

Suppression and overexpression of *Lsi1* induce differential gene expression in rice under ultraviolet radiation

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Abstract Low silicon rice gene 1 (*Lsi1*) belongs to a member of *Nod26*-like major intrinsic protein (*NIP*) subfamily and is thought to control silicon (Si) accumulation in rice. In order to further elucidate its regulatory mechanisms in the defense of rice plants to enhanced ultraviolet-B (UV-B) radiation stress, *Lsi1* was subjected to suppressed and overexpressed treatments in a UV-B tolerance rice accession Lemont as well as to overexpressed treatment in a UV-B sensitive rice accession Dular. The results showed that transcript levels of *Lsi1* increased in *Lsi1*-overexpressed transgenic lines of rice accessions Lemont and Dular, but down-regulated in *Lsi1*-RNAi transgenic line of Lemont in comparison with their wild types (WT). A similar tendency was found in the root Si uptake and the Si concentrations in leaves. Further, the different transgenic rice lines and their WT were exposed to enhance UV-B radiation, study by suppression subtractive hybridization (SSH) found that *Lsi1* not only could regulate the expression of phenylalanine ammonia-lyase (*PAL*), 4-coumarate-CoA ligase (*4CL*) and photolyase (*PL*), but also could induce other signal transduction-, detoxification-, resistance-, and photosynthesis-related genes. The findings suggested that the regulation of silicon nutrient by enhancing/inhibiting expression of *Lsi1* could effectively induce the transcription of the mRNAs relative to tolerance in rice.

Keywords *Lsi1* · Rice (*Oryza sativa* L.) · Silicon · UV-B radiation

Abbreviations

4CL	4-coumarate-CoA ligase
<i>Lsi1</i>	Low silicon rice 1
PL	Photolyase
PAL	Phenylalanine ammonia-lyase
Si	Silicon
SSH	Suppression subtractive hybridization
UV-B	Ultraviolet-B
WT	Wild types

Introduction

Depletion of the stratospheric ozone (O_3) layer due to the emission of chlorofluorocarbons and other trace gases has resulted in increased solar ultraviolet-B (UV-B: 280–315 nm) radiation on the earth's surface (Ganguly et al. 2009; Blumthaler and Ambach 1990), which would reduce aboveground biomass and plant height, and increased DNA damage, and the effects of UV-B radiation on plants showed that they responded similarly in Arctic, Antarctic and lower latitudes (Newsham and Robinson 2009). Thus, there is an urgent need to find out effective ways to alleviate such damages on plant growth and development

Silicon (Si) is the second most prevalent element in the soil. Although Si is not considered to be an essential element for higher plants, it is beneficial to plant growth and contributes to plant defense against many biotic and abiotic stresses, such as salt tolerance (Liang et al. 2006), drought tolerance (Gong et al. 2005) and tolerance to toxic metals in plant (Liang et al. 1994; Epstein 1999; Ma 2004; Ma and

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Yamaji 2006). Si is considered as a modulator in enhancing plant resistance. Improvement of Si nutrient could invoke plant produce phenolics and phytoalexins, which could increase plant's capability to defend stressful factors (Fawe et al. 1998). The related mechanism was that Si might decrease the oxidative damage in plants subjected to environmental stresses (Saqib et al. 2008). The roles of Si in enhancing plant tolerance to biotic stresses (e.g. fungal diseases) are also attributed partly to Si-enhanced antioxidant defense capacity. It deposited beneath the cuticle to form a cuticle-Si double layer which acts as a physical barrier to avoid fungal penetration, and supplement with Si in a *Magnaporthe grisea*-infected susceptible rice resulted in accumulation of the momilactone phytoalexins (Rodrigues et al. 2003) and up-regulated glucanase, peroxidase and PR-1 transcripts (Rodrigues et al. 2005).

Si may also help alleviate physical stress caused by UV. Our previous study (Wu et al. 2007) has found that the amount of silica on the adaxial surface in UV-B tolerance rice Lemont was higher than that in the UV-B sensitive type Dular, and showed increasing tendency in the tolerance rice when exposed to UV-B radiation. The reverse was true in its counterpart Dular, suggesting that the characteristics of silicic accumulation might be one of the mechanisms for rice to defense enhanced UV-B radiation. Li et al. (2004) also found that rice plants grown in hydroponics with Si supplement could enhance UV-B resistance compared to that in Si-deficient condition, which was believed to be attributed to increased phenolic compounds in epidermis of rice plants in responding to silicon supplement. Si is taken up through plant roots in the form of silicic acid and transport to the shoot via the transpiration stream, and then polymerized and accumulated on the shoot tissues as silica (Takahashi and Hino 1978; Ma and Takahashi 2002). Ma et al. (2006, 2007) reported that the uptake of Si in rice is mediated by at least two transporters, low silicon rice 1 (*Lsi1*) and low silicon rice 2 (*Lsi2*). *Lsi1* is an influx transporter, while *Lsi2* is an efflux transporter of Si. Therefore, it suggests that *Lsi1* functions in controlling Si accumulation in rice, meaning that inhibiting the gene expression would reduce the uptake of Si in rice, which in turn might result in decreased plant tolerance to many stresses. So, it is hypothesized that by regulating the transcript level of *Lsi1* may increase plant tolerance to various stressful factors.

Based on the hypothesis, the present study was conducted to determine whether *Lsi1* plays a role in regulating rice tolerance to UV-B stress by employing gene suppression and overexpression approaches. A comparison of molecular changes between transgenic lines including *Lsi1* suppression and its overexpression lines, and their wild-types (*WT*) was conducted in the responses to enhanced UV-B radiation, and the molecular response network was

further detected by using suppression subtractive hybridization (SSH) approach (Diatchenko et al. 1996).

Materials and methods

Plant materials

We used the rice accessions, Lemont (UV-B tolerant, introduced from USA) and Dular (UV-B sensitive, from India) in this study (Fang et al. 2009a). The grain of the rice accession, Lemont were surface-sterilized with NaClO for 30 min, placed in Petri dishes in a temperature-controlled growth chamber and then sown in separate seedling plates. At the 3-leaf stage, uniform seedlings from each plant were selected and transferred into a Styrofoam plate (with holes spaced at $5 \times 6 \text{ cm}^2$); the seedlings were affixed to the plate by inserting a cotton plug into each hole on the plate. The Styrofoam plate was allowed to float in a pot ($45 \times 35 \times 15 \text{ cm}^3$) filled with 10 l rice (*Oryza sativa* L.) culture solution (Si solution) and had the following composition: 482 mg $(\text{NH}_4)_2\text{SO}_4$, 248 mg KH_2PO_4 , 185 mg KNO_3 , 149 mg K_2SO_4 , 864.3 mg $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1350.6 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2,000 mg $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$, 457 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 484.4 mg EDTA, 14.3 mg H_3BO_3 , 0.4 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.1 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 9.05 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.45 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, which is minor optimization from Yoshida et al. (1976), and the solution was changed every week. The pot was continuously aerated and its outer side was painted black to prevent algal growth. The pH value of the solution was maintained at 5.5 throughout the experiment. After 7 days, the root tissue of Lemont was sampled and then immediately frozen in liquid nitrogen and stored at -80°C for the isolation of total RNA.

Total RNA from the roots of Lemont was isolated using the Trizol method (Invitrogen) according to the manufacturer's instructions; the genomic DNA was digested using DNase I (Takara). Complementary DNA (cDNA) was synthesized using the ExScript RT reagent Kit (Takara). A 300 base pairs (bp) partial coding region from 900 bp downstream of ATG of rice *Lsi1* (Genbank accession No. AB222272) was cloned with a forward primer 5'-cgGGATCCtgcgccgacttctccctc-3' and a reverse primer 5'-ggGGTACCtgcgtccggtgaactgc-3' containing *BamHI* and *KpnI* sites (underlined), respectively. The same fragment was again amplified with the primers containing different recognition sites for *SpeI* and *SacI*, respectively, in forward primer 5'-ggACTAGTatcgctccggtgaactgc-3' and reverse primer 5'-cGAGCTCtgcgccgacttctccctc-3'. The full-length coding region of rice *Lsi1* was amplified with gene specific forward primer (5'-cgGGATCCatggccagcaacaactcg-3') and reverse primer (5'-ggGGTACCtcacacttgatgttctccatc-3') containing *BamHI* and *KpnI* sites. Before construction of the

stability vectors, all the gene fragments were subsequently cloned in pMD18-T vector (Takara) and transformed in *Escherichia coli* (strain DH5 α) and sent to Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., China for sequencing. The obtained DNA sequences were analyzed at NCBI (the National Center for Biotechnology Information, USA, www.ncbi.nlm.nih.gov) to further confirm the sequence similarity with *Lsi1*. Both fragments were inserted into pTCK303 to create a *Lsi1*-RNAi stability vector (*pTCK303-Lsi1*), and the ORF of rice *Lsi1* was inserted into Ubiquitin-1301 to create a *Lsi1*-overexpression stability vector (*Ubiquitin-1301-Lsi1*).

Mobilization of constructs in *Agrobacterium tumefaciens*

The recombinant vectors *pTCK303-Lsi1* and *Ubiquitin-1301-Lsi1* were, respectively, mobilized into *A. tumefaciens* disabled strain EHA105 by freeze and thaw method (Höfgen and Willmitzer 1988). Transformants were selected on YEB-agar containing kanamycin (50 $\mu\text{g/ml}$) and rifampicin (20 $\mu\text{g/ml}$).

Transformation of rice plants

The protocols of *A. tumefaciens*-mediated transformation of rice plants were performed following the methods described by Supartana et al. (2005) with minor modification. The grain of UV-B tolerant rice, Lemont was sterilized with ethanol and sodium hypochlorite and soaked at 20°C for 36 h. Water was replaced once during soak. After 36 h of soak, the embryo region of the grain turned white. At this stage, neither shoots nor roots had appeared. To inoculate *A. tumefaciens* contained with the recombinant vector *pTCK303-Lsi1* or *Ubiquitin-1301-Lsi1* into the embryonic apical meristem in the soaked grain, respectively, a site of the husk where a shoot would later emerge was pierced to a depth of 1–1.5 mm with a needle ($\Phi 0.70$ mm; Kangyou Medical Instrument Co., Ltd. Jiangsu, China) that had been dipped in the *A. tumefaciens* inoculum. The inoculated grain were placed on filter papers on wet vermiculite in flasks covered with aluminum foil and incubated at 23°C in the dark for 5 days. Then, the germinated seedlings were transplanted in a Styrofoam plate (with holes spaced at 5×6 cm²) float in a pot ($45 \times 35 \times 15$ cm³) filled with 10 l culture solution which is described in section “Plant materials”. To further confirm that the overexpression of *Lsi1* functions in enhancing rice UV-B tolerance, we inoculate *A. tumefaciens* contained with *Ubiquitin-1301-Lsi1* onto the UV-B sensitive rice Dular following the same procedure as mentioned above. Simultaneously, either the strain EHA105 with a blank-pTCK303 vector or a blank-Ubiquitin1301 vector was transformed into the embryonic

apical meristem in the soaked grain of Lemont, and only the strain EHA105 with a blank-Ubiquitin1301 vector into that of Dular as the controls of *Lsi1* overexpressed transgenic lines.

Histochemical analysis of *GUS* gene expression in transgenic rice lines

GUS dyeing was carried out according to the method described by Jefferson (1989). Segments of leaves sampled from transgenic rice lines were incubated in a solution containing 100 mM Na₃PO₄ (pH 7.0), 0.1% Triton X-100, 10 mM EDTA, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, and 1 mg·mL⁻¹ 5-bromo-4-chloro-3-indolyl- β -D-GlcUA (X-Gluc) at 37°C for 12 h. The stained tissues were then transformed into 95% ethanol for 24 h to remove chlorophylls. The leaves of the *WT*, e.g. the rice accessions, Lemont and Dular, were used for *GUS* dyeing as control. An 800-bp *GUS* gene contained in *pTCK303* and *Ubiquitin-1301* vectors was amplified from the positive transgenic rice, the sequence of *GUS* primers is 5'-gcattgtacgtcctgtagaaacc-3' and 5'-caagccagtaaagttagaacggt-3'. The stability of the transgenic plants was checked by germinating in a hygromycin B water solution.

Detection of *Lsi1* gene expression, Si uptake and Si concentrations in transgenic rice lines

In this section, the identified transgenic rice lines and their *WT* were transplanted into the pots, each pot contained 0.5 l Si solution and one rice plant. After 7 days, total RNAs of transgenic rice lines and *WT* were isolated and cDNA was synthesized following the method as described in section “Plant materials”. Gene expression levels of *Lsi1* were detected in both transgenic rice lines and *WT*, respectively, according to qRT-PCR methods. The forward primer 5'-tcgccgactcttccctc-3' and reverse primer 5'-atcgctccggtgaactgc-3' were used. Further, the root uptake of Si and Si concentration in transgenic rice lines and *WT* leaves were determined by the colorimetric molybdenum blue method, following the protocols as describe by Ma et al. (2003).

UV-B treatment

The identified transgenic rice lines and their *WT* were then transplanted, respectively, into the new pots for recovery in the solution culture above. After 7 days, each transgenic rice line and *WT* were exposed to enhance UV-B radiation, the treatment was followed by the method as described by Fang et al. (2009a), the procedures were described as follows, fluorescent lamps (40 W, Beijing Electric Light Sources Research Institute, China) were used as the source of UV-B radiation, these lamps were suspended above the

rice plants and UV-radiation of wavelength less than 280 nm (UV-C) was eliminated by wrapping the lamps in a 0.1-mm cellulose diacetate film (West Design Product Co., Ltd, United Kingdom). The distance between the rice canopy and the lamps was 30 cm and 5 lamps were used. The rice seedlings were irradiated with UV-B for a period of 1 week. The UV-B radiation was applied at the top of the leaf canopy for a period of 7 h per day (from 09:00 to 16:00 h) at an intensity of $18.6 \text{ kJ m}^{-2} \text{ d}^{-1}$ (It equals to the enhance dose of UV-B radiation when 25% depletion of the stratospheric O_3 layer occurred in the summer solstice in Fuzhou). Rice under solar radiation was taken as control. The second leaves from the apex (fully open leaf at that point of time) of the two treated rice accessions and their corresponding controls were randomly sampled on days 7 after UV-B treatment. The obtained samples were immediately frozen in liquid nitrogen and stored at -80°C prior to SSH analyze. SSH was carried out using the PCR-Select cDNA Subtraction Kit (Clontech) according to the manufacturer's introduction (Fang et al. 2009a, b; Song et al. 2008).

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using SPSS. Means were tested by the least significant difference at $P < 0.05$ ($\text{LSD}_{0.05}$).

Results

Lsi1 expression in transgenic rice lines

GUS dyeing and amplification of *GUS* gene were used to identify the transgenic rice (Fig. 1). To detect the expression level of *Lsi1* in transgenic rice lines, qRT-PCR was performed on the roots of the transgenic rice together with *WT*. As expected, *Lsi1* expression was inhibited in *Lsi1*-RNAi transgenic line of UV-B resistant rice Lemont,

showing 2.10 times down-regulation in comparison with its *WT*; the reverse was true in the case of *Lsi1*-overexpressed transgenic line of Lemont, showing 2.25-folds up-regulation in *Lsi1* transcript level. In terms of *Lsi1*-overexpressed transgenic line of UV-B sensitive rice Dular, it was up-regulated by 1.87 times in *Lsi1* expression compared to its *WT*. However, there was no difference in *Lsi1* expression between *WT* plants and blank vector control lines (Fig. 2). The sterilized T_1 – T_3 grain were also confirmed germinating in a hygromycin B water solution ($20 \mu\text{g/ml}$), showing the stability of the transgenic plants (data not shown). The result confirmed that it was successful in *Lsi1* suppression and its overexpression in rice with the aid of the molecular biological technique.

Si uptake and Si concentrations in transgenic rice lines

We also investigated the Si transport activity in transgenic rice lines and *WT*, the result showed that Si uptake was significantly reduced in *Lsi1*-RNAi transgenic line of Lemont compared with its *WT*. On 7 days, the accumulation of Si was 18.90 mg in 1 g dry root that sampled from *Lsi1*-RNAi transgenic line of Lemont in hydroponics with Si supplement, while 49.65 mg in that of *WT*. Si uptake capacity was significantly enhanced in *Lsi1*-overexpressed transgenic line of Lemont, the amount of Si uptake was 93.87 mg on 7 days. As far as the UV-B sensitive rice, Dular was concerned, the overexpression of *Lsi1* also significantly increased Si transport capacity in the roots of the transgenic line of Dular, the result showed that the amount of Si uptake was 55.16 mg in the transgenic line on 7 days, showing much higher than that (28.38 mg) in *WT* (Fig. 3I). The same trend was found in Si concentrations in the leaves of rice lines, showing that 10.17 mg Si in 1 g leaf dry weight in *Lsi1*-RNAi transgenic line of Lemont, 21.08 mg g^{-1} in the *WT* and 38.32 mg g^{-1} in *Lsi1*-overexpressed transgenic line of Lemont; For Dular rice accession, Si concentration in *Lsi1*-overexpressed transgenic line was 22.34 mg g^{-1} , which was significantly

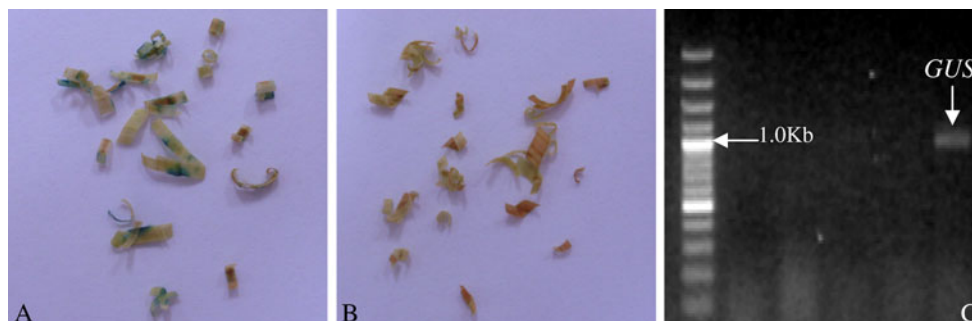


Fig. 1 Identification part of *Lsi1* transgenic rice by β -glucuronidase activity and PCR amplification. **a** Transgenic rice; **b** Wide type **c** Amplification of *GUS* gene in transgenic rice

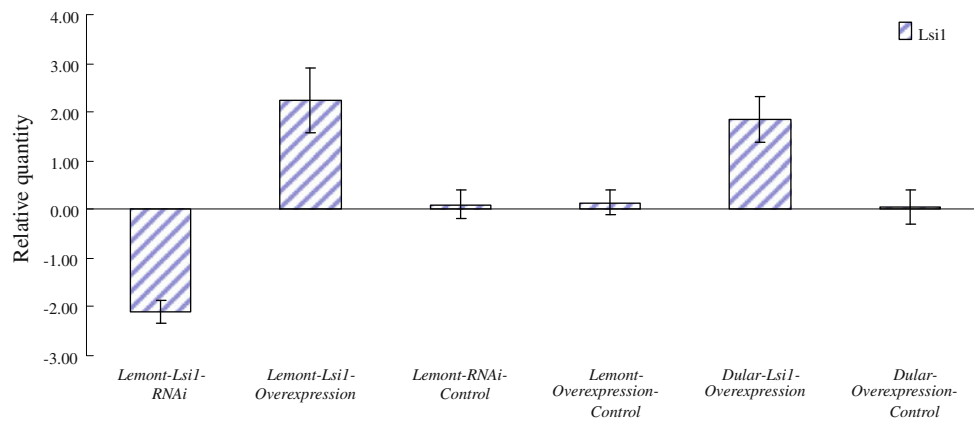


Fig. 2 qRT-PCR analysis *Lsi1* expression in transgenic rice plants and WT. *Lemont*, *Dular*: Rice cultivars; *Lsi1*-RNAi: *Lsi1*-RNAi transgenic rice line; *Lsi1*-overexpression: *Lsi1*-overexpression transgenic rice line; *RNAi-Control*: the control, RNAi vector transgenic rice line; *Overexpression-Control*: the control, overexpression vector transgenic rice line; The same below

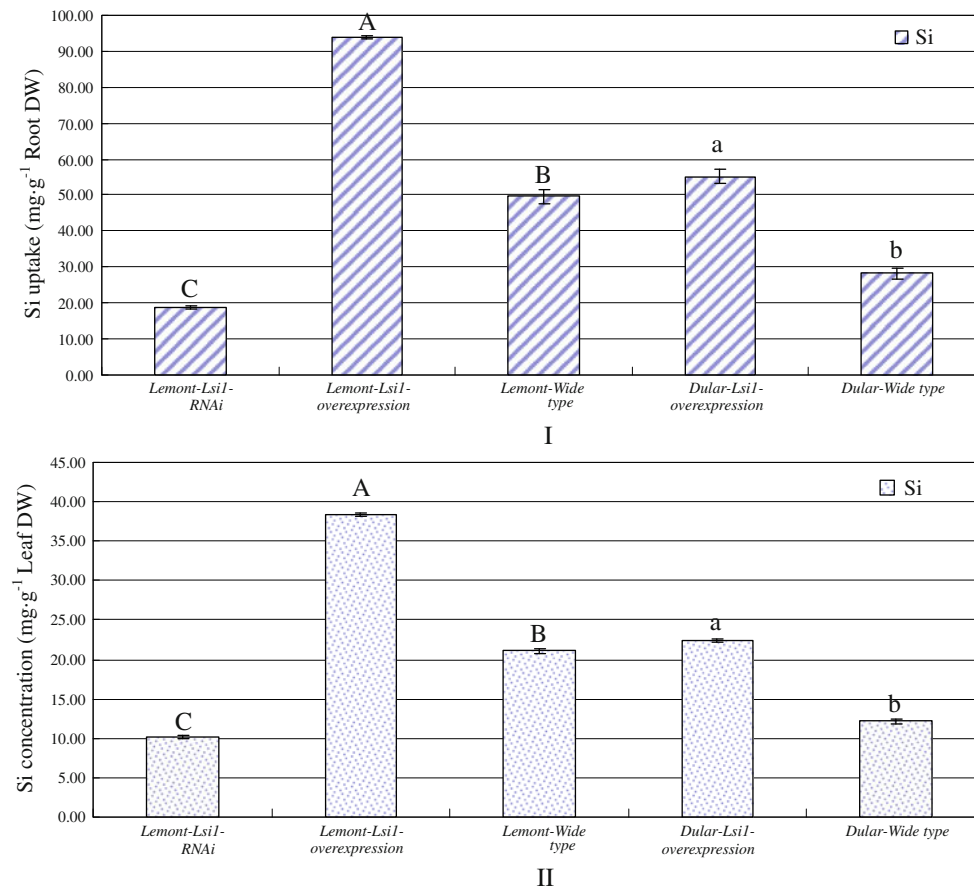


Fig. 3 Root Si uptake and leaf Si concentration in transgenic rice and WT. Values are means \pm SE based on three replicates. Different letters in each column indicate significant differences between means at $P < 0.05$ according to LSD. Small letters denote significant

differences between *Lsi1*-overexpressed line of Dular and WT; Large letters designate the differences between *Lsi1*-RNAi, *Lsi1*-overexpressed lines of Lemont and WT ($P < 0.05$). Values with the same letter are not significantly different ($P < 0.05$). FW: Fresh weight

higher than that (12.15 mg g^{-1}) in its WT (Fig. 3II). This result confirmed that *Lsi1* functions indeed in controlling silicon accumulation in rice.

The result also found that the *Lsi1*-overexpressed transgenic lines of Lemont and Dular were not phenotypically different from WT, but the yellow specks on the

leaves of *Lsi1*-RNAi transgenic lines of Lemont were detected as shown in Fig. 4, implying that *Lsi1* might be involved in the defense to UV-B stress in some way.

Analysis of differential gene expression by using SSH

To unravel the regulatory network of *Lsi1* in the defense response to UV-B irradiation, we isolated down-regulated genes in *Lsi1*-RNAi transgenic line of Lemont, as well as up-regulated genes in *Lsi1*-overexpressed transgenic lines of Lemont and Dular in the comparison with *WT* under enhanced UV-B radiation treatment using SSH. The positive gene clones were selected for reverse RNA blot after PCR (Fig. 5).

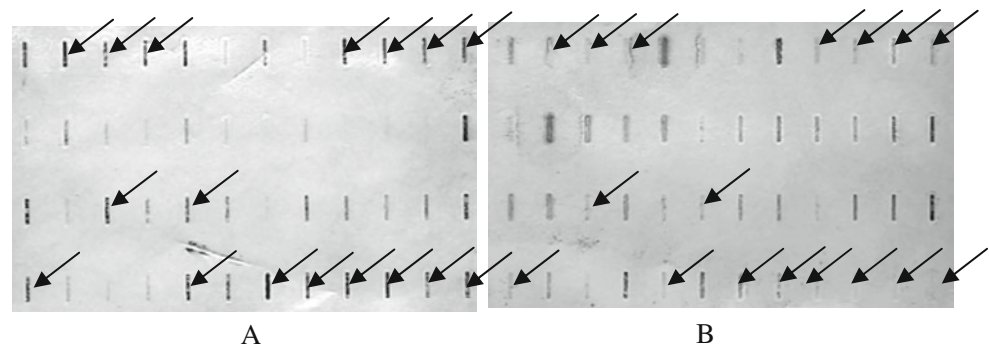
These clones were sequenced, and the cDNA sequences were searched against standard databases (www.ncbi.nlm.nih.gov). According to nucleic acid homologies and encoded protein sequences of the rice (*O. sativa* L.) database, the putative functions of the assigned expressed sequence tags (ESTs) are given in Table 1. The results indicated that several genes showed various expression trends in different transgenic rice lines. The identified genes were given as follow: A resistance-related non-coding RNA, which has been reported to be look like the inducible RNA in planthopper-infested *O. sativa*, was up-regulated in *Lsi1*-overexpressed transgenic lines of Lemont and Dular, but down-regulated in *Lsi1*-RNAi transgenic line of Lemont compared with *WT* when exposed to enhance UV-B radiation. The same was true in some genes, such as the gene encoding DNA repair associated enzyme putative CPD photolyase (*PL*, EC 4.1.99.3), the detoxification-related genes coding for metallothionein-like protein type 1 and glutathione S-transferase (*GST*, EC

2.5.1.18), and the genes for 4-coumarate-CoA ligase-like protein (*4CL*, EC 6.2.1.12) and phenylalanine ammonia-lyase (*PAL*, EC 4.3.1.5), which are associated with phenolics and flavonoids synthesis. However, some genes in rice plants in question showed different expression patterns in the responses to UV-B radiation, such as the genes encoding rice photosynthesis-related proteins, including photosystem I reaction centre subunit N, chlorophyll a-b binding protein 2, photosystem ii 10 kDa polypeptide, ribulose-bisphosphate carboxylase activase large isoform precursor protein and ribulose bisphosphate carboxylase/oxygenase activase appeared to decrease transcript levels in *Lsi1*-RNAi transgenic line of Lemont, reverse was true in *Lsi1*-overexpressed transgenic lines of Lemont and Dular. The genes coding for protein kinase domain containing protein, zinc-finger protein Lsd1, and dof zinc finger protein, which are all involved in signal transduction, were down-regulated in *Lsi1*-RNAi transgenic lines of Lemont, but up-regulated in *Lsi1*-overexpressed transgenic lines of Lemont and Dular. A down-regulated gene encoding constitutive photomorphogenic (COP8) homolog was detected only in *Lsi1*-RNAi transgenic line of Lemont, and the gene coding for *NPH3* domain containing protein, which is a *NPH1* photoreceptor-interacting protein, was up-regulated in *Lsi1*-overexpressed transgenic lines of Lemont and Dular. Some down-regulated genes which are related to cell physiology, such as cell elongation protein, peptide chain release factor 2 family protein and K⁺ potassium transporter family protein were detected only in *Lsi1*-RNAi transgenic line of Lemont, however, the genes encoding amino acid selective channel protein, 16 kDa membrane protein and membrane related protein-like were up-regulated in *Lsi1*-overexpressed transgenic line of

Fig. 4 Phenotypes of different Lemont transgenic rice lines and their wild type



Fig. 5 Part of screening of positive clones by reverse northern blot. **a** Forward hybridization **b** Reverse hybridization. * *Arrows* refer to positive clones



Lemont and Dular. This result provided us the valuable information to outline the regulatory network how to increase the tolerant ability to defense UV-B radiation via the feedback regulation of *Lsi1* to increase the activities of *PAL* and *PL* etc. in rice responding to UV-B radiation stress.

Discussion

The previous studies have shown that as a vital role involves in regulating plant growth and enhancing plant tolerance to abiotic and biotic stresses, accumulation of Si in rice plants is mediated by a member of *Nod26*-like major intrinsic protein (*NIP*) subfamily encoded by *Lsi1*, and the suppression of *Lsi1* in rice would result in reduced Si accumulation (Ma et al. 2006). In this study, we suppressed *Lsi1* transcript level in UV-B resistance rice Lemont and enhanced transcript level of *Lsi1* in Lemont and UV-B-sensitive rice Dular using RNAi and overexpression technique, respectively. The results certified that the absorptive capability of Si was significantly increased in *Lsi1*-overexpressed transgenic lines of Lemont and Dular compared to *WT*. When the transgenic rice lines and *WT* were exposed to UV-B irradiation, we found that a resistance-related non-coding RNA (nc1) was up-regulated in *Lsi1*-overexpressed transgenic lines of Lemont and Dular, but down-regulated in *Lsi1*-RNAi transgenic line of Lemont compared to their responsive *WT* plants. It has been reported that resistance-related non-coding RNA (ncRNA1) was an inducible RNA detected in planthopper-infested *O. sativa* L. spp *indica*. The present result suggested that the regulation of rice UV-B tolerance by *Lsi1* might be based on the differential expression ways of ncRNA, which acts as a regulator in rice to trigger off the activation of plant defense system in the tolerance response to UV-B radiation. The tentative cassette reaction might be to increase messenger RNA (mRNA) expression of the antioxidant enzymes glutathione S-transferase and metallothionein-like protein type 1, which was up-regulated in *Lsi1*-overexpressed transgenic lines of Lemont and Dular, in the comparison with *WT*, when they

were exposed to UV-B radiation. It has been reported that the up-regulated expression of the genes coding for the two antioxidant enzymes may contribute to prevent from the oxidative damage caused by UV-B radiation etc. (Marrs 1996). The result was further confirmed in the expression pattern of the gene encoding glutathione S-transferase and metallothionein-like protein type 1 in *Lsi1*-RNAi transgenic rice, showing down-regulation in the mRNA expression level of the two enzymes in the *Lsi1*-suppressed transgenic line under UV-B radiation supplement.

The present study also showed that the overexpression of *Lsi1* was able to significantly promote the up-regulation of *PAL* and *PL* in rice. As *PAL* is a key enzyme in plant phenylalanine metabolism pathway, it catalyzes the conversion of phenylalanine to cinnamate and initiates all secondary phenolic biosynthesis (Zon and Amrhein 1992), meanwhile, and also shows the activity to convert tyrosine into p-coumarate, following by *4CL* converting p-coumarate into its coenzyme-A ester, which in turn triggers off the reaction with malonyl CoA in the flavonoid biosynthetic pathway starting with the condensation of one molecule of 4-coumaroyl-CoA and three ones of malonyl-CoA to produce naringenin chalcone (Bohm 1998; Harborne and Williams 2000), and the production of various secondary substances, which known as primarily UV-B absorbing compounds, participating in plant responses to enhanced UV-B radiation (Barabás et al. 1998). The analysis of the gene differential expression and its related metabolites showed that the up-regulated transcripts level of *PAL* and *4CL* in *Lsi1*-overexpressed transgenic lines of Lemont and Dular resulted in significantly increased content of UV-B absorptive substances including flavonoids and total phenolics in the leaves of the two transgenic rice lines, and this in turn led to enhanced UV-B tolerance in rice (McClure 1975).

Our previous study has also reported that the activation of a metabolic response to promote the synthesis of UV-protective substances, flavonoids and phenolics, would in turn reduce the UV-induced damage or trigger the molecular processes involved in the repair of UV-B-damaged DNA, and the two steps involved in the process of the molecular regulation to up-regulate transcript level

Table 1 Differential expression of genes in *Lsi1* transgenic rice accessions compared to *WT* when exposed to UV-B radiation

Gene accession no.	Best homologue in database	Expression trend		
		<i>Lsi1-RNAi</i> rice cultivar Lemont	<i>Lsi1-overexpression</i> rice cultivar Lemont	<i>Lsi1-overexpression</i> rice cultivar Dular
Detoxification-related				
NM_001075076	Metallothionein-like protein type 1 cell rescue defense and virulence	Down-regulated	Up-regulated	Up-regulated
NM_001066130	Glutathione S-transferase	Down-regulated	Up-regulated	Up-regulated
Resistance-related				
AY166969	Resistance-related non-coding RNA	Down-regulated	Up-regulated	Up-regulated
Photosynthesis-related				
NM_001072866	Photosystem I reaction centre subunit N, chloroplast precursor	Down-regulated	Up-regulated	Up-regulated
NM_001050028	Chlorophyll a-b binding protein 2, chloroplast precursor	Down-regulated	Up-regulated	Up-regulated
EF575937	Photosystem ii 10 kDa polypeptide	Down-regulated	Up-regulated	Up-regulated
AB110180	Ribulose-bisphosphate carboxylase activase large isoform precursor protein	Down-regulated	Up-regulated	Up-regulated
EF576577	Ribulose bisphosphate carboxylase/oxygenase activase	Down-regulated	Up-regulated	Up-regulated
Flavonoids, phenolics synthesis				
NM_001060839	4-coumarate-CoA ligase-like protein	Down-regulated	Up-regulated	Up-regulated
NM_001062143	Phenylalanine ammonia-lyase	Down-regulated	Up-regulated	Up-regulated
Cell physiology				
NM_001071069	Cell elongation protein	Down-regulated	Not found	Not found
NM_001066459	Peptide chain release factor 2 family protein	Down-regulated	Not found	Not found
NM_001069852	K ⁺ potassium transporter family protein	Down-regulated	Not found	Not found
NM_001060981	Amino acid selective channel protein	Not found	Up-regulated	Up-regulated
AK058848	16 kDa membrane protein	Not found	Up-regulated	Up-regulated
NM_001058601	Membrane related protein-like	Not found	Up-regulated	Up-regulated
Signal transduction				
NM_001055807	COP8 (Constitutive photomorphogenic) homolog (CSN complex subunit)	Down-regulated	Not found	Not found
NM_001072128	NPH3 domain containing protein (A NPH1 Photoreceptor-Interacting Protein)	Not found	Up-regulated	Up-regulated
NP_001047432	Protein kinase domain containing protein	Down-regulated	Up-regulated	Up-regulated
NM_001067577	Zinc-finger protein Lsd1	Down-regulated	Up-regulated	Up-regulated
NM_001054524	Dof zinc finger protein	Down-regulated	Up-regulated	Up-regulated
DNA repair				
NM_195034	Putative CPD photolyase	Down-regulated	Up-regulated	Up-regulated

of *PAL*, *4CL* and *PL* etc. as mentioned above, seems to be vital for the development of tolerance in the rice cultivar (Fang et al. 2009a). The present study certified that enhanced UV-B radiation also resulted in induced the up-regulation of the gene coding for *PL*, which functions in splitting the cyclobutane ring and repairs the damaged DNA caused by UV (Mees et al. 2004). It is therefore suggested that the activation of the mRNA expression level of *PL* would contribute to enhance DNA repairing capability, and consequently enhance rice resistance to UV-B radiation stress.

The present result suggested that *Lsi1* affect rice photosynthesis and photomorphogenesis under UV-B radiation. One case in this study can be taken for the example. As we know, the ribulose bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), which involves the ATP-dependent carboxylation of the epsilon-amino group of lysine leading to the formation of a carbamate structure, is a key enzyme for photosynthesis in the leaves of rice. The detection indicated that it was significantly suppressed by elevated UV-B radiation at the transcription step during early leaf stages (Takeuchi et al. 2006); however, the

present result showed that the mRNA expression of Rubisco in *Lsi1*-overexpressed lines of Lemont and Dular was higher than that of *WT* when exposed to UV-B radiation. The same tendency was found in the gene expression level of other photosynthesis-related proteins, such as photosystem I reaction centre subunit N, which functions in mediating the binding of the antenna complexes to the PSI reaction center and core antenna, and plays an important role in docking plastocyanin to the PSI complex (Haldrup et al. 1999), chlorophyll a-b binding protein 2 known as light-harvesting complex (LHC), which works as a light receptor, in capturing and delivering excitation energy to photosystems (Liu and Shen 2004), and photosystem II 10 kDa polypeptide, which is associated with the oxygen-evolving complex of photosystem II (Lautner et al. 1988). While in *Lsi1*-RNAi transgenic line of Lemont, the expression level of these genes was lower than that of *WT* when exposed to UV-B radiation. The results further supported the hypothesis that the higher *Lsi1* expression level is beneficial for the enhancement of UV-B tolerance in rice.

With respect to photomorphogenic responses of different transgenic lines to the stressful condition, the different transgenic rice lines showed various sensitivities to the supplement of UV-B radiation. UV-B radiation induced photomorphogenic responses in *Lsi1*-overexpressed transgenic lines of Lemont and Dular by up-regulating the expression of nonphototropic hypocotyl 3 (NPH3) gene encoding a NPH1-interacting protein, which is a member of a large protein family, apparently specific to higher plants, and may function as an adapter or scaffold protein to bring together the enzymatic components of a NPH1-activated phosphorelay. In *Arabidopsis thaliana* seedlings, the phototropism is initiated by NPH1, a light-activated serine-threonine protein kinase (Motchoulski and Liscum 1999). However, in *Lsi1*-RNAi transgenic line of Lemont, the supplement of UV-B radiation inhibited the photomorphogenesis, which was attributed to down-regulation of constitutive photomorphogenic 8 (CSN complex subunit), a photo receptor in photomorphogenesis, which involves the expansion of leaves and accumulation of chlorophyll etc. (Kim et al. 1998).

It was also found that the overexpression of *Lsi1* in rice could activate amino acid selective channel protein, membrane related protein, which contribute to strengthen the membrane structure in cell, protein kinase domain containing protein, and zinc-finger protein, which are associated with signal transduction in the pathway to increase UV-B tolerance in rice through the feedback regulation of *Lsi1* function, but the reverse was true in *Lsi1*-RNAi transgenic line of Lemont when exposed to UV-B radiation. In addition, the inhibited expression of *Lsi1* in rice also resulted in down-regulated mRNA

transcripts level of cell elongation protein, peptide chain release factor 2 family proteins and K⁺ potassium transporter family protein, which are involved in cell physiology. These findings suggested that *Lsi1* may play different roles in the feedback regulation through the positive or negative regulatory machinery of the gene.

In conclusion, rice UV-B tolerance could be regulated by *Lsi1*, and the overexpression of *Lsi1* contributes to enhance rice UV-B tolerance and the resistance-related non-coding RNA acts as a regulator in the channel, the active effect involves enhancing gene expression level of *PAL*, *4CL* etc. Increasing accumulation of Si in rice can also activate *PL* and the related antioxidant enzymes to increase DNA repair ability to reduce the damage caused by UV-B radiation. At the same time, the photosynthesis and detoxification-related pathways were also strengthened in the leaves of *Lsi1*-overexpressed lines of UV-B resistant rice Lemont and its counterpart Dular, and all of them contribute to enhanced rice defense to UV-B irradiation. Further investigation should be put on the changes in grain yield and nutrient content in the grain products of different transgenic rice accessions grown in the supplement of UV-B radiation to aim at further understanding the possibility to obtain rice accessions with the high yielding and strong resistance in plant breeding by using overexpression technique of functional genes.

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