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Overexpression of rice *OsLOL2* gene confers disease resistance in tobacco to *Pseudomonas syringae* pv. *tabaci*

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Abstract

LSD1-related proteins of *Arabidopsis* with LSD1-like zinc finger domains regulate disease resistance and programmed cell death (PCD). We cloned a rice *OsLOL2* gene, orthologous to LSD1 of *Arabidopsis* and expressed it in a tobacco plant. Transgenic tobacco lines displayed enhanced disease resistance to a virulent bacterium *Pseudomonas syringae* pv. *tabaci* (*Pst*). RT-PCR analysis showed that overexpression of *OsLOL2* in transgenic tobacco lines resulted in upregulation of two pathogenesis-related (PR) protein genes, *PR2* and *PR5*. Our results suggest that overexpression of *OsLOL2* in transgenic tobacco enhances the resistance through the induction of PR proteins and hypersensitive response-like reaction.

Keywords: *OsLOL2*; Zinc finger protein/domain; Hypersensitive response (HR); *Pseudomonas syringae* pv. *tabaci*; Disease resistance

1. Introduction

Plants are sessile and confronted with a variety of harsh environmental stresses during their lifetime, which adversely affect physiological processes. Several biotic (viruses, bacteria, fungi and insects) and abiotic (light, temperature and water availability) stresses affect metabolism, growth and yield of higher plants [1,2], and plants have evolved defense mechanisms accordingly [3]. It is desirable to introduce disease resistance genes to confer resistance in plants against pathogens threat. However, host–pathogen recognition scope has already been broadened [4–6]. To date, numerous disease resistance genes of plants involving recognition of invading pathogens have been cloned and characterized, but the success claim remains to be questioned [7].

Plants possess a variety of defense mechanisms for pathogens recognition and protect themselves from pathogens' attack [3]. Usually, the defense reaction commences with pathogens' avirulence (Avr) proteins recognition by the plant disease resistance (R) proteins that can be represented as a gene-for-gene model [8]. This in turn activates a cascade disease resistance signaling events [9]. The R-Avr interaction results in a hypersensitive response (HR), a strong defense mechanism recognized as a swift, local programmed cell death (PCD) reaction. The PCD subsequently results in mobility arrest, growth retardation and confinement of invading pathogen at the infection site, because of an incompatible pathogen–plant interaction. The HR is often coupled with evoking plant immune responses and systemic acquired resistance (SAR). SAR may be characterized by production of reactive oxygen species (ROS) [10], activation of peroxidases [5,7], assembly of phytoalexins [11], flavonoids [12], lipid peroxidation [13], and rise in salicylic acid (SA) level [14], aggravation of pathogen-related (*PR*) genes transcription and augmenta-

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tion of resistance against a number of virulent pathogens [15].

An LSD1-related zinc finger protein class has been found to regulate the PCD and transmit the signals for disease resistance in *Arabidopsis* [16,17]. The *LSD1* gene encodes a novel class of zinc finger protein due to its unique (C-X2-C-X14-C-X2-C) zinc finger domain [17]. The *lsd1* mutants exhibit lesions to intensify the disease resistance response under long-day conditions. However, *PR* genes expressed under such circumstances and the *lsd1* mutants showed resistance to the virulent pathogens [16]. The *lsd1* mutants need NIM1/NPR1 and EDS1/PAD4, the disease resistance signaling expression as well as accumulation of SA essentially for cell death [18,19]. *LOL1* encodes an LSD1-related protein acting as a positive regulator of cell death [20]. Antisense repression of *LOL1* led to a decline resistance in *Arabidopsis* against avirulent pathogens, but the overexpressing *LOL1* transgenic lines displayed more resistance to virulent pathogens [20]. Seven *LSD1*-related genes have been documented in the rice genome at present. Among, the regulatory phenomenon of *OsLSD1* in cell death and callus differentiation has been well established [21]. More recently, we characterized another *LSD1* ortholog *OsLOL2*. Overexpression and antisense expression of *OsLOL2* in transgenic rice suggest that *OsLOL2* is involved in rice growth and disease resistance [22]. In this paper, we provide evidence that expression of *OsLOL2* gene triggers disease resistance in tobacco against virulent *Pst*. Enhanced resistance of *OsLOL2* transgenic tobacco to *Pst* may result from an induction of PR proteins and hypersensitive response-like reaction.

2. Materials and methods

2.1. Gene cloning and construction of expression vector

The rice *OsLOL2* was cloned and the expression vector p*OsLOL2*S was constructed according to the method of Xu and He [22].

2.2. Tobacco transformation

Agrobacterium tumefaciens EHA105 bacteria harboring pCAMBIA1301 and p*OsLOL2*S [22] were used to transform *Nicotiana tabacum* cv. SR1 leaflets according to the method proposed by Horsch et al. [23] and then transferred to soil for seed setting. T1 progeny plants from independent T0 transformants seeds were made to raise on a 1/2 MS medium with a selective antibiotic, the hygromycin, and then flush green plantlets with roots were transferred to soil. The T2 plants grown under greenhouse conditions were used for disease resistance assay.

2.3. PCR amplification assay

The PCR assay of T0 and T1 generations was conducted for the detection of transgene in putative transgenic tobacco plants. The genomic DNA from fresh fully expanded tobacco leaves was used for PCR analysis. The amplification of hygromycin resistance gene (*Hyg*) and *OsLOL2* transgene products was made by adjusting PCR conditions for 30 cycles. The *Hyg* forward Hyg-F 5′-TGC GCCCAAGCTGCATCAT-3′ and reverse Hyg-R 5′-TGA ACTCACCGCGACGTCTGT-3′ and for *OsLOL2* gene, the forward OsL-F 5′-TCAGCTACCACTTGCATCGC-3′ and reverse OsL-R 5′-GTTGCTCGATTGATAACCG A-3 primers pairs were used for amplification of the desired gene products.

2.4. RT-PCR analysis

The 4 μg tobacco total RNA extracted from fresh fully expanded leaves was used for reverse transcription with the hexamer oligonucleotide primer using RT-PCR kit (Fermentas, China) in a 20 μl reaction according to the manufacturer's instructions. Two microliters of cDNA solution was used for PCR amplification (94 °C 50 s, 60 °C 1 min, 72 °C 1 min, 28 cycles). RT-PCR for *OsLOL2* was performed with the forward primer OsL-F 5′-TCAGC-TACCACTTGC ATCGC-3′ and reverse primer OsL-R 5′-GTTCTTGTGGGTTGCTCGAT-3′. The fragments of *PR2* gene were amplified using the primer pair PR2-F 5′-TCTTGCAGCTGCCCT TGTACT-3′ and PR2-R 5′-GA AGGCCAGCCACTTTCAGAT-3′. The fragments of *PR5* gene were amplified using the primer pair PR5-F 5′-CCGTATCAGGAATGCTGCAAG-3′ and PR5-R 5′-G GCCAAAGCCTAACAAGTGC-3′. The fragments of *PAR-1b* (photo assimilate respiratory) gene were amplified using the primer pair PA-b-F 5′-TCAAGTA CTGGGAA GCGTTGC-3′ and PA-b-R 5′-AGCCATTGCCGGAGTA ATCA-3′. The fragments of the tobacco *Actin* gene were amplified from the same cDNAs as a control to normalize the amount of cDNA using the forward ANF 5′-CAATGA ACTTCGTGTGGCTCC-3′ and reverse ANR 5′-CGGAA TCTCTCAGCACCAATG-3′ primer pair.

2.5. Bacterial infection, disease score, growth measurement and HR assay

The two strains (11528 and 6605) of *Pst* were used for bacterial wildfire disease infection assay on tobacco. The strains were allowed to grow for 24 h on King's medium B (20 g/l peptone, 10 g/l glycerol, 1.5 g/l K_2HPO_4, 1.96 g/l KH_2PO_4. $3H_2O$, and 1.5 g/l $MgSO_4$ 16 g/l bacto agar, pH 7.2) [24], washed twice and re-suspended in 10 mM $MgCl_2$ solution. Inoculum concentration was determined spectrophotometrically and adjusted at OD600 to 0.1–0.2 and was also confirmed by serial dilutions method on the same KMB plates. Bacterial solutions were infiltrated into fully expanded tobacco leaves using a 5 ml needleless plastic syringe [25]. The inoculum was injected into lamina locale between two veins after puncturing the abaxial leaf surface. The disease score (1–4) was assigned starting from 2nd to 7th day of post-inoculation (DPI). Photographs

were taken of the inoculated leaves on the 7th day of post-inoculation.

For bacterial growth determination, the tobacco leaves were inoculated as described above using an inoculum concentration of (10^6 cfu/ml). At each time point, six 1.25 cm^2 leaf discs were cut from the inoculated area of each treatment from transgenic and control plants. The discs were washed twice and ground in 1.5 ml eppendorf tube containing 10 mM $MgCl_2$. The bacterial population in the tobacco leaves was determined by a serial dilution plate method on King's medium B. Tobacco plants were kept in the greenhouse at 28–30 °C and 16/8 h light/dark.

The bacteria on liquid Kings' medium B were grown for 24 h at 28 °C, and 2-month-old plants raised in the greenhouse conditions were inoculated by a pressure inoculation method using a needleless syringe. The inoculum at 10^8 cfu/ml concentration was used for HR assay. The inoculated transgenic and control tobacco leaves were photographed at 12, 24 and 36 h post-inoculation.

3. Results

3.1. Generation of OsLOL2 transgenic tobacco plants

To investigate the physio-pathological role of *OsLOL2*, we generated transgenic tobacco lines bearing *OsLOL2* gene in sense sequence under the control of CaMV35 S promoter. Seven independent hygromycin resistant *OsLOL2* transgenic tobacco lines were obtained. These transgenic tobacco plants were proven to have *OsLOL2* gene by PCR (Fig. 1).

3.2. Expression of OsLOL2 transgene

Expressions of *OsLOL2* transgene in randomly selected two lines (L3 and L6) were analyzed using RT-PCR. The results indicated that *OsLOL2* was highly expressed in these transgenic lines (Fig. 2). To find the correlation between HR and SAR, the expression level of pathogenesis-related (PR) genes was determined using RT-PCR.

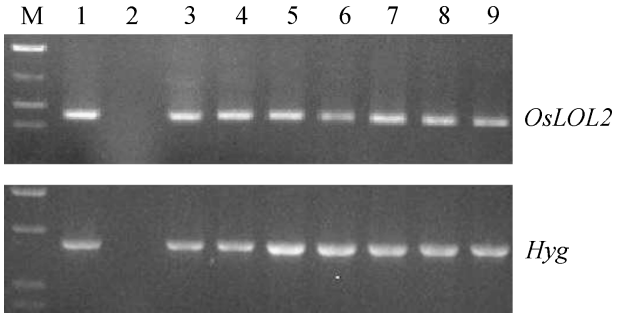

Fig. 1. PCR analysis of transgene *OsLOL2* and plant selection marker hygromycin resistance gene (*Hyg*). M, 1 kb DNA molecular weight marker; 1, vector p*OsLOL2*S plasmid DNA as positive control; 2, nontransgenic tobacco as negative control; 3–9, the transgenic tobacco lines.

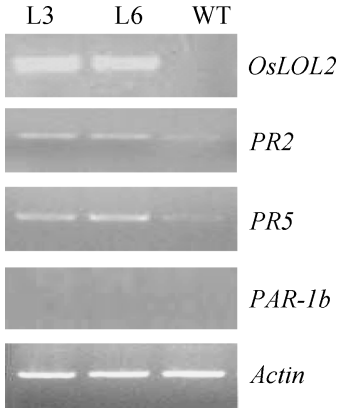

Fig. 2. RT-PCR analysis of transcripts of *OsLOL2*, *PR2*, *PR5* and *PAR-1b* genes in transgenic and nontransgenic tobacco lines. Normalized expression of *Actin* gene was used as control. WT, non-transgenic tobacco; L3 and L6, transgenic lines.

SAR may be due to the production of antimicrobial compounds by the disease resistance plants. Some of the PR gene's translational products have antimicrobial activity and provide mechanical resistance to strengthen cell wall to retard pathogen attack [26]. The expressed PR proteins probably play a role in resistance, either directly through antimicrobial activity or indirectly through signal transduction [27]. Enhanced disease resistance is often associated with PR genes expression. Among them, *PR2* gene (encoding β-1, 3-glucanase) is known to be involved in fortification of host cell wall for the reinforcement of the mechanical barrier. Expression of *PR2* gene has been confirmed for conferring disease resistance in tobacco [28]. PR5 proteins have antifungal activity, which may be involved in the enhancement of fungal membrane permeability [29–31]. The *PR2* and *PR5* genes expression was found to be upregulated in transgenic lines, which was comparable with control plants (Fig. 2). However, *PAR-1b* gene was expressed neither in the transgenic lines nor in the control plant. The equal level of *Actin* gene transcripts was obtained for normalization of the desired gene expression.

3.3. Disease resistance assay of transgenic tobacco infected with Pseudomonas syringae pv. tabaci

The homozygous T2 progeny of two *OsLOL2* transgenic lines was used for disease resistance assay. Two strains (11528 and 6605) of *Pst* were challenged for disease score assay according to proposed method [32]. The results indicated that necrotic as well as chlorotic symptoms were relatively less prominent in transgenic plants than in control (Fig. 3). The disease score on 2nd and 3rd DPI of the transgenic lines for both the *Pst* strains was relatively lower than that of the control. On the 4th day, the disease score of transgenic lines was significantly lower ($p = 0.01$) than that of the control. Nevertheless, from 5th to 7th DPI, the disease score was significantly lower ($p = 0.001$) than that of

Fig. 3. Representative disease symptoms of transgenic and nontransgenic tobacco plants. Fully expanded tobacco leaves were inoculated with *Pseudomonas syringae* pv. *tabaci* (*Pst*) at 10^6 cfu/ml inoculum. The experiment was repeated twice with similar results. Photos were taken on 10 DAI.

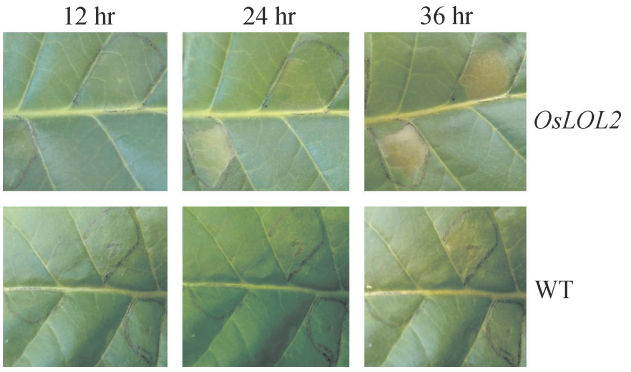

Fig. 5. Host response in transgenic lines and control to *Pst*. HR-like reactions were observed in *OsLOL2* transgenic tobacco 24 h after inoculation with *Pst* strain 11528. Similar results were observed in independent experiment inoculated with *Pst* strain 6605.

the control (Fig. 4). The results apparently indicated that the transgenic lines exhibit higher resistance against *Pst* than the control.

The HR can be seen by the quick crumple and death of the infected host tissue usually within 24 h, owing to incompatible pathogen–host interplay [33]. As a result of parchedness, the infected tissue becomes brown and then dies out. This leads to a decline in the bacterial population and the residual bacteria are restricted to the inoculated vicinity. Fully expanded tobacco leaves were subjected to infiltration with *Pst* strains at 10^8 cfu/ml. The earliest HR cropped up after 10–12 h post-inoculation as it was apparent by thinness and wrinkling of the inoculated tissue in the transgenic lines, however, at that time point, it was not visible in the control plants (Fig. 5). The HR convoyed with cellular death was more visible in transgenic plants than in wild type between 12 and 24 h post-inoculation. At 36 h the infected tissue necrosis delimiting the healthy adjoining tissue was more prominent in the transgenic lines, whereas a mild necrosis was observable in the control tissue. Our results demonstrated that the transgenic lines showed HR-like phenomenon, which may result in an enhanced disease resistance.

The bacterial growth in the infected leaf tissues was determined on 0–5 DPI with *Pst* at 10^6 cfu/ml by a needleless syringe. Using serial dilution plate method, it was recorded that the growth of both the *Pst* strains was almost equal in transgenic and control plants on zero DPI. However, the growth rate of *Pst* was relatively low on 1st and 2nd DPI in transgenic lines than that of the control (Fig. 6). On the subsequent days, the growth of *Pst* was significantly lower ($p = 0.001$) in transgenic lines than that of control (Fig. 6). These results further suggest that overexpression of *OsLOL2* in tobacco may restrict bacterial growth, which in turn enhances disease resistance.

4. Discussion

In *Arabidopsis*, 176 proteins have been discovered which contain one or more zinc finger domains, so they are among the largest family of putative transcriptional regulators found in plants [34]. Several proteins containing zinc finger domains have been characterized and were found in regulation of a variety of phenomena including stress responses [34]. The *LSD1* of *Arabidopsis* acts as a negative

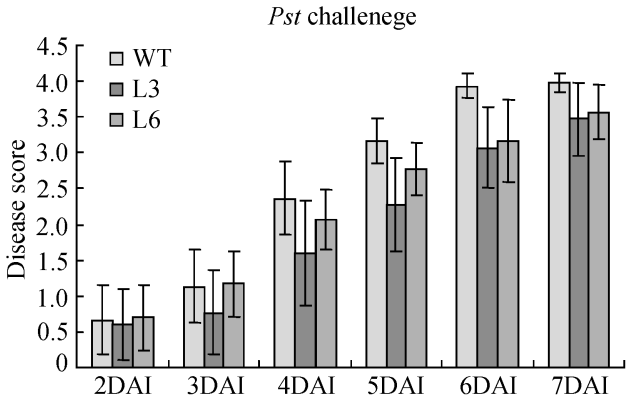

Fig. 4. Disease score of tobacco leaves challenged with *Pst*. Nontransgenic and *OsLOL2* transgenic tobacco were inoculated with *Pst* strains at 10^6 cfu/ml inoculum. Disease severity was scored at different time points after inoculation. Values are means ± standard error ($n = 15$). The disease score assay was carried out three times with similar results.

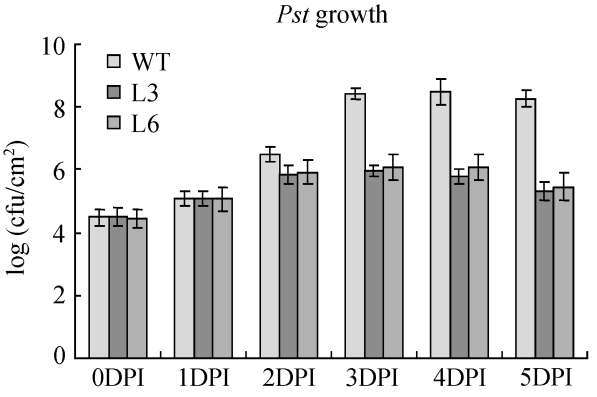

Fig. 6. Representative of *Pst* growth in transgenic and WT tobacco leaves. The population of *Pst* was measured daily for six days. Each time point represents the bacterial population recovered from six 1.25 cm^2 leaf discs from different leaves. Values presented are means of three independent experiments each with nine leaf samples and vertical bars represent standard deviations. DPI, days post-inoculation.

regulator of cell death. The *lsd1* mutant showed cell death and lesion symptoms on exposure to pathogens under long-day conditions. Nevertheless, *LOL1*, a paralog of *Arabidopsis LSD1*, acts as a positive regulator of cell death. Overexpression of *LOL1* may yield adequate signals for cell death initiation, suggesting that *LSD1*-like zinc finger domains are engaged in disease resistance [20].

Unlike *LSD1* and *LOL1* genes of *Arabidopsis,* the rice *OsLOL2* gene contains two, instead of three, zinc finger domains. It was hypothesized that *OsLOL2* might be involved in disease resistance against pathogens [22]. In this paper, we provide the evidence that *OsLOL2* is involved in cell death that in turns confers disease resistance in transgenic tobacco against *Pst*. These findings agree with the previously reported evidence that *OsLOL2* was involved in disease resistance against rice bacterial blight *Xanthomonas oryzae* pv. *oryzae* [22]. These results are also in agreement with the findings that *LOL1* of *Arabidopsis* incur disease resistance against *Pseudomonas syringae* [20]. In plants, based on the structural and functional characteristics, different families of PR proteins have been identified, which perform various functions such as cell wall fortification, signal transduction and antimicrobial activity [27]. The upregulation of *PR2* and *PR5* genes in transgenic lines suggests that *OsLOL2* may have some contributions in conferring disease resistance against virulent and/or avirulent pathogens. Nevertheless, *PAR-1b* gene was not expressed in both transgenic lines and control plants. *PAR-1b* gene expression is somewhat dependent on SA accumulation, which probably did not occur under the absence of pathogen challenge [35]. The disease symptoms were more prominent in control plants than that in transgenic lines inoculated with *Pst*. The disease score for two *Pst* strains was significantly higher in control than in transgenic plants indicating that *OsLOL2* transgene shows an elevated level of disease resistance.

We predict a functional homology existence among *OsLOL2* transgene and *PR* genes to confer disease resistance because a prominent HR-like reaction was visible in transgenic plants, commencing from 12 to 36 h after inoculation of both the strains (11528 and 6605) of *Pst*. The HR stimulation caused by *OsLOL2* transgene expression may trigger disease resistance against pathogens. The OsLOL2 protein found distinctively localized in the nucleus of a plant cell [22] signifying its probable role as a transcriptional regulation of defense-related genes. More resistance of *OsLOL2* transgenic tobacco lines against *Pst* might be due to the upregulated *PR* gene's transcription to trigger endogenous disease defense mechanism, similar to the role of *LSD1* in transcription regulation [18].

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