

# Overexpression of miR529a confers enhanced resistance to oxidative stress in rice (*Oryza sativa* L.)

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## Abstract

**Key message** Overexpressing miR529a can enhance oxidative stress resistance by targeting *OsSPL2* and *OsSPL14* genes that can regulate the expression of their downstream SOD and POD related genes.

**Abstract** MicroRNAs are involved in the regulation of plant developmental and physiological processes, and their expression can be altered when plants suffered environment stresses, including salt, oxidative, drought and Cadmium. The expression of microRNA529 (miR529) can be induced under oxidative stress. However, its biological function under abiotic stress responses is still unclear. In this study, miR529a was overexpressed to investigate the function of miR529a under oxidative stress in rice. Our results demonstrated that the expression of miR529a can be induced by exogenous H<sub>2</sub>O<sub>2</sub>, and overexpressing miR529a can increase plant tolerance to high level of H<sub>2</sub>O<sub>2</sub>, resulting in increased seed germination rate, root tip cell viability, reduced leaf rolling rate and chlorophyll retention. The expression of oxidative stress responsive genes and the activities of superoxide dismutase (SOD) and peroxidase (POD) were increased in miR529a overexpression plant, which could help to reduce redundant reactive oxygen species (ROS).

Furthermore, only *OsSPL2* and *OsSPL14* were targeted by miR529a in rice seedlings, repressing their expression in miR529aOE plants could lead to strengthen plant tolerance to oxidation stress. Our study provided the evidence that overexpression of miR529a could strengthen oxidation resistance, and its target genes *OsSPL2* and *OsSPL14* were responsible for oxidative tolerance, implied the manipulation of miR529a and its target genes regulation on H<sub>2</sub>O<sub>2</sub> related response genes could improve oxidative stress tolerance in rice.

**Keywords** miR529a · Oxidative stress · SQUAMOSA promoter-binding protein like (SPL) · H<sub>2</sub>O<sub>2</sub> · Rice

## Introduction

Rice is usually subjected to various abiotic stresses in the process of growth and development, such as high and low temperature, drought, heavy metals and salt (Cui et al. 2015; Dionisio-Sese and Tobita 1998; Nigam et al. 2015; Tang et al. 2014). The plants exhibit growth retardation under stresses, which can lead to reduce crop yield and quality, even cause death. Reactive oxygen species (ROS) including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide radical (O<sub>2</sub><sup>·-</sup>), and hydroxyl radicals (.OH) are by-products of aerobic metabolism, which are controlled by antioxidant defense systems (Gill and Tuteja 2010; Mittler 2002; You and Chan 2015). When plants constantly suffer from environmental stresses, the generated ROS will be beyond the ability of the active oxygen scavenging system, which will cause oxidative damage, eventually resulting in cell apoptotic death. H<sub>2</sub>O<sub>2</sub>, acting as a signaling molecule or a second messenger (Quan et al. 2008; Vranova et al. 2002), can adjust plant development, adaptation of environmental

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stresses, respiration and programmed cell death (PCD) (Neill et al. 2002; Orozco-Cardenas et al. 2001).

Recently, many H<sub>2</sub>O<sub>2</sub> stress related response genes have been discovered. Constitutive expression of pathogenesis-related genes 5 (*CPR5*), a new type of regulatory factor (Orjuela et al. 2013), is involved in the regulation of seed germination, seedling development and plant ROS signaling pathway of oxidative stress (Bowling et al. 1997; Gao et al. 2011). The vacuole was known to affect oxidative stress response, and the vacuolar processing enzymes (VPEs) are participated in H<sub>2</sub>O<sub>2</sub>-induced PCD pathway (Deng et al. 2011). In addition, *Os04g0376100* and *Os10g0519700*, encoding for translocase of the inner/out mitochondrial membrane (TIM/TOM) complexes, were also found to be related to H<sub>2</sub>O<sub>2</sub> signaling pathway (Pegoraro et al. 2012).

MicroRNA (miRNA) is one of the most important regulators to control gene expression, which is involved in many fundamental biological processes, and plays the key role in the process of plant development (Jones-Rhoades et al. 2006). A lot of miRNAs have emerged as important players in plant responses to biotic and abiotic stresses (Sunkar et al. 2007). Among them, miR398 mediated responses to oxidative stress through regulating the expression of its target genes (Dugas and Bartel 2008; Lu et al. 2011). MiR529 was down-regulated in response to cold and drought stresses (Gupta et al. 2014; Zhou et al. 2010), and it was identified to be responsive to exogenous H<sub>2</sub>O<sub>2</sub> stress in rice (Li et al. 2011). However, the mechanism of miR529 response to oxidative stress is still not clear. MiR529 was present in the common ancestor of embryophytes but lost in eudicots (Cuperus et al. 2011), which contains miR529a and miR529b two members (Zhang et al. 2015). MiR529 targets SQUAMOSA promoter-binding protein like (*SPL*) genes, a plant specific transcription factor in regulating plant growth and development (Chen et al. 2010). Previous studies showed that *SPLs*, especially *SPL2* and *SPL11* promote the expression of heat stress (HS)-responsive genes, which is critical only after exposure to HS, and *SPL3*, *SPL14* and *SPL17* are involved in regulating the expression of cold stress responsive genes (Cui et al. 2014, 2015; Stief et al. 2014). Whereas, the network of miR529a-*SPLs* in plant ROS signaling pathway of oxidative stress is still not clear.

Here, we reported a systematic investigation of H<sub>2</sub>O<sub>2</sub>-regulated miR529a and its target genes in rice. Overexpressing miR529a showed that only *OsSPL2* and *OsSPL14* two genes were targeted by miR529a in rice seedlings. The expression of H<sub>2</sub>O<sub>2</sub>-responsive genes were increased by repressing the expression of miR529a targeted genes *OsSPL2* and *OsSPL14*, which could lead to increase the expression of ROS-responsive genes and

finally elevate the activities of SOD and POD to strengthen plant tolerance to oxidation stress. Our results suggested that manipulation of miR529a and its target genes could improve oxidative stress tolerance in rice.

## Materials and methods

### Plants materials and growth conditions

Two japonica cultivars Nipponbare and Aikawa1 were used in this study. Seeds were shelled and sterilized in 1% NaClO for 20–30 min, washed with distilled water for at least four times, placed on sterile MS solid culture medium, and kept at the conditions of 28 °C/21 °C, 14 h light/10 h dark, and 75% relative humidity in growth cabinet for germination and growth. The seedlings were then placed in MS liquid culture solution after 7 days growth.

### Vector construction and rice transformation

To overexpress miR529a in rice, the 456 bp pri-miR529a sequence including stem-loop structure was amplified by PCR with specific primers (Table S1), PCR product was then cloned into the *SacI*–*SmaI* sites of the binary vector pCAMBIA1300, which was driven by a maize *Ubiquitin* promoter (Fig. S1). And the constructed vector was then transferred to *Agrobacterium tumefaciens* strain EHA105 for transformation. A modified high-efficiency transformation system by co-cultivating rice calli with *Agrobacterium* was used in our experiment (Ozawa 2009). The transgenic rice plants carrying *MIR529a* was confirmed by PCR using the specific primers (Table S1). The homozygotes of T3 generation of transgenic plants (miR529aOE3, OE5, and OE6) were chosen for further study.

### Seed germination test and phenotype analysis of rice seedling

Rice seeds were soaked at 37 °C in the dark for 24 h, were then moved on filter paper wetted by ddH<sub>2</sub>O supplemented with different concentrations of H<sub>2</sub>O<sub>2</sub> in petri dishes, and incubated at 27 °C with 14/12 h light/dark cycles for three days. The germination rate was calculated using 30 seeds in each treatment (Deng et al. 2011).

The four-leaf seedlings were immersed in the solutions of different concentrations of H<sub>2</sub>O<sub>2</sub> (0, 0.6, 3, and 15 mM) for 6 h to detect phenotype response. The number of rolling leaf was calculated and equal amounts of leaves (0.15 g) were harvested and frozen in liquid nitrogen, then stored at –80 °C.

### Detection of ROS formation in root tip with FDA staining

The rice root tips were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> for 3 days, and the primary roots were then incubated in 20 μM H2DCF-DA [initially dissolved in dimethyl sulfoxide (DMSO)] at 4 °C for 1 h (Foreman et al. 2003). The roots were washed and immersed with 0.1 mM KCl and 0.1 mM CaCl<sub>2</sub> (pH 6.0) at 22 °C for 1 h, and were immediately mounted and photographed under a fluorescence microscope (Nikon AZ100, Japan) equipped with a filter set (Fluorescence was detected using wavelength of 488 nm excitation and emission wavelength of 505–545 nm band pass filter).

### The activities of antioxidant enzymes analysis

Leaf was grounded to fine powder by grinding machine (Shanghai Jingxin, China), then transferred to 10 mL tubes and added 5 mL of 50 mM phosphate buffer (PBS, pH 7.8). The samples were then violently oscillated for 1 min, and homogenates were centrifuged at 8000g for 25 min at 4 °C. Supernatants were then collected into fresh tubes and stored at 4 °C for enzyme analysis.

SOD (EC 1.15.1.1) activity was measured according to nitro blue tetrazolium (NBT) method by measuring the photo-reduction of NBT at OD<sub>560</sub> (Beers and Sizer 1952; Qiao et al. 2010); activity of POD (EC1.11.1.7) was assayed by monitoring guaiacol at 470 nm by spectrophotometry (Egley et al. 1983).

### Histochemical detection of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup>

The content of H<sub>2</sub>O<sub>2</sub> in the leaf was measured spectrophotometrically as described previously (Willekens et al. 1997). And 3, 3'-diaminobenzidine (DAB) and NBT staining were used to determine the accumulation of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup>, respectively (Wohlgemuth et al. 2002).

For H<sub>2</sub>O<sub>2</sub> detection, the treated rice leaf was immediately immersed into 1 mg/mL DAB solution (pH 3.8) and incubated for 6 h in light at 25 °C. And leaf was then bleached in 95% boiling ethanol for at least 10 min. This step decolorized the leaf with the exception of the brown polymerization spots produced by the reaction of DAB with H<sub>2</sub>O<sub>2</sub>. As for observation of the visualized O<sub>2</sub><sup>•-</sup> accumulation, NBT staining solution was prepared by dissolving NBT with 25 mM HEPES free acid (pH 7.8) to a concentration of 0.5 mg/mL. Leaf was then collected and completely submerged into NBT solution. The following steps were the same as the procedures of DAB staining except for incubation in dark for 6 h instead of light.

### Chlorophyll content assay

The flag leaf was treated with varied concentrations of H<sub>2</sub>O<sub>2</sub> (0, 0.6, 3, and 15 mM) for 5 days. 0.1 g leaf was grind into powder, and then infiltrated in 10 mL 95% ethanol solution at room temperature in dark for 3–5 days. A<sub>665</sub> and A<sub>649</sub> of the extracts were monitored using a Multifunction Microplate Reader (BioTek Synergy H1, Vermont, USA). The chlorophyll content was calculated using the formula:

$$[Chl_a] = 13.95A_{665} - 6.88A_{649}$$

$$[Chl_b] = 24.96A_{649} - 7.32A_{665}$$

$$[Chl_t] = [Chl_a] + [Chl_b]$$

The total content of chlorophyll (mg/g) = [chlorophyll concentration × extraction volume/sample (fresh weight)]. All experiments were repeated three times.

### RNA extraction, qRT-PCR and stem-loop qRT-PCR

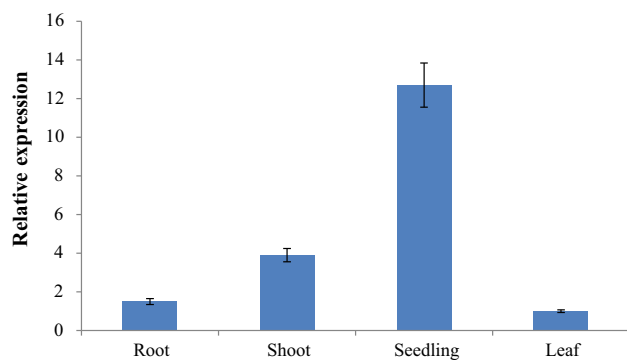
Total RNA was extracted from rice seedlings using TRIzol reagent (Invitrogen, USA). PrimeScript<sup>TM</sup> RT reagent Kit with gDNA Eraser (Takara, Japan) was used to remove residues genomic DNA, then the first strand of cDNA was synthesized. The qRT-PCR was performed on a Roche LightCycler<sup>®</sup> 96 Real-time PCR System (Roche, Switzerland) by using SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> II (Tli RNaseH Plus) (Takara, Japan). The conditions were as follows: denature at 95 °C for 30 s, 40 cycles at 94 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s. *Osef1-α* (*LOC\_Os03g08050*) was used as an internal reference. To enable statistical analysis, three independent biological replicates were obtained and subjected to qRT-PCR in triplicate.

Stem-loop qRT-PCR was performed to measure the expression of miR529a (Chen et al. 2005; Kramer 2011; Varkonyi-Gasic and Hellens 2011). U6 snRNA was used as the internal control for normalizing the raw data. Gene-specific primers are shown in supporting information Table S1.

## Results

### Expression profile of miR529a and miR529a overexpression

At vegetative stage, miR529a was highly expressed in seedling, and lowly expressed in shoot, root and leaf (Fig. 1). Previous study showed that miR529a was up regulated under H<sub>2</sub>O<sub>2</sub> treatment (Li et al. 2011), four-leaf stage rice seedling was then treated with different concentrations of H<sub>2</sub>O<sub>2</sub> (0, 0.6, 3, and 15 mM) for 6 h, the expression of miR529a was increased gradually with the



**Fig. 1** The expression patterns of miR529a in different rice tissues. The materials were obtained from rice root (19 days growth after planting), shoot (7 day-old plants), seedling (2-week-old plants), and leaf (6-week-old plants)

rising of concentration of  $H_2O_2$  (Fig. 2a), which was consistent with the previous study (Li et al. 2011).

To clarify the function of miR529a responding to  $H_2O_2$ , miR529a overexpression (miR529aOE) vector was constructed to generate transgenic plants, six independent transgenic lines with similar phenotype were obtained (Fig. S1b). The results displayed that the expression of miR529a was around 15 times higher in miR529aOE plants than in WT plants (Fig. 2b), while there was no significant difference in the phenotype of WT and miR529aOE plants at seedling stage.

### miR529a overexpression promotes plant tolerance to $H_2O_2$ stress

No significant difference of germination capacity was exhibited in WT, miR529aOE and Aikawa1 under normal conditions. While the germination rate was dramatically reduced in WT ( $58 \pm 1.4\%$ ) and Aikawa1 ( $38.9 \pm 9.4\%$ ),

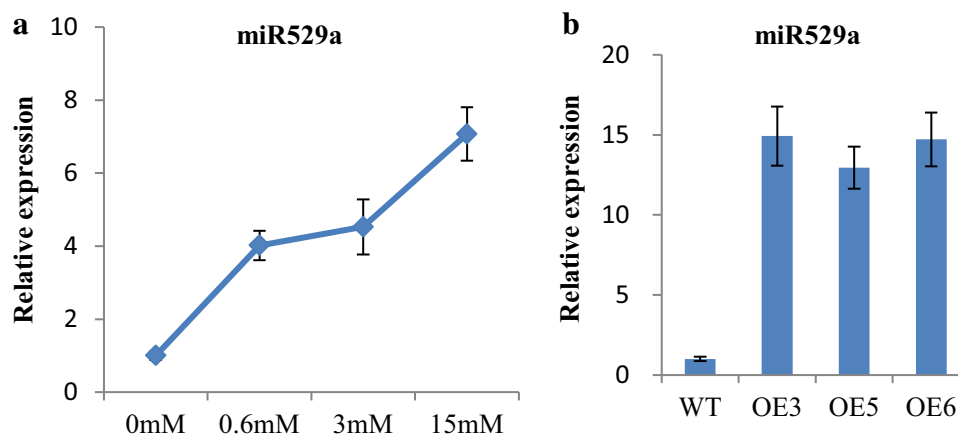
compared to miR529aOE ( $83.3 \pm 6.9\%$ ) after treated with 15 mM  $H_2O_2$  for 3 days (Fig. 3).

The fluorescein diacetate (FDA) method was performed to detect the ROS formation in root tip, and the miR529aOE maintained higher cell viability after exposure to 15 mM  $H_2O_2$  for 5 days compared to WT, while Aikawa1 showed the lowest cell viability (Fig. 3c).

The WT showed obvious severely wilted symptoms, such as water loss and leaf roll, whereas miR529aOE remained relatively healthy when the seedlings (four-leaf stage) were subjected to oxidation stress (15 mM  $H_2O_2$ ) for 6 h (Fig. 4a). Statistical analysis showed that miR529aOE plants exhibited significantly less rolling leaf than WT under oxidative stress (Fig. 4b).

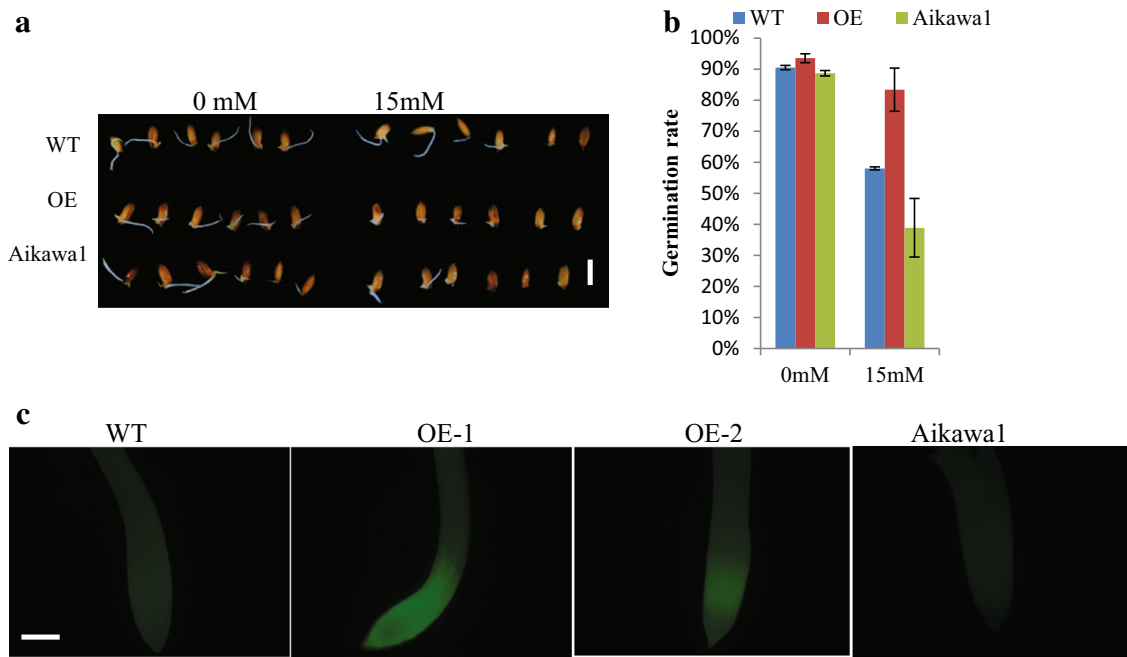
The ROS levels can be controlled by synthesize antioxidant enzymes to prevent their toxicity. Therefore, the activities of two enzymes SOD (the first enzyme scavenges oxygen radical) and POD (the inducible enzyme) were detected, which can eliminate the metabolic process of hydrogen peroxide (Jacob and Burri 1996). The SOD and POD activities were increased under oxidation stress, while the activities of SOD and POD in miR529aOE were much higher than that in WT after  $H_2O_2$  treatment, especially under 15 mM (Fig. 5a, b), suggesting that overexpressing miR529a could be endowed with the ability to keep balance of ROS. Furthermore, the accumulation of  $H_2O_2$  and  $O_2^-$  in young leaf from WT and miR529aOE was assessed by DAB and NBT staining, respectively. The WT leaf displayed more staining spots than the leaf from miR529aOE (Fig. 5c, d), demonstrating that the former had higher ROS than the latter. Subsequently, quantitative measurement of  $H_2O_2$  showed that the endogenous  $H_2O_2$  was significantly higher in WT than in miR529aOE (Fig. 5e).

The flag leaf was treatment with 15 mM  $H_2O_2$  for 5 days that led to severe chlorosis in WT, while maintained



**Fig. 2** The expression levels of miR529a. **a** The expression levels of miR529a under different concentration of exogenous  $H_2O_2$  treatment. The four-leaf stage seedlings were treated with different concentrations of  $H_2O_2$  (0, 0.6, 3 and 15 mM) for 6 h. **b** The expression levels

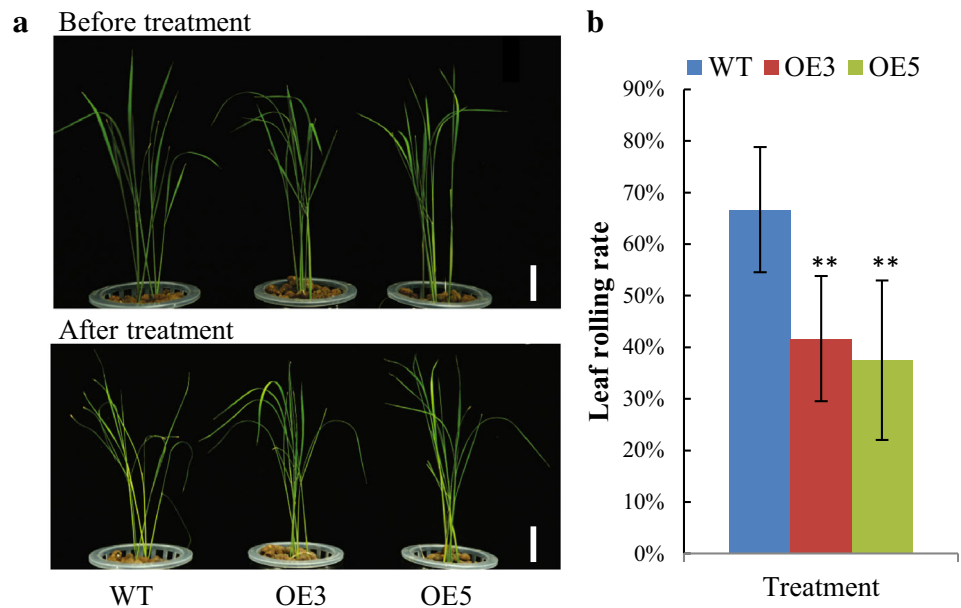
of miR529a in WT and miR529aOE. The expression of miR529a was performed with stem-loop qRT-PCR using *OsU6* as an internal control. Values represent the means of three independent biological replicates and three technical replicates



**Fig. 3** The effect of exogenous H<sub>2</sub>O<sub>2</sub> on plant seed germination and root tip cell viability. **a** Phenotypes of WT and miR529aOE plant seed germination response to H<sub>2</sub>O<sub>2</sub>. **b** Statistical analysis of seed germination rates, which were calculated from the plants of WT

and miR529aOE ( $n = 30$ ,  $*P < 0.05$ ,  $**P < 0.01$  by student's  $t$  test). **c** Intracellular ROS level after treatment with 15 mM H<sub>2</sub>O<sub>2</sub> in the WT and miR529aOE lines. DCFH-DA was used to probe intracellular ROS in root tips [scale bars 8 mm (a), 0.4 mm (c)]

**Fig. 4** The effect of exogenous H<sub>2</sub>O<sub>2</sub> on the plant of WT and miR529aOE at four-leaf stage seedlings. **a** Phenotypes of WT and miR529aOE plants in response to H<sub>2</sub>O<sub>2</sub> (scale bars 2 cm). **b** Statistical analysis of the leaf blade rolling rates, which were calculated from the plants of WT and miR529aOE ( $n = 18$ ,  $*P < 0.05$ ,  $**P < 0.01$  by student's  $t$  test). The hydroponic-cultured seedlings were treated with 15 mM H<sub>2</sub>O<sub>2</sub> for 6 h



green in miR529aOE (Fig. 5f), and the chlorophyll level in miR529aOE was significantly higher than that in WT (Fig. 5g).

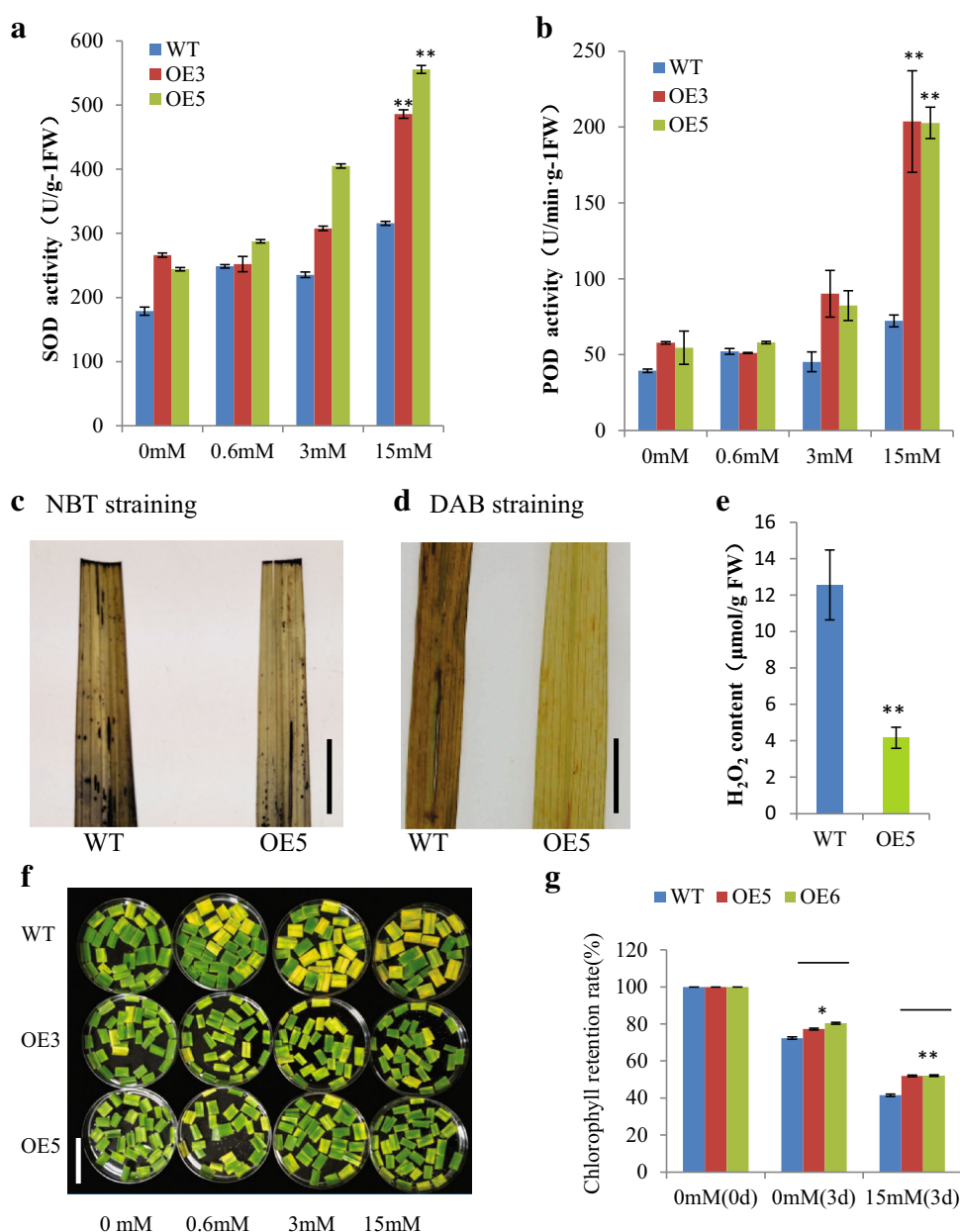
**Expression patterns of H<sub>2</sub>O<sub>2</sub> responsive genes**

Above results showed a potential role of miR529a in ROS scavenging. To gain more insight into the role of miR529a

in response to H<sub>2</sub>O<sub>2</sub> stress, we further examined the expression patterns of ROS-responsive genes in both WT and miR529aOE, including one organic osmotic regulation related gene *LOC\_Os05g38150* (*OsP5CS*) and two scavenging ROS genes *LOC\_Os01g22249* and *LOC\_Os04g59150* (Chen et al. 2009; Cui et al. 2015; Maruyama et al. 2014), which can be response to adverse stimulus. All three genes were up-regulated under H<sub>2</sub>O<sub>2</sub>



**Fig. 5** The activities of the antioxidant enzymes and ROS accumulation analyses. The activities of **a** SOD and **b** POD in the leaf of WT and miR520aOE under different concentration of exogenous  $H_2O_2$  (0, 0.6, 3 and 15 mM) were measured by NBT and guaiacol methods, respectively. The accumulations of **c**  $O_2^-$  and **d**  $H_2O_2$  were histochemically detected using NBT and DAB staining, respectively, and the content of  $H_2O_2$  was determined by a spectrophotometric assay (**e**). **f** Detached flag leaf of WT, the transgenic lines OE3 and OE5 were exposed to 0, 0.6, 3 and 15 mM  $H_2O_2$  solutions for 5 days. **g** Chlorophyll was extracted with 95% ethanol, and chlorophyll content was determined by spectrophotometrically at 665 and 649 nm [(scale bars 1 cm, **c**, **d**; 4 cm, **f**), asterisks indicate a significant difference between WT and transgenic line, \* $P < 0.05$ , \*\* $P < 0.01$  by student's *t* test]



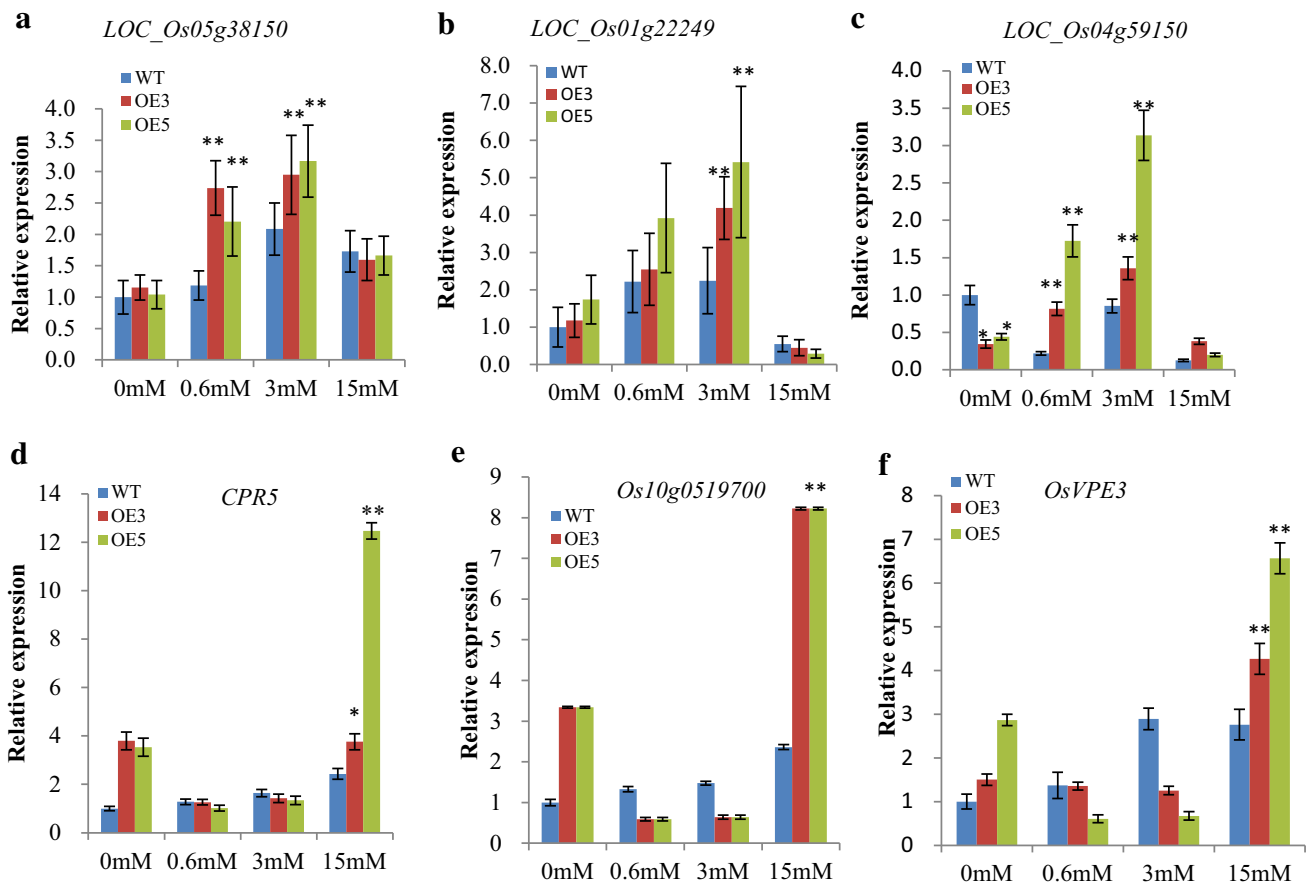
stress and displayed similar expression profile in miR529aOE, with the highest expression level at concentration of 3 mM after 6 h  $H_2O_2$  treatment (Fig. 6). However, the expression of genes at 15 mM after 6 h treatment were decreased, even significantly lower than untreated (Fig. 6a–c), indicating that scavenging enzymes had been accumulated enough at concentration of 15 mM.

Additionally,  $H_2O_2$ -induced closely related genes were further selected to discover the possible path of  $H_2O_2$  responses. *CPR5*, *Os10g0519700* and *OsVPE3* three genes displayed up-regulation after exposure to  $H_2O_2$  treatment for 6 h in WT, while in miR529aOE, the expression were much higher than in WT at 15 mM (Fig. 6d–f), but not in others (Fig. S2), these results demonstrated that *CPR5*,

*Os10g0519700* and *OsVPE3* might directly respond to  $H_2O_2$  stress.

### Expression patterns of miR529a target genes

MiR529a was predicted to target five *SPL* genes-*OsSPL2*, *OsSPL14*, *OsSPL16*, *OsSPL17* and *OsSPL18* using psRNATarget software with the cut-off threshold as 2.5 (Dai and Zhao 2011). However, only *OsSPL2* and *OsSPL14* genes were down-regulated in the seedling of miR529aOE compared to WT (Fig. 7a), indicating that only *OsSPL2* and *OsSPL14* genes were targeted by miR529a in rice seedlings. And the expression of *OsSPL2* and *OsSPL14* was decreased with the increasing of the



**Fig. 6** The expression of H<sub>2</sub>O<sub>2</sub> responsive genes in the seedlings of WT and miR529aOE under different concentration of exogenous H<sub>2</sub>O<sub>2</sub> (0, 0.6, 3, 15 mM). The expression of a proline synthase gene *LOC\_Os05g38150* (a), two ROS scavenging genes

*LOC\_Os01g22249* (b) and *LOC\_Os04g59150* (c), the *CPR5* gene (d), a *TIM/TOM* genes of *Os04g0519700* (e) and a vacuolar processing enzymes genes, *OsVPE3* (f), which were performed by qRT-PCR. \**P* < 0.05, \*\**P* < 0.01 by student's *t* test

concentration of exogenous H<sub>2</sub>O<sub>2</sub> (Fig. 7b, c), resulting from the increased expression of miR529a (Fig. 1b). The expression of *OsSPL2* and *OsSPL14* were then investigated under oxidative stress with 15 mM H<sub>2</sub>O<sub>2</sub> in WT and miR529aOE, their expression were significantly lower in miR529aOE than in WT after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 7d, e), which implied that low expression of *SPLs* significantly contribute to resist oxidation, and they were negatively responded to H<sub>2</sub>O<sub>2</sub> stress.

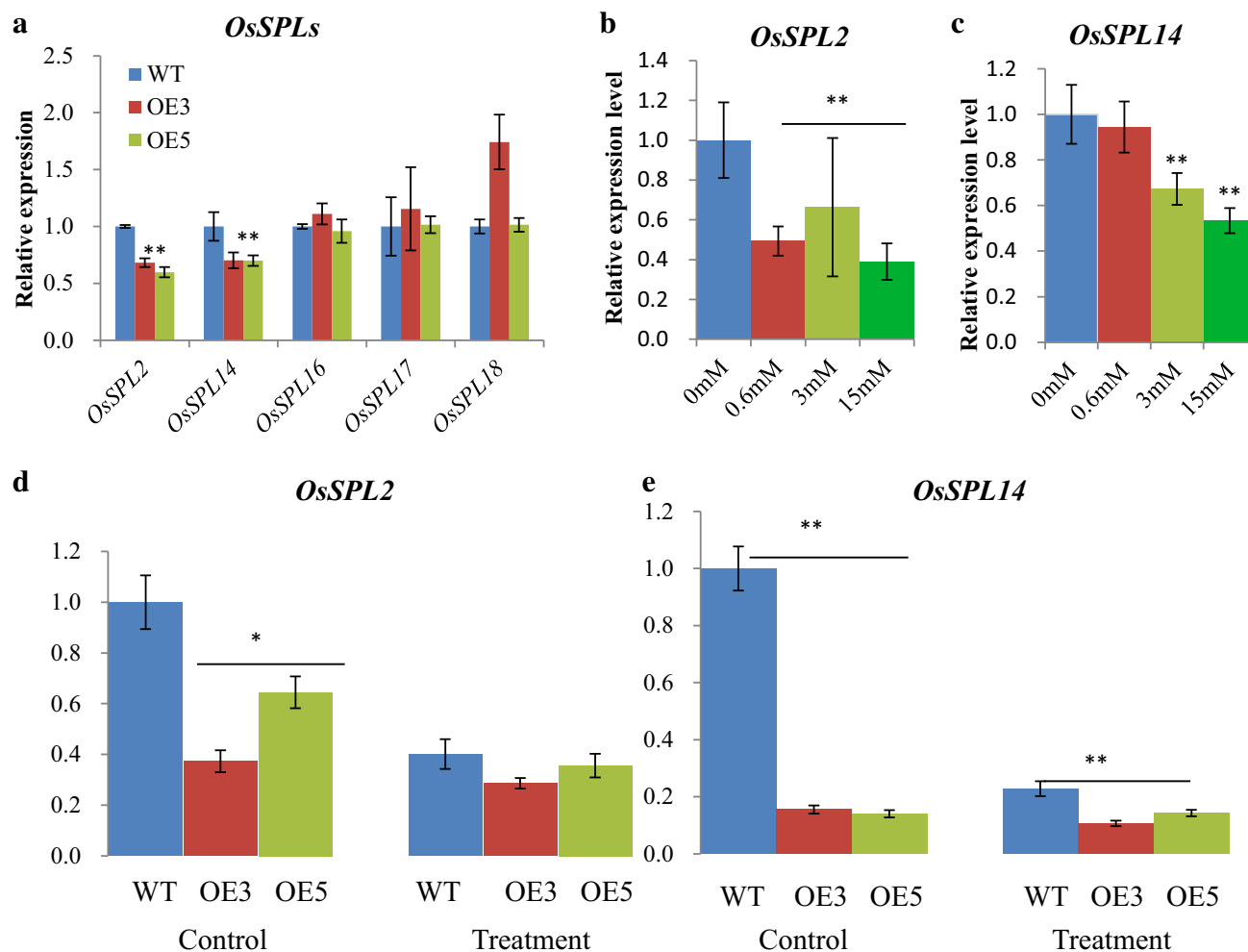
#### Up-regulation of miR529a target gene *OsSPL14* can weaken oxidative tolerance

The seedlings of Aikawa1 and Nipponbare were treated with 15 mM H<sub>2</sub>O<sub>2</sub> to test oxidative tolerance, the result showed that Aikawa1 has a severe syndrome (Fig. 8a), and the leaf rolling rate of Aikawa1 was significantly higher than Nipponbare (Fig. 8b). Moreover, the expression level of *OsSPL14* in Aikawa1 seedling of both control and H<sub>2</sub>O<sub>2</sub> treatment was much higher than in Nipponbare (Fig. 8c). These results implied that *OsSPL14* was a negative

regulator to control ROS balance, and down-regulated *OsSPL14* by overexpression of miR529a could enhance oxidative tolerance.

## Discussion

Many studies showed that miRNAs not only were involved in plant growth and development, but also affected plant stress responses (Liu et al. 2008; Sunkar and Zhu 2004; Wang et al. 2013; Zhou et al. 2008). MiR529 is an ancient microRNA in plants, which is present from moss to vascular plant, especially enriched in monocots (Chavez Montes et al. 2014). It has miR529a and miR529b two members in rice (Liu et al. 2005; Zhu et al. 2008). The expression of miR529a was much higher than miR529b, and it is the miR529a-5p (miR529a\*), not miR529a-3p, acts as an executive in rice development (Jeong et al. 2011). Recent studies have shown that the expression of miR529a can be induced by the environmental stimuli of H<sub>2</sub>O<sub>2</sub> and Cd stresses, which could help to reduce



**Fig. 7** The expression of miR529a putative target genes. **a** The expression levels of five putative targeted genes in the plants of WT and miR529aOE. **b, c** The expression patterns of *OsSPL2* and *OsSPL14* directly regulated by miR529a at different concentration of

$H_2O_2$  in WT. **d, e** The expression of *OsSPL2* and *OsSPL14* under different concentrations of  $H_2O_2$  treatment. Relative transcript levels were determined by qRT-PCR using *OsEfl-a* as an internal. \* $P < 0.05$ , \*\* $P < 0.01$  by student's  $t$  test

redundant ROS (Li et al. 2011; Tang et al. 2014). The expression of miR529a was up-regulated under  $H_2O_2$  treatment, and the higher the concentration of  $H_2O_2$ , the higher the expression of miR529a (Fig. 2a), demonstrating that miR529a was positive response to  $H_2O_2$  stress and the induced ROS by  $H_2O_2$  was significantly dependent on exogenous  $H_2O_2$  (Deng et al. 2011), while miR529 was negatively response to drought, arsenate and arsenite stresses (Sharma et al. 2015; Zhou et al. 2010).

ROSs are by-products of the aerobic metabolic pathways including respiration and photosynthesis in plant chloroplast, mitochondria and peroxisome (D'Autreaux and Toledano 2007). Furthermore, exogenous  $H_2O_2$  can also induce a typical ROS in the suspension cultured cells (Desikan et al. 1998; Houot et al. 2001). The plant has its own system to modulate the internal ROS level to maintain its homeostasis (Del Rio and Lopez-Huertas 2016). Once the balance is broken, the high level of ROS will cause

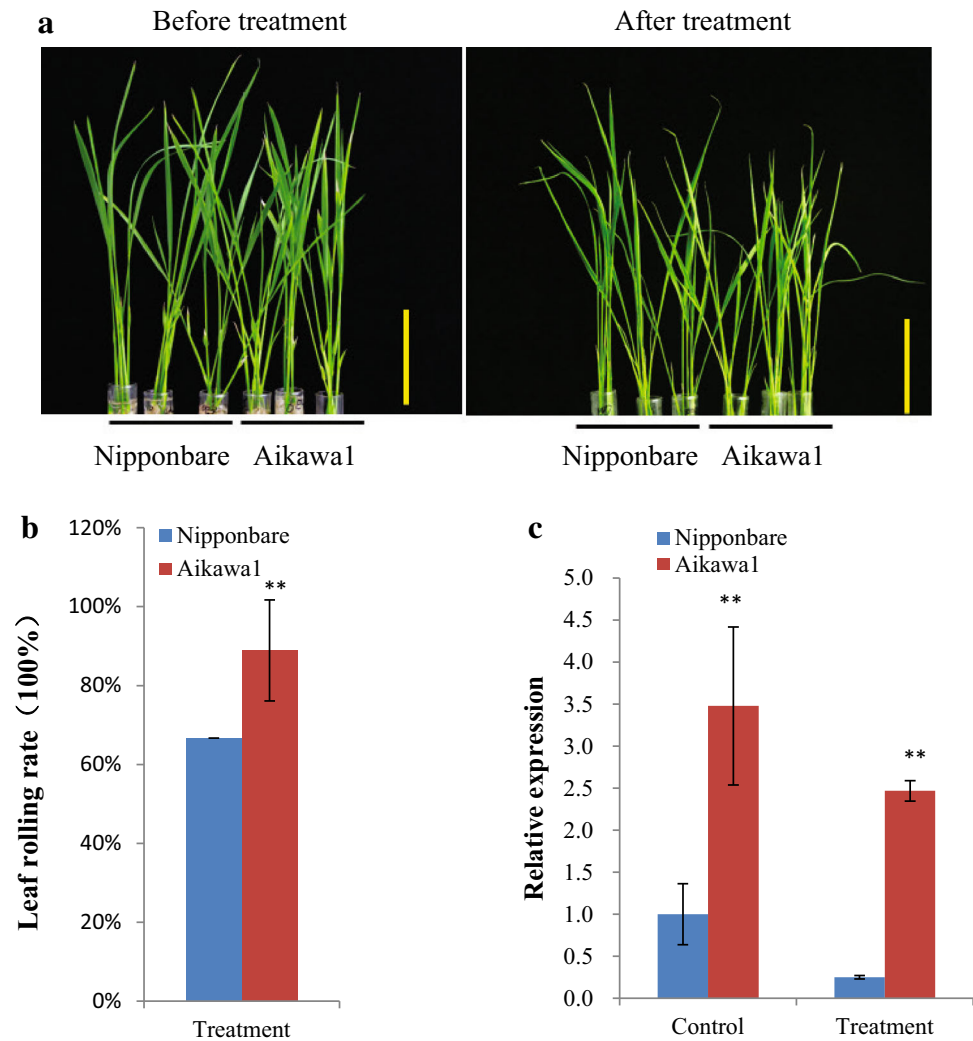
plant oxidative stress. The flag leaf of WT accumulated much more ROS than transgenic line (Fig. 5c, d); which had less chlorosis degree treated with exogenous  $H_2O_2$  (Fig. 5f), suggested that overexpressing miR529a had the ability to keep ROS at suitable levels.

The high concentration of ROS is reactive and toxic in plants, although the accumulation of stress-induced ROS can be counteracted by intrinsic antioxidant systems. The antioxidant systems include various enzymatic scavengers, which can convert toxic superoxide radicals to harmless ion, and at last eliminate hydrogen peroxide in plants (Mittler et al. 2004). SOD and POD are two important ROS-scavengers in antioxidant defense system (Bansal and Kanwar 2013; Wang et al. 2016), their activities in transgenic line were significantly higher than that in WT (Fig. 5a, b), which were confirmed by highly expressed ROS scavenging genes (Fig. 6b, c). In other respect, the free proline accumulation can directly protect plants from various stresses (Szabados



**Fig. 8** The effect of exogenous  $H_2O_2$  on the seedlings of Nipponbare and Aikawa1.

**a** Phenotypes of Nipponbare and Aikawa1 plants in response to  $H_2O_2$ . The hydroponic-cultured seedlings were treated with 15.0 mM  $H_2O_2$  for 6 h (scale bars 5 cm). **b** Statistical analysis of the leaf blade rolling rates. The leaf blade rolling rates were calculated from the plants of Nipponbare and Aikawa1 ( $n = 18$ ) under  $H_2O_2$  treatment for 6 h. **c** The expression levels of *OsSPL14* in Nipponbare and Aikawa1. Relative transcript levels were determined by qRT-PCR using *OsEfl-a* as an internal. \* $P < 0.05$ , \*\* $P < 0.01$  by student's *t* test



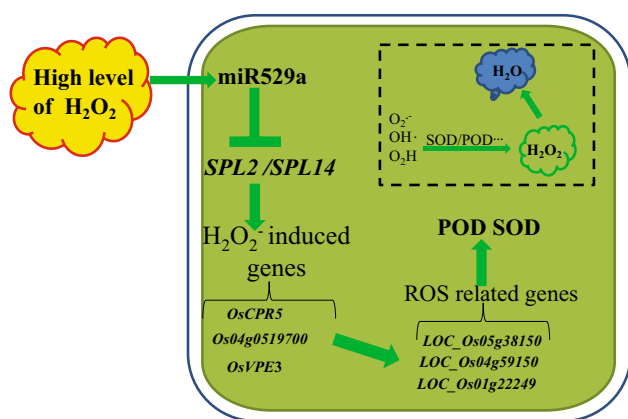
and Savoure 2010). And then, the expression of proline synthase gene (*LOC\_Os05g38150*) was significantly higher in transgenic line than WT, which may partly explain the increased tolerance to oxidative stress (Fig. 6a). These results revealed that miR529a could enhance the tolerance ability to exogenous  $H_2O_2$  stress.

Not surprisingly, the expression of  $H_2O_2$ -responsive genes (Liu et al. 2013) in rice seedling were up-regulated in transgenic lines at the concentration of 15 mM  $H_2O_2$  (Fig. 6d–h), and these genes might positive regulate  $H_2O_2$  stress in ROS signaling pathway.

The *SPL* genes family is a class of highly conserved transcription factors involved in multiple physiological and biochemical processes, including plant morphosis, flowering time, juvenile-to-adult transition and stresses response (Chen et al. 2010; Gou et al. 2011; Wang et al. 2009; Yang et al. 2008), which can be targeted by miR529 (Ling and Zhang 2012; Morea et al. 2016; Zhang et al. 2015). Five *SPL* genes were predicted to be targeted by miR529a, while overexpressing miR529a only led to decrease the

expression of *OsSPL2* and *OsSPL14* in rice seedlings (Fig. 8a). It indicated that *OsSPL2* and *OsSPL14* might be targets of miR529a in rice seedlings, while *OsSPL14* and *OsSPL17* were regulated by miR529a to affect tillering and panicle branching (Wang et al. 2015), implying that *SPL* genes were tissue-specifically regulated by miR529a. In addition, the expression of *OsSPL2* and *OsSPL14* were decreased with the increasing of  $H_2O_2$  concentration (Fig. 7b, c), and their expression were all significantly low in WT and transgenic line under  $H_2O_2$  treatment (Fig. 7d, e), suggested that miR529a might play an important role in response to oxidative stress in rice, and its targets are negatively response to exogenous  $H_2O_2$  in rice seedlings.

The japonica cultivar Aikawa1 has a single nucleotide change from C to A at the miR156-targeted site in *OsSPL14* gene (Miura et al. 2010), displayed higher transcript of *OsSPL14*, low seed germination rate and low ROS level of root tip, suffered more severe rolling syndrome than Nipponbare under  $H_2O_2$  stress because of increased transcript level of *OsSPL14* (Fig. 8a). Furthermore,



**Fig. 9** The likely miR529a regulation network in ROS pathway under  $H_2O_2$  stress in plant cells

*OsSPL14* was reported to be regulated directly by miR529a and miR156 (Jeong et al. 2011). These findings confirmed that *OsSPL14* gene was likely the target of miR529 and negatively regulated exogenous  $H_2O_2$  stress.

Overall, the expression of  $H_2O_2$  responsive genes *CPR5*, *Os10g0519700* and *OsVPE3* were increased by repressing the expression of miR529a target genes *OsSPL2* and *OsSPL14*, which could lead to increase the expression of *LOC\_Os05g38150*, *LOC\_Os01g22249* and *LOC\_Os04g59150*, and finally elevated SOD and POD activities to strengthen the plant tolerance to oxidation stress (Fig. 9). These results indicated a potential complex network of miR529a-SPLs-downstream genes in ROS signaling pathway of oxidative stress response (Fig. 9).

**Author contribution statement** JHX and EY designed research; EY, ZL, CL, YL, and QL performed research; EY, ZL, CL, and JHX analyzed data; EY and JHX wrote and edited the manuscript.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- Bansal N, Kanwar SS (2013) Peroxidase(s) in environment protection. *Sci World J* 2013:714639
- Beers RF Jr, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* 195:133–140
- Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X (1997) The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9:1573–1584

- Chavez Montes RA, de Fatima Rosas-Cardenas F, De Paoli E, Accerbi M, Rymarquis LA, Mahalingam G, Marsch-Martinez N, Meyers BC, Green PJ, de Folter S (2014) Sample sequencing of vascular plants demonstrates widespread conservation and divergence of microRNAs. *Nat Commun* 5:3722
- Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 33:e179
- Chen S, Li XQ, Zhao A, Wang L, Li X, Shi Q, Chen M, Guo J, Zhang J, Qi D, Liu G (2009) Genes and pathways induced in early response to defoliation in rice seedlings. *Curr Issues Mol Biol* 11:81–100
- Chen X, Zhang Z, Liu D, Zhang K, Li A, Mao L (2010) SQUAMOSA promoter-binding protein-like transcription factors: star players for plant growth and development. *J Integr Plant Biol* 52:946–951
- Cui LG, Shan JX, Shi M, Gao JP, Lin HX (2014) The miR156–SPL9–DFR pathway coordinates the relationship between development and abiotic stress tolerance in plants. *Plant J* 80:1108–1117
- Cui N, Sun X, Sun M, Jia B, Duanmu H, Lv D, Duan X, Zhu Y (2015) Overexpression of *OsmiR156k* leads to reduced tolerance to cold stress in rice (*Oryza sativa*). *Mol Breed* 35:1–11
- Cuperus JT, Fahlgren N, Carrington JC (2011) Evolution and functional diversification of MIRNA genes. *Plant Cell* 23:431–442
- Dai X, Zhao PX (2011) psRNATarget: a plant small RNA target analysis server. *Nucleic Acids Res* 39:W155–W159
- D’Autreaux B, Toledano MB (2007) ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 8:813–824
- Del Rio LA, Lopez-Huertas E (2016) ROS generation in peroxisomes and its role in cell signaling. *Plant Cell Physiol* 57:1364–1376
- Deng M, Bian H, Xie Y, Kim Y, Wang W, Lin E, Zeng Z, Guo F, Pan J, Han N, Wang J, Qian Q, Zhu M (2011) Bcl-2 suppresses hydrogen peroxide-induced programmed cell death via *OsVPE2* and *OsVPE3*, but not via *OsVPE1* and *OsVPE4*, in rice. *FEBS J* 278:4797–4810
- Desikan R, Reynolds A, Hancock JT, Neill SJ (1998) Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in *Arabidopsis* suspension cultures. *Biochem J* 330(Pt 1):115–120
- Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. *Plant Sci* 135:1–9
- Dugas DV, Bartel B (2008) Sucrose induction of *Arabidopsis* miR398 represses two Cu/Zn superoxide dismutases. *Plant Mol Biol* 67:403–417
- Egley GH, Paul RN Jr, Vaughn KC, Duke SO (1983) Role of peroxidase in the development of water-impermeable seed coats in *Sida spinosa* L. *Planta* 157:224–232
- Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422:442–446
- Gao G, Zhang S, Wang C, Yang X, Wang Y, Su X, Du J, Yang C (2011) *Arabidopsis* *CPR5* independently regulates seed germination and postgermination arrest of development through LOX pathway and ABA signaling. *PLoS One* 6:e19406
- Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 48:909–930
- Gou JY, Felippes FF, Liu CJ, Weigel D, Wang JW (2011) Negative regulation of anthocyanin biosynthesis in *Arabidopsis* by a miR156-targeted SPL transcription factor. *Plant Cell* 23:1512–1522

- Gupta OP, Meena NL, Sharma I, Sharma P (2014) Differential regulation of microRNAs in response to osmotic, salt and cold stresses in wheat. *Mol Biol Rep* 41:4623–4629
- Houot V, Etienne P, Petitot AS, Barbier S, Blein JP, Suty L (2001) Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner. *J Exp Bot* 52:1721–1730
- Jacob RA, Burri BJ (1996) Oxidative damage and defense. *Am J Clin Nutr* 63:985S–990S
- Jeong DH, Park S, Zhai J, Gurazada SG, De Paoli E, Meyers BC, Green PJ (2011) Massive analysis of rice small RNAs: mechanistic implications of regulated microRNAs and variants for differential target RNA cleavage. *Plant Cell* 23:4185–4207
- Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. *Annu Rev Plant Biol* 57:19–53
- Kramer MF (2011) Stem-loop RT-qPCR for miRNAs. In: *Current protocols in molecular biology*. Wiley, New York
- Li T, Li H, Zhang YX, Liu JY (2011) Identification and analysis of seven H(2)O(2)-responsive miRNAs and 32 new miRNAs in the seedlings of rice (*Oryza sativa* L. ssp. *indica*). *Nucleic Acids Res* 39:2821–2833
- Ling LZ, Zhang SD (2012) Exploring the evolutionary differences of SBP-box genes targeted by miR156 and miR529 in plants. *Genetica* 140:317–324
- Liu B, Li P, Li X, Liu C, Cao S, Chu C, Cao X (2005) Loss of function of OsDCL1 affects microRNA accumulation and causes developmental defects in rice. *Plant Physiol* 139:296–305
- Liu HH, Tian X, Li YJ, Wu CA, Zheng CC (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA (New York, NY)* 14:836–843
- Liu F, Shu X, Zheng YH, Li ZJ, Yang CW, Wang YQ (2013) Involvement of rice CPR5 in response to oxidative stress. *J Trop Subtrop Bot* 21:305–314
- Lu YZ, Feng Z, Bian LY, Xie H, Liang JS (2011) miR398 regulation in rice of the responses to abiotic and biotic stresses depends on CSD1 and CSD2 expression. *Funct Plant Biol* 38:44–53
- Maruyama K, Urano K, Yoshiwara K, Morishita Y, Sakurai N, Suzuki H, Kojima M, Sakakibara H, Shibata D, Saito K, Shinozaki K, Yamaguchi-Shinozaki K (2014) Integrated analysis of the effects of cold and dehydration on rice metabolites, phytohormones, and gene transcripts. *Plant Physiol* 164:1759–1771
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7:405–410
- Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. *Trends Plant Sci* 9:490–498
- Miura K, Ikeda M, Matsubara A, Song XJ, Ito M, Asano K, Matsuoka M, Kitano H, Ashikari M (2010) OsSPL14 promotes panicle branching and higher grain productivity in rice. *Nat Genet* 42:545–549
- Morea EG, da Silva EM, e Silva GF, Valente GT, Barrera Rojas CH, Vincentz M, Nogueira FT (2016) Functional and evolutionary analyses of the miR156 and miR529 families in land plants. *BMC Plant Biol* 16:40
- Neill S, Desikan R, Hancock J (2002) Hydrogen peroxide signalling. *Curr Opin Plant Biol* 5:388–395
- Nigam D, Kumar S, Mishra DC, Rai A, Smita S, Saha A (2015) Synergistic regulatory networks mediated by microRNAs and transcription factors under drought, heat and salt stresses in *Oryza sativa* spp. *Gene* 555:127–139
- Orjuela J, Deless EF, Kolade O, Cheron S, Ghesquiere A, Albar L (2013) A recessive resistance to rice yellow mottle virus is associated with a rice homolog of the CPR5 gene, a regulator of active defense mechanisms. *Mol Plant Microbe Interact* 26:1455–1463
- Orozco-Cardenas ML, Narvaez-Vasquez J, Ryan CA (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell* 13:179–191
- Ozawa K (2009) Establishment of a high efficiency Agrobacterium-mediated transformation system of rice (*Oryza sativa* L.). *Plant Sci* 176:522–527
- Pegoraro C, Krüger MM, Santos RSD, Mertz LM, Maia LCD, Rombaldi CV, Oliveira ACD (2012) Transcription of mitochondrial and chloroplast related genes in rice plants under anoxic stress. *Plant Omics* 5:547–552
- Qiao Y, Jiang W, Lee J, Park B, Choi MS, Piao R, Woo MO, Roh JH, Han L, Paek NC, Seo HS, Koh HJ (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 Phytol* 185:258–274
- Quan LJ, Zhang B, Shi WW, Li HY (2008) Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *J Integr Plant Biol* 50:2–18
- Sharma D, Tiwari M, Lakhwani D, Tripathi RD, Trivedi PK (2015) Differential expression of microRNAs by arsenate and arsenite stress in natural accessions of rice. *Metallomics* 7:174–187
- Stief A, Altmann S, Hoffmann K, Pant BD, Scheible WR, Baurle I (2014) Arabidopsis miR156 regulates tolerance to recurring environmental stress through SPL transcription factors. *Plant Cell* 26:1792–1807
- Sunkar R, Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell* 16:2001–2019
- Sunkar R, Chinnusamy V, Zhu J, Zhu JK (2007) Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends Plant Sci* 12:301–309
- Szabados L, Savoure A (2010) Proline: a multifunctional amino acid. *Trends Plant Sci* 15:89–97
- Tang M, Mao D, Xu L, Li D, Song S, Chen C (2014) Integrated analysis of miRNA and mRNA expression profiles in response to Cd exposure in rice seedlings. *BMC Genom* 15:835
- Varkonyi-Gasic E, Hellens RP (2011) Quantitative stem-loop RT-PCR for detection of microRNAs. *Methods Mol Biol (Clifton, NJ)* 744:145–157
- Vranova E, Inze D, Van Breusegem F (2002) Signal transduction during oxidative stress. *J Exp Bot* 53:1227–1236
- Wang Y, Hu Z, Yang Y, Chen X, Chen G (2009) Function annotation of an SBP-box gene in Arabidopsis based on analysis of co-expression networks and promoters. *Int J Mol Sci* 10:116–132
- Wang M, Wang Q, Zhang B (2013) Response of miRNAs and their targets to salt and drought stresses in cotton (*Gossypium hirsutum* L.). *Gene* 530:26–32
- Wang L, Sun S, Jin J, Fu D, Yang X, Weng X, Xu C, Li X, Xiao J, Zhang Q (2015) Coordinated regulation of vegetative and reproductive branching in rice. *Proc Natl Acad Sci USA* 112:15504–15509
- Wang W, Xia MX, Chen J, Yuan R, Deng FN, Shen FF (2016) Gene expression characteristics and regulation mechanisms of superoxide dismutase and its physiological roles in plants under stress. *Biochem Biokhimiia* 81:465–480
- Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M, Inze D, Van Camp W (1997) Catalase is a sink for H<sub>2</sub>O<sub>2</sub> and is indispensable for stress defence in C3 plants. *EMBO J* 16:4806–4816
- Wohlgemuth H, Mittelstrass K, Kschieschan S, Bender J, Weigel HJ, Overmyer K, Kangasjarvi J, Sandermann H, Langebartels C (2002) Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. *Plant Cell Environ* 25:717–726
- Yang Z, Wang X, Gu S, Hu Z, Xu H, Xu C (2008) Comparative study of SBP-box gene family in Arabidopsis and rice. *Gene* 407:1–11

- You J, Chan Z (2015) ROS regulation during abiotic stress responses in crop plants. *Frontiers Plant Sci* 6:1092
- Zhang SD, Ling LZ, Zhang QF, Xu JD, Cheng L (2015) Evolutionary comparison of two combinatorial regulators of SBP-Box genes, MiR156 and MiR529, in plants. *PLoS One* 10:e0124621
- Zhou X, Wang G, Sutoh K, Zhu JK, Zhang W (2008) Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochem Biophys Acta* 1779:780–788
- Zhou L, Liu Y, Liu Z, Kong D, Duan M, Luo L (2010) Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. *J Exp Bot* 61:4157–4168
- Zhu QH, Spriggs A, Matthew L, Fan L, Kennedy G, Gubler F, Helliwell C (2008) A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. *Genome Res* 18:1456–1465