length, and ran the gel at a constant 100 W for 4 h. The tRNA band was cut from the gel and eluted with 0.5 M NaAc (pH 5.2) at room temperature (three times), ethanol precipitated at 20°C after two phenol/chloroform extractions, and dissolved in 5 mM MgCl$_2$. The tRNA was denatured at 80°C for 5 min and slowly cooled to room temperature (Fang et al., 2014).

ATP-PPI Exchange and Aminoacylation

The adenosine triphosphate (ATP)–pyrophosphate (PPI) exchange reaction mixture contains 100 mM Tris–HCl (pH 7.8), 12 mM MgCl$_2$, various concentrations of tRNA$^{Glu}$, 5 mM L-glutamate, 4 mM ATP, 2 mM $[^{32}P]$ tetradsodium pyrophosphate (PerkinElmer, Waltham, MA, US), 0.1 mg/ml BSA, and 100 nM His-OsERS1 with or without 100 nM MBP-OsARC (or MBP-ΔOsARC, deleted for GST_C). The formation of $[^{32}P]$ ATP at 37°C was followed at various time intervals in 9 μl aliquots. Samples at specific time-points were terminated using 1 ml of quenching buffer containing 2% (w/v) activated charcoal, 3.5% (v/v) HClO$_4$, and 50 mM tetradsodium pyrophosphate.
The charcoal suspension was put through a Whatman GF/A filter, washed four times with 5 ml of water, and rinsed with 10 ml of 100% ethanol. The charcoal powder on the filters was dried and the synthesized [\(^{32}\)P] ATP was quantified using a scintillation counter (Beckman Coulter) (Chen et al., 2000).

We performed aminoacylation reactions in a mixture of 100 mM Tris–HCl (pH 7.8), 30 mM KCl, 12 mM MgCl\(_2\), 2 mM dithiothreitol, 4 mM ATP, 20 mM [\(^{3}\)H] glutamate (15 Ci/mM, PerkinElmer, Waltham, MA, US), 15 \(\mu\)M tRNA\(_{\text{Glu}}\), and 100 nM His-OsERS1 with or without 100 nM MBP-OsARC (or MBP-\(\Delta\)OsARC, deleted for GST_C) at 37°C. We used 0.5–20 \(\mu\)M tRNA\(_{\text{Glu}}\) for kinetic constants analyses of OsERS1 and its variants (mutations of three key motifs). Reactions were initiated with addition of the enzyme and were conducted in a 37°C heat block. Aliquots (9 \(\mu\)l) were taken at different time points and quenched on Whatman filter pads that had been presoaked with 5% trichloroacetic acid (TCA). The pads were washed three times for 10 min each time with cold 5% TCA, and once with cold 100% ethanol; the washed pads were then dried. Radioactivity was quantified in the scintillation counter. The mixture of OsERS1 and OsARC was incubated at room temperature for 30 min to allow complexation before ATP-PPi and aminoacylation assays (Chen et al., 2011).

**Y2H, Pull-down, BiFC, and Co-localization assays**

We performed yeast two-hybrid experiments according to the manufacturer's instructions (Clontech). The coding sequences of OsERS1 and \(\Delta\)OsERS1 (deleted for GST_C) were fused in-frame with the GAL4 DNA-binding domain of
the bait vector \( pGBKT7 \). The coding sequences of \( OsARC \) and \( \Delta OsARC \) were cloned in the prey vector \( pGADT7 \). Each bait–prey pair was co-transformed into the yeast strain AH109. To analyze protein–protein interactions using histidine auxotrophy assays, yeast colonies were patched in duplicate onto His– and His+ plates and kept at 30°C for 2–3 days.

Amylose resin beads containing MBP, MBP-OsARC, and MBP-\( \Delta OsARC \) were incubated with His-OsERS1 in pulldown buffer (15 mM HEPES-NaOH at pH 7.9, 50 mM potassium glutamate, 5 mM magnesium chloride, 5% glycerol, 0.1% NP40, 1 \( \mu \)g/\( \mu \)l BSA, and 1 mM dithiothreitol). We rotated the mixture at 4°C for 1 h, and washed the beads five times with wash buffer (15 mM HEPES-NaOH at pH 7.9, 50 mM potassium glutamate, 5 mM magnesium chloride, and 0.1% NP40). Proteins were eluted from the beads by boiling in SDS-PAGE sample buffer, and the eluate was analyzed by immunoblot detected with anti-MBP (1:1,000 dilution) antibodies.

The cDNAs of \( OsERS1 \), \( \Delta OsERS1 \), and \( OsARC \) were individually cloned into \( pSAT1-nEYFP-N1 \) and \( pSAT1-cEYFP-C1-B \) vectors that contain either amino- or carboxy-terminal eYFP fragments. The method for bimolecular fluorescence complementation assays using onion epidermal cells is described above (subcellular localization). YFP fluorescence was imaged at an excitation wavelength of 514 nm and emission wavelengths of 525–546 nm.

For co-localization between OsERS1 and OsARC1, we constructed \( 35Spro::OsERS1\cdot eGFP \) and \( 35Spro::OsARC\cdot mcherry \) by cloning cDNA
sequences of OsERS1 and OsARC into p1301. The method for co-localization assays using onion epidermal cells is described above (subcellular localization).

**Metabolomics Analysis**

Over 120 mg of rice anther from wild-type and osers1 plants were collected, frozen with liquid nitrogen, and stored at –80°C until metabolomics analysis. We ground samples into a fine powder and analyzed methanol extracts from 40 mg sample using the analytical platform composed of ultra-high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) and gas chromatography (GC)-MS. METABOLON performed the experiments and raw data analysis ([http://www.metabolon.com](http://www.metabolon.com)). Metabolites were named after standard references ([http://www.kegg.jp](http://www.kegg.jp)).

**Reactive Oxygen Species Measurement and Hydrogen Peroxide Staining**

We collected rice spikelets from wild-type and osers1 plants and sorted them according to spikelet length. Samples were ground into a fine powder and processed according to the instructions of the Hydrogen Peroxide Assay Kit (abcom, catalogue no. ab102500). Fluorometric readings were collected from a POLARstar OPTIMA (BMG Labtech). Three independent experiments were performed to calculate mean value and standard deviation (SD). Aliquots of sample powder were suspended in 65 mM K-phosphate buffer (pH 7.8) to extract superoxide radicals. Following a modified method (Wang and Jiao, 2000), the reaction mixture (0.5 ml extract, 0.7 ml K-phosphate buffer, and 0.3
ml 10 mM hydroxylammonium chloride) was incubated at 25°C for 60 min. Then
0.5 ml of the above reaction mixture was added to 0.5 ml of 19 mM sulfanilic acid and 0.5 ml of 1.0% α-naphthylamine. After a 20-min coloring reaction at 25°C, the optical density was determined at 530 nm in a spectrophotometer. The linear standard curve was determined by 0–5 μM NaNO₂ reacted with sulfanilic acid and α-naphthylamine.

Freshly collected anthers were carefully immersed in the fixative solution O.C.T. Compound (Sakura) and frozen in liquid nitrogen. Embedded fresh tissues were sectioned into 9-μm thick sections using a Leica CM1800 cryostat. Production of H₂O₂ was performed by incubating sectioned anthers in the DAB solution (Vector Laboratories) for 3 to 4 min optimized from the manufacturer’s instructions and photographed with a Nikon Ni-E optical microscope.

**Anther Treatments**

We removed the outermost whorls of leaves from wild-type and osers¹ stems to locate the inflorescences. Next, 1 mM KI and 100 μM H₂O₂ solutions were freshly prepared and injected through leaf whorls using an 18-gauge needle into the airspace about 5 cm above the first stem node, where the young inflorescence was developed (Supplemental Fig. S9). Water injection served as a control. After 72 h of treatment, rice spikelets were fixed, embedded in resin, and sectioned as described above.

**Accession Numbers**
OsERS1, LOC_Os10g22380; OsERS2, LOC_Os01g16520; OsERS3, LOC_Os02g02860; OsARC, LOC_Os01g60660; MSP1, LOC_Os01g68870; H4, LOC_Os09g38020; CDKB2;1, LOC_Os08g40170; AlaRS3, LOC_Os10g10244; ArgRS2, LOC_Os05g07030; AsnRS1, LOC_Os01g27520; AspRS3, LOC_Os02g46130; CysRS1, LOC_Os03g04960; GlnRS1, LOC_Os01g09000; GlyRS3, LOC_Os08g42560; LysRS2, LOC_Os03g38980; MetRS2, LOC_Os06g31210; ProRS2, LOC_Os12g25710; ThrRS2, LOC_Os08g19850; TrpRS2, LOC_Os12g35570.

Supplemental Data

Supplemental Figure S1. Phenotypic analysis of the osers1 mutant.

Supplemental Figure S2. Cytological analysis of wild-type and osers1 anthers.

Supplemental Figure S3. Expression of genes that encode regulators of early anther development in osers1 anthers.

Supplemental Figure S4. Molecular characteristics of OsERS1.

Supplemental Figure S5. Alignment of conserved motifs among GluRSs.

Supplemental Figure S6. Shift of major metabolic pathways in osers1 anthers.

Supplemental Figure S7. RT-qPCR detection of expression levels of aminoacyl-tRNA synthetase genes.

Supplemental Figure S8. DAB staining analysis for hydrogen peroxide in anther lobes.

Supplemental Figure S9. Chemical treatments to anthers.

Supplemental Figure S10. Full scan figures of the gel and western blot.

Supplemental Table S1. Alignment of GluRS amino acid sequences.
**Supplemental Table S2.** Heat map of metabolite changes between the wild type and the *osers1* mutant.

**Supplemental Table S3.** RT-qPCR detection of the expression changes of genes in metabolic pathways between the wild type and the *osers1* mutant.

**Supplemental Table S4.** Sequences of primers used in the study.

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**FIGURE LEGENDS**
**Figure 1.** OsERS1 is essential for cell proliferation and organization in early rice anther development. (A) to (H) Cytological comparison of anther development between the wild type and the osers1 mutant. S3–S5 indicate anther developmental stages. (A) and (B) are anthers in stage 3, (C) and (D) are magnifications of the boxed region in (A) and (B), respectively, (E) and (F) are anther lobes in stage 4, (G) and (H) are anther lobes in stage 5. Yellow asterisks indicate archesporial cells at S3 and sporogenous cells at S4–S5. Bars = 15 μm. (I) Diagrams of anther development from stages 1 to 5 in the wild type and the osers1 mutant. Disorganization of somatic cell layers and excess archesporial/sporogenous-like cells are shown in the mutant. (J) and (K) Quantification of cell over-proliferation in osers1 anther. (J) Average L2-d cell counts of anther cross-sections, (K) average archesporial/sporogenous cell counts of the anther cross-sections. All error bars represent SD. S3 wild type, n=11; S4 wild type, n=13; S5 wild type, n=15; S3 osers1, n=12; S4 osers1, n=14; S5 osers1, n=13. Student’s t-test: *P < 0.05; **P < 0.01. (L) to (Q) In situ hybridization of cell-division marker genes in stage 4 anthers. Bars = 50 μm. Ar, archesporial cell; eAr, excess archesporial-like cell; Sp, sporogenous cell; eSp, excess sporogenous-like cell; L2-d, Layer 2-derived cell; PPC, primary parietal cell; SPC, secondary parietal cell; E, epidermis; En, endothecium; ML, middle layer; T, tapetum.

**Figure 2.** Molecular identification of OsERS1. (A) Fine mapping of OsERS1 to chromosome 10. Molecular markers used for mapping are indicated. Numbers
in brackets indicate the number of recombinants. AC numbers are rice genomic DNA accession numbers of bacterial artificial chromosome clones. (B) A schematic representation of OsERS1 and functional domains. Red letter ‘A’ indicates an insertion after A⁵³⁹ in oser1. NT, nucleotides. Black rectangles represent exons. The asterisk means stop codon. GST_C, Glutathione S-transferase C-terminal domain; GluRS_core, catalytic core domain of glutamyl-tRNA synthetase; tRNA-synt_C, tRNA synthetases class I anti-codon binding domain. (C) to (E) Complementation of the osers1 mutant. (C) Inflorescences with mature seeds. (D) Average seed-setting rates in panicles (n=20 for each set). Error bars represent SD. (E) Mature anthers. Bars = 2 cm in (C) and 1 mm in (E). COM, complementation.

Figure 3. Expression pattern and phylogenetic analysis of OsERS1. (A) and (B) RT-qPCR data showing (A) relative expression level of OsERS1 in various tissues and (B) anthers at various stages. For (B), total RNA was extracted from whole rice flowers before stage 6 and from anthers after stage 7. All Error bars represent SD (n=3). (C) In situ hybridization of OsERS1 in the wild-type anther at different stages. A sense probe was used as the control. Bars = 15 μm. Ar, archesporial cell; Sp, sporogenous cell; PPC, primary parietal cell; SPC, secondary parietal cell; ML, middle layer; T, tapetum. (D) Subcellular localization of OsERS1 in rice protoplasts. GFP was used as a control. Vector CD3-991 containing the mitochondria-specific protein ScCOX4 fused with mCherry served as the organelle marker. Chl, chloroplast; BF, bright field. Bars
= 10 μm. (E) A neighbor-joining tree of OsERS1 and its homologs showing progressive recruitment of novel functional domains. AA, amino acids; GST_C, C terminus of glutathione S-transferase; GluRS_core, glutamyl-tRNA synthetase catalytic core; tRNA-synt_C, tRNA synthetase class I anti-codon binding domain; ProRS, prolyl-tRNA synthetase.

Figure 4. OsERS1 functions as a ligase of glutamate and tRNAGlu. (A) Conserved sites in the catalytic domain of OsERS1 used for site-directed mutagenesis. The figure was generated from 40 homologs of OsERS1 by WebLogo (http://weblogo.berkeley.edu). (B) ATP–PPi exchange rate in the presence of increased concentrations of tRNAGlu. (C) tRNAGlu aminoacylation by wild-type and mutant (M1-M3) OsERS1. (D) Kinetics of tRNAGlu aminoacylation by OsERS1WT and OsERS1M1 under increased tRNAGlu concentrations. OsERS1WT, R² = 0.99, OsERS1M1, R² = 0.97. (E) Kinetic constants determined by tRNAGlu charging assays. Kinetics of the OsERS1 and OsERS1M1-M3 aminoacylation reaction was measured using varied concentrations of tRNAGlu, and reactions were conducted for 10 min under standard conditions. Reciprocal initial velocity was plotted against the reciprocal tRNAGlu concentration according to Lineweaver–Burk to calculate the corresponding Km values. All error bars represent SD (n=3).

Figure 5. The complex of OsERS1 and OsARC enhances OsERS1’s catalytic activity. (A) Domain structures of OsERS1 and OsARC and their truncations. (B) OsERS1 and OsARC interact in yeast. Interaction is verified by growth of yeast
strain in selective medium. (C) Pull-down assays between His-OsERS1 and MBP-OsARC in vitro. The precipitated MBP-OsARC was detected by α-MBP antibody. MBP-tagged ΔOsARC was used as a control to show the interaction in a GST_C domain-dependent manner. CBB, coomassie brilliant; WB, western blot. (D) Bimolecular fluorescence complementation (BiFC) assays in onion epidermal cells showing the interaction between OsERS1 and OsARC. Bars = 50 μm. nYFP, N-terminal split of yellow fluorescence protein (YFP); cYFP, C-terminal split of YFP. (E) Co-localization of OsERS1-eGFP and OsARC-mCherry in onion epidermal cells. Bar = 50 μm. (F) Influence of the OsERS1-OsARC complex on ATP-PPi exchange. 15 μM tRNAGlu was used as the substrate. (G) Influence of the OsERS1–OsARC complex on tRNAGlu aminoacylation. All error bars represent SD (n=3).

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Figure 1. OsERS1 is essential for cell proliferation and organization in early rice anther development. (A) to (H) Cytological comparison of anther development between the wild type and the osers1 mutant. S3–S5 indicate anther developmental stages. (A) and (B) are anthers in stage 3, (C) and (D) are magnifications of the boxed region in (A) and (B), respectively, (E) and (F) are anther lobes in stage 4, (G) and (H) are anther lobes in stage 5. Yellow asterisks indicate archesporial cells at S3 and sporogenous cells at S4–S5. Bars = 15 μm. (I) Diagrams of anther development from stages 1 to 5 in the wild type and the osers1 mutant. Disorganization of somatic cell layers and excess archesporial/sporogenous-like cells are shown in the mutant. (J) and (K) Quantification of cell over-proliferation in osers1 anther. (J) Average L2-d cell counts of anther cross-sections, (K) average archesporial/sporogenous cell counts of the anther cross-sections. All error bars represent SD. S3 wild type, n=11; S4 wild type, n=13; S5 wild type, n=15; S3 osers1, n=12; S4 osers1, n=14; S5 osers1, n=13. Student’s t-test: *P < 0.05; **P < 0.01. (L) to (Q) In situ hybridization of cell-division marker genes in stage 4 anthers. Bars = 50 μm. Ar, archesporial cell; eAr, excess archesporial-like cell; Sp, sporogenous cell; eSp, excess sporogenous-like cell; L2-d, Layer 2-derived cell; PPC, primary parietal cell; SPC, secondary parietal cell; E, epidermis; En, endothecium; ML, middle layer; T, tapetum.
Figure 2. Molecular identification of OsERS1. (A) Fine mapping of OsERS1 to chromosome 10. Molecular markers used for mapping are indicated. Numbers in brackets indicate the number of recombinants. AC numbers are rice genomic DNA accession numbers of bacterial artificial chromosome clones. (B) A schematic representation of OsERS1 and functional domains. Red letter ‘A’ indicates an insertion after A\(^{539}\) in oser1. NT, nucleotides. Black rectangles represent exons. The asterisk means stop codon. GST_C, Glutathione S-transferase C-terminal domain; GluRS_core, catalytic core domain of glutamyl-tRNA synthetase; tRNA-synt_C, tRNA synthetases class I anti-codon binding domain. (C) to (E) Complementation of the oser1 mutant. (C) Inflorescences with mature seeds. (D) Average seed-setting rates in panicles (n=20 for each set). Error bars represent SD. (E) Mature anthers. Bars = 2 cm in (C) and 1 mm in (E). COM, complementation.
Figure 3. Expression pattern and phylogenetic analysis of OsERS1. (A) and (B) RT-qPCR data showing (A) relative expression level of OsERS1 in various tissues and (B) anthers at various stages. For (B), total RNA was extracted from whole rice flowers before stage 6 and from anthers after stage 7. All Error bars represent SD (n=3). (C) In situ hybridization of OsERS1 in the wild-type anther at different stages. A sense probe was used as the control. Bars = 15 μm. Ar, archesporial cell; Sp, sporogenous cell; PPC, primary parietal cell; SPC, secondary parietal cell; ML, middle layer; T, tapetum. (D) Subcellular localization of OsERS1 in rice protoplasts. GFP was used as a control. Vector CD3-991 containing the mitochondria-specific protein ScCOX4 fused with mCherry.
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**Figure 4.** OsERS1 functions as a ligase of glutamate and tRNA\textsuperscript{Glu}. (A) Conserved sites in the catalytic domain of OsERS1 used for site-directed mutagenesis. The figure was generated from 40 homologs of OsERS1 by WebLogo (http://weblogo.berkeley.edu). (B) ATP–PPi exchange rate in the presence of increased concentrations of tRNA\textsuperscript{Glu}. (C) tRNA\textsuperscript{Glu} aminoacylation by wild-type and mutant (M1-M3) OsERS1. (D) Kinetics of tRNA\textsuperscript{Glu} aminoacylation by OsERS1\textsubscript{WT} and OsERS1\textsubscript{M1} under increased tRNA\textsuperscript{Glu} concentrations. OsERS1\textsubscript{WT}, $R^2 = 0.99$, OsERS1\textsubscript{M1}, $R^2 = 0.97$. (E) Kinetic constants determined by tRNA\textsuperscript{Glu} charging assays. Kinetics of the OsERS1 and OsERS1\textsubscript{M1-M3} aminoacylation reaction was measured using varied concentrations of tRNA\textsuperscript{Glu}, and reactions were conducted for 10 min under standard conditions. Reciprocal initial velocity was plotted against the reciprocal tRNA\textsuperscript{Glu} concentration according to Lineweaver–Burk to calculate the corresponding Km values. All error bars represent SD (n=3).
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