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Original Paper

The Arabidopsis NSL2 negatively controls systemic acquired resistance via hypersensitive response

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Abstract The Arabidopsis *nsl2* (*necrotic spotted lesion 2*) mutant, which has been originally reported as the *cad1* (*constitutively activated cell death 1*), shows a phenotype that mimics a hypersensitive response (HR)-like cell death. The NSL2 protein is suggested to negatively control the salicylic acid (SA)-mediated pathway of HR-like cell death in plant immunity. The induction of systemic acquired resistance (SAR) results in the induction of pathogenesis-related (*PR*) genes in systemic organs triggered by the local HR. In this report, we establish a *NSL2* knockdown system in transgenic Arabidopsis based on constitutive or dexamethasone (DEX)-induced RNAi. The former showed a *nsl2*-like phenotype. In DEX-induced RNAi, localized knockdown resulted in the induction of *PR1* gene expression and the restriction of bacterial growth in both DEX-treated and systemic leaves. These results indicate that NSL2 negatively controls SAR via HR.

Key words: Arabidopsis, hypersensitive response, lesion mimic mutant, local inducible silencing, *Pseudomonas syringae* pv. *tomato* DC3000.

Plants respond to pathogen infection by activating a defense mechanism known as plant immunity. One of the most efficient and immediate resistance reactions against pathogen attack in plants is the hypersensitive response (HR), which leads to rapid local cell death at the site of pathogen entry that is characterized by restricted growth and spread of the pathogen (Heath 2000a; Heath 2000b; Lam 2001; Morita-Yamamuro et al. 2005). Concomitant with the appearance of the HR, a secondary response known as systemic acquired resistance (SAR) is induced in uninfected tissue.

SAR provides long-lasting resistance throughout the plant to prevent subsequent infection by a broad range of pathogens. The establishment of SAR is associated with elevated levels of salicylic acid (SA) both at the site of infection and at the systemic tissue. Eliminating SA accumulation in transgenic plants by expressing the bacterial salicylate hydroxylase (*nahG*) gene has been shown to prevent SAR to pathogen infection (Gaffney et al. 1993; Ryals et al. 1996). *SID2/EDS16* encodes a putative *chloroplast-localized isochorismate synthase 1* (*ICS1*), which is tightly related to the pathogen-response of SA biosynthesis. Furthermore, mutations of the *ICS1* gene reduce SA accumulation after infection and compromise systemic resistance (Wildermuth et al. 2001).

Pathogen infection induces expression of pathogenesis related (*PR*) genes as well as SA synthesis. Most of the PR proteins have been shown to possess antimicrobial activity *in vitro* or an ability to enhance disease resistance when overexpressed in transgenic plants (Ryals et al. 1996). The *cprl* and *cpr5* (*constitutive expresser of PR genes*) mutants that were isolated based on elevated expression of the endogenous PR genes are resistant to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), indicating a constitutively activated SAR (Bowling et al. 1994; Bowling et al. 1997). Furthermore, SA-insensitive mutants such as *npr1* (*non-expressor of PR genes 1*), which suppresses expression of the PR genes, exhibits compromised SAR to pathogen infection (Cao et al. 1994; Cao et al. 1997; Glazebrook et al. 1996).

The *nsl2* (*necrotic spotted lesion 2*) mutant, which was originally reported as the *cad1* and which showed a constitutive HR consistent with pathogen infection, leads to a 32-fold increase in SA content and shows restricted growth of *Pst* DC3000 (Morita-Yamamuro et al. 2005). The cell death phenotype of *nsl2* is partially suppressed by crossing with transgenic plants expressing *nahG*. Thus, the NSL2 (=CAD1) protein seems to negatively

Abbreviations: DEX, dexamethasone; HR, hypersensitive response; MACPF, membrane attack complex and perforin; PCD, programmed cell death; PCR, polymerase chain reaction; PR genes, pathogenesis-related genes; *Pst* DC3000, *Pseudomonas syringae* pv. *tomato* DC3000; SA, salicylic acid; SAR, systemic acquired resistance

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control the SA-mediated pathway of SAR in plant immunity.

To clarify the physiological roles of NSL2 in plant immunity, we generated transgenic plants with both constitutive and DEX-induced NSL2 knockdown and examined the occurrence of HR and SAR. Based on the data presented here, we conclude that NSL2 is a factor that controls the SAR.

Materials and methods

Rename of NSL2 gene from the CAD1 as At1g29690

We originally reported the knockout mutant of At1g29690 as *cad1* (*constitutively activated cell death 1*) (Morita-Yamamuro et al. 2005), also seen in other related papers (Tsutsui et al. 2006; Tsutsui et al. 2008). However, "*cad*" was named after the cadmium insensitive mutant. Therefore, the name has been changed to *nsl2* (*necrotic spotted lesion 2*), since *nsl2* showed a similar phenotype to *nsl1*, and the NSL2 protein shares a strong similarity to the NSL1-containing MACPF domain (Noutoshi et al. 2006).

Plant materials and growth conditions

The wild-type Columbia-0 and all other materials used in this study were grown at 22°C. For germination, seeds were surface sterilized and placed on Murashige and Skoog medium supplemented with 20 g L^{-1} sucrose (MS medium). After an overnight cold treatment to synchronize germination, seeds were grown at 22°C with 50% relative humidity under a 16/8 h light/dark cycle.

Plasmid construction

Gene construction was performed according to the protocol supplied with the GATEWAY system (http://www.invitrogen.co.jp/gateway). The pNSL2::NSL2::myc construct using pGWB16 (Nakagawa et al. 2007) for complementation of *nsl2-1* (=*cad1-1*; SALK_002259) contained a 2-kb fragment encompassing the 5′ region upstream from the translation site of the *NSL2* gene, which was translationally fused to the myc tag. The *NSL2* gene fragment used to hairpin construct was amplified by the primer set, NSL2 KD-1 and NSL2 KD-2 (Supplemental Table 1), introduced into the TOPO cloning vector pENTR™/D-TOPO. For knock down of *NSL2* transcription, pHELLSGATE12 and pOpOff2 (kan), as described previously (Helliwell et al. 2003; Wielopolska et al. 2005), were used as the destination vector. The LR recombination reaction mix contained 2 μl LR clonase buffer, 1 μl of the *NSL2* gene fragment (100 ng) with the attL1 and attL2 amplified using the M13 primer set, M13 forward and M13 reverse (Supplemental Table 1), 2 μl of the destination vector (150 ng), 3 μl TE buffer, and 2 μl LR clonase (Invitrogen). The mix was incubated for 2 hrs at room temperature (25°C). At the end of incubation, the reaction mix was treated with 1 μl proteinase K mix for 10 min at 37°C. Three microliters were then used for transformation to α.

Arabidopsis transformation

The fusion plasmid was transformed into *Agrobacterium tumefaciens* GV2260 by electroporation, and then introduced into wild-type Arabidopsis plants using Agrobacterium-mediated transformation methods, as described previously (Sonoda et al. 2007). To select for pNSL2::NSL2 transgenic progeny, T1 seeds from primary transformants were planted on germination medium containing 50 μg L^{-1} kanamycin and 50 μg L^{-1} hygromycin. Otherwise, to select for *NSL2* gene knock down construction, T1 seeds were plated on germination medium containing 50 μg L^{-1} kanamycin. In order to introduce pNSL2::NSL2::myc into the *nsl2* mutant, pollen from heterozygous *nsl2* plants was used to fertilize homozygous transgenic progeny.

RNA isolation, RT-PCR

Total RNA was isolated from whole plants with an acid guanidium thiocyanate-phenol-chloroform extraction protocol. Single-stranded cDNA was synthesized from total RNA. First-strand cDNA synthesis was performed with reverse transcriptase using 1 μg of total RNA and oligo (dT) primer (RNA PCR kit, TaKaRa SHUZO CO., LTD. Siga, Japan). PCR (total volume, 20 μl) was performed using 0.2 units of Taq DNA polymerase (Taq, Biolabs CO., LTD. England). Gene-specific primers were designed to produce DNA fragments of the PR2 and EF1α; genes. The primers used (Supplemental Table 1) were designated NSL2-1 and NSL2-2, NSL2 KD-1 and PDK-intron, PR1-1 and PR1-2, PDF1.2-1 and PDF1.2-2, GUS-1 and GUS-2, EF1α-1 and EF1α-2 were used to amplify DNA fragments of endogenous *NSL2* (At1g29690), exogenous *NSL2* (NSL2 KD.), *PR1*, *PDF1.2*, *GUS* and *EF1*α, respectively, from their cDNAs by PCR. The amount of template cDNA required and the number of PCR cycles necessary were determined in preliminary experiments to ensure that amplification occurred in the linear range and allowed accurate quantification of the amplified products.

GUS staining and DEX treatment

GUS staining was performed as described previously (Morita-Yamamuro et al. 2005). The treatment plants grown on plates were germinated in MS medium containing 10 μM DEX. For treatment of soil-grown plants, 20 μM DEX was diluted in 0.05% agar, and was applied on rosette leaves of 2–3 week-old plants.

Infection of Pseudomonas syringae pv. tomato DC3000

The virulent bacterial leaf pathogen *P. syringae* pv. *tomato* DC3000, which causes bacterial speck disease, was grown overnight at 28°C in liquid NBY medium. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a final density of 10^5 colony-forming units ml^{-1}. For the inoculation of plants by leaf dip, the surfactant Silwet-77 was added to a final concentration 0.01%. For each sample, three leaf discs from treated or systemic leaves were pooled three times per data point. Leaf discs were bored from the infiltrated area, ground in distilled water, and serially diluted to measure bacterial numbers (Tsutsui et al. 2008).

Results

The nsl2 mutant phenotype was completely restored by NSL2 expression

The *nsl2* mutant was isolated from a T-DNA mutagenised Arabidopsis population based on a phenotype of spontaneously activated cell death. The *nsl2* mutant showed a dwarf phenotype with normal cotyledons, and dark brown or black-colored cell death lesions on the true leaves with accelerated leaf senescence. The *nsl2-1* (=*cad1-1*; SALK_002259) mutant phenotype was shown to be due to a loss-of-function mutation of the *NSL2* gene (At1g29690), as no *NSL2* transcripts were detectable in the *nsl2-1* mutant and the three additional alleles, *nsl2-2* (=*cad1-2*; GABI_192A09), *nsl2-3* (=*cad1-3*; GABI_385H08) and *nsl2-4* (=*cad1-4*; FLAG_120C12), all showed the same phenotypes (Morita-Yamamuro et al. 2005). In order to further evaluate the role of NSL2, transgenic plants were generated expressing a pNSL2::NSL2 construct in the *nsl2-1* mutant background. This construct, containing a native 2-kb promoter sequence lying directly upstream of the translation start site of the *NSL2* gene, which showed functional regions including a W-box (Tsutsui et al. 2006), was transcriptionally fused to the *NSL2* cDNA with a *nos* terminator. In 4-week-old plants, these transgenic plants (designated as pNSL2::NSL2) were indistinguishable from the wild-type, indicating that the cell death and dwarf phenotypes observed in the *nsl2-1* mutant were completely restored to the wild type (Figure 1A). The *NSL2* transcript was detectable in pNSL2::NSL2 but not in the original *nsl2-1* mutant (Figure 1B). Furthermore, the *nsl2* showed enhanced expression of both SA-related *PR1* and jasmonic acid related *PDF1.2* (Morita-Yamamuro et al. 2005) as well as the *cpr5* gene (Clarke et al. 2000). These genes were transcriptionally repressed in pNSL2::NSL2 as well as in the wild-type. Since the *PR1* and *PDF1.2* are well-known markers for the hypersensitive response, the cell death phenotype seen in the *nsl2-1* mutant was completely restored in the pNSL2::NSL2 complementation. Furthermore, overexpression of the *NSL2* under control of the constitutive 35S promoter was generated for analysis of the transgenic plants or complementation of the *nsl2-1* mutant. Although various constructs were used, and several transformation experiments were conducted, no overexpressor plants were detected, suggesting that overexpression of the NSL2 gene is lethal (data not shown). These results indicate that temporal and/or spatial expression patterns are critical for NSL2 function.

The extent of nsl2 phenotype corresponds to NSL2 transcript accumulation

There are three proteins sharing homology with NSL2 in the Arabidopsis genome that contain the MACPF (membrane attach complex and perforin) domain, which are conserved in perforin and complement components (Morita-Yamamuro et al. 2005). To clarify NSL2 function in cell death, NSL2 knock-down transformants (NSL2 KD) were generated by introducing a construct derived from the RNAi generating vector pHELLSGATE12 (Helliwell et al. 2003). This construct produced a hairpin RNA containing approximately 500 bp of *NSL2* coding region that does not share identity with other NSL2 family members. Three-week-old NSL2 KD plants were indistinguishable from wild-type (data not shown), however, at 4 weeks some of the plants did exhibit accelerated leaf senescence and brown cell death as observed in *nsl2* (Figure 2A-b, c, d). NSL2 KD plants at five weeks showed various intensities of the *nsl2* phenotype from mild to severe. In the severe NSL2 KD plants (6-1 line), cauline leaves as well as the rosette leaves had brown cell death (Supplemental Figure 1). RT-PCR analysis demonstrated that exogenously expressed *NSL2* transcript derived from hairpin RNA for NSL2 was much more abundant in the severe 6-1 plants compared to the mild plants (25-1; Figure 2 B). Consistent with this, endogenous *NSL2* transcript accumulation was less abundant in the severe 6-1 plants, indicating that the extent of NSL2 silencing corresponds to the severity of the *nsl2*-like phenotype. RT-PCR analysis also revealed that *PR1* is transcriptionally activated in the severe NSL2 KD plant as well as in *nsl2*, but not in the mild plants.

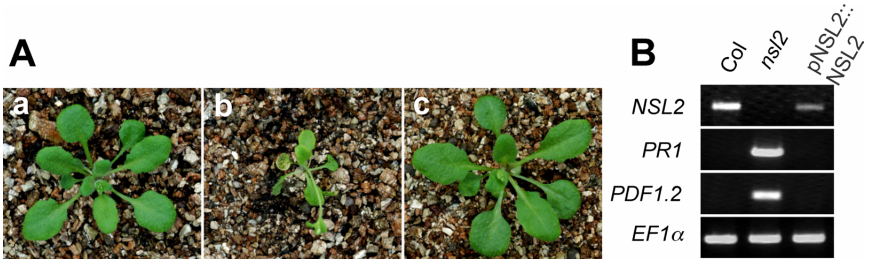

Figure 1. Complementation of the *nsl2* mutant. (A) Anatomy of 4 week-old Col (a), *nsl2-1* (b), and transgenics expressing pNSL2::NSL2 in a *nsl2-1* mutant background (c). All images are at the same scale. (B) RT-PCR analysis on the complementation. RT-PCR was performed on mRNA isolated from Col, *nsl2-1*, and the pNSL2::NSL2 transformant using specific primers for *NSL2*, *PR1*, *PDF1.2*, and EF1α; (control) (see Supplemental Table 1).

These results are consistent with the previous report (Tsutsui et al. 2006) that the *nsl2* phenotype corresponds to the reduction in *NSL2* transcript, whereas no effect of transcription for the NSL2 homolog genes was observed in the NSL2 KD plants (data not shown).

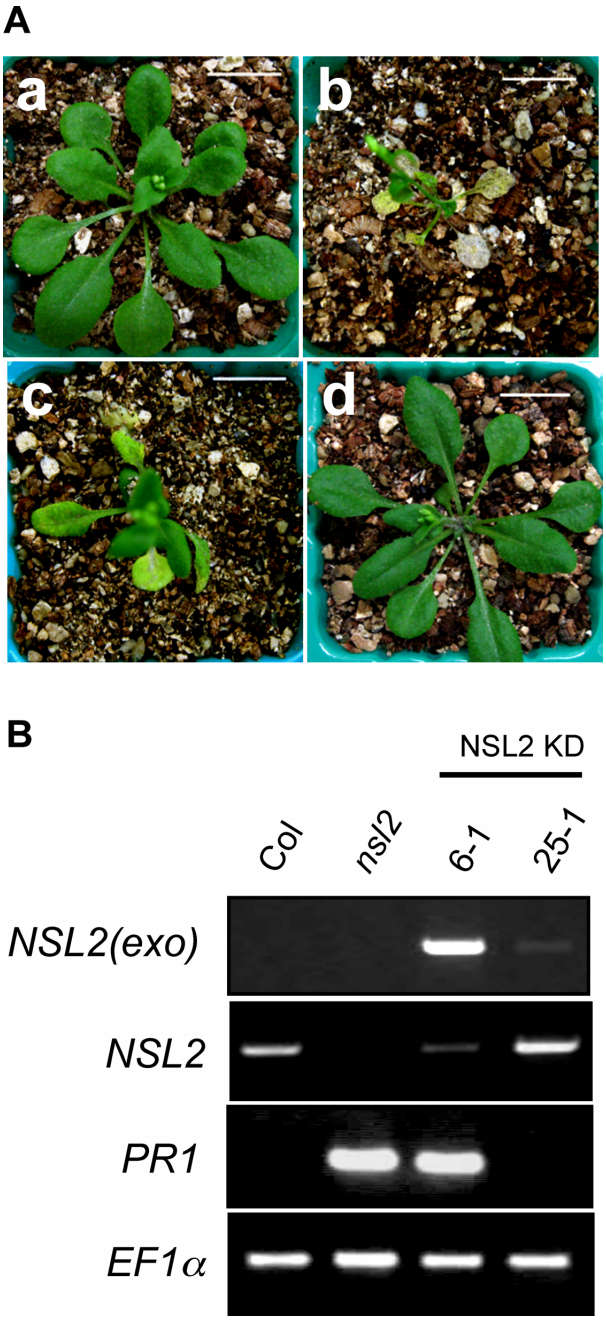

Figure 2. Phenotypic characterization of NSL2 KD. (A) Images of 4 week-old Col (a), *nsl2-1* (b), NSL2 KD (c) 6-1 as severe and (d) 25-1 as mild phenotypes. Scale bar=1 cm. (B) RT-PCR analysis of the NSL2 KD plants. RT-PCR was performed on mRNA isolated from the Col, *nsl2-1*, and NSL2 KD (6-1, 25-1) using specific primers for *PR1*, exogenous *NSL2* as "*NSL2(exo)*", endogenous *NSL2* as "*NSL2*", and *EF1α*; as control (see Supplemental Table 1).

Dexamethasone-triggered NSL2 silencing induces cell death

We have previously demonstrated that NSL2 negatively regulates SA accumulation and expression of the genes related to SA-inducible defense (Morita-Yamamuro et al. 2005; Tsutsui et al. 2006). To evaluate the possibility that NSL2 is associated with SAR, we generated the *NSL2* knock-down transformants in which transcription for hairpin NSL2 RNA could be regulated in a conditional manner by DEX treatment. The construction was made from the pOpOff2 vector which produces hairpin RNA containing a *NSL2* coding region identical to that used in the generation of NSL2 KD plants. In the pOpOff2 construct, DEX induces transcripts for hairpin RNA to reduce NSL2-specific mRNA and the GUS gene is used to visualize where the hairpin RNA is produced. In the absence of DEX, transgenic plants containing pOpOff2-NSL2 were anatomically indistinguishable from those of wild-type (Figure 3A). When grown on MS medium containing DEX for 2 weeks, the plants showed a dwarf phenotype similar to the *nsl2* mutant, even though the severe plants had a limited number of lesions. RT-PCR analysis demonstrated that DEX treatment promotes *NSL2* repression and *GUS* induction, in correlation with the dwarf phenotype (Figure 3A, B), indicating that the dwarf phenotype was due to *NSL2* silencing. *PR1* expression remained negatively correlated with *NSL2* expression and was transcriptionally promoted in pOpOff2-NSL2 when treated with DEX.

Subsequently, the spatial control of NSL2 silencing was examined by monitoring GUS activity and the cell death phenotype. DEX containing soft-agar was applied on single rosette leaves and GUS activity was detected at 3 days after treatment. As shown in Figure 4B, GUS staining was exclusively observed in leaves treated with DEX, but not in untreated leaves. Furthermore, trypan blue staining at 2 weeks after treatment revealed death of vascular cells in the region treated with DEX (Figure 4 D). These results indicate that DEX treatment of pOpOff2-NSL2 plants induces *NSL2* silencing and cell death.

Local NSL2 silencing induces resistance to bacterial infection in systemic leaves

To determine whether local NSL2 silencing transcriptionally induces the *PR* gene in systemic leaves before cell death, rosette leaves were treated with DEX and carried out with RT-PCR analysis at 3 days after treatment (Figure 5). NSL2 silencing was detectable in pOpOff2-NSL2 leaves (T) treated with DEX, but not in the systemic leaves (S). Additionally, DEX treatment promoted *PR1* expression in the systemic leaves as well as in the treated leaves. These results indicate that the *NSL2* silencing triggers activation of the *PR* gene in systemic regions as well as in localized areas where HR

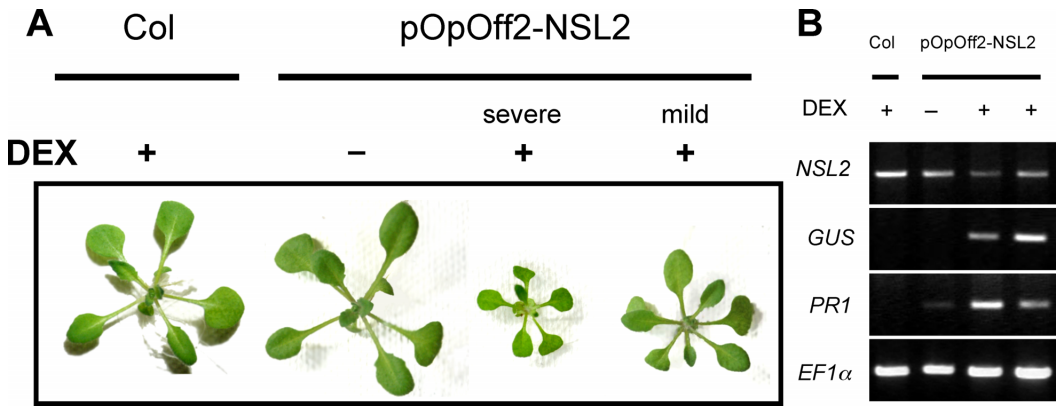

Figure 3. Effect of DEX treatment on phenotype and gene expression in pOpff2-NSL2 plants. (A) Two week-old Col and pOpOff2-NSL2 plants grown in MS medium with DEX (+) or without DEX (−). (B) RT-PCR analysis of the pOpff2-NSL2 plants. RT-PCR was performed on mRNA isolated from the Col and pOpOff2-NSL2 plants using specific primers for *PR1*, *NSL2*, *GUS*, and *EF1α*; as control (see Supplemental Table 1).

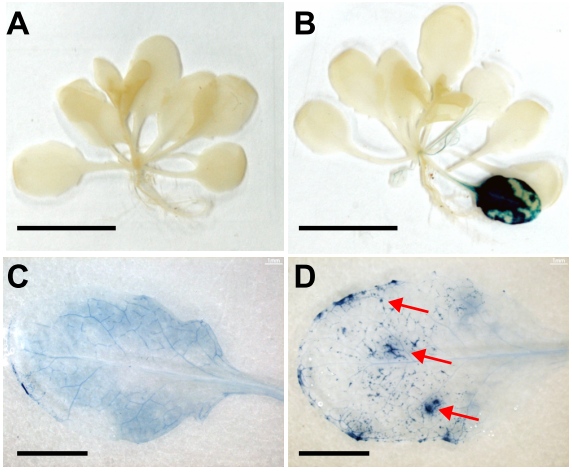

Figure 4. Effect of DEX treatment on GUS activity and cell death in pOpff2-NSL2 plants. (A)–(D) pOpff2-NSL2 plants after DEX treatment. Scale bar=1.5 cm (A, B), 0.5 cm (C, D). Plants were grown on MS medium without DEX and then treated with DEX. DEX solution (20 μM) was applied on the indicated rosette leaf. GUS staining of Col (A) and pOpff2-NSL2 plants (B) for 3 d after DEX treatment. Images were taken at the same scale. Trypan blue staining of Col (C) and pOpff2-NSL2 plant (D) for 2 weeks after DEX treatment. Red arrows show lesions.

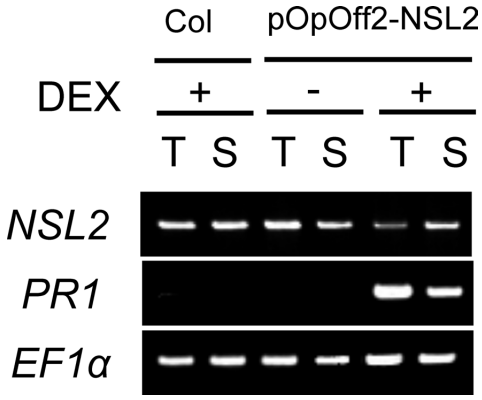

Figure 5. RT-PCR analysis of treated and systemic leaves in pOpff2-NSL2 plants. RT-PCR was performed on mRNA isolated 3 d after DEX treatment from Col and pOpOff2-NSL2, which were grown on MS medium without DEX, and then treated with DEX on the rosette leaf for 3 d. DEX solution (20 μM) was applied on the treated rosette leaf. The following primers were used: *NSL2*, *PR1* and *EF1α*; as control. T, treated leaf; S, untreated systemic leaf.

cell death takes place.

cpr mutants constitutively display both *PR* gene expression and SAR activation (Bowling et al. 1994; Bowling et al. 1997). Accumulation of *PR1* gene transcripts in systemic leaves is principally associated with SAR (Cameron et al. 1999). To evaluate whether local NSL2 silencing induces SAR, bacterial infection tests were carried out with pOpOff2-NSL2 plants. Plants were tested for disease tolerance to pathogen growth following challenge with *Pst* DC3000 after treatment of the rosette leaves with DEX. Because Kang and coworkers reported that DEX induces defense-related genes (Kang et al. 1999), Col with DEX treatment for the control was used. A marked increase in bacterial numbers was detectable at 15 hrs after inoculation in the Col with DEX treatment for 2 d, which was reduced to approximately one-fifth in pOpOff2-NSL2 leaves treated with DEX (Figure 6A). Bacterial numbers in systemic leaves were also monitored at 24 hrs after inoculation (Figure 6B). Growth in systemic leaves of pOpOff2-NSL2 was reduced to approximately half of that in the wild-type, indicating that local NSL2 silencing affects the tolerance to bacterial growth in systemic leaves. Since bacterial numbers at 2 days after inoculation were the same level between pOpOff2-NSL2 and wild-type (data not shown), these data were consistent with the fact that mRNA expression of the target gene gradually increases following removal from DEX (Wielopolska et al. 2005). These results suggested that induced RNAi leads to transient restriction of bacterial growth.

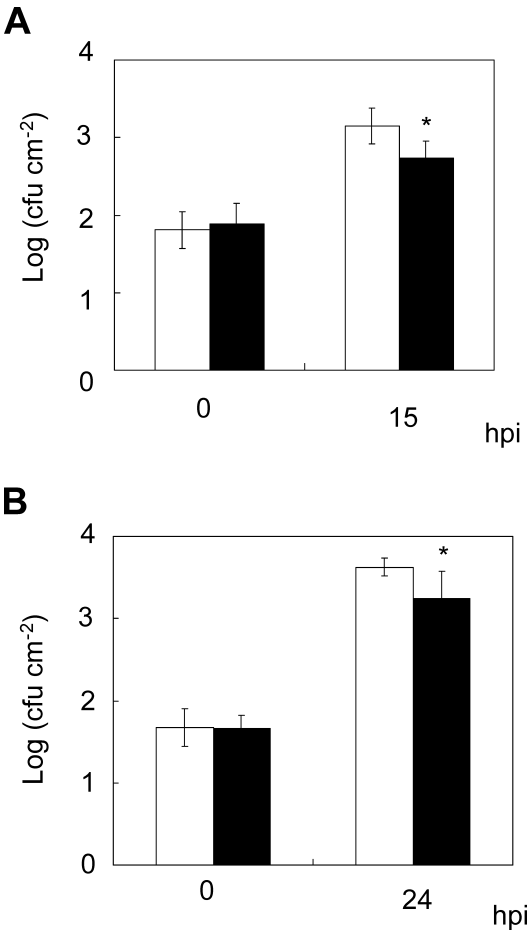

Figure 6. Analysis of the bacterial growth. Analysis of the growth (*P. syringae* pv. *tomato* DC3000) on treated (A) and systemic leaves (B) of Col (white bar) and DEX-treated pOpOff2-NSL2 (black bar) 2 d after DEX treatment. Error bars show SDs. Asterisk indicates statistically significant differences compared with Col (student's *t* test; $P<0.001$, $n=20–35$). Each experiment was repeated twice with similar results.

Discussion

Relation between HR and SAR

HR, which leads to rapid local cell death and is characterized by restricted plant growth and pathogen spread, is one of the most efficient and immediate resistance reactions in plant immunity. Mutants called lesion-mimics display phenotypes that resemble pathogen-inducible HR cell death (Lorrain et al. 2003). The recessive *lesion simulating disease 1* (*lsd1*) mutant, which is one of the lesion mimic mutants, exhibited hypersensitivity to SA, impaired control, and spread cell death following HR. LSD1 is a plant-specific zinc-finger protein, and the mutant phenotype indicated that it functions as a negative regulator of plant immunity via retention of other proteins (Dietrich et al. 1994; Kaminaka et al. 2006). The recessive *cpr5* mutant displays constitutive expression of SAR, exhibits spontaneous chlorotic lesions and constitutive resistance to virulent pathogens, which indicates that CPR5 has an unknown function as a negative regulator of plant immunity (Bowling et al. 1997; Yoshida et al. 2002). These mutants and many other lesion-mimic mutants demonstrate the effect of constitutive SAR activation. There is less information available on spatial interactions.

The recessive *nsl2* mutant also exhibits SA over-production and resistance to pathogens, indicating that NSL2 is a negative regulator of plant immunity. Park and coworkers suggested that methyl salicylic acid is converted from SA and acts as a mobile signal for SAR in tobacco (Park et al. 2007). These reports imply that NSL2 is the possible suppressor for SAR via SA regulation.

NSL2 function in terms of SAR

Taken together, the data presented here implies that NSL2 controls SAR via HR. First, the *nsl2* mutant phenotype was completely restored by NSL2 expression (Figure 1A) and NSL2 silencing resulted in an *nsl2* phenotype of HR-like cell death (Figures 2A, 3A, 4D). Secondly, the *PR1* gene was transcriptionally promoted in correlation with NSL2 silencing in constitutive knockdown plants (Figures 2B, 3B). Thirdly, induction of local NSL2 silencing resulted in disease tolerance to pathogen growth as well as *PR1* induction in treated leaves (Figures 5, 6A). Finally, local NSL2 silencing also resulted in *PR1* induction (Figure 5) and disease tolerance to pathogen growth (Figure 6B) in systemic leaves as well as in treated leaves.

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