Mutation of \textit{SPOTTED LEAF3 (SPL3)} impairs abscisic acid-responsive signalling and delays leaf senescence in rice

Seung-Hyun Wang$^{1,*}$, Jung-Hyun Lim$^{1,*}$, Sang-Sook Kim$^{1}$, Sung-Hwan Cho$^{1,†}$, Soo-Cheul Yoo$^{2}$, Hee-Jong Koh$^{1}$, Yasuhito Sakuraba$^{1,‡}$, and Nam-Chon Paek$^{1,3,‡}$

$^1$ Department of Plant Science, Plant Genomics and Breeding Institute, and Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 151–921, Korea
$^2$ Department of Plant Life and Environmental Science, Hankyong National University, Ansung 456-749, Korea
$^3$ Crop Biotechnology Institute, GreenBio Science and Technology, Seoul National University, Pyeongchang 232-916, Korea

* These authors contributed equally to this work.
† Present address: Divisions of Biochemistry and Plant Sciences, University of Missouri, Columbia, MO 65211, USA
‡ To whom correspondence should be addressed. E-mail: sakuraba0425@gmail.com; ncpaek@snu.ac.kr

Received 17 June 2015; Revised 29 July 2015; Accepted 30 July 2015

Editor: Christine Foyer

Abstract

Lesion mimic mutants commonly display spontaneous cell death in pre-senescent green leaves under normal conditions, without pathogen attack. Despite molecular and phenotypic characterization of several lesion mimic mutants, the mechanisms of the spontaneous formation of cell death lesions remain largely unknown. Here, the rice lesion mimic mutant \textit{spotted leaf3 (spl3)} was examined. When grown under a light/dark cycle, the \textit{spl3} mutant appeared similar to wild-type at early developmental stages, but lesions gradually appeared in the mature leaves close to heading stage. By contrast, in \textit{spl3} mutants grown under continuous light, severe cell death lesions formed in developing leaves, even at the seedling stage. Histochemical analysis showed that hydrogen peroxide accumulated in the mutant, likely causing the cell death phenotype. By map-based cloning and complementation, it was shown that a 1-bp deletion in the first exon of \textit{Oryza sativa Mitogen-Activated Protein Kinase Kinase Kinase1 (OsMAPKKK1)/OsEDR1/OsACDR1} causes the \textit{spl3} mutant phenotype. The \textit{spl3} mutant was found to be insensitive to abscisic acid (ABA), showing normal root growth in ABA-containing media and delayed leaf yellowing during dark-induced and natural senescence. Expression of ABA signalling-associated genes was also less responsive to ABA treatment in the mutant. Furthermore, the \textit{spl3} mutant had lower transcript levels and activities of catalases, which scavenge hydrogen peroxide, probably due to impairment of ABA-responsive signalling. Finally, a possible molecular mechanism of lesion formation in the mature leaves of \textit{spl3} mutant is discussed.

Key words: Abscisic acid, catalase activity, lesion mimic mutant, MAPKKK, reactive oxygen species, rice, \textit{spotted leaf3}.

Introduction

Lesion mimic mutants display cell death in normal conditions and have a common phenotype similar to the pathogen infection-induced hypersensitive response. The study of lesion mimic mutants has provided insights on the mechanisms of programmed cell death. Lesion mimic mutants have been isolated and characterized in many plants, including maize (Hoisington et al., 1982), barley (Wolter et al., 1993), \textit{Arabidopsis} (Noutoshi et al., 2006), and rice (Wu et al., 2008).
Previous studies revealed that lesion mimic mutant genes encode distinct functional proteins such as a heat stress transcription factor (Yamanouchi et al., 2002), membrane-associated proteins (Lorrain et al., 2004; Noutoshi et al., 2006), an ion channel family member (Balague et al., 2003; Rostoks et al., 2006; Mosher et al., 2010), zinc finger proteins (Dietrich et al., 1997; Wang et al., 2005), an E3 ubiquitin ligase (Zeng et al., 2004), a clathrin-associated adaptor protein (Qiao et al., 2010), and splicing factor 3b subunit 3 (Chen et al., 2012).

Thus, the molecular mechanisms of lesion formation seem to be very complicated in plants. Over-accumulation of reactive oxygen species (ROS), such as superoxide radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), is closely associated with lesion formation, as confirmed in several lesion mimic mutants (Qiao et al., 2010; Shirsekar et al., 2014). It is also reported that lesion mimic phenotypes are affected by the light-intensity and light/dark diurnal cycle (Kusumi et al., 2000). Thus, endogenous signalling pathways to external stress stimuli are likely impaired in several lesion mimic mutants.

The highly conserved mitogen-activated protein kinase (MAPK) cascade functions in the response to external environmental stimuli, acting in the transduction of extracellular cues to intercellular targets (Widmann et al., 1999). The MAPK cascade comprises MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs) (Schaeffer et al., 2002). Arabidopsis thaliana genome has 20 MAPKs, 10 MAPKKs, and more than 80 MAPKKKs (Colcombet and Hirt, 2008). By contrast, the rice genome encodes 75 MAPKKKs, 8 MAPKKs, and 17 MAPKs (Agrawal et al., 2003, Hamel et al., 2006, Rao et al., 2010). Arabidopsis and rice have many more MAPKKKs than MAPKs or MAPKKs, leading to complicated and variable regulatory cascades. Several Arabidopsis MAPKKKs have been characterized, and they regulate various biological processes, such as cytokinesis (Takahashi et al., 2010), stomatal development (Kim et al., 2012), and the responses to biotic (Kieber et al., 1993; Frye et al., 2001) and abiotic stresses (Giao and Xiang, 2008; Huang et al., 2014). However, studies of MAPKKKs in rice remain limited.

Phytohormone signalling pathways also have important roles in responding to external stress stimuli. Jasmonic acid (JA)-, salicylic acid (SA)-, and ethylene-responsive signalling pathways are tightly associated with the resistance to biotrophic and necrotrophic pathogens (Robert-Seilanianzt et al., 2007), and are important for the response to abiotic stresses (Clarke et al., 2009; Brossa et al., 2011; Lei et al., 2011). Abscisic acid (ABA) positively regulates the response to several abiotic stresses, e.g. drought and osmotic stresses, by promoting the closure of stomata (Radin, 1984; Fujita et al., 2005). Arabidopsis transgenic plants overexpressing several ABA-induced bZIP transcription factors, such as ABA-responsive element (AREB) binding protein (AREB) and ABRE binding factor (ABF), exhibited tolerance to drought and/or osmotic stresses (Fujita et al., 2005, 2009; Yoshida et al., 2010). In addition to abiotic stress response, ABA also controls various developmental processes, including seed germination, root elongation, leaf senescence, and seed development (De Smet et al., 2006; Nakashima et al., 2013). These phytohormone signalling pathways are, at least in part, regulated by specific MAPK cascades. One of the Arabidopsis MAPKKKs, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) modulates ethylene signalling by promoting ETHYLENE INSENSITIVE3 (EIN3) transcription, together with MAPKK9-MAPK5/MAPK6 (Yoo and Sheen, 2008). Another Arabidopsis MAPKKK, ENHANCED DISEASE RESISTANCE1 (EDR1), negatively regulates the SA-dependent defence pathway. The edr1 knockout mutant showed resistance to powdery mildew disease caused by Erysiphe cichoracearum, but SA-deficient or SA signalling-related mutants completely suppressed this phenotype (Frye et al., 2001). However, the relationship between phytohormone signalling and MAPK pathways largely remains unknown.

In this study, the rice spl3 mutant, which produces spontaneous cell death lesions on its leaf blades and shows excessive accumulation of H$_2$O$_2$, was analysed. Map-based cloning showed that the spl3 locus encodes a putative kinase protein, OsMAPKKK1. The spl3 mutant is strongly insensitive to ABA treatment and delays leaf senescence, probably due to reduced expression of ABA signalling-related genes. In the spl3 leaves, a significant decrease of catalase activity, which functions in scavenging H$_2$O$_2$ in the cells, was found. These spl3 data provide insights into the molecular function of SPL3 in ABA-responsive signalling in plants.

Materials and methods

Plant materials and growth conditions

The spl3 mutant was originally generated by γ-ray irradiation of a Japanese japonica rice cultivar ‘Norin8’ (Yoshimura et al., 1997). The wild-type Norin8 and spl3 mutant were grown in the paddy field (natural long day conditions at 37° N latitude, Suwon, Korea) or in the growth chambers. The chamber experiments were performed under short day (SD) conditions [10-h light with normal intensity (300 μmol m$^{-2}$ sec$^{-1}$) at 30°C and 14-h dark at 20°C], or continuous light with 30°C for 10h and 20°C for 14h. For phenotypic characterization and map-based cloning of spl3 mutant, all the plants were grown in the paddy field.

Detection of ROS

The detection of ROS accumulation was conducted as previously described (Sakuraba et al., 2013). To determine hydrogen peroxide (H$_2$O$_2$) and superoxide anion (O$_2^-$), leaf samples of 1-month-old plants grown in the growth chamber under SD or continuous light conditions were transferred in DAB staining solution containing 0.1% 3,3-diaminobenzidine or in nitroblue tetrazolium (NBT) staining solution including 0.05% nitroblue tetrazolium chloride in 50mM sodium phosphate buffer, and incubated for 6h with gentle shaking. After staining, chlorophyll was completely removed by incubation with 90% ethanol at 80°C. H$_2$O$_2$ accumulation was also measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Life Technologies, USA) according to the manufacturer’s protocol.

Genetic analysis and map-based cloning

For genetic analysis, an F$_2$ population was developed from crossing between a japonica-type spl3 mutant and Korean Tongil-type cultivar Milyang23, which was derived from hybridization of indica × japonica
rice cultivars. The spl3 locus was previously mapped to the short arm of chromosome 3 (Yoshimura et al., 1997). In this study, a mapping population of 1800 F2 individuals from the cross between spl3 and Milyang23 was used for locating and fine mapping of the spl3 locus. Genomic DNA was extracted from young leaves of each F2 individual line. The newly designed markers using Milyang23 sequence data (Lim et al., 2014) were used to narrow down the genomic region of spl3 locus on chromosome 3; these markers included sequence-tagged-site (STS) markers (Supplementary Table S1 at JXB online).

**Stress treatments**

Stress treatments were performed as described (Kim et al., 2003). Two-week-old wild-type (WT) seedlings (cv. Norin8) were treated with dehydration, NaCl (150 mM), mannitol (500 mM), SA (100 μM), methyl jasmonate [MeJA (100 μM)], 1-aminocyclo-propane-1-carboxylic acid [ACC (10 mM)], and different concentrations of ABA (5–50 μM). To check the senescence phenotype of WT and spl3 leaves under treatments of four senescence-promoting phytohormones (ABA, ACC, SA, and MeJA), detached leaf discs from 1-month-old plants were floated on the 3 mM MES (pH 5.8) buffer supplemented with 50 μM ABA, 10 mM ACC, 100 μM SA, and 100 μM MeJA and incubated for 4 d under continuous light conditions. To check the phenotype under drought and osmotic stresses, WT and spl3 plants were grown under LD conditions for 2 month. For drought stress treatment, plants were dehydrated for 5 d at 25 °C and 50% humidity, and then rehydrated again for investigating the recovery of wilting phenotype. For the salt stress assay, plants were transferred to 500 mM mannitol and incubated for 5 d.

**Chlorophyll measurement**

For the measurement of total chlorophyll (Chl) concentration, photosynthetic pigments were extracted from the leaf tissues with 80% ice-cold acetone. Chl concentrations were determined by spectrophotometry as described previously (Porra et al., 1989).

**SDS-PAGE and immunoblot analysis**

Total protein extracts were prepared from the leaf tissues. To extract total proteins, leaf tissues of rice grown in the paddy field were ground in liquid nitrogen and 10 mg aliquots were homogenized with 100 μl of sample buffer (50 mM Tris, pH 6.8; 2 mM EDTA; 10% glyceral; 2% SDS; and 6% mercaptoethanol). Homogenates were centrifuged at 10 000 × g for 3 min, and supernatants were denatured at 80 °C for 5 min. Four microlitres of each sample was subjected to 12% (w/v) SDS-PAGE and resolved proteins were electroblotted onto a Hybond-P membrane (GE Healthcare, USA). Antibodies against the photosystem proteins Lhcb1, Lhcb2, Lhcb4, Lhca1, Lhca2, and D1 (Agrisera, Sweden) were used for immunoblot analysis. The level of each protein was examined using the ECL system (WESTSA VE kit, AbFRONTIER, Korea) according to the manufacturer’s protocol.

**Measurement of ion leakage rates**

Ion leakage rates were measured as described previously (Lee et al., 2015). Briefly, membrane leakage was determined by the amount of electrolytes (or ions) leaking from rice leaf discs (1 cm²). Three leaf discs from each treatment were immersed in 6ml of 0.4M mannitol at room temperature with gentle shaking for 3 h, and initial conductivity of the solution was measured with a conductivity meter (CON 6 METER, LaMotte Co., USA). Total conductivity was determined after sample incubation at 85 °C for 20 min. Ion leakage rate is expressed as the percentage of initial conductivity divided by the total conductivity.

**Stomatal aperture analysis**

The stomatal aperture of abaxial leaf epidermal strips was analysed as previously described (Xing et al., 2007) with minor modifications.

Leaf discs from 3-week-old plants grown under SD conditions were incubated in 3 mM MES buffer (pH 6.15) containing 50 mM KCl (MES-KCl) for 2 h under light (22 °C) to open stomata. Leaf discs were then transferred to the MES-KCl buffer containing 5 μM ABA for 4 h. Stomatal cells were observed by Field-Emission Scanning Electron Microscopy (AURIGA, Carl ZEISS, Germany).

**Complementation test**

For complementation of the spl3 mutation, a full-length cDNA of SPL3 was ligated into the pMDC32 Gateway binary vector containing the 35S promoter (Curtis and Grossniklaus, 2003). The 35S:SPL3 construct in the pMDC32 plasmid was introduced into the calli generated from the mature embryos of spl3 mutant seeds by Agrobacterium (strain LBA4404)-mediated transformation (Jeon et al., 2000). Transformants were confirmed by PCR using the specific primers listed in Supplementary Table S1 at JXB online.

**Reverse transcription-quantitative real-time PCR analysis**

Total RNA was extracted from the 4-week-old plants with the MG Total RNA Extraction Kit (Macrogen, Korea), including DNase I treatment step for removing the possible contamination of genomic DNAs. First-strand cDNAs were synthesized with 2 μg of total RNA in a 25 μl volume using M-MLV reverse transcriptase and oligo(dT)12 primer. The 20 μl of reverse transcription (RT)-quantitative real-time PCR (qPCR) mixture contained 2 μl of the RT mixture, 10 μl of 2X GoTar PCR mix (Roche), and 0.25 μl of the primers. The qPCR was performed on the Light Cycler 2.0 (Roche Diagnostics, Germany). The qPCR conditions were 95 °C for 2 min, followed by 45 cycles at 95 °C for 5 s, 59 °C for 15 s, and 72 °C for 10 s. The relative expression of each gene was calculated using the 2−ΔΔCt methods (Livak and Schmittgen, 2001). The primers used for qPCR are listed in Supplementary Table S1 at JXB online.

**Catalase assay**

Catalase activity in the rice leaves was analysed using the Sigma Catalase Assay kit (Sigma-Aldrich, USA) following the manufacturer’s instructions.

**Results**

**Phenotypic characterization of the spl3 mutant**

A single recessive spl3 mutant in rice was isolated from the M2 population of japonica cultivar ‘Norin8’ irradiated with gamma rays (Yoshimura et al., 1997). First, the spl3 phenotype was examined in the paddy field. At early tillering stage [50 d after seeding (DAS)], the phenotype of spl3 mutant appeared to be quite similar to that of the WT, without any lesion development (Fig. 1, left panels). At late-tillering stage (90 DAS), lesions gradually appeared, mainly in the old leaves and from the tip region (Supplementary Fig. S1 at JXB online), with agronomic traits, especially spikelet fertility and panicle length (Supplementary Fig. S2 at JXB online). These results indicate that lesion formation in spl3 mutant is closely related with the leaf age, between late-tillering and heading stages.
Excessive accumulation of ROS causes development of cell death lesions in leaf blades

To examine the effect of the diurnal light-dark cycle on lesion formation in the spl3 mutant, WT (cv. Norin8) and spl3 mutant were grown in the growth chamber (300 μmol m⁻² s⁻¹) under SD; 10-h light, 30 °C/14-h dark, 20 °C) or continuous light (CL). Under SD, the spl3 mutant developed no lesions on the leaves even after 30 DAS (Fig. 2A). Under CL conditions, however, reddish spots with some necrotic lesions appeared in the spl3 leaves (Fig. 2A).

Excessive accumulation of ROS causes leaf variegation and/or necrotic lesions in some variegated-leaf mutants in rice (Li et al., 2010; Han et al., 2012; Sakuraba et al., 2013). Thus, the levels of two kinds of ROS, O₂⁻ and H₂O₂, were examined in the spl3 leaves grown under SD or CL conditions, using two staining methods: NBT for O₂⁻ and DAB for H₂O₂. Under SD, the O₂⁻ and H₂O₂ levels in the spl3 leaves were almost the same as those of the WT. However, O₂⁻ and H₂O₂ accumulated in the spl3 leaves under CL compared with the WT leaves (Fig. 2B, C). H₂O₂ production under SD and CL conditions was also confirmed by quantification using the hydrogen peroxide/peroxidase assay kit. Consistent with the results of DAB staining (Fig. 2B), the spl3 leaves had significantly higher H₂O₂ levels under CL conditions (Fig. 2D). Furthermore, it was found that at 90 DAS, the spl3 leaves had much higher H₂O₂ levels than the WT leaves, especially in the 3rd and 4th leaves, which had many lesions (Fig. 2E).

Map-based cloning of SPL3

To isolate the SPL3 gene, a map-based cloning was performed using 1771 F2 plants that were generated from a cross of spl3 (japonica) mutant and Milyang23 (a Tongil-type indica/japonica hybrid cultivar). The spl3 locus was mapped to a 415-kb interval between RM14395 and RM14423 on chromosome 3 (Fig. 3A). Using two STS and one simple sequence repeat (SSR) markers, the spl3 locus was further delimited to a 161.2-kb interval between S3015.2 and SSR-5, in the BAC clones AC099401 and AC119797 (GenBank accession number) (Fig. 3B). In this genomic region, 16 candidates were found based on the Rice Functional Genomic Expression Database of the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/RiceGE). Sixteen expressed genes were cloned by RT-PCR or genomic PCR. As a result, a 1-bp deletion was identified in the first exon of a candidate gene, LOC_Os03g06410 (Fig. 3C, D), resulting in a frameshift mutation (Supplementary Fig. S1 at JXB online) that leads to premature translational termination (Supplementary Fig. S4 at JXB online). LOC_Os03g06410 encodes a putative MAPKKK, which is orthologous to AtEDR1 (Arabidopsis thaliana Enhanced Disease Resistance1; Frye et al., 2001). Based on this orthology, the locus had been named OsEDR1 (Kim et al., 2003; Shen et al., 2011), although the same group renamed it OsACDR1 (Oryza sativa Accelerated Cell Death and Resistance1) because the function of OsEDR1 was considerably different from that of AtEDR1 (Kim et al., 2009a). It was also named OsMAPKK1 (Rao et al., 2010). SPL3/
OsEDR1/OsACDR1/OsMAPKKK1 comprises 1018 amino acids with a protein kinase domain at the C-terminal region, which was abrogated in the spl3 allele (Supplementary Fig. S3 at JXB online).

To confirm that the mutation in SPL3 caused the spl3 phenotype, a complementation test was performed. As a result, five independent transgenic lines did not show any lesion mimic phenotype at 60 DAS, when spots developed clearly on the mature leaves of spl3 mutant (Supplementary Fig. S5 at JXB online). These results indicate that the 1-bp deletion in the exon 1 of OsMAPKKK1 is responsible for the spl3 mutation.

**The transcript accumulation of SPL3 is dependent on leaf age**

To examine SPL3 function, SPL3 expression was examined in various tissues of 2-week-old WT plants by RT-qPCR. SPL3 transcripts accumulated the most in the leaf sheath and leaf blade (Supplementary Fig. S6A at JXB online). Lesions in spl3 mutant are predominant in the tip area of older leaf blades both in the field and in growth chamber conditions (Figs 1 and 2; Supplementary Fig. S1). Thus, SPL3 expression was subsequently examined in two different sections (top and middle) of three different stages of leaf blades (flag, 2nd, and 3rd leaves). In all leaves, SPL3 transcript levels were significantly lower in the top area, especially in flag leaves (Supplementary Fig. S6B). Together, these data indicate that SPL3 has an important role in protecting young leaves from the generation of necrotic lesions.

**ABA-responsive signalling is impaired in the spl3 mutant**

SPL3 expression was shown to be rapidly and transiently regulated by diverse environmental stresses over a short time-frame of 30–120 min (Kim et al., 2003). To further study the SPL3 function under abiotic stresses, the time-course expression of SPL3 was examined for 24 h in response to three abiotic stresses (drought, mannitol, and salt) and four hormones (ABA, ethylene, SA, and JA) (Supplementary Fig. S7 at JXB online). SPL3 transcript levels were found to decrease rapidly in response to ABA, SA, and MeJA, and decrease slowly in response to ethylene (ACC) and three abiotic stresses, suggesting that SPL3 has an important role in both hormone-responsive and abiotic stress-responsive signalling. Thus, the response of spl3 mutant to senescence-promoting hormones, including ABA, ethylene, SA, and MeJA (Kusaba et al., 2013), was examined. It was found that the spl3 mutant showed a stay-green phenotype under ABA- and ACC-induced senescence conditions, but no significant phenotypic difference under SA- and MeJA-induced senescence conditions (Supplementary Fig. S8 at JXB online), indicating that both ABA and ethylene signalling pathways were impaired in the spl3 leaves.
Next, the senescence phenotype of \textit{spl3} mutant in the field was checked. During the pre-senescent phase, the leaf colour of the \textit{spl3} mutant was almost the same as that of the WT (Fig. 1). At the senescent phase [40 d after heading (DAH)], however, the \textit{spl3} leaves exhibited a strong stay-green phenotype (Fig. 4A), even though the heading date of \textit{spl3} mutant was the same as that of the WT (Supplementary Fig. S9 at \textit{JXB} online). Consistent with this, Chl and photosynthesis-related proteins (D1, Lhcb1, Lhcb2, Lhcb4, Lhca1, Lhca2, and RbcL) were retained in the \textit{spl3} leaves (Fig. 4B, C). In parallel, the $Fv/Fm$ ratio, and the photosynthetic efficiency of photosystem II, were also retained in the \textit{spl3} leaves compared with the WT (Fig. 4D). The expression of three typical senescence-associated genes (SAGs) was also investigated for Chl catabolism: \textit{STAYGREEN} (SGR; Park \textit{et al.}, 2007), \textit{NON-YELLOW COLOURING1} (NYC1; Kusaba \textit{et al.}, 2007), and \textit{OsNAP} (Liang \textit{et al.}, 2014). The transcript levels of these three SAGs were down-regulated in the \textit{spl3} mutant at 40 DAS compared with the WT (Fig. 4E).

The stay-green phenotype of the \textit{spl3} mutant was also confirmed during dark-induced senescence. After 4 d of dark incubation (4 DDI), the leaf discs of the WT turned completely yellow, while those of the \textit{spl3} mutant remained green (Fig. 4F), with higher Chl levels (Fig. 4G) and lower ion leakage rates (Fig. 4H). Furthermore, \textit{SPL3} expression increased during both natural and dark-induced senescence (Supplementary Fig. S10 at \textit{JXB} online).

\textbf{ABA signalling-related phenotype of the \textit{spl3} mutant}

Next, it was tested whether the \textit{spl3} mutant shows altered phenotypes for ABA-related processes. The root phenotype of \textit{spl3} mutant in ABA-containing media (Fig. 5A–C) was investigated. The WT showed remarkably retarded root development in the presence of ABA; however, the \textit{spl3} mutant produced longer primary roots and more adventitious roots than the WT. Next, the phenotype of the \textit{spl3} mutant was examined under abiotic stress conditions, such as drought and osmotic stresses. During 5 d of dehydration, the \textit{spl3} mutant wilted much earlier than the WT and did not recover after rehydration (Fig. 5D, E). Similarly, the \textit{spl3} mutant was more sensitive to osmotic...
stress (500 mM mannitol) (Supplementary Fig. S11 at JXB online). The stomata in the spl3 leaf surfaces was also observed, which revealed that stomatal closure in the spl3 mutant was nearly insensitive to ABA treatment (Fig. 5F, G). In contrast to these ABA-related phenotypes, which differed between the WT and spl3 mutant, the seed germination rate did not differ between the WT and spl3 (Supplementary Fig. S12 at JXB online). Taken together, these data indicate that SPL3 is involved in some, but not all ABA-responsive pathways.

Altered expression of ABA signalling-related genes in the spl3 mutant

Because of ABA insensitivity in root development, leaf senescence, and abiotic stresses, it was hypothesized that ABA-responsive signalling is severely compromised in the spl3 mutant. To examine this, the expression levels of the ABA signalling-associated genes were compared between the WT and spl3 mutant after 6 h of ABA treatment. Based on previous reports of ABA signalling in rice (Zhou et al.,...
Wang et al. 2008; Park et al. 2010; Tseng et al. 2013), the ABA signalling-associated genes involved in seed germination (OsABI1, OsABI3, OsABI4, and OsDSG1), seed germination and development (ABI5), abiotic stress responses (OsAREB1, OsbZIP23, OsSAPK8, and OsSAPK9), and root development (OsSAPK6, OsRePRP, and OsDSR1) were investigated. Among them, several ABA-associated genes, including ABI1, ABI4, ABI5, OsbZIP23, and OsSAPK9, OsSAPK6, and OsRePRP were significantly down-regulated in the spl3 mutant after ABA treatment (Fig. 6). Thus, it is probable that the strong repression of ABA signalling-associated genes leads to the ABA insensitivity in the spl3 mutant. By contrast, the expression of OsABI3 (Fig. 6B) and OsDSG1 (Fig. 6D), key regulators of seed germination in rice (Park et al. 2010), were not down-regulated in the spl3 mutant.

In parallel, ABA signalling-associated genes (OsABI1, OsABI3, OsbZIP23, and OsSAPK9) were also found to be down-regulated in the spl3 mutant during natural senescence (Supplementary Fig. S13 at JXB online), suggesting that the down-regulation of these genes, in addition to OsNAP (Fig. 4E), is also associated with delayed senescence of spl3 leaves. A previous study reported that the expression levels of genes related to ethylene biosynthesis were severely suppressed in the knockout mutant of OsEDR1 (Shen et al. 2011). During
Rice spl3 mutant impairs ABA-responsive signalling

senescence, two ethylene-signalling-related SAGs, ETHYLENE INSENSITIVE2 (EIN2) and EIN3, were found to be down-regulated in the spl3 mutant, as were two ethylene biosynthetic genes, ACC SYNTHASE1 (ACS1) and ACS2 (Supplementary Fig. S14 at JXB online), suggesting that the impairment of both ABA- and ethylene-responsive signalling pathways results in the delayed leaf senescence phenotype of the spl3 mutant.

ABA insensitivity in the spl3 mutant leads to down-regulation of catalase expression

Catalase (CAT) scavenges H2O2, and its physiological functions have been widely studied in Arabidopsis (Mhamdi et al., 2010). In Arabidopsis, ABA treatment induces expression of the three CAT genes (Xing et al., 2007). Because ABA signalling is compromised in the spl3 mutant (Figs 4, 6), it was next tested whether gene expression and enzymatic activity of catalases are also significantly down-regulated in the spl3 mutant.

To this end, the expression levels of rice catalase genes was investigated in the spl3 mutant. Rice has three CAT homologues, OsCatA, OsCatB, and OsCatC (Iwamoto et al., 2000; Mhamdi et al., 2010). The three OsCAT genes were found to be significantly up-regulated in the WT, in response to ABA treatment (Fig. 7A, C), similar to Arabidopsis catalases (Xing et al., 2007). Among these three rice catalases, OsCatA and
OsCatC mRNA levels were significantly down-regulated compared with the WT (Fig. 7A, C), whereas OsCatB mRNA levels were not altered in the spl3 mutant after ABA treatment (Fig. 7B). Furthermore, the CAT activities of spl3 and WT leaves were compared, and the spl3 leaves were found to have significantly lower CAT activity than the WT (Fig. 7D).

Discussion

By map-based cloning, the SPL3 locus was found to encode OsMAPKK1, a rice homologue of Arabidopsis EDR1 (Kim et al., 2003). Thus, it was also termed OsEDR1 (Kim et al., 2003; Shen et al., 2011). Previous studies of OsEDR1 mainly focused on its function in the biotic stress-responsive pathway; transgenic rice plants overexpressing OsEDR1 (OsEDR1-OX) displayed spontaneous hypersensitive response-like spots on the mature leaves, and concurrent up-regulation of defence-related genes and accumulation of phenolic compounds and phytoalexins. As a result, the OsEDR1-OX plants gained enhanced resistance to the rice blast fungal disease Magnaporthe grisea (Kim et al., 2009a). Moreover, another group reported that osedr1 knockout plants have enhanced resistance to the bacterial blight disease Xanthomonas oryzae pv. oryzae. This resistance was closely associated with increased accumulation of SA and JA and thus up-regulation of SA- and JA-associated gene expression, in parallel with decreased accumulation of the direct ethylene precursor ACC and down-regulation of ethylene-related gene expression (Shen et al., 2011). Finally, the authors concluded that OsEDR1 is not a functional homologue of AtEDR1 because of the different responses to pathogen attacks (Frye et al., 2001; Tang et al., 2005). Here it is shown that SPL3/OsMAPKK1/OsEDR1 functions as a transducer of ABA-responsive signalling and that the spl3 mutant showed strong ABA insensitivity, leading to several interesting phenotypes, such as delayed leaf senescence (Fig. 4; Supplementary Fig. S15 at JXB online).

In this study, it was found that the spl3 mutant was insensitive to ABA in several ABA-responsive processes. For example, in ABA-containing media, spl3 mutant produced longer primary roots and more adventitious roots than the WT (Fig. 5A–C), and showed hypersensitivity to drought (Fig. 5D, E) and osmotic stresses (Supplementary Fig. S11). Furthermore, stomatal closure in the spl3 leaves was insensitive to ABA treatment (Fig. 5F, G). All these results demonstrate that SPL3 is involved in ABA signal transduction.

It was also found that many ABA signalling-associated genes were significantly down-regulated in the spl3 leaves (Fig. 6). Among these genes, the physiological functions of several genes have been studied using rice overexpressing and/or antisense transgenic lines. OsABI5 knockdown lines showed low spikelet fertility because of aberrant pollen development (Zou et al., 2008). Similarly, the spl3 mutant showed low spikelet fertility (Supplementary Fig. S2) and OsABI5 was strongly down-regulated in the spl3 leaves in response to ABA treatment (Fig. 6E), suggesting that OsABI5 may be one of the important genes downstream of SPL3 in seed maturation. OshZIP23 (Fig. 6G) is considered a functional homologue of Arabidopsis AREB genes, based on physiological and phylogenetic studies (Xiang et al., 2008). The transgenic lines overexpressing OshZIP23 showed tolerance to drought and high salinity stresses, and the T-DNA-insertion knockout lines
showed sensitive phenotypes (Xiang et al., 2008). OsSAPK9 (Fig. 6l), a functional homologue of Arabidopsis SnRK2 (Kobayashi et al., 2005), was also significantly down-regulated in the spl3 leaves. The Arabidopsis SnRK2 proteins participate in ABA signal transduction by directly phosphorylating ABA-responsive element (ABRE)-binding factors, including AREB1 (Fujita et al., 2009). Similarly, OsSAPK9 phosphorylates one of the rice AREB homologues, TRAB1 (Kobayashi et al., 2005). Thus, it appears that the hypersensitivity of the spl3 mutant to drought and osmotic stresses may result from the down-regulation of OsbZIP23 and OsSAPK9.

It was also found that OsRePRP2.1, a positive regulator of ABA-dependent root growth inhibition (Tseng et al., 2013), was substantially reduced in the spl3 mutant (Fig. 6k), which probably contributes to the insensitivity of spl3 roots to ABA-mediated root growth inhibition (Fig. 5a–c). However, the spl3 seeds did not show higher germination rates in the presence of ABA, compared with the WT (Supplementary Fig. S12), indicating that SPL3 is involved in some, but not all of the ABA-responsive pathways, acting by indirectly regulating several ABA signalling-associated genes (Supplementary Fig. S15). Notably, SPL3 expression was down-regulated in response to ABA treatment (Supplementary Fig. S7), suggesting that SPL3 contributes only to activating the early phase of ABA signalling when SPL3 transcripts are abundant.

SPL3 regulates ROS production via the ABA signalling pathway

In addition, it was found that this strong ABA insensitivity of the spl3 mutant leads to the differential expression of OsCAT genes; expression of OsCatA and OsCatC is down-regulated in the spl3 mutant. Catalases in plants can be classified into three classes by organ/tissue specificity and expression pattern (Willekens et al., 1995; Mhamdi et al., 2010). Class I CATs are highly expressed in photosynthetic tissues while class II catalases in vascular tissues, and class III catalases in seeds and reproductive tissues (Mhamdi et al., 2010). Among the three OsCATs, OsCatC and OsCatA are classified class I and class II, respectively (Mhamdi et al., 2010). In Arabidopsis, the class I catalase cat2 mutant showed a severe necrotic lesion phenotype under long day conditions (Queval et al., 2007). Although the class II catalase cat1 mutant did not show a necrotic phenotype, a cat1 cat2 double mutant showed a much more severe lesion phenotype than the cat1 single mutant (Mhamdi et al., 2010), indicating that both CAT1 and CAT2 contribute to scavenging H2O2 in the leaves. Thus, it is possible that down-regulation of OsCatA and OsCatC in the spl3 mutant causes increased concentration of ROS in the mature leaves (Fig. 2), which leads to cell-death lesion formation around the heading stage (Fig. 1). Similar to the Arabidopsis cat2 mutant, the expressivity of the spl3 mutation is somewhat photoperiod-dependent; under CL conditions, the lesion mimic phenotype of the spl3 mutant is very severe compared with the mutant grown under SDs (Fig. 2). Taking these results together, it was concluded that the mRNA levels of two OsCAT genes (OsCatA and OsCatC) and catalase activity in the spl3 leaves were greatly suppressed, which probably leads to accumulation of excessive H2O2 and formation of lesions on the spl3 leaves (Supplementary Fig. S15).

Similar to the three CAT genes in Arabidopsis, the three OsCAT genes were also induced by ABA treatment (Fig. 7). Thus, it seems that down-regulation of OsCatA and OsCatC in the spl3 mutant is caused by an impairment of ABA signalling. In Arabidopsis, the MAPKK1-MAPK6 signalling cascade regulates metabolism of H2O2 scavenging by promoting CAT1 expression (Xing et al., 2008), similar to SPL3 function. A mutant of MEKKI, one of the Arabidopsis MAPKKKs, accumulates high levels of ROS and develops a local lesion mimic phenotype (Teige et al., 2004). Furthermore, Ning et al. (2010) reported that a mutant of DROUGHT-HYPERSENSITIVE MUTANT1 (DSM1), one of the OsMAPKKKs, accumulates excessive amounts of H2O2 under methyl viologen (MV)-induced oxidative stress, which is closely associated with down-regulation of two peroxidase genes, POX22.3 and POX8.1. These results indicate that several MAPK cascades play critical roles in controlling H2O2 scavenging in plants. To the authors’ knowledge, either OsMAPKs or OsMAPKKs that act downstream of SPL3/OsMAPKKK1 and regulate OsCAT expression have not yet been identified, although several MAPK genes, such as OsMAPK5, OsMAPK12, OsMAPK1, and OsMAPK2, are induced by ABA treatment (Xiong and Yang, 2003; You et al., 2007).

SPL3 promotes ABA and ethylene signalling pathways in leaf senescence

It was also found that the spl3 mutant showed delayed leaf yellowing during both natural and dark-induced senescence (Fig. 5). Concurrently, SPL3 expression increased during senescence (Supplementary Fig. S10), indicating that SPL3 contributes to promoting leaf senescence. In Arabidopsis, a few MAPK components have been revealed to be involved in the leaf senescence. Knockout mutants of MAPKK9 and MAPK6, which are known to form a MAPK cascade together, showed commonly delayed senescence, indicating that the MAPKK9-MAPK6 cascade definitely promotes leaf senescence (Zhou et al., 2009). MAPK6 accelerates SA-mediated leaf senescence by promoting the activity of NONEXPRESSOR OF PATHOGENESIS-RELATED GENE 1 (NPR1), which acts as a major component of SA-responsive signalling and as an inducer of leaf senescence (Ogawa et al., 2005; Chai et al., 2014). Arabidopsis EDR1 also functions in leaf senescence; the leaves of the edr1 mutant senesce early during ethylene-induced leaf senescence (Fry et al., 2001; Tang and Innes, 2002). Recently, Matsuoka et al. (2015) reported that Arabidopsis MAPKKK18 is also involved in leaf senescence, because the loss-of-function mutant of MAPKKK18 showed a delayed senescence phenotype (Matsuoka et al., 2015), indicating that MAPKKK18 positively regulates leaf senescence, similar to SPL3.

In the present study, it has been revealed how SPL3 exerts its function in the promotion of leaf senescence. When the rice plants enter the senescence phase, several ethylene- and ABA-associated genes are down-regulated in the spl3 mutant.
(Supplementary Figs. S13 and S14), which probably leads to delaying leaf yellowing during natural and dark-induced senescence (Fig. 4). ABA and ethylene promote leaf yellowing (Gepstein and Thimann, 1981; Nooden, 1988), and Arabidopsis mutants or transgenic plants overexpressing genes that are related to ABA or ethylene signalling exhibited different senescence phenotypes (Kusaba et al., 2013). In this study, it was found that OsABI5, OsEIN2, and OsEIN3 were down-regulated in the spl3 mutant during senescence (Supplementary Figs S13 and S14). Although previous work reported that ethylene synthesis is impaired in the osedr1 mutant (Shen et al., 2011), the finding that OsEIN2 and OsEIN3 are down-regulated in the spl3 mutant indicates that both ethylene-synthesis and -signalling pathways are impaired in the spl3 mutant during senescence. Arabidopsis homologues of these three genes were previously identified as senescence-promoting transcription factors. Arabidopsis ABI5 and EIN3 directly promote the expression of ORESARA1 (ORE1; Kim et al., 2014; Sakuraba et al., 2014), which encodes a key senescence-promoting NAC transcription factor (Kim et al., 2009b). EIN2 also activates OREI expression by repressing the expression of miR164, which cleaves the ORE1 mRNA; EIN2 also functions by a miR164-independent pathway (Kim et al., 2009b; Li et al., 2013). Furthermore, EIN3 also directly promotes the expression of NAP (Kim et al., 2014), another key senescence-promoting NAC transcription factor (Guo and Gan, 2006). The rice homologue OsNAP was found to be down-regulated in the spl3 mutant during senescence (Fig. 4E). OsNAP is induced by ABA treatment and directly activates ABA-responsive genes (Chen et al., 2014; Liang et al., 2014), similar to AtNAP function (Zhang and Gan, 2012; Yang et al., 2014). Thus, it is probable that down-regulation of OsNAP expression in the spl3 mutant was caused by an impairment of both ABA- and ethylene-responsive signalling pathways.

Here, it has been shown that SPL3 positively regulates the ABA-responsive signalling pathway, which affects several important processes, including root elongation, abiotic stress responses, stomatal closure, and leaf senescence (Figs 4 and 5; Supplementary Fig. S15). SPL3 indirectly promotes ABA and ethylene signalling (Fig. 6; Supplementary Figs S13 and 14), while suppressing both SA- and JA-associated defence signalling (Kim et al. 2009a; Shen et al., 2011). Thus, SPL3 has a vital role in the crosstalk among important phytohormone signalling-associated processes, such as abiotic stress signalling, leaf senescence, and defence against pathogens. Because SPL3 is one of the OsMAPKKs (Kim et al., 2003), SPL3 likely regulates specific MAPKKs and MAPKs in the ethylene and ABA signalling pathways. Large-scale interactome analysis between OsMAPKKs and OsMAPKs will be necessary to reveal the SPL3-dependent MAPK cascade, as described in the previous study of OsMAPKKs and OsMAPKs (Singh et al., 2012).

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Lesion mimic phenotype of the spl3 mutant is predominant in the tip region of leaf blades.

Fig. S2. Difference in plant height of the WT and spl3 mutant.

Fig. S3. Agronomic traits of the spl3 mutant.

Fig. S4. Amino acid sequence alignment of SPL3 and its homologues in other plant species.

Fig. S5. Complementation of the spl3 mutant by transformation with J55:SPL3.

Fig. S6. Expression of SPL3 in different organs of rice plants.

Fig. S7. Expression of SPL3 under different abiotic stress conditions.

Fig. S8. Senescence phenotype of the spl3 leaves under ABA, ACC, MeJA, and SA treatments.

Fig. S9. No difference of heading date in the WT and spl3 mutant in the paddy field.

Fig. S10. Expression of SPL3 during natural and dark-induced senescence.

Fig. S11. The spl3 mutant is hypersensitive to osmotic stress.

Fig. S12. The effect of ABA on the germination rate of WT and spl3 seeds.

Fig. S13. Altered expression of ABA-responsive genes in the spl3 mutant during natural senescence.

Fig. S14. Altered expression of ET signalling- and synthesis-related genes in the spl3 mutant during natural senescence.

Figure S15. Tentative model of the role of SPL3 in ABA-responsive signalling pathways.

Table S1. Primers used in this study.

**Accession numbers**

Sequence data from this article can be found in the National Center for Biotechnology Information (NCBI) or GenBank/EMBL databases under the following accession numbers: OsABI1, Os09g0532400; OsABI3, Os01g091170; OsABI4, Os05g0351200; OsABI5, Os09g0456200; OsACSI, Os01g0978100; OsACS2, Os04g0578000; OsAREB1, Os06g0211200; OsbZIP23, Os02g0766700; OsCatA, Os02g0115700; OsCatB, Os06g0727200; OsCatC, Os03g0131200; OsDSG1, Os09g0434200; OsDSR1, Os10g0177200; OsEIN2, Os07g0155600; OsEIN3, Os03g0324200; OsNAP, Os03g0327800; NYC1, Os01g0227100; OsRePRP2.1, Os07g0418700; OsSAPK6, Os02g0551100; OsSAPK8, Os03g0764800; OsSAPK9, Os12g0586100; SGR, Os09g0532000; OsUBQ5, Os01g0328400; SPL3, Os03g0160100.

**Acknowledgements**

We thank Prof. Nam-Soo Jwa for donating the T-DNA insertion osedr1 mutant seeds. This work was carried out with the support of ‘Next-Generation BioGreen21 Program for Agriculture & Technology Development (Project No. PJ01106301)’, Rural Development Administration, Republic of Korea. The authors declare no competing financial interests.

**Conflict of interest disclosure**

The authors declare that they have no conflict of interest.

**References**

Agrawal GK, Iwahashi H, Rakwal R. 2003. Rice MAPKs. Biochemical and Biophysical Research Communications 302, 171–180.
Balogue C, Lin B, Alcon C, Flottes G, Malmstrom S, Kohler C, Neuhaus G, Pelletier G, Gaymand F, Roby D. 2003. HLM1, an essential signalling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. Plant Cell 15, 365–379.

Brossa R, López-Carbonell M, Juby-Mari T, Alegre L. 2011. Interplay between abscisic acid and jasmonic acid and its role in water-deficit stress in wild-type, ABA-deficient, JA-deficient, and ascorbate-deficient Arabidopsis plants. Journal of Plant Growth Regulation 30, 322–333.

Chai J, Liu J, Zhou J, Xing D. 2014. Mitogen-activated protein kinase 6 regulates NPR1 gene expression and activation during leaf senescence induced by salicylic acid. Journal of Experimental Botany 65, 6513–6528.

Chen X, Hao L, Pan J, Zheng X, Jiang G, Yin Y, Gu Z, Qian Z, Zhai W, Ma B. 2012. SPL5, a cell death and defense-related gene, encodes a putative splicing factor 3b subunit 3 (SF3b3) in rice. Molecular Breeding 30, 939–949.

Chen X, Wang Y, Lv B, Li J, Luo L, Lu S, Zhang X, Ma H, Ming F. 2014. The NAC family transcription factor OsNAP confers abiotic stress response through the ABA pathway. Plant and Cell Physiology 55, 604–619.

Clarke SM, Cristescu SM, Miersch O, Harren FJ, Westnack C, Mur LA. 2009. Jasmonates act with salicylic acid to confer basal thermotolerance in Arabidopsis thaliana. New Phytologist 182, 175–187.

Colcombet J, Hirt H. 2008. Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. Biochemical Journal 413, 217–226.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiology 133, 462–469.

De Smet I, Zhang H, Inze D, Beeckman T. 2006. A novel role for abscisic acid emerges from underground. Trends in Plant Science 11, 434–439.

Dietrich RA, Richberg MH, Schmidt R, Dean C, Dangl JL. 1997. A novel zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as a negative regulator of plant cell death. Cell 88, 685–694.

Frye CA, Tang D, Innes RW. 2001. Negative regulation of defense responses in plants by a conserved MAPK/ kinase. Proceedings of the National Academy of Sciences USA 98, 373–378.

Fujita Y, Fujita M, Satoh R, et al. 2005. ABE1 is a transcription activator of novel ABR-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. Plant Cell 17, 3470–3488.

Fujita Y, Nakashima K, Yoshida T, et al. 2009. Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. Plant and Cell Physiology 50, 2123–2132.

Gao L, Xiang CB. 2008. The genetic locus At1g73660 encodes a putative MAPK/kinase and negatively regulates salt tolerance in Arabidopsis. Plant Molecular Biology 67, 125–134.

Gepstein S, Thimann KV. 1981. The role of ethylene in the senescence of oat leaves. Plant Physiology 68, 349–354.

Guo Y, Gan S. 2006. AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant Journal 46, 601–612.

Hamel LP, Nicole MC, Sritubtim S, et al. 2006. Ancient signals: comparative genomics of plant MAPK and MAPKK gene families. Trends in Plant Science 11, 192–198.

Han SH, Sakuraba Y, Koh HJ, Paek NC. 2012. Leaf variegation in the rice zebra2 mutant is caused by photoperiodic accumulation of tetra-cis-lycopene and singlet oxygen. Molecules and Cells 33, 87–97.

Hoisington DA, Neuffer MG, Walbot V. 1982. Disease lesion mimics in maize. I. Effect of genetic background, temperature, developmental age, and wounding on necrotic spot formation with Les1. Developmental Biology 93, 381–398.

Huang Y, Li CY, Qi Y, Park S, Gibson SL. 2014. SiS8, a putative mitogen-activated protein kinase protein kinase, regulates sugar-resistant seedling development in Arabidopsis. Plant Journal 77, 577–588.

Iwamoto M, Higo H, Higo K. 2000. Differential diurnal expression of rice catalase genes: the 5′-flanking region of CatA is not sufficient for circadian control. Plant Science 151, 39–46.

Jeon JS, Lee S, Jung KH, et al. 2000. T-DNA insertional mutagenesis for functional genomics in rice. Plant Journal 22, 561–570.

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR. 1993. CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell 72, 427–441.

Kim JA, Agrawal GK, Rakwal R, Han KS, Kim KN, Yun CH, Heu S, Park SY, Lee YH, Jwa NS. 2003. Molecular cloning and mRNA expression analysis of a novel rice (Oryza sativa L.) MAPK kinase gene, OsERD1, an ortholog of Arabidopsis AERD1, reveals its role in defense/stress signalling pathways and development. Biochemical and Biophysical Research Communications 300, 868–876.

Kim JA, Cho K, Singh R, et al. (2009a) Rice OsACD1 (Oryza sativa Accelerated Cell Death and Resistance 1) is a potential positive regulator of fungal disease resistance. Molecules and Cells 28, 431–439.

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang DG, Nam HG. 2009b. Tricufate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. Science 323, 1053–1057.

Kim TW, Michniewicz M, Bergmann DC, Wang ZY. 2012. Brassinosteroid regulates stomatal development by GSK3-mediated inhibition of a MAPK pathway. Nature 482, 419–422.

Kim HJ, Hong SH, Kim YW, et al. 2014. Gene regulatory cascade of senescence-associated NAC transcription factors activated by ETHYLENE-SENSITIVE2-mediated leaf senescence signalling in Arabidopsis. Journal of Experimental Botany 65, 4023–4036.

Kobayashi Y, Murata M, Minami H, Yamamoto S, Kagaya Y, Hobe T, Yamamoto A, Hattori T. 2005. Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. Plant Journal 44, 939–949.

Kusaba M, Ito H, Morita R, et al. 2007. Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. Plant Cell, 19, 1362–1375.

Kusaba M, Tanaka A, Tanaka R. 2013. Stay-green plants: what do they tell us about the molecular mechanism of leaf senescence. Photosynthesis Research 117, 221–234.

Kusum K, Komori H, Satoh H, Iba K. 2000. Characterization of a zebrafish mutant of rice with increased susceptibility to light stress. Plant and Cell Physiology 41, 158–164.

Lee SH, Sakuraba Y, Lee T, Kim KY, An G, Lee HY, Paek NC. 2015. Mutation of Oryza sativa CORONATINE INSENSITIVE1b (OsCOI1b) delays leaf senescence. Journal of Integrative Plant Biology 57, 562–576.

Lei G, Shen M, Li ZG, et al. 2011. EIN2 regulates salt stress response and interacts with a MA3 domain-containing protein ECIP1 in Arabidopsis. Plant, Cell and Environment 34, 1678–1692.

Li J, Pandeya N, Nath K, et al. 2010. ZEBRA-NECROSIS, a thylakoid-bound protein, is critical for the photoprotection of developing chloroplasts during early leaf development. Plant Journal 62, 715–725.

Li Z, Peng J, Wen X, Guo H. 2013. Ethylene-insensitive3 is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing miR164 transcription in Arabidopsis. Plant Cell 25, 3311–3328.

Liang C, Wang Y, Zhu Y, et al. 2014. OsNAP connects abscisic acid and leaf senescence by fine-tuning abscisic acid biosynthesis and directly targeting senescence-associated genes in rice. Proceedings of the National Academy of Sciences USA 111, 10013–10018.

Ligterink W. 2000. MAP kinases in plant signal transduction: how many, and what for? Results and Problems in Cell Differentiation 27, 11–27.

Lim JH, Yang HJ, Jung KH, Yoo SC, Paek NC. 2014. Quantitative trait locus mapping and candidate gene analysis for plant architecture traits using whole genome re-sequencing in rice. Molecules and Cells 37, 149–160.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408.

Lorrain S, Lin B, Auriac MC, Krog T, Saindrenan P, Nicole M, Balagué C, Roby D. 2004. VASCULAR ASSOCIATED DEATH1, a novel GRAM domain-containing protein, is a regulator of cell death and defense responses in vascular tissues. Plant Cell 16, 2217–2232.

Matsuoka D, Yasufuku T, Furuya T, Nanmori T. 2015. An abscisic acid inducible Arabidopsis MAPKKK, MAPKKK18 regulates leaf senescence via its kinase activity. Plant Molecular Biology 87, 565–575.
Mhamdi A, Queval G, Chaouch S, Vanderauwera S, Van Breusegem F, Noctor G. 2010. Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. Journal of Experimental Botany 61, 4197–4220.

Mosher S, Moeder W, Nishimura N, Jikumaru Y, Jou, Sh, Urquhart W, Klessig DF, Kim SK, Nambara E, Yoshiba K. 2010. The lesion-mimic mutant cpr22 shows alterations in abscisic acid signalling and abscisic acid insensitivity in a salicylic acid-dependent manner. Plant Physiology 152, 1901–1913.

Nakashima K, Yamaguchi-Shinozaki K. 2013. ABA signalling in stress-response and seed development. Plant Cell Reports 32, 959–970.

Ning J, Li X, Hicks LM, Xiong L. 2010. A Raf-like MAPKKK gene DSM1 mediates drought resistance through reactive oxygen species scavenging in rice. Plant Physiology 152, 876–890.

Nooden LD. 1988. Postlude and prospects. Senescence and aging in plants (Academic Press), pp 499–517.

Noutoshi Y, Kuromori T, Wada T, et al. 2006. Loss of Nectroc Spotted Lesions 1 associates with cell death and defense responses in Arabidopsis thaliana. Plant Molecular Biology 62, 29–42.

Ogawa T, Pan L, Kawai-Yamada M, et al. 2005. Functional analysis of Arabidopsis ethylene-responsiveness element binding protein conferring resistance to Bax and abiotic stress-induced plant cell death. Plant Physiology 138, 1436–1445.

Park GG, Park JJ, Yoon J, Yu SN, An G. 2010. A RING finger E3 ligase gene, Oryza sativa Delayed Seed Germination 1 (OsdSg1), controls seed germination and stress responses in rice. Plant Molecular Biology 74, 467–478.

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

Porra R, Thompson W, Kriedemann P. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta—Bioenergetics 975, 384–394.

Qiao Y, Jiang W, Tang D, Christiansen KM, Innes RW. 2005. Catalase function in plants: a focus on Arabidopsis photorespiratory mutant cat2 demonstrate that redox state of the cytoplasmic H2O2 pool regulates rice bacterial resistance via activation of ethylene biosynthesis. Plant Molecular Biology 58, 525–536.

Shirouzu M, Iwata N, Ideta O, Iwata N. 2010. Identification and characterization of suppressor mutants of spl11-mediated cell death in rice. Molecular Plant-Microbe Interactions 27, 528–536.

Singh R, Singh J, Lee MO, Lee JE, et al. 2012. Rice mitogen-activated protein kinase interactome analysis using the yeast two-hybrid system. Plant Physiology 160, 477–487.

Takahashi Y, Soyano T, Kosetsu K, Sasabe M, Machida Y. 2010. HINKEL kinase, ANP MAPKKKs and MKK6/ANQ MAPKK, which phosphorylates and activates MPK4 MAPK, constitute a pathway that is required for cytokinesis in Arabidopsis thaliana. Plant and Cell Physiology 51, 1766–1776.

Tang D, Innes RW. 2002. Overexpression of a kinase-deficient form of the EDR1 gene enhances powdery mildew resistance and ethylene-induced senescence in Arabidopsis. Plant Journal 32, 975–983.

Tang D, Christiansen KM, Innes RW. 2005. Regulation of plant disease resistance, stress responses, cell death, and ethylene signalling in Arabidopsis by the EDR1 protein kinase. Plant Physiology 138, 1018–1026.

Teige M, Schei M, Eulgem T, Doebeli M, Schmid M, Shinozaki K, Hirt H. 2003. The MKK2 pathway mediates cold and salt stress signalling in Arabidopsis. Molecular Cell 15, 141–152.

Tseng IC, Hong CY, Yu SM, Ho TH. 2013. Abscisic acid- and stress-induced highly proline-rich glycoproteins regulate root growth in rice. Plant Physiology 163, 118–134.

Wang L, Pei Z, Tian Y, He C. 2005. OsLSD1, a rice zinc finger protein, regulates programmed cell death and cellus differentiation. Molecular Plant-Microbe Interactions 18, 357–384.

Widmann C, Gibson S, Jarpe MB, Johnson GL. 1999. Mitogen-activated protein kinases: conservation of a three-kinase module from yeast to human. Physiological Reviews 79, 143–180.

Willekens H, Inzé D, Van Montagu M, Van Camp W. 1995. Catalases in plants. Molecular Breeding 1, 207–228.

Wolter M, Hollricher K, Salamin F, Schulze-Lefert P. 1993. The mi resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. Molecular and General Genetics 239, 122–128.

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

Xiang Y, Tang N, Hu H, Ye H, Xiong L. 2008. Characterization of OsZIP23 as a key player of the basic zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. Plant Physiology 148, 1938–1952.

Xing Y, Jia W, Zhang J. 2007. AtMEK1 mediates stress-induced gene expression of CAT1 catalase by triggering H2O2 production in Arabidopsis. Journal of Experimental Botany 58, 2969–2981.

Xing Y, Jia W, Zhang J. 2008. AtMKS1 mediates ABA-induced expression of H2O2 production via ATRIPK6-coupled signalling in Arabidopsis. Plant Journal 54, 440–451.

Xiong L, Yang Y. 2003. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell 15, 745–759.

Yamanouchi U, Yano M, Lin H, Ashikari M, Yamada K. 2002. A rice spotted leaf gene, Sp17, encodes a heat stress transcription factor protein. Proceedings of the National Academy of Sciences USA 99, 7530–7535.

Yang J, Worley E, Udvardi M. 2014. A NAP-AAC3 regulatory module promotes chlorophyll degradation via ABA biosynthesis in Arabidopsis leaves. Plant Cell 26, 4862–4874.

Yoo SD, Sheen J. 2008. MAPK signalling in plant hormone ethylene signal transduction. Plant Signalling and Behavior 3, 848–849.

Yoshida T, Fujita Y, Sayama H, Kidokoro S, Maruyama K, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K. 2010. AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABA-dependent ABA signalling involved in drought stress tolerance and require ABA for full activation. Plant Journal 61, 672–685.

Yoshimura A, Ideta O, Iwata N. 1997. Linkage map of phenotype and AFLP markers in rice. Plant Molecular Biology 36, 49–60.
You MK, Oh S, Ok SH, Cho SK, Shin HY, Jeung JU, Shin JS. 2007. Identification of putative MAPK kinases in Oryza minuta and O. sativa responsive to biotic stresses. *Molecules and Cells* **23**, 108–114.

Zeng LR, Qu S, Bordeos A, Yang C, Baraoidan M, Yan H, Xie Q, Nahm BH, Leung H, Wang GL. 2004. Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. *Plant Cell* **16**, 2795–2808.

Zhang K, Gan SS. 2012. An abscisic acid-AtNAP transcription factor-SAG113 protein phosphatase 2C regulatory chain for controlling dehydration in senescing Arabidopsis leaves. *Plant Physiology* **158**, 961–969.

Zhou C, Cai Z, Guo Y, Gan S. 2009. An Arabidopsis mitogen-activated protein kinase cascade, MKK9-MPK6, plays a role in leaf senescence. *Plant Physiology* **150**, 167–177.

Zhou J, Zhang H, Yang Y, Zhang Z, Zhang H, Hu X, Chen J, Wang XC, Huang R. 2008. Abscisic acid regulates TSRF1-mediated resistance to Ralstonia solanacearum by modifying the expression of GCC box-containing genes in tobacco. *Journal of Experimental Botany* **59**, 645–652.

Zou M, Guan Y, Ren H, Zhang F, Chen F. 2008. A bZIP transcription factor, OsABI5, is involved in rice fertility and stress tolerance. *Plant Molecular Biology* **66**, 675–683.