Functional inactivation of UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1) induces early leaf senescence and defence responses in rice

Zhaohai Wang1, Ya Wang1, Xiao Hong1, Daoheng Hu1, Caixiang Liu2, Jing Yang1, Yang Li1, Yunqing Huang3, Yuqi Feng3, Hanyu Gong1, Yang Li1, Gen Fang1, Huiru Tang2,4 and Yangsheng Li1,*

1 State Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Hubei 430072, China
2 Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, Wuhan Centre for Magnetic Resonance, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, China
3 Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), Department of Chemistry, Wuhan University, Hubei 430072, China
4 State Key Laboratory of Genetic Engineering, Metabolomics Laboratory, School of Life Sciences, Fudan University, Shanghai 200433, China

Received 28 April 2014; Revised 17 October 2014; Accepted 20 October 2014

Abstract

Plant leaf senescence and defence responses are important biological processes, but the molecular mechanisms involved are not well understood. This study identified a new rice mutant, spotted leaf 29 (spl29). The SPL29 gene was identified by map-based cloning, and SPL29 was confirmed as UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1) by enzymatic analysis. The mutant spl29 lacks UAP activity. The biological phenotypes for which UAP is responsible have not previously been reported in plants. The spl29 mutant displayed early leaf senescence, confirmed by chlorophyll loss and photosystem II decline as physiological indicators, chloroplast degradation as a cellular characteristic, and both upregulation of senescence transcription factors and senescence-associated genes, and downregulation of photosynthesis-related genes, as molecular evidence. Defence responses were induced in the spl29 mutant, shown by enhanced resistance to bacterial blight inoculation and upregulation of defence response genes. Reactive oxygen species, including O$_2^-$ and H$_2$O$_2$, accumulated in spl29 plants; there was also increased malondialdehyde content. Enhanced superoxide dismutase activity combined with normal catalase activity in spl29 could be responsible for H$_2$O$_2$ accumulation. The plant hormones jasmonic acid and abscisic acid also accumulated in spl29 plants. ROS and plant hormones probably play important roles in early leaf senescence and defence responses in the spl29 mutant. Based on these findings, it is suggested that UAP1 is involved in regulating leaf senescence and defence responses in rice.

Key words: Defence responses, leaf senescence, reactive oxygen species (ROS), rice (Oryza sativa), SPL29, UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1).

Introduction

Leaf senescence is a complex physiological event that constitutes the last stage of leaf development, integrating multiple developmental and environmental signals (Lim and Nam, 2005; Balazadeh et al., 2011). Although it has been investigated many...
times, the mechanisms involved are still not well understood. Early leaf senescence has been described as one of the characteristics of lesion mimic mutants (LMMs) (Qiao et al., 2010; Jiao et al., 2012). Leaf senescence in LMMs usually happens rapidly, providing a unique tool for dissecting possible regulatory genes or pathways during the progression of leaf growth and development to senescence. For example, RLS1, encoding an uncharacterized nucleotide-binding-site-containing protein, was identified and shown to be involved in autophagy-mediated chloroplast degradation during leaf senescence in rice (Jiao et al., 2012). Characterization of more LMMs will help to enrich our understanding of leaf senescence.

Defence responses are important for plants in combating pathogen invasion (Dangl and Jones, 2001). Both resistance genes and many regulatory genes are involved in defence pathways. LMMs develop spontaneous lesions that resemble disease symptoms in the absence of pathogen attack, and interestingly these plants usually have a significantly enhanced resistance to disease (Jung et al., 2005; Wu et al., 2008). It is believed that cloning the genes responsible for LMMs is the key to deciphering the defence pathways and may aid the development of broad-spectrum pathogen resistance in plants.

UDP-N-acetylglucosamine pyrophosphorylase (UAP), also termed UTP:N-acetylglucosamine-1-P uridylyltransferase or N-acetylglucosamine-1-phosphate uridylyltransferase, is widely distributed in living organisms. Researchers have found that both of the human UAP1 isoforms, UAPA and UAPB (also termed AGX1 and AGX2, respectively), can use N-acetylglucosamine-1-phosphate (GlcNAc1P) or N-acetylgalactosamine-1-phosphate (GalNAc1P) as substrates for producing UDP-N-acetylglucosamine (UDP-GlcNAc) or UDP-N-acetylgalactosamine (UDP-GalNAc) (Peneff et al., 2001). Enzymatic experiments with UAPs from Arabidopsis thaliana also showed that AtUAP1 and AtUAP2 (also termed GlcNAc1pUT-1 and GlcNAc1pUT-2, respectively) catalyze the reversible reactions (Yang et al., 2010):

\[
\text{UTP + GlcNAc-1-P / GalNAc-1-P} \\
\rightarrow \text{UDP - GlcNAc / UDP - GalNAc + PPI}
\]

UAP is essential for organisms to grow normally. Some UAP mutants have been described. In Escherichia coli, GlmU has UAP activity, and inactivation of the GlmU gene rapidly reduces peptidoglycan synthesis, leading to alteration in cell shape and cell lysis (Mengin-Lecreulx and van Heijenoort, 1993). In yeast (Saccharomyces cerevisiae), the cells of the ScUAP null mutant displayed aberrant morphology in which most cells were fully swollen and some were lysed (Mio et al., 1998). In Drosophila melanogaster, mutants of DmUAP (also termed mummy) exhibit defects in dorsal closure, central nervous system fasciculation, and eye development (Schimmelpeng et al., 2006). However, no known phenotype caused by UAP gene mutations has been reported in plants. In addition, the gene encoding UAP has not been identified in rice.

In order to further elucidate the molecular mechanisms of leaf senescence and defence responses in rice, a new LMM termed spotted leaf 29 (spl29) was isolated. This exhibits spotted leaves and rapid leaf senescence from the seedling stage throughout the rest of its life cycle. The SPL29 gene was identified using a map-based cloning strategy. It was confirmed that SPL29 is UAP1, with UAP activity, and that there is no enzymatic activity in the spl29 mutant. Both early leaf senescence and defence responses were investigated and confirmed in spl29 plants. Our data suggest that UAP1 is probably involved in regulating leaf senescence and defence responses in rice.

Materials and methods

Plant material and growth conditions

The LMM mutant line spl29 was isolated in 2008 from regenerated plants derived from a tissue culture of the rice cultivar Zhonghua 11 (ZH11, Oryza sativa spp. japonica). Its mutant phenotype, lesion mimic leaf spots and rapid leaf senescence, was stably inherited over multiple generations. M6 generation seeds of spl29 were used in this study. Germinated wild-type and spl29 seeds were grown in soil in a plant growth chamber (light cycle: 14 h light/10 h dark, 28/26°C) for use at the seedling stage, when the second-emerging leaves were used 28 days after germination. For plants used from the tillering to ripening stages, germinated wild-type and spl29 seeds were grown in an experimental field under natural summer conditions. Leaf samples used at the tillering stage were the third leaves from the top of the main tiller of plants 50 days after germination.

Genetic analysis and map-based cloning

For genetic analysis, the leaf phenotypes of F1 and F2 plants were observed from hybridizations of Guangzhan63s, 10N056, and Yuehui9113 with spl29, respectively. The F2 population from the cross between Guangzhan63s and spl29 was used for preliminary and fine mapping of the SPL29 locus. Using 44 mutant plants obtained in the F2 population, preliminary mapping was performed with simple sequence repeat markers, which were well distributed across all 12 chromosomes and allowed Guangzhan63s and spl29 to be distinguished. The data for preliminary mapping were analyzed using Mapmaker 3.0 software. A total of 870 F2 mutant plants were used for the fine mapping of SPL29. Nine markers (S1, S6, S8, S15, S19, S26, S32, S33, and S40) were developed for fine mapping based on DNA sequence differences between indica and japonica rice varieties. The molecular marker primer sequences are listed in Supplementary Table S1.

Complementation of SPL29

The G-to-T mutation was identified by PCR amplification and sequencing using the primer pair SPL29Mutation (Supplementary Table S2). For complementation of the mutant phenotype, three DNA fragments of LOC_Os08g10600, the 2214-bp upstream promoter, 4674-bp gene region, and 1000-bp downstream terminator, were used in this study. The 2214-bp upstream promoter, 4674-bp gene region, and 1000-bp downstream terminator were used in this study. The 2214-bp upstream promoter, 4674-bp gene region, and 1000-bp downstream terminator were used in this study. The 2214-bp upstream promoter, 4674-bp gene region, and 1000-bp downstream terminator were used in this study. The 2214-bp upstream promoter, 4674-bp gene region, and 1000-bp downstream terminator were used in this study. The 2214-bp upstream promoter, 4674-bp gene region, and 1000-bp downstream terminator were used in this study. The 2214-bp upstream promoter, 4674-bp gene region, and 1000-bp downstream terminator were used in this study. The 2214-bp upstream promoter, 4674-bp gene region, and 1000-bp downstream terminator were used in this study.
Bioinformatic analysis and construction of the phylogenetic tree

The gene sequence and structure of SPL29 were used to search the Rice Genome Annotation Project (RGAP; http://rice.plantbiology.msu.edu/). A gene family search was conducted using Pfam (http://pfam.sanger.ac.uk/). To identify the specific UDP-N-acetylglucosamine pyrophosphorylase encoded by SPL29, BLASTP searches were performed with the full-length amino acid sequence on NCBI (http://www.ncbi.nlm.nih.gov/). Sequence identities were also analysed by BLASTP.

Multiple full-length amino acid sequences were aligned using ClustalW prior to phylogenetic analysis. MEGA 5 was utilised to construct the consensus phylogenetic tree with 1000 random bootstrap replicates.

Recombinant protein construction, expression, and purification

To generate the glutathione S-transferase (GST) gene fusion constructs GST-SPL29/spl29, the 1470-bp full-length coding sequence (CDS) of SPL29 or spl29 was amplified from the cDNA of ZH11 or spl29 leaves, respectively (primers GST-SPL29/spl29 CDS in Supplementary Table S2). PCR products were inserted into pGEX-6P-1 using the restriction enzyme sites BamHI and EcoRI. The recombinant vectors were transferred into E. coli DH5α and sequenced to check for correct construction. The empty vector pGEX-6P-1 and the recombinant vectors were then transferred into E. coli BL21 for protein expression. Strains harbouring GST, GST-SPL29, or GST-spl29 were cultured in Luria-Bertani medium at 37°C. At an optical density at 600 nm of 0.5, isopropylthigalactopyranoside was added to a final concentration of 0.5 mM. Strains were then cultured at 18°C for 20 h (200 rpm) to induce gene expression. The cells were centrifuged and resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4). After sonication, the lysates were centrifuged. Protein purification was conducted using a column with Glutathione SepharoseTM 4 Fast Flow (GE Healthcare) according to the manufacturer’s instructions. Proteins were detected by SDS-PAGE analysis and the concentration of purified protein was determined using bovine serum albumin as a standard.

1H-nuclear magnetic resonance (1H-NMR) analysis of SPL29/spl29 activities in situ

The examination of enzymatic activities was performed as described previously (Yang et al., 2010) with some modifications. The forward reactions were carried out in a final volume of 540 μL, and the mixture consisted of 2H₂O/H₂O at a ratio of 8:1 (v/v), Na/K phosphate buffer (80 mM K₂HPO₄, 20 mM Na₂HPO₄, pH 7.4), 5 mM MgCl₂, 0.2 mM UTP, 0.2 mM GlcNAc-1-P, 1.5 units of yeast inorganic pyrophosphatase, and recombinant enzyme (1.86 μg of GST, GST-SPL29, or GST-spl29). The reverse reactions were carried out in a similar manner, in a solution containing 2H₂O/H₂O at a ratio of 8:1, Na/K phosphate buffer, 5 mM MgCl₂, 0.2 mM P₆, 0.2 mM UDP-GlcNAc (or UDP-GalNAc), and recombinant enzyme (1.86 μg of GST, GST-SPL29, or GST-spl29). Examination of GlcNAc-1-P/GalNAc-1-P and UDP-GlcNAc/UDP-GalNAc was performed by 1H-NMR as described previously (Zhang et al., 2011). Data acquisition started at 10 min and 60 min after the addition of enzyme to the reaction mixture.

Gene expression analysis

Samples were collected and immediately frozen in liquid nitrogen, then stored at –80°C. Total RNA was extracted using TRIZol reagent (Invitrogen). After RNAase-free DNase treatment, 5 μg of RNA was used for cDNA synthesis using M-MLV reverse transcriptase (Promega) in a 50-μL reaction mixture. The quantitative, real-time reverse transcription polymerase chain reaction (qRT-PCR) technique was performed using 2×SYBR Green Master Mix reagent (Bio-Rad) in a 96-well plate using a Bio-Rad CFX96 real-time PCR system. Three technological replicates were used for each biological sample. Six rice reference genes (from our unpublished data on the selection of stable reference genes to normalise gene expression in rice) were assessed for their potential as stable internal standards by geNorm as previously described (Vandesompele et al., 2002). These genes were T(LOC_Os01g05490), ARF (LOC_Os05g41060), EF-1α (LOC_Os03g08020), UBC (LOC_Os02g42314), Profilin-2 (LOC_Os06g05880), and Actin1 (LOC_Os03g50885). As a result of this analysis, UBC, Profilin-2, and Actin1 were selected as internal standards for all leaf samples (Supplementary Figure S1). All primers used for qRT-PCR analysis are listed in Supplementary Table S3, with good PCR efficiencies (85–105%) assessed using a 10-fold dilution series of total cDNA.

Chlorophyll content and chlorophyll a fluorescence

Chlorophyll was extracted from leaves with ice-cold 80% acetone, and the chlorophyll content per gram of leaf fresh weight (FW) was determined as previously described (Lichtenthaler, 1987).

Chlorophyll a fluorescence transients from dark-adapted plants were measured using a Handy Plant Efficiency Analyser (Handy PEA, Hansatech, UK). The description and calculation formulae of parameters are listed in Supplementary Table S4.

Transmission electron microscopy

Leaf segments were fixed with 2.5% glutaraldehyde in sodium phosphate buffer (pH 7.2) for 4 h at 4°C. Ultrathin samples were made as previously described (Zhou et al., 2013), and viewed using a transmission electron microscope.

Inoculations with bacterial blight pathogen

Xanthomonas oryzae pv. oryzae strain PXO99 (Philippine race 6) was cultured on pressure-sensitive adhesive medium. The second and third youngest fully expanded leaves from both the main and lateral tillers of 5–7 independent wild-type and spl29 plants were inoculated with PXO99 suspensions (optical density of 0.5 at 600 nm) using the leaf clipping method (Manosalva et al., 2011). Lesion lengths on inoculated plants were measured 12 days after inoculation.

Histochemical staining

Leaves in wild-type and spl29 plants were sampled at the tillering stage (about 45 days after germination under natural summer field conditions). Histochemical assays for determining reactive oxygen species (ROS) accumulation were conducted as previously described (Qiao et al., 2010). Three independent experiments were performed.

Lipid peroxidation and ROS-scavenging enzyme assays

Plant samples were ground in liquid nitrogen. Crude protein extraction, measurement of malondialdehyde (MDA) contents, and assays for the activities of superoxide dismutase (SOD) and catalase (CAT) were conducted according to previously described methods (Wang et al., 2012).

Quantification of jasmonic acid and abscisic acid

Plant leaves were collected, weighed, immediately frozen in liquid nitrogen, and stored at –80°C. Extraction and measurement of jasmonic acid (JA) and abscisic acid (ABA) were conducted following a previously described method (Chen et al., 2012).

Results

Characterization of the spl29 mutant

Small, dark-brown LM leaf spots (followed by rapid leaf senescence) appeared on spl29 plants from the seedling stage
to the ripening stage (Fig. 1A, B, and C). However, newly emerging leaves showed no difference to wild-type plants. During the seedling stage, abundant leaf spots had appeared on spl29 plants by 28 days after germination, with leaves exhibiting chlorosis beside the spots (Fig. 1D). After the appearance of leaf spots and chlorosis, the leaves withered from the tip to the base in about five days (Supplementary Figure S2). The severity of leaf mutant phenotypes varied for different leaves of a single spl29 plant at the tillering stage 50 days after germination (Fig. 1E). No spots were present on the first new fully expanded leaf, whereas the third leaf from the top of the main tiller had larger and more numerous spots and associated chlorosis than the second leaf, and at this point the fourth leaf was already withered and dead. By contrast, the corresponding leaves on the wild-type plant grew normally. When the last emerging flag leaf was filled with spots (Fig. 1F), the spl29 plants began to senesce far earlier than wild-type plants. In addition to the obvious leaf phenotype, the plant height, panicle length, total grain number, filled grain number, seed-setting rate, and thousand-grain weight of the spl29 mutants were all significantly decreased when compared with the wild-type plants (Table 1).

**Map-based cloning of the SPL29 gene**

For genetic analysis, three indica cultivars, Guangzhan63s, 10N056, and Yuehui9113, were selected for hybridisation with spl29. All F1 plants displayed the wild-type leaf phenotype. In all three F2 populations, the wild-type and LM mutant phenotypes segregated at a ratio of 3:1 ($\chi^2 < \chi^2_{0.05} = 3.84$; $P > 0.05$; see Supplementary Table S5), which suggested that the phenotype of spl29 was controlled by a single recessive nuclear gene.

The F2 population from the cross between Guangzhan63s and spl29 was used to map the SPL29 gene. Using 44 F2 mutant plants, the SPL29 gene was closely linked with marker M1077, and mapped inside markers M1037 and M1230 on chromosome 8, with an equal genetic distance of 1.4 cM to each (Fig. 2A). For fine mapping of the SPL29 gene, more than 3000 F2 individuals were generated and new markers between M1037 and M1230 were designed according to sequence differences between indica and japonica rice. Nine markers showing polymorphisms between Guangzhan63s and spl29 (Supplementary Table S1) were used to screen the recombinants. Using 870 F2 mutant plants, the SPL29 gene was eventually limited to a 97-kb region between the new

---

**Fig. 1.** Leaf phenotype of wild-type and spl29 mutant plants. (A) Plants at the seedling stage (28 days after germination). (B) Plants at the tillering stage (50 days after germination). (C) Plants at the ripening stage (115 days after germination). (D) Phenotype of the second-emerged leaf after germination of plants at the seedling stage, corresponding to (A). (E) Phenotype of the first, second, third, and fourth fully expanded leaves from the tip to the base of the main tiller of plants at the tillering stage, corresponding to (B). (F) Flag-leaf phenotype of plants 90 days after germination. The spl29 leaf area in the red square frame is magnified on the right.
Table 1. Agronomic traits of wild-type and spl29 plants

|                      | Plant height (cm) | Panicle length (cm) | Total grain number | Filled grain number | Seed-setting rate (%) | Thousand-grain weight (g) |
|----------------------|-------------------|---------------------|--------------------|---------------------|------------------------|--------------------------|
| WT                   | 89.9 ± 2.5        | 19.2 ± 0.8          | 942 ± 116          | 871 ± 104           | 92.5 ± 2.3             | 24.59 ± 0.32             |
| spl29                | 68.8 ± 3.5***     | 15.3 ± 0.9***       | 601 ± 113***       | 249 ± 44***         | 41.4 ± 1.2***          | 21.97 ± 0.22***          |

*Data represent the mean ± SD of nine (WT) or seven (spl29) independent plants (Student's t-test, ***: P < 0.0005). WT, wild type.*

Fig. 2. Map-based cloning and functional complementation of SPL29. (A) Preliminary mapping of the SPL29 locus. (B) Fine mapping of the SPL29 locus. (C) Ten putative ORFs are located in the 97-kb region identified by fine mapping. (D) Gene structure of the SPL29 candidate LOC_Os08g10600. Exons, introns, and upstream/downstream regions are displayed. The point mutation of G to T on the eighth exon is indicated by the red arrow, leading to the amino acid exchange of Gly to Cys. (E) Schematic diagram of vectors for functional complementation. The pSPL29C functional complementation vector contains the promoter, gene region, and terminator of LOC_Os08g10600; pEmvC is the empty vector control. The pSPL29C and pEmvC vectors were transformed into spl29 calli. LB, left border; RB, right border; 35S, cauliflower mosaic virus 35S promoter; Bar, the phosphinothricin gene; Tnos, the nopaline synthase terminator. (F–G) Transgenic plants of pSPL29C and pEmvC about 40 days after regeneration. Arrows indicate leaves with the mutant phenotype. (H) Clear leaf phenotype in transgenic plants of pSPL29C and pEmvC. (I) Sequence analysis of the G-to-T mutation site in transgenic plants of pSPL29C and pEmvC.
markers S8 and S26, with one recombinant for each marker (Fig. 2B). Ten putative open reading frames (ORFs) were predicted according to the RGAP website (Fig. 2C). All ten genes were amplified and sequenced. By comparing gene sequences between wild-type and spl29 plants, one point mutation was identified on the fifth gene, LOC_Os08g10600. No DNA sequence changes were found in the other nine genes or the putative promoter region (about 2.2 kb) of LOC_Os08g10600. The single nucleotide substitution of guanine (G) to thymine (T) occurred in the eighth exon of LOC_Os08g10600 (at a position of 712 bp in the CDS), resulting in a single amino acid change from glycine (Gly) to cysteine (Cys) (Fig. 2D).

For preliminary confirmation that the G-to-T mutation was responsible for the phenotype of the spl29 mutant, this nucleotide site was examined in different rice cultivars, F2 non-lesion-mimic (NLM) plants, and F2 LM plants (Supplementary Table S6). The nucleotide at this site was T for spl29 and all tested F2 LM plants, but G for 10 different normal rice cultivars. Four of the F2 NLM plants had a G at this position, while G and T existed together at this site in seven of the F2 NLM plants. Thus, LOC_Os08g10600 was a likely SPL29 candidate.

Functional complementation with LOC_Os08g10600 in the spl29 mutant

A 7888-bp genomic fragment of wild-type Zh11, containing the 2214-bp upstream promoter, the 4674-bp gene region, and the 1000-bp downstream terminator of LOC_Os08g10600, was constructed using the plasmid pSPL29C and transferred into the Spl29 calli by Agrobacterium tumefaciens-mediated transformation. The empty vector pEmVC was introduced to spl29 calli as the control (Fig. 2E). About 40 days after plant regeneration, all 22 independent transgenic lines containing the wild-type SPL29 gene showed a complete rescue of the mutant phenotype (Fig. 2F), while all 16 independent transgenic lines with the empty vector failed to complement the spl29 mutant (Fig. 2G). Leaf phenotypes of the transgenic plants with pSPL29C or pEmVC are shown in Fig. 2H. Successfully transformed plants were confirmed by PCR amplification of the screening marker Bar gene (Fig. 2I). Sequencing the G-to-T mutation site of the SPL29 gene revealed that plants transformed with pSPL29C had both G and T at the mutant nucleotide site, whereas the pEmVC control plants only had the mutant T nucleotide (Fig. 2J). Therefore, functional complementation with the wild-type candidate gene rescued the mutant phenotype of spl29 plants, and it was concluded that LOC_Os08g10600 was the SPL29 gene.

SPL29 encodes a putative UAP1

As annotated on RGAP, LOC_Os08g10600 (SPL29) encodes a putative 489-amino acid protein. Pfam analysis with its predicted amino acid sequence showed that SPL29 belongs to the UDP-glucose pyrophosphorylase gene family (PF01704). NCBI BLASTP revealed that SPL29 is a putative UAP1 gene in rice (OsUAP1), but not the UDP-glucose pyrophosphorylase (UGP) gene. In the rice genome, one homologue of SPL29 is also annotated as UAP (LOC_Os04g52370, termed OsUAP2). To examine the evolutionary relationships between SPL29 and its homologues in different species, a bootstrap consensus phylogenetic tree was constructed by the neighbour-joining method (Fig. 3). All UAPs in different eukaryotic organisms clustered separately from the UGPs. The protein EcGlmU, which shows UAP activity in E. coli, showed no amino acid homology with SPL29, and was thus located furthest away. UAPs in plants were tightly clustered, with separate groups for monocots and dicots, indicating that gene divergence occurred after diversification of these two clades. In addition, SPL29 showed over 30% amino acid identity with all eukaryotic UAPs analysed by NCBI BLASTP (Fig. 3). These results all suggest that SPL29 encodes a putative UAP1 in rice.

SPL29 shows UAP enzymatic activities but spl29 eliminates these enzymatic functions

UAP has not been identified in rice before. In order to reveal whether SPL29 and the mutant spl29 have UAP activity, recombinant proteins of GST-SPL29 and GST-spl29 were produced. Theoretically, the molecular weights are 26 kDa for GST, 54.071 kDa for SPL29, and 54.117 kDa for spl29. GST, GST-SPL29 (about 80 kDa), and GST-spl29 were highly expressed after induction (Supplementary Figure S3, lanes 2–4). Column-purified proteins were used to detect the enzymatic reactions (Supplementary Figure S3, lanes 5–7).

1H-NMR spectroscopy was used to monitor the enzymatic reaction of SPL29 and spl29 in situ. In the time-gradient enzymatic progression at 10 min and 60 min, forward conversion of GlcNAc-1-P (5.36 ppm) to UDP-GlcNAc (5.52 ppm) was observed with GST-SPL29 (Fig. 4A, lines 3 and 4), but not with the GST control (Fig. 4A, lines 1 and 2). Interestingly, GST-spl29 was unable to catalyse this reaction (Fig. 4A, lines 5 and 6). Similarly, reverse conversion of UDP-GlcNAc (5.52 ppm) to GlcNAc-1-P (5.36 ppm) was observed with GST-SPL29 (Fig. 4B, lines 3 and 4), but not with GST (Fig. 4B, lines 1 and 2) or GST-spl29 (Fig. 4B, line 5 and 6). GST-SPL29 could also catalyse the reverse conversion of UDP-GalNAc (5.55 ppm) to GalNAc-1-P (5.39 ppm) (Fig. 4C, lines 3 and 4), whereas GST (Fig. 4C, lines 1 and 2) and GST-spl29 (Fig. 4C, lines 5 and 6) could not. The forward reaction for GalNAc-1-P could not be tested since GalNAc-1-P was commercially unavailable.

These NMR-based assays provide unambiguous evidence that SPL29 has UAP activity whereas the mutation of SPL29 to spl29 eliminates this enzymatic function. To date, UAP has not been well studied in plants. The spl29 mutant can be used to reveal the biological significance of UAP.

Early leaf senescence in the spl29 mutant is identified by physiological indicators

The decrease of chlorophyll content is commonly used as a physiological indicator related to plant leaf senescence (Jiao et al., 2012). At the seedling stage, chlorophyll content decreased from 1240 μg g−1 FW in wild-type leaves to 861 μg
g$^{-1}$ FW in spl29 leaves (Fig. 5A). A similar reduction was also observed at the tillering stage, when chlorophyll contents were 1362 μg g$^{-1}$ FW in the wild type and 786 μg g$^{-1}$ FW in spl29 (Fig. 5B). The senescence-induced \textit{STAY GREEN (SGR)} gene plays an important role in regulating chlorophyll degradation (Park et al., 2007). Analysis by qRT-PCR showed that the expression of \textit{SGR} was greatly upregulated in spl29 leaves at the seedling and tillering stages (Fig. 5C–D).

Photosystem II (PSII) function is usually reduced during senescence. The chlorophyll \textit{a} fluorescence transient can be examined to reveal the activities of PSII machinery on light absorption, energy transformation, and electron transfer, thus this technique is commonly used to monitor leaf senescence (Zhang et al., 2012; Panda and Sarkar, 2013). Wild-type and spl29 plants at the seedling stage were analysed. In both lines, chlorophyll \textit{a} fluorescence transients showed a typical polyphasic rise with the basic steps O-J-I-P, but the fluorescence intensity was reduced in spl29 plants (Fig. 5E). Greater amplitude of ΔW, increased K (300 μs), J (2 ms), and I (30 ms) steps, positive AL$_{\text{band}}$, and the maximum amplitude of the IP phase implied PSII reduction (Supplementary Figure S4). Data analysis of chlorophyll \textit{a} fluorescence transients is shown in Fig. 5F. Absorption flux (ABS/CS$_o$ and ABS/CS$_m$), trapped energy flux (TR$_o$/CS$_o$ and TR$_m$/CS$_m$), electron transport flux (ET$_o$/CS$_o$ and ET$_m$/CS$_m$), and dissipated energy flux (DI$_o$/CS$_o$ and DI$_m$/CS$_m$) per excited cross section (CS) were all decreased in spl29 plants, indicating the destruction of the entire PSII machinery. Interestingly, although the density of reaction centres (RCs) (RC/CS$_m$ and RC/CS$_o$) decreased, the capacities per RC (ABS/RC, TR$_o$/RC, ET$_o$/RC, and DI$_o$/RC) increased in spl29 plants. However, increased capacities per RC did not rescue the capacity of the PSII machinery in spl29, which was completely reduced as clearly reflected by performance indices (PI$_{ABS}$, PI$_{CSm}$, PI$_{tol-ABS}$, PI$_{tol-CSm}$, and PI$_{tol-CSm}$). These results demonstrated that the PSII units were damaged and could not function properly in spl29 leaves.

The decreased chlorophyll content and reduced PSII capacity indicated that the spl29 plants had entered senescence at the physiological level.

Irreversible degradation of chloroplasts in early-senescence leaves of spl29 mutants

The main cellular characteristic of leaf senescence is the occurrence of chloroplast degradation (Lim et al., 2007; Jiao et al., 2012). To investigate whether the chloroplasts were affected in the early-senescing leaf of the spl29 mutant,
the ultrastructures of wild-type and spl29 cells in 28-day-old seedling leaves were compared using transmission electron microscopy. In wild-type leaves, well-developed mesophyll cells were observed with fully developed and membrane-intact chloroplasts (Fig. 6A). However, three different chloroplast states were observed among spl29 mesophyll cells. Fully developed and membrane-intact chloroplasts could be observed in many spl29 mesophyll cells (Fig. 6B), while in some cells the chloroplast envelope was observed to be breaking (Fig. 6C and Supplementary Figure S5A and B). Unsurprisingly, mesophyll cells which had lost chloroplasts but retained mitochondria were present abundantly in spl29 (Fig. 6D and Supplementary Figure S5C and D).

These results suggest that chloroplasts developed normally in spl29 at first, but were completely degraded during early leaf senescence. Chloroplast breakage may be one mechanism causing this degradation. Drastic, irreversible, and complete degradation of chloroplasts in spl29 leaves is coincident with the phenotype of rapid early leaf senescence (leading to death).

Upregulated expression levels of senescence-associated transcription factors and senescence-associated genes, and downregulated expression levels of photosynthesis-related genes, are molecular evidence of early leaf senescence in the spl29 mutant

Leaf senescence is a complex process controlled by a large number of different genes. Many genes that encode transcription factors are upregulated to induce leaf senescence (Zhou et al., 2013). To further confirm that senescence occurred in the spl29 plants, gene expression analysis of three senescence-associated transcription factors (OsWRKY23, OsWRKY72, and OsNAC2) was performed by qRT-PCR. At the seedling stage, transcripts of OsWRKY23, OsWRKY72, and OsNAC2 in spl29 leaves increased 40.7-, 8.5-, and 2.1-fold, respectively, in comparison to those of wild-type leaves (Fig. 7A); at the tillering stage their levels in spl29 leaves were increased 110.0-, 12.0-, and 1.5-fold, respectively, compared to the wild type (Fig. 7B). The expression levels of the senescence-associated transcription factors investigated here were all upregulated, according with the early leaf senescence of the spl29 mutant.

Senescence-associated genes (SAGs) are upregulated during senescence and thought to be involved in triggering the process or controlling its rate of progression (Lee et al., 2001). Expression levels of four SAGs (Osl2, Osl30, Osl43 and Osl85) were determined by qRT-PCR. At the seedling stage, in spl29 leaves, Osl2, Osl30, Osl43, and Osl85 mRNAs were 2.6-, 1.5-, 494.4-, and 1324.4-fold higher, respectively, than they were in wild-type leaves (Fig. 7C); at the tillering stage their levels in spl29 leaves were 110.0-, 12.0-, and 1.5-fold, respectively, compared to the wild type (Fig. 7B). The expression levels of the senescence-associated transcription factors investigated here were all upregulated, according with the early leaf senescence of the spl29 mutant.

The upregulated expression patterns of SAGs further support the notion that early leaf senescence occurred in spl29 plants.
Leaf senescence is accompanied by the decreased expression of genes related to photosynthesis (Lim et al., 2007). The expression levels of three nuclear encoded genes (rbcS, lhcA, and lhcB) and six chloroplast-encoded genes (rbcL, psaA, psbA, petD, ndhA, and atpA) associated with photosynthesis were assessed by qRT-PCR. At the seedling stage, expression levels of these nine genes in spl29 were 0.18-, 0.26-, 0.27-, 0.40-, 0.18-, 0.41-, 0.52-, 0.41-, and 0.26-fold, respectively, of those in the wild type (Fig. 7E). At the tillering stage, these genes were expressed 0.18-, 0.24-, 0.19-, 0.16-, 0.25-, 0.29-, 0.44-, 0.22-, and 0.26-fold, respectively, as much as those in the wild type (Fig. 7F). Downregulation of these photosynthesis-associated genes also provided molecular evidence for early leaf senescence in spl29 plants.

Plant resistance to the bacterial blight pathogen is enhanced and expression levels of defence signalling-related genes are induced in the spl29 mutant

The appearance of LM spots in spl29 plants resembles the hypersensitive response (HR), an important resistance mechanism in plants (Dangl and Jones, 2001; Lam et al., 2001). To test their resistance to pathogens, spl29 and wild-type plants were inoculated with the bacterial blight strain PXO99 at the tillering stage. The wild-type plants showed a typical response to bacterial blight disease, while the spl29 plants exhibited significantly enhanced resistance. The average leaf lesion lengths following PXO99 inoculation were 9.87 cm in wild-type plants and 1.42 cm in spl29 plants (Fig. 8A).

Activation of defence-response gene expression has previously been observed during the development of lesions in some rice LMMs (Yin et al., 2000; Manosalva et al., 2011). Therefore, we examined the expression of four defence signalling-related genes (PR1a, PBZ1, PO-C1, and OsWRKY45) in wild-type and spl29 plants by qRT-PCR. At the seedling stage, levels of PR1a, PBZ1, PO-C1, and OsWRKY45 mRNAs in the spl29 mutant were 1627.5-, 96.2-, 419.5-, and 21.1-fold, respectively, the amount of those in wild-type plants (Fig. 8B); at the tillering stage these genes were expressed 71.9-, 21.2-, 11.6-, and 2.6-fold, respectively, as much as those in wild-type plants (Fig. 8C). The upregulation of defence signalling-related genes indicates that the plant defence responses are induced, which may contribute to enhancing disease resistance in the spl29 mutant.

ROS and MDA accumulate in the spl29 mutant accompanied by increased SOD activity and normal CAT activity

ROS generation is one of the earliest responses of plant cells during senescence (Khanna-Chopra, 2012). ROS also play...
important roles in plant–pathogen interactions (Wojtaszek, 1997; Apel and Hirt, 2004). This prompted us to investigate whether ROS accumulation occurred in spl29 plants. Leaves at the tillering stage were used for the ROS assay. The pattern of nitro blue tetrazolium (NBT) staining reflected the formation of blue formazan precipitates and was indicative of O$_2^-$ accumulation. There was extensive NBT staining in the leaf area surrounding lesions in spl29 plants, whereas staining was minimal in wild-type leaves (Fig. 9A). When 3,3’-diaminobenzidine (DAB) staining was used as an indicator of H$_2$O$_2$ accumulation, intense brown staining was seen correlating with lesion formation in spl29 mutant leaves, but no such signal was detected in wild-type leaves (Fig. 9B). These results demonstrated that ROS accumulated in spl29 plants.

Lipid peroxidation by ROS was analysed by measuring contents of MDA, an end-product of oxidized lipids. At the seedling stage, the MDA content of 103 nmol g$^{-1}$ FW in spl29 leaves was more than double the 49 nmol g$^{-1}$ FW in wild-type leaves (Fig. 9C). At the tillering stage, an MDA content of 378 nmol g$^{-1}$ FW was generated in the leaves of spl29 mutant plants, much higher than the 209 nmol g$^{-1}$ FW in wild-type leaves (Fig. 9D). Increased MDA contents suggested that lipid peroxidation occurred in spl29 plants, which provided further evidence of the ROS accumulation and membrane damage in spl29 plants.

During oxidative stress, plants synthesize anti-oxidative enzymes, such as SOD and CAT, to remove ROS (Miller et al., 2010). SOD catalyses the dismutation of O$_2^-$ to produce H$_2$O$_2$, and CAT is the major H$_2$O$_2$-scavenging enzyme. Thus SOD and CAT activities were examined to explore the ROS metabolic process in spl29 plants. At the seedling stage, SOD activity was 8926 U g$^{-1}$ FW in spl29 leaves, which was much higher than the 3208 U g$^{-1}$ FW in the wild-type leaves (Fig. 9E). A similar result was observed in leaves at the tillering stage, with a SOD activity of 12260 U g$^{-1}$ FW in spl29 leaves compared with 2167 U g$^{-1}$ FW in the wild type (Fig. 9F). However, there was no obvious difference in CAT activity between spl29 and wild-type leaves at both the seedling stage and the tillering stage (Fig. 9G–H). The increase of SOD activity suggests that the spl29 mutant may actively respond to the O$_2^-$ accumulation and produce more H$_2$O$_2$, while the normal CAT activity may not be enough to scavenge the additional H$_2$O$_2$, leading to its accumulation in spl29.

**JA and ABA accumulate in the spl29 mutant**

Plant hormones, such as JA and ABA, are reported to play roles in both leaf senescence and defence responses (Lim et al., 2007; Robert-Seilaniantz et al., 2011). This prompted us to investigate the contents of JA and ABA in spl29. At the seedling stage, the content of JA was 110.6 ng g$^{-1}$ FW in spl29, whereas JA in the wild type was hardly detectable, with a content of 0.3 ng g$^{-1}$ FW (Fig. 10A). Similar results were also found at the tillering stage, with a JA content of 53.5 ng g$^{-1}$ FW in spl29, but 0.2 ng g$^{-1}$ FW in the wild type (Fig. 10B).
The content of ABA at the seedling stage was 49.6 ng g⁻¹ FW in spl29, compared with 8.0 ng g⁻¹ FW in the wild type (Fig. 10C). At the tillering stage, the content of ABA was 34.7 ng g⁻¹ FW in spl29, higher than the 11.0 ng g⁻¹ FW in the wild type (Fig. 10D). These results show that JA and ABA accumulate in spl29 plants, probably playing a role in early leaf senescence and defence responses in this mutant.

Discussion

SPL29 is the UAP1 gene in rice and functional inactivation of OsUAP1 is responsible for the mutant phenotypes

From the seedling stage, the spl29 mutant showed LM spots on every leaf. The leaf then exhibited chlorosis and soon withered (Fig. 1 and Supplementary Figure S2). It was decided that examining spl29 might show a novel gene involved in early leaf senescence and defence responses. The SPL29 gene was identified as LOC_Os08g10600 by map-based cloning (Fig. 2), and an NCBI BLASTP search revealed it to be a putative UAPI gene. Phylogenetic analysis showed that SPL29 belongs to the cluster of UAPs, which are separate from the cluster of UGPs (Fig. 3). SPL29 was confirmed as UAP1 in rice, able to perform UAP enzymatic activity (Fig. 4). The mutant phenotype of spl29 results from its UAPI functional inactivation (Fig. 4). UAP is an important enzyme, widely distributed in living organisms; UAP mutants have been discovered in E. coli, S. cerevisiae, and D. melanogaster, and all show abnormal development (Mengin-Lecreulx and van Heijenoort, 1993; Mio et al., 1998; Schimmelpfeng et al., 2006). Until now, there has been no report of UAP mutants in plants, nor has the UAPI gene been identified in rice. This study identified the UAPI gene in rice and showed that the functional inactivation of OsUAPI induces early leaf senescence and defence responses.

Early leaf senescence is induced by functional inactivation of OsUAPI

Only a few studies describe senescence as a phenotype of spl mutant plants, and the senescence syndrome has not been fully
Senescence-induced SGR expression in spl29 (Fig. 5C and D) may play an important role in regulating chlorophyll degradation (Park et al., 2007). Reduced PSII capacity is another manifestation of leaf senescence at the physiological level (Zhang et al., 2012; Panda and Sarkar, 2013). The PSII machinery in spl29 was degraded and its function was fully depressed (Fig. 5E and F; Supplementary Figure S4). Surprisingly, spl29 had increased capacity in each RC (ABS/RC, TR/RC, ET/RC, and DI/RC), although this was previously seen in the senescence-induced PSII alterations of Cucumis sativus cotyledons (Prakash et al., 2003).

The earliest and most significant change during leaf senescence at the cellular level is chloroplast breakdown (Lim et al., 2007). Since the leaves initially grew normally in spl29, well-developed chloroplasts could be observed in many spl29 mesophyll cells even when early leaf senescence occurred (Fig. 6B). However, chloroplasts were completely degraded in abundant dying spl29 mesophyll cells (Fig. 6D; Supplementary Figure S5C and D), with chloroplast breakage potentially being involved in this degradation process (Fig. 6C; Supplementary Figure S5A and B). Chloroplast degradation in spl29 is different from the autophagy-mediated process described in previous reports (Jiao et al., 2012), but the drastic and irreversible degradation process is nevertheless coincident with the rapid leaf senescence phenotype in spl29.

At the molecular level, leaf senescence is mediated by a large number of genes. The senescence-associated transcription factors OsWRKY23, OsWRKY72, and OsNAC2 are all upregulated during leaf senescence, caused by knockdown of the H subunit gene of the glycine decarboxylase complex (Zhou et al., 2013). Four SAGs (OsI2, OsI30, OsI43, and OsI85) are upregulated during dark-induced or natural leaf senescence (Lee et al., 2001). Also the expression of photosynthesis-related genes usually decreases during leaf senescence (Lim et al., 2007). Upregulation of senescence-associated transcription factors (Fig. 7A and B) and SAGs (Fig. 7C and D) and down-regulation of photosynthesis-related genes (Fig. 7E and F) in spl29 together support the hypothesis that early leaf senescence in this mutant is not a passive and unregulated degenerative process, but an active and well-controlled genetic programme.

The physiological, cellular, and molecular evidence all suggest that early leaf senescence happens in spl29 plants. Functional inactivation of OsUAP1 results in the appearance of early leaf senescence, suggesting a role for OsUAP1 in the regulation of this process.

Defence responses are induced by functional inactivation of OsUAP1

LMMs frequently have upregulated defence gene expression and spontaneous appearance of HR-like lesions, both of which may contribute to enhanced resistance to pathogens (Yin et al., 2000). A point mutation in OsUAP1 results in the induction of HR-like lesions and enhanced resistance against the bacterial blight pathogen (Fig. 8A).

**Fig. 8.** Detection of disease resistance and expression of defence signalling-related genes in wild-type and spl29 plants. (A) Mean lesion length after inoculation of plant leaves with bacterial blight pathogen PXO99. Data represent the mean ± SD from 5–7 independent plants at the tillering stage (Student’s t-test: ***, P < 0.0005). (B, C) Relative expression of four defence signalling-related genes in wild-type and spl29 plants at the seedling and tillering stages analysed by qRT-PCR. The expression level of each gene in the wild type was normalised to 1; data represent the mean ± SD of three biological replicates (Student’s t-test: ***, P < 0.0005).

analysed in these mutants (Qiao et al., 2010). Additionally, senescence has not been reported in UAP mutants in other species. Therefore, it was necessary to determine that early leaf senescence does indeed occur in spl29 plants.

The major physiological change during leaf senescence is the gradual loss of chlorophyll (Jiao et al., 2012; Zhang and Zhou, 2013). After the mutant phenotypes appeared, chlorophyll contents were indeed decreased in spl29 (Fig. 5A-B).
defence responses can also be reflected by the upregulation of defence response-associated genes. *PR1a* and *PBZ1* have commonly been used as molecular markers for rice defence responses (Campbell and Ronald, 2005; Tang *et al.*, 2011). Constitutively overexpressing rice *PO-C1* enhanced disease resistance and induced transcript levels of pathogenesis-related genes in transgenic carrot (Wally and Punja, 2010). Overexpression of *OsWRKY45* markedly enhanced fungal and bacterial disease resistance (Shimono *et al.*, 2012). These four defence response-associated genes (*PR1a*, *PBZ1*, *PO-C1*, and *OsWRKY45*) were all upregulated in *spl29* (Fig. 8B and C), and probably play important roles in its enhanced resistance to bacterial blight. Defence responses are induced in *spl29*, which implies a role for *OsUAP1* in disease resistance by regulating the defence response.

ROS, JA, and ABA may play important roles in early leaf senescence and defence responses induced by *OsUAP1* inactivation

ROS (O$_2^-$ and H$_2$O$_2$) accumulated in *spl29* (Fig. 9A and B), and the mutant also had elevated levels of MDA (Fig. 9C and D), a product of lipid peroxidation. Increased SOD activity, but normal CAT activity (Fig. 9E–H), may result in the H$_2$O$_2$ accumulation in *spl29* plants. Despite increased SOD activity to remove O$_2^-$, O$_2^-$ still accumulated in the mutant, and therefore the ROS accumulation in *spl29* can probably be attributed to the overproduction of O$_2^-$.

ROS may play several important roles in the leaf senescence of *spl29*. ROS-mediated chloroplast degradation happens during leaf senescence (Khanna-Chopra, 2012), while lipid peroxidation by ROS has been proposed as the major cause of membrane deterioration in plant senescence (Pauls and Thompson, 1980). Thus, ROS accumulation is the most likely reason for the lipid peroxidation (Fig. 9C and D) and chloroplast membrane breakage (Fig. 6C; Supplementary Figure S5A and B) in *spl29* leaves. The onset of plant senescence can be promoted by ROS (Navabpour *et al.*, 2003). Expression patterns of the senescence-related transcription factors *OsWRKY23*, *OsWRKY72*, and *OsNAC2*, as well as the SAG *Osl85*, were all upregulated after H$_2$O$_2$ treatment (Zhou *et al.*, 2013). Upregulation of these four genes was also found in *spl29* plants (Fig. 7A–D). ROS probably also play a key role in the defence responses of *spl29*. The oxidative burst, a rapid and transient production of huge amounts of ROS, is one of the earliest observable aspects of a plant’s defence strategy (Wojtaszek, 1997). ROS have been implicated not only in anti-pathogen roles, but also in cellular signalling associated with the induction of defence gene expression (Desikan *et al.*, 2000). In *spl29*, the bacterial blight resistance was enhanced (Fig. 8A) and defence response-associated genes were upregulated (Fig. 8B and C).

In addition, JA and ABA levels were significantly elevated in *spl29* plants (Fig. 10). The JA and ABA signalling

---

**Fig. 9.** ROS accumulation in wild-type and *spl29* leaves. (A-B) Histochemical detection of O$_2^-$ by NBT staining and H$_2$O$_2$ by DAB staining: purple formazan precipitate indicates the location of O$_2^-$ and brown precipitate indicates the location of H$_2$O$_2$. Plant leaves at the tillering stage were analysed; results are representative of three independent experiments. (C, D) MDA contents. (E, F) SOD activities. U: 1 U = 1 SOD activity unit (inhibition of 50%). (G, H) CAT activities. For C–H, leaf samples were analysed at the seedling and tillering stages; data represent the mean ± SD of three biological replicates (Student’s t-test: ***, *P < 0.0005*). FW, fresh weight.
pathways are widely considered to play a role in modulating leaf senescence and defence responses (Lim et al., 2007; Robert-Seilaniantz et al., 2011; Zhang and Zhou, 2013). The accumulated JA and ABA in spl29 (Fig. 10) are probably involved in its early leaf senescence and defence responses.

In conclusion, functional inactivation of OsUAPI induces early leaf senescence and defence responses in spl29. ROS, JA, and ABA are all likely to be involved in these two biological processes or pathways in the mutant. It is suggested that OsUAPI may play an important role in regulating leaf senescence and defence responses in rice.

Supplementary material

Supplementary data can be found at JXB online.

Supplementary Table S1. List of PCR-based molecular markers used for map-based cloning of SPL29.

Supplementary Table S2. Primers for the detection of the mutation site, construction of the functionally complementary vector, and confirmation of positive transgenic plants.

Supplementary Table S3. All primers used for qRT-PCR analysis.

Supplementary Table S4. Formulae and glossary of terms used in the analysis of chlorophyll a fluorescence transients.

Supplementary Table S5. Segregation of F₂ populations from three crosses.

Supplementary Table S6. Sequencing of the mutation site in different rice cultivars and F₂ plants.

Supplementary Figure S1. Determination of reference genes for normalisation in qRT-PCR analysis.

Supplementary Figure S2. Phenotype of the second-emerged leaf after germination in wild-type and spl29 plants.

Supplementary Figure S3. SDS-PAGE of proteins.

Supplementary Figure S4. Different expression of relative variable fluorescence.

Supplementary Figure S5. Ultrastructure in mesophyll cells of spl29.

Funding

This work was supported by the Key Grant Project of the Chinese Ministry of Education (Grant No. 313039), Specialized Research Fund for the Doctoral Programme of Higher Education (20130141110069), National Programme of Transgenic Variety Development of China (Grant Nos 2011ZX08001-001 and 2011ZX08001-004), and the National Natural Science Foundation of China (91017013, 91217309, 20825520, and 31200208).

Acknowledgements

The authors thank Drs Bingyuan Chen and Xiaobing Deng for critical reading of the manuscript, and Jie Jin for preparing the bacterial blight pathogen.

References

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55, 373–399.

Balazadeh S, Kwasniewski M, Caldana C, Mehrnia M, Zanor MI, Xue GP, Mueller-Roemer B. 2011. ORS1, an H2O2-responsive NAC transcription factor, controls senescence in Arabidopsis thaliana. Molecular Plant 4, 346–360.

Campbell MA, Ronald PC. 2005. Characterization of four rice mutants with alterations in the defence response pathway. Molecular Plant Pathology 6, 11–21.

Chen ML, Fu XM, Liu JQ, Ye TT, Hou SY, Huang YQ, Yuan BF, Wu Y, Feng YQ. 2012. Highly sensitive and quantitative profiling of acidic phytohormones using derivatization approach coupled with nano-LC-ESI-Q-TOF-MS analysis. Journal of Chromatography B 905, 67–74.

Dangl JL, Jones JD. 2001. Plant pathogens and integrated defence responses to infection. Nature 411, 826–833.

Desikan R, Neill SJ, Hancock JT. 2000. Hydrogen peroxide-induced gene expression in Arabidopsis thaliana. Free Radical Biology and Medicine 28, 773–778.

Jiao BB, Wang JJ, Zhu XD, Zeng LJ, Li Q, He ZH. 2012. A novel protein RLS1 with NB-ARM domains is involved in chloroplast degradation during leaf senescence in rice. Molecular Plant 5, 205–217.

Jung YH, Lee JH, Agrawal GK et al. 2005. The rice (Oryza sativa) blast lesion mimic mutant, blm, may confer resistance to blast pathogens by triggering multiple defense-associated signaling pathways. Plant Physiology and Biochemistry 43, 397–406.

Khanna-Chopra R. 2012. Leaf senescence and abiotic stresses share reactive oxygen species-mediated chloroplast degradation. Protoplasma 249, 469–481.

Lam E, Kato N, Lawton M. 2001. Programmed cell death, mitochondria and the plant hypersensitive response. Nature 411, 848–853.

Lee RH, Wang CH, Huang LT, Chen SC. 2001. Leaf senescence in rice plants: cloning and characterization of senescence up-regulated genes. Journal of Experimental Botany 52, 1117–1121.

Lichtenthaler HK. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymology 148, 350–382.
Lim PO, Kim HJ, Nam HG. 2007. Leaf senescence. Annual Review of Plant Biology 58, 115–136.

Lim PO, Nam HG. 2005. The molecular and genetic control of leaf senescence and longevity in Arabidopsis. Current Topics in Developmental Biology 67, 49–83.

Manosalva PM, Bruce M, Leach JE. 2011. Rice 14-3-3 protein (GF14e) negatively affects cell death and disease resistance. The Plant Journal 68, 777–787.

Mengin-Lecreux D, van Heijenoort J. 1993. Identification of the glmU gene encoding N-acetylglucosamine-1-phosphate uridyltransferase in Escherichia coli. Journal of Bacteriology 175, 6150–6157.

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. 2008. The eukaryotic UDP-N-acetylglucosamine pyrophosphorylases. Gene cloning, protein expression, and catalytic mechanism. The Journal of Biological Chemistry 273, 14392–14397.

Mio T, Yabe T, Arisawa M, Yamada-Okabe H. 1998. The eukaryotic UDP-N-acetylglucosamine pyrophosphorylases. Gene cloning, protein expression, and catalytic mechanism. The Journal of Biological Chemistry 273, 14392–14397.

Navabpour S, Morris K, Allen R, Harrison E, S AH-M, Buchanan-Webb et al. 2003. Expression of senescence-enhanced genes in response to oxidative stress. Journal of Experimental Botany 54, 2285–2292.

Panda D, Sarkar RK. 2013. Natural leaf senescence: probed by chlorophyll fluorescence, CO₂ photosynthetic rate and antioxidant enzyme activities during grain filling in different rice cultivars. Physiology and Molecular Biology of Plants 19, 43–51.

Park SY, Yu JW, Park JS et al. 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. The Plant Cell 19, 1649–1664.

Pauls KP, Thompson JE. 1980. In vitro simulation of senescence-related membrane damage by ozone-induced lipid peroxidation. Nature 283, 504–506.

Peneff C, Ferrari P, Charrrier V, Taburet Y, Monnier C, Zamboni V, Winter J, Harnois M, Fassy F, Bourne Y. 2001. Crystal structures of two human pyrophosphorylase isoforms in complexes with UDPGlc(Gal)Nac: role of the alternatively spliced insert in the enzyme oligomeric assembly and active site architecture. The EMBO Journal 20, 6191–6202.

Prakash JS, Srivastava A, Strasser RJ, Mohanty P. 2003. Senescence-induced alterations in the photosystem II functions of Cucumis sativus cotyledons: probing of senescence driven alterations of photosys tem II by chlorophyll fluorescence induction O-J-I-P transients. Indian Journal of Biochemistry and Biophysics 40, 160–168.

Qiao Y, Jiang W, Lee J et al. 2010. SPL28 encodes a clathrin-associated adaptor protein complex 1, medium subunit micro 1 (AP1M1) and is responsible for spotted leaf and early senescence in rice (Oryza sativa). New Phytologist 185, 258–274.

Robert-Seilaniantz A, Grant M, Jones JD. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annual Review of Phytopathology 49, 317–343.

Schimmelpfeng K, Strunk M, Stork T, Klambt C. 2006. Mummy encodes an UDP-N-acetylglucosamine-diphosphorylase and is required during Drosophila dorsal closure and nervous system development. Mechanisms of Development 123, 487–499.

Shimono M, Koga H, Akagi A et al. 2012. Rice WRKY45 plays important roles in fungal and bacterial disease resistance. Molecular Plant Pathology 13, 83–94.

Tang J, Zhu X, Wang Y, Liu L, Xu B, Li F, Fang J, Chu C. 2011. Semi-dominant mutations in the CC-NB-LRR-type R gene, NLS1, lead to constitutive activation of defense responses in rice. The Plant Journal 66, 996–1007.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, RESEARCH0034.

Wally O, Punja ZK. 2010. Enhanced disease resistance in transgenic carrot (Daucus carota L.) plants over-expressing a rice cationic peroxidase. Plants 232, 1229–1239.

Wang X, Fang G, Li Y, Ding M, Gong H, Li Y. 2012. Differential antioxidant responses to cold stress in cell suspension cultures of two subspecies of rice. Plant Cell, Tissue and Organ Culture 113, 353–361.

Wojtaszek P. 1997. Oxidative burst: an early plant response to pathogen infection. Biochemical Journal 322, 681–692.

Wu C, Bordeos A, Madamba MR, Baraoidan M, Ramos M, Wang GL, Leach JE, Leung H. 2008. Rice lesion mimics mutants with enhanced resistance to diseases. Molecular Genetics and Genomics 279, 605–619.

Yang T, Echols M, Martin A, Bar-Peled M. 2010. Identification and characterization of a strict and a promiscuous N-acetylglucosamine-1-P uridylyltransferase in Arabidopsis. Biochemical Journal 430, 275–284.

Yin Z, Chen J, Zeng L, Goh M, Leung H, Khush GS, Wang GL. 2000. Characterizing rice lesion mimic mutants and identifying a mutant with broad-spectrum resistance to rice blast and bacterial blight. Molecular Plant Microbe Interactions 13, 869–876.

Zhang H, Zhou C. 2013. Signal transduction in leaf senescence. Plant Molecular Biology 82, 539–545.

Zhang J, Zhang Y, Du Y, Chen S, Tang H. 2011. Dynamic metabolic responses of tobacco (Nicotiana tabacum) plants to salt stress. Journal of Proteome Research 10, 1904–1914.

Zhang Z, Li G, Gao H, Zhang L, Yang C, Liu P, Meng Q. 2012. Characterization of photosynthetic performance during senescence in stay-green and quick-leaf-senescence Zea mays L. introbed lines. PLoS ONE 7, e42936.

Zhou Q, Yu Q, Wang Z, Pan Y, Lv W, Zhou L, Chen R, He G. 2013. Knockdown of GDCH gene reveals reactive oxygen species-induced leaf senescence in rice. Plant, Cell and Environment 36, 1476–1489.