Identification of a novel mitochondrial protein, short postembryonic roots 1 (SPR1), involved in root development and iron homeostasis in Oryza sativa

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Summary

• A rice mutant, Oryza sativa short postembryonic roots 1 (Osspr1), has been characterized. It has short postembryonic roots, including adventitious and lateral roots, and a lower iron content in its leaves.
• OsSPR1 was identified by map-based cloning. It encodes a novel mitochondrial protein with the Armadillo-like repeat domain.
• Osspr1 mutants exhibited decreased root cell elongation. The iron content of the mutant shoots was significantly altered compared with that of wild-type shoots. A similar pattern of alteration of manganese and zinc concentrations in shoots was also observed. Complementation of the mutant confirmed that OsSPR1 is involved in post-embryonic root elongation and iron homeostasis in rice. OsSPR1 was found to be ubiquitously expressed in various tissues throughout the plant. The transcript abundance of various genes involved in iron uptake and signaling via both strategies I and II was similar in roots of wild-type and mutant plants, but was higher in the leaves of mutant plants.
• Thus, a novel mitochondrial protein that is involved in root elongation and plays a role in metal ion homeostasis has been identified.

Introduction

Iron (Fe) is an essential co-factor for several enzymes involved in crucial cellular processes ranging from oxygen and electron transport to hormone production and DNA synthesis (Briat & Lobreaux, 1997). In addition, plants require Fe for several processes that occur in plastids and an adequate Fe supply is essential to maintain photosynthetic function at optimal rates (Briat et al., 2007). However, excess Fe generates hydroxyl radicals via the Fenton reaction, which can damage biological molecules (Grotz & Guerinot, 2006).

Although abundant in soils, Fe often forms insoluble ferric hydroxide precipitates that limit its uptake by plants. Therefore, higher plants have evolved two distinct strategies to solubilize and acquire Fe from the rhizosphere (Jeong & Guerinot, 2009; Morrissey & Guerinot, 2009). Nongraminaceous plants use the strategy I system, which involves the induction of membrane-bound Fe(III)-chelate reductases, which reduce Fe(III) to the more soluble form of Fe(II), followed by uptake of Fe(II) via the Fe(II) transporter iron-regulated transporter 1 (IRT1) (Eide et al., 1996). By contrast, graminaceous plants use strategy II, which is mediated by the synthesis and secretion of natural Fe chelators, the mugineic acid (MA) family of phytosiderophores. The secreted MAs solubilize Fe(III) in the rhizosphere, and the resulting Fe(III)–MA complexes are absorbed into the root cells by the yellow-stripe-like (YSL) transporters (Curie et al., 2001). Rice (Oryza sativa) can use both strategies I and II for Fe uptake (Cheng et al., 2007).

Several genes involved in Fe uptake have been identified in rice. Loss-of-function mutants of OsNAAT (Nicotianamine Aminotransferase) were found to have a low assimilation capacity for Fe(III), but a normal assimilation capacity for Fe(II) (Cheng et al., 2007). OsSUL15 and OsSUL1 were reported to be Fe(III)–MA and Fe(II) transporters in rice,
respectively (Bughio et al., 2002; Inoue et al., 2009). YSL family members are thought to transport metal–MA complexes (Curie et al., 2001). There are 18 YSL genes in the rice genome (Koike et al., 2004). Of these, OsYSL15 was reported to be an Fe(III) transporter, which plays roles in Fe uptake from the rhizosphere and Fe distribution in rice plants (Inoue et al., 2009; Lee et al., 2009). OsFRDL1 (Oryza sativa FRD3-Like1;FRD3 (FERRIC REDUCTASE DEFECTIVE 3)) is a citrate transporter required for efficient translocation of Fe in rice, and knockouts of this gene resulted in leaf chlorosis, a lower leaf Fe concentration and a higher root Fe concentration (Yokosho et al., 2009). The rice genome encodes two proteins related to the Arabidopsis strategy I transporter IRT1 (OsIRT1 and OsIRT2) that are specifically up-regulated in roots of Fe-deficient plants and have been found to be responsible for Fe uptake in a yeast functional complementation assay (Bughio et al., 2002; Ishimaru et al., 2006). OsIRT1 has been confirmed to be an Fe transporter in rice (Lee & An, 2009).

Fe is translocated to multiple parts of the plant and is compartmentalized into specific organelles in the cell. Fe moves symplastically through the interconnected cytoplasm of the root, perhaps diffusing along a concentration gradient (Morrisey & Guerinot, 2009). At the pericycle, Fe is effluxed into the xylem and moves toward the shoot through the transpiration stream. Fe(III)–citrate is the major form of Fe present in xylem exudates, and citrate is thought to be involved in long-distance Fe transport from roots to shoots (Grotz & Guerinot, 2006). In contrast, YSL family members are thought to transport metal–MA complexes. It has been suggested that the eight Arabidopsis YSL transporters transport Fe chelated by the phytosiderophore (PS) precursor nicotianamine (NA) in and out of the phloem (Curie et al., 2009). Damage to the phloem appeared to impair Fe movement to young leaves but not to old leaves (Tsukamoto et al., 2009). This provides evidence that young leaves receive Fe primarily from the phloem, while older leaves receive Fe from the xylem. In rice, OsYSL15 and OsYSL18 transporters have also been shown to be involved in Fe distribution in plants (Aoyama et al., 2009; Lee et al., 2009).

The three major cell compartments that have key roles in Fe homeostasis are vacuoles, chloroplasts and mitochondria. Vacuoles are crucial compartments for Fe storage and sequestration within plant cells. In Arabidopsis, vacuolar iron transporter 1 (VIT1) was identified as a vacuolar Fe(II) transporter that functions in vacuolar Fe storage (Kim et al., 2006), while Arabidopsis thaliana natural resistance-associated macrophage protein 2 (AtNRAMP3) and AtNRAMP4 were both found to export Fe from vacuoles (Lanquart et al., 2005). Recently, a chloroplast ferric chelate reductase, Arabidopsis thaliana Ferric Reduction Oxidase 7 (AtFRO7), was characterized as a candidate chloroplast Fe transporter (Pilon et al., 2009). A putative Fe transporter, permease in chloroplasts 1 (PIC1), has also been identified which localizes to the chloroplast (Duy et al., 2007). Although Fe plays important roles in mitochondrial metabolism, little is known about mitochondrial Fe homeostasis. Recently, the Fe-binding proteins ferritin and frataxin have been localized to mitochondria (Morrisey & Guerinot, 2009). Knockdown T-DNA insertional mutants in Arabidopsis frataxin showed that Fe accumulation occurred in mitochondria as well as in plastids (Martin et al., 2009). AtATM3, also known as STARIK (STA1), is one of three orthologs of the yeast ATP-binding cassette (ABC) transporter of mitochondria 1 (ATM1). It is thought to export Fe–sulphur (S) clusters in planta, and sta1 mutant plants are dwarf and chlorotic (Chen et al., 2007). Recently, the cloning of a mitochondrial gene involved in Fe homeostasis mitochondrial iron-regulated (MIR) was reported. The mir mutant showed twofold Fe accumulation in both shoot and root compared with the wild type (Ishimaru et al., 2009).

In this study, a novel mitochondrial protein, Oryza sativa short postembryonic roots 1 (OsSPR1), was identified and shown to be involved in root development and Fe homeostasis. OsSpr1 mutants showed lower Fe content in leaves, aberrant root development and increased cell death in the root tip.

Materials and Methods

Plant material and growth conditions

The OsSpr1 mutant was isolated from an ethyl methylsulfonate mutagenized (EMS)-generated rice mutant library (India cv Kasalash) and maintained in a solution culture with Yoshida nutrient solution. Phenotypic characterization of the wild-type and mutant plants was performed in a growth chamber at 30 : 22°C (day : night) and 60–70% humidity with a photoperiod of 12 h. For hydroponics, seedlings were transferred to a plastic net floating on the Yoshida nutrient solution containing 35 µM citrate-Fe(III) or 125 µM EDTA-Fe(II) or without Fe, as described by Cheng et al. (2007).

Reactive oxygen species (ROS) and cell death detection

In situ H$_2$O$_2$ production was detected using an endogenous peroxidase-dependent staining procedure with 3,3-diaminobenzidine (DAB). Roots were detached and placed in a solution of 1 mg ml$^{-1}$ DAB, pH 3.8, for 6 h. H$_2$O$_2$ production was visualized as a reddish-brown coloration at the site of DAB polymerization. Nitroblue tetrazolium chloride (NBT) was used to stain for the site of superoxide production (Carol et al., 2005). Roots were covered with freshly made NBT solution (0.5 mg ml$^{-1}$ NBT in 0.1 M potassium phosphate with 0.1 M NaCl at pH 7) for 2 h at room temperature for the color to develop. O$_2^-$ was visualized as a blue color at the site of NBT precipitation.
Genetic analysis and marker development

Genomic DNA was extracted and analyzed for co-segregation from F2 plants using available simple sequence repeats (SSRs). New SSR markers were developed based on the Nipponbare genome sequence from the National Center for Biotechnology Information database by searching for simple repeat sequences with the program SSRIT (http://www.gramene.org/db/markers/ssrtool). The markers used for genetic mapping are listed in Supporting Information Table S2.

Complementation of the spr1 mutant

For complementation of the spr1 mutation, a full-length cDNA fragment encoding SPR1 was amplified by RT-PCR using the primers 5'-GGATCCAGAAGATGGGGGTGA TGT-3' and 5'-GGATCCCTATCAGCAGCCTCTC-3' from wild type (the added BamHI recognition sites are underlined). PCR products were cloned into the Pmd19-T vector (TaKaRa). The SPR1 cDNA fragment from wild-type plants was digested with BamHI and ligated into the BamHI sites of the modified binary vector pCAMBIA1300 harboring a hygromycin-resistant gene. The resulting pCAMBIA1300-SPR1 plasmid, which contained the SPR1 coding sequence driven by the CaMV 35 S promoter, was transformed into Agrobacterium tumefaciens strain EHA105 harboring a hygromycin-resistant gene. The resulting vector was named pCAMBIA1305.1. The resulting vector was then introduced into the pCAMBIA1305.1 vector by EHA105-mediated transformation (Cheng et al., 2003).

Transient expression of OsSPR1-GFP

The subcellular localization of SPR1 was determined by cloning the coding region of the first 107 and 150 amino acids in front of GFP using gateway cloning techniques (Invitrogen, Carlsbad, CA, USA) as previously described (Carrie et al., 2009). Biolistic transformations of both onion epidermal cells and Arabidopsis suspension cells were carried out using a PDS-1000/He system gene gun (Bio-Rad) as previously described (Carrie et al., 2009). The targeting signal of the soybean (Glycine max) alternative oxidase (AOX) fused to RFP (AOX-RFP) was used as a control for mitochondrial localization (Murcha et al., 2007). Fluorescence patterns were observed after transformation using an Olympus BX61 fluorescence microscope (Arabidopsis cell suspension) as previously described (Carrie et al., 2009) and a Zeiss LSM 510 confocal microscope (onion epidermal cells).

Phylogenetic analysis

The phylogenetic relationship of the sequences was analyzed using the neighbor-joining method with MEGA 4 (http://www.megasoftware.net/mega.html). Sequence alignments were performed using CLUSTALW (http://align.genome.jp/). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the relationship of the sequences analyzed. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The tree in Fig. 5 was drawn to scale, with branch lengths given in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the data set (complete deletion option).

Analysis of OsSPR1 expression in planta

To construct an OsSPR1-promoter-β-glucuronidase (GUS) chimeric gene, the GUS-plus fragment obtained from pCAMBIA1305.1 was cloned between the BamHI and SacI sites of PBI101.3 and the resulting vector was named PBI101-GUS. The promoter region of OsSPR1 was obtained by PCR amplification using the primers 5'-AAGCTTGTAGCTACACAGCGA-3' and 5'-GGATCCCTAGAAAACCTAAT-3' and then cloned into PBI101-GUS. The resulting vector was transformed into Kasalash (Kas) rice plants by Agrobacterium tumefaciens-mediated transformation (Cheng et al., 2007).

Measurement of chlorophyll content

SPAD values (total chlorophyll content) were determined on the fully expanded youngest leaves of 10-d-old seedlings with a portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, Sakai, Osaka, Japan).

Metal ion measurements

Shoots and roots of 15-d-old seedlings were washed three times for 5 min with distilled water, drained and dried at 70°C for 2 d. Four 50-ml aliquots of dried plant material were acid-digested for 6 h at 130°C in 3 ml of ultrapure HNO3 in a clean glass tube. After digestion, the samples were diluted to 25 ml with distilled water. Metal concentrations were measured using inductively coupled plasma mass spectrometry (Agilent 7500ce, Agilent Technologies, Palo Alto, CA, USA).

RT-PCR analysis

Total RNA was extracted from the roots and leaves of 2-wk-old seedlings grown in culture solution with a supply of Fe(III) or Fe(II), or without Fe. The first-strand cDNA was synthesized using Superscript II (Invitrogen) and was used as RT-PCR templates. The RT-PCR was repeated at least three times for separately harvested samples using
gene-specific primers. The primers and amplification cycle numbers for the selected genes are listed in Table S3.

Statistical analysis of data

The data shown in Fig. 6 were analyzed using a two-sample $t$-test assuming unequal variances, with all samples compared with the wild type. Significance was defined as $P \leq 0.05$.

Results

Isolation of the spr1 mutant

Screening of an EMS rice mutant library identified a chlorotic mutant with short lateral and adventitious roots, named Oryza sativa short postembryonic root 1 (OsSpr1). In total three mutant alleles were identified. OsSpr1 showed a defect in postembryonic root development, with lateral and adventitious root elongation arrested under normal solution culture conditions (Fig. 1a,b). Initiation of lateral roots occurred normally but elongation was retarded (Fig. 1b). The average lateral root length of 7-d-old mutant seedlings was $0.04 \pm 0.005$ cm compared with $0.33 \pm 0.02$ cm for the wild type (Fig. 1b, Table S1). Importantly, lateral root initiation and lateral root number were not affected by the loss of OsSpr1 function (Fig. 1b, Table S1). This indicates that the effect of OsSpr1 on lateral root development was specific to elongation. Longitudinal sections of primary and lateral roots of 7-d-old seedlings revealed that the length of cells in the maturation zone of mutant roots was dramatically reduced compared with that of wild-type roots, while the axial and radial patterns of cell organization in the root apical meristem (RAM) were not affected in the mutant (Fig. 1c,e). This indicated that the process of cellular differentiation that defines root formation was not affected in OsSpr1. However, the average tiller number in the mutant was significantly reduced compared with that of the wild type (Fig. 1d). Seed set in the mutant was normal and comparable to that in wild-type plants (data not shown).

Increases in ROS concentration and cell death were observed in the root tips of spr1 mutants

The roots of spr1 mutants were more fragile than those of the wild type, and were broken more easily. To investigate the cause of this, a variety of physiological and anatomical analyses were performed. Staining of 7-d-old seedling roots with Evans blue (Gaff & Okong’O-gola, 1971) revealed large patches of stained cells along the roots of spr1 mutant plants (Fig. 1f,g), especially near the root tips, indicating that cell death had occurred. To investigate the relationship between cell death and ROS concentration, the amount and distribution of ROS in root tissue from the wild-type and mutant plants were examined. In situ detection of $O^-$ was performed using NBT staining (Carol et al., 2005), whereas $H_2O_2$ was analyzed using DAB staining (Veljovic-Jovanovic et al., 2002). The results showed that basal $H_2O_2$ concentrations were higher in the roots of the spr1 mutant plants compared with wild-type seedlings (Fig. 1j,k). $H_2O_2$ accumulated to the greatest extent at the root tip (in the elongating zone) where cell death was observed, as evidenced by Evans blue staining (Fig. 1f,g). The degree of NBT staining, and hence the amount of superoxide in the roots, was similar in the wild type and spr1 (Fig. 1h,i).

Identification of the OsSPR1 gene

To identify the gene disrupted in the mutant plants, the OsSpr1 mutants were crossed with Nipponbare (japonica). The F1 seedlings displayed a wild-type phenotype and their F2 progenies showed a segregation ratio of 3 : 1 (normal seedlings : mutant seedlings, $\chi^2 < \chi_{0.05}^2 = 3.84; P > 0.05$), suggesting that the short lateral root phenotype in the OsSpr1 mutant plants is controlled by a single recessive nuclear gene. Fine mapping of the OsSPR1 locus was achieved using 1511 OsSpr1 mutant plants, selected from 6150 F2 progeny. The OsSPR1 locus was localized to a region of 18 kb between two sequence-tagged site (STS) markers, STS1 and STS2, on BAC P0506A10 on the long arm of chromosome 1 (Fig. 2a). Two open reading frames (ORFs) were predicted in this region according to The Institute for Genomic Research (TIGR) annotation (http://rice.plantbiology.msu.edu/). Sequencing of the two ORFs in one of the OsSpr1 mutant plants and the wild-type plant revealed a single point mutation in an ORF encoding a protein of unknown function (LOC_Os01g67290). The point mutation in exon 17 at 5153 bp changes cytosine to thymine, resulting in a premature stop codon. The OsSPR1 gene is 5767 bp in length, and contains 19 exons and 18 introns. The protein-coding region of OsSPR1 is 2943 bp, with a predicted protein of 980 amino acids with a calculated molecular mass of 109 kDa. This experimentally deduced gene structure is consistent with the annotated version generated by the Rice Genome Initiative (http://rice.plantbiology.msu.edu/).

To confirm that OsSPR1 caused the mutant phenotype, the other two spr-like mutants were sequenced. This analysis indicated that both contained a point mutation in the SPR1 gene, resulting in premature stop codons. According to the position of each mutation, the three mutant alleles were subsequently named spr1-1, spr1-2 and spr1-3, respectively (Fig. 2b). Unless specified, the spr1 mutant in this study refers to spr1-1. To further confirm that the mutation in SPR1 was the direct cause of the phenotype, the cDNA of OsSPR1, driven by the 35S cauliflower mosaic virus (CaMV) promoter, was transferred into the spr1 mutant by A. tumefaciens-mediated transformation. A series of
Fig. 1 Morphological characterization of the short postembryonic roots 1 (spr1) mutant. (a) Seven-day-old seedlings of wild-type (Kasalath; left) and spr1 mutant (right) plants grown in normal culture solution in chambers with a 12 h light (30°) : 12 h dark (24°) photoperiod. (b) Phenotype of lateral roots from 7-d-old seedlings of the wild type (Kasalath; left) and the mutant (right). (c) Longitudinal sections of primary roots (wild type, left; spr1, right). (d) Phenotype of wild type (left) and Oispr1 mutant (right) mature plants. (e) Longitudinal sections of lateral root (wild type, left; spr1, right; bar, 50 μm). (f, g) Evans blue staining for root cell death in primary root tips (f) and lateral root tips (g) of the wild type (left) and spr1 mutant (right). (h, i) Nitroblue tetrazolium chloride (NBT) staining for superoxide in primary root tips (h) and lateral root tips (i) of the wild type (left) and spr1 mutant (right). (j, k) 3,3-Diaminobenzidine (DAB) staining for hydrogen peroxide in primary root tips (j) and lateral root tips (k) of the wild type (left) and spr1 mutant (right). Bars: (a) 2 cm; (b, f, g–k) 1 mm; (c) 100 μm; (d) 15 cm; (e) 50 μm.
progenies from these transformants displayed a wild-type phenotype and the short root system (primary root, adventitious root and lateral root) was restored to the levels of wild-type plants (Fig. 2d). Two independent transgenic lines are shown in Fig. 2(d). Southern blotting indicated they were independent single copy transgenic lines (Fig. 2e). The empty vector transformants did not rescue the spr1 mutation (data not shown). Thus, three independent mutant alleles and restoration of phenotype confirmed that OsSPR1 encodes a protein that, if mutated, results in the observed phenotype.

**OsSPR1 expression and localization**

The expression pattern of OsSPR1 was determined using the GUS gene driven by the OsSPR1 promoter transferred into wild-type plants. GUS staining in transgenic plants indicated that OsSPR1 was ubiquitously expressed in all plant organs, including the root, leaf, stem and spikelet (Fig. 3a–r). OsSPR1 was expressed in primary and lateral roots, both in mature regions and in root tips. Strong expression was observed in the root tip (in the meristem and lateral root initiation regions), where dividing cells are present. The expression of OsSPR1 in the root is mainly associated with vascular tissues (Fig. 3b,d,h). Semi-quantitative RT-PCR analysis using SPR1-specific primers showed that SPR1 was expressed at relatively similar levels in all tissues, including the root, stem base, stem, leaf and panicles (Fig. S3), consistent with the GUS staining patterns observed.

**OsSPR1 encodes a mitochondrial protein of unknown function**

The SPR1 protein was found to contain a predicted mitochondrial targeting signal (http://psort.ims.u-tokyo.ac.jp/form.html). To determine the subcellular localization of OsSPR1, the first 107 and 150 amino acids were fused in-frame with the green fluorescent protein (GFP) and transiently expressed in onion epidermal and Arabidopsis suspension cells. GFP fluorescence was visualized by confocal microscopy. GFP in onion epidermal cells and Arabidopsis culture cells showed subcellular co-localization with AOX:RFP, a well-characterized mitochondrial marker (Fig. 4) (Carrie et al., 2009). These results strongly suggest that OsSPR1 is localized in the mitochondria.

Searching of public databases with OsSPR1, using BLASTp, failed to detect proteins with high levels of amino acid sequence identity. Only the N-terminal region of the OsSPR1 protein shares a low level of sequence similarity to the protein Emerging Flux Region 3 (EFR3) homolog Conserved Membrane Protein at 44E (Cmp44E) in Drosophila melanogaster (20% identity and 41% similarity in an 496 amino acid region). The Cmp44E gene in
D. melanogaster encodes a transmembrane protein essential for cell viability (Huang et al., 2004). Using InterProScan, OsSPR1 was found to contain a predicted Armadillo repeat domain (http://www.ebi.ac.uk/Tools/InterProScan/). Using the TMPRED program, OsSPR1 was predicted to contain two transmembrane domains, one between residues 423 and 447 and one between residues 500 and 518 (http://www.ch.embnet.org/software/TMPRED_form.html), and
a coiled-coil domain between amino acid residues 740 and 835 (http://www.ch.embnet.org/software/COILS_form.html). These results suggest that OsSPR1 is a novel mitochondrial membrane protein in plants.

Four additional genes that display 32 to 66% amino acid sequence identity with OsSPR1 were found in the rice genome, located on chromosomes 2, 3 and 7 (Fig. S1). Based on BLASTp searching, 24 sequences of SPR1-like proteins that displayed > 30% similarity from a variety of organisms were identified (Fig. 5). Phylogenetic analysis using the full protein sequences demonstrated that all these proteins from various species form a monophyletic group, which can be separated into plants (including clades 1, 2 and 3), animal (clade 6), and unicellular organisms (clades 4 and 5) (Fig. 5). This suggests that all OsSPR1-like genes evolved from a single ancestral gene that underwent duplication after species divergence.

Altered Fe content in spr1 mutant and over-expressing lines

Two-week-old spr1 mutant plants displayed chlorotic leaves when grown in 35 μM Fe [Fe(III)] nutrient solution, but normal green leaves when grown in 125 μM Fe [Fe(II)] culture solution (Fig. 6a,b). The average chlorophyll content (SPAD-502 meter readings) in mutant leaves was significantly lower (c. 40%) than that in leaves of wild-type plants supplied with Fe(III) (Fig. 6c) This is consistent with Fe deficiency symptoms. These results suggest that the mutants have impaired Fe uptake or homeostasis, which resulted in Fe-deficiency symptoms in the leaves.

The leaf chlorosis observed in spr1 mutant plants growing in Fe(III) solution indicated that the Fe content in the spr1 mutant plants had changed compared with that in the wild-type plants. The shoot Fe concentration of mutant plants was 55% that of the wild type in Fe(II) solution and 30% that of the wild type in Fe(III) solution (Fig. 6d). The Fe content of roots from spr1 plants was comparable to that of wild-type plants regardless of the Fe species and concentration supplied (Fig. 6e). The Fe content of shoots over-expressing OsSPR1 did not increase compared with the wild type, and was slightly decreased under Fe(III) conditions (Fig. 6d). The Fe content of roots in over-expressing OsSPR1 plants increased almost twofold under Fe(II) conditions, while it was unaltered under Fe(III) conditions compared with that of wild-type roots (Fig. 6e). These results showed that ectopic over-expression of SPR1 increases Fe accumulation in roots, suggesting that over-expression of SPR1 may promote Fe uptake from the rhizosphere.

Analysis of other metals showed that the zinc (Zn) content was also dramatically decreased in spr1 leaves (Fig. 6f) and showed no significant difference between the Fe(II) and Fe(III) conditions. A similar trend was observed for manganese (Mn) content (Fig. 6h). Furthermore, the Zn and Mn

![Fig. 5 Phylogenetic analysis of plant Oryza sativa short postembryonic roots 1 (OsSPR1) homologs from different organisms: Oryza sativa (Os), Arabidopsis thaliana (At), Vitis vinifera (Vv), Monodelphis domestica (Md), Cucumis sativus (Cs), Physcomitrella patens (Pp), Chlamydomonas reinhardtii (Cr), Dictyostelium discoideum (Dd), Caenorhabditis briggsae (Cb), Caenorhabditis elegans (Ce), Danio rerio (Dr), Anopheles gambiae (Ag), Aedes aegypti (Aa), Drosophila pseudoobscura (Dp) and Drosophila melanogaster (Dm). A phylogenetic analysis in MEGA 4.0 was performed using the neighbor-joining tree with 1000 replications. OsSPR1 is indicated by the arrowhead. Numbers on branches indicate the probability that a given grouping is correct. Bar, substitutions/site.](image)
contents were reduced in mutant roots compared with the wild type (Fig. 6g,i). Ectopic over-expressing OsSPR1 plants showed increased Zn and Mn contents in both roots and leaves (Fig. 6f,g). These results suggest that disruption of OsSPR1 could also affect metal ion uptake.

Up-regulation of genes involved in Fe(II) and Fe(III) uptake in the Ospr1 mutant

To evaluate the effect of OsSPR1 disruption on the rice Fe uptake and signaling system, RT-PCR analysis was performed on the leaves and roots of 15-d-old wild-type and spr1 seedlings grown in Fe(III), Fe(II) or no Fe culture solutions. The results showed that Fe-deficiency responsive genes (Oryza sativa iron-responsive operator2 (OsIRO2), OsIROPT1, OsYSL15, nicotianamine synthase 1 (OsNAS1), OsNAS2, OsIRT2 and OsNRAMP4) were induced in both the wild type and the spr1 mutants (Fig. 7), indicating that spr1 does not affect the Fe-deficiency response signaling pathway.

Two NAS genes, OsNAS1 and OsNAS2, and the Fe(III)-deoxymugineic acid (DMA) transporter gene OsYL15, which is involved in Fe uptake strategy II, were expressed in the roots, but very weakly expressed in the leaves of wild-type plants under Fe supply conditions. However, the expression of all three genes was up-regulated under Fe-deficient conditions (Fig. 7). In the spr1 mutant, these genes had similar expression levels in roots compared with...
the wild type, but were strongly up-regulated in leaves compared with the wild type under Fe(II) and Fe(III) conditions (Fig. 7), consistent with the low Fe concentrations in leaves of spr1 plants. OsNAAT1, which participates in DMA synthesis, showed no detectable difference between the wild type and mutant in roots and shoots (Fig. 7). In rice, OsIRO2, encoding a basic Helix-Loop-Helix (bHLH) protein, has been identified as an Fe-deficiency-induced gene, and regulates the PS-mediated Fe uptake system, but not the additional Fe(II) uptake mechanism (Walker & Connolly, 2008). Induction of OsIRO2 was also observed in mutant leaves compared with wild-type leaves. OsIROPT1, encoding an oligo peptide transporter, was up-regulated in the spr1 mutant in both roots and shoots (Fig. 7). Together, these results indicate that the Fe(III) uptake system was up-regulated in the leaves of the mutant plants, consistent with low Fe concentrations.

We also examined the expression pattern of Fe uptake strategy I genes, including OsIRT1 and OsIRT2. The results showed little difference in OsIRT1 transcript abundance in leaves between the wild type and the mutant but a significant decrease in abundance in the roots of the mutant plants (Fig. 7). By contrast, the expression of OsIRT2 was up-regulated in spr1 plants, in both roots and shoots, compared with wild-type plants, irrespective of the Fe status of the growth medium. These results indicate that the strategy I system was also up-regulated in mutant leaves. OsNRAMP4, a member of the NRAMP family, exports Fe(II) from vacuoles and its expression is known to be induced under Fe deficiency (Lanquar et al., 2005). Expression of OsNRAMP4 was also found to be significantly induced in mutant leaves under both Fe(II) and Fe(III) conditions compared with the wild type (Fig. 7).

**Discussion**

This study has identified a novel mitochondrial protein, OsSPR1, that is involved in maintaining Fe homeostasis in rice. The large reduction of Fe content observed in the leaves but not the roots of Ospr1 mutant plants under Fe(II) and Fe(III) nutrient supply, and the normal Fe deficiency response for both strategy I and II marker genes in the leaves but not the roots indicate a defect in Fe homeostasis. In addition, root development was also compromised in Ospr1 mutant plants. It is likely that the defective roots of Ospr1 plants will have a secondary effect on nutrient uptake. However, if reduced Fe uptake as a result of damaged roots was the primary reason for the reduced Fe content in the leaves, the Fe content and the Fe deficiency response would have been reduced and activated, respectively, in the roots. However, the Fe content in roots of Ospr1 mutant plants was similar to that of wild-type plants (Fig. 6e). Also, no Fe deficiency response was observed in roots (Fig. 7). However, a lower Fe content was observed in the mutant leaves, which was inconsistent with the induction of Fe-responsive genes (Figs 6c, 7). These results indicate that OsSPR1 plays a role in maintaining Fe (and other metal) homeostasis between roots and leaves.

OsFRDL1, which has been recently characterized in rice, functions in citrate transport in the xylem and regulates Fe translocation from roots to shoots. A loss of function of OsFRDL1 results in leaf chlorosis, a lower leaf Fe
concentration and precipitation of Fe in the root stele (Yokosho et al., 2009). Although these features are similar to those of the Ospr1 mutant, differences exist in that the concentrations of Zn, Mn and some other metals were also decreased in the shoots of the Ospr1 mutant. Furthermore, OsFRDL1 is a citrate transporter whose expression is restricted to pericycle cells, while OsSPR1 is more widely expressed in various tissues and cell types. Ectopic over-expression of OsSPR1 in rice results in a significantly increased Fe content in the roots under Fe(II) supply conditions, whereas expression was not significantly changed in Fe(III) medium. However, the Fe content in the leaves of over-expression lines was comparable to that of the wild type. These results suggest that over-expression of OsSPR1 promotes Fe(II) uptake in roots. This is also consistent with the observed up-regulation of OsIRT2 expression in the mutant roots, while strategy II genes were not significantly up-regulated in the roots (Fig. 7).

Mutation of OsSPR1 inhibits root elongation

Root elongation is determined by two successive processes: cell elongation and cell proliferation (Beemster et al., 2003). In rice, several genes controlling division and differentiation in root growth and development have been identified through genetic screens (Reboulillat et al., 2009). Here, we cloned OsSPR1, a novel mitochondrial gene involved in root cell elongation. Loss of function of OsSPR1 resulted in defective root elongation, including primary root, adventitious root and lateral root elongation; notably, the defects were more severe in lateral roots and adventitious roots. Analysis of the different root zones revealed similar arrangements of cells in those zones compared with the wild type, but reduced cell length was observed in the spr1 roots, suggesting that reduced root length in spr1 resulted from reduced cell elongation.

Fe plays an important role in regulating rice root elongation. The Osnaat1 mutant, which cannot assimilate Fe(III), also displays short roots (short primary, adventitious and lateral roots) in Fe(III) supply solution. However, Osnaat1 grows normally under Fe(II) supply conditions (Cheng et al., 2007). By contrast, in Ospr1 mutant plants supply of Fe(II) or Fe(III) does not rescue the phenotype. Additionally, as OsNAAT1 is normally expressed in spr1 mutant plants, this suggests that OsNAAT1 is not the cause of the observed root growth phenotype. Recently, a mir mutant was reported to be involved in Fe homeostasis. The mir mutant accumulated higher Fe concentrations in both shoots and roots and also showed shorter root length compared with the wild type (Ishimaru et al., 2009). As the concentration of Fe in roots is normal under Fe(II) and Fe(III) supply (Fig. 6d,e), the root phenotype observed in Ospr1 plants cannot be attributed to the Fe content of roots directly.

It has been reported that ROS can regulate root cell elongation and development (Forester et al., 2003). The strong reddish-brown color of DAB staining in the Ospr1 mutant root elongation zone suggests increased H$_2$O$_2$ production. Given that intracellular Fe depletion promotes the transcription of pathogenesis-related genes and the production of H$_2$O$_2$, this indicates that Fe is an underlying factor associated with the oxidative burst (Liu et al., 2006). Increased H$_2$O$_2$ and up-regulation of AOX3 were detected in the Ospr1 mutant roots (Fig. S2), indicating oxidative stress (Van Aken et al., 2009). The observed H$_2$O$_2$ accumulated to the greatest extent at the root tip elongation zone, where cell death was observed by Evans blue staining.

While the OsSPR1 protein appears to be a novel protein, it contains an Armadillo domain in the N-terminal half of the protein. Armadillo domains belong to a larger class of helix-forming proteins that mediate protein–protein interactions (Groves & Barford, 1999). Armadillo domain-containing proteins play integral roles in development, cell division and cell death in a variety of organisms (Brembeck et al., 2006), including lateral root development in Arabidopsis, where they are proposed to target an inhibitor of lateral root development for degradation (Coates et al., 2006). Additionally, the Arabidopsis ortholog of OsSPR1 has been found to be phosphorylated with early elicitor signaling (Benschop et al., 2007). A mitochondrial protein in Saccharomyces cerevisiae (yeast) that displays 30% identity with OsSPR1 in the Armadillo domains is also a phosphorylated protein (Reinders et al., 2007). The loss of function of OsSPR1 results in a complex phenotype, characterized by a loss of cell elongation, resulting in a severe lateral root growth phenotype. This is accompanied by a dysfunction of metal homeostasis, production of ROS (H$_2$O$_2$) and cell death. The integral role of mitochondria in programmed cell death and the ability of this protein to be phosphorylated upon elicitor induction suggest a role in signaling developmental processes in roots that is interlinked with defense and nutrient signaling. The identification of this novel mitochondrial protein adds to the group of Armadillo type proteins that appear to regulate root development in plants. This protein differs from those previously characterized in that it is located in mitochondria, in contrast to previous Armadillo domain-containing proteins that have been found to be located in the nucleus (Coates et al., 2006).

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References


Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Amino acid alignments of rice (*Oryza sativa*) short postembryonic roots 1 (OsSPR1) and its homologs.

**Fig. S2** Analysis of alternative oxidase (AOX) transcript abundance in wild-type and *Oryza sativa* short postembryonic roots 1 (OspR1) mutant plants.

**Table S1** The phenotypic parameters of 7-d-old seedlings

**Table S2** Sequence of primers used for map-based cloning

**Table S3** Primer sequences of iron (Fe)-responsive genes for RT-PCR

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