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Loss of *NECROTIC SPOTTED LESIONS 1* associates with cell death and defense responses in *Arabidopsis thaliana*

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Abstract We isolated a lesion mimic mutant, n*ecrotic* s*potted* l*esions 1* (*nsl1*), from *Ds*-tagged *Arabidopsis thaliana* accession No-0. The *nsl1* mutant exhibits a growth retardation phenotype and develops spotted necrotic lesions on its rosette and cauline leaves. These phenotypes occur in the absence of pathogens indicating that *nsl1* mutants may constitutively express defense responses. Consistent with this idea, *nsl1* accumulates high levels of callose and autofluorescent phenolic compounds localized to the necrotic lesions. Furthermore RNA gel blot analysis revealed that genes associated with disease resistance activation are upregulated in the *nsl1* mutants and these plants contain elevated levels of salicylic acid (SA). Crossing *nsl1* with an SA deficient mutant, *eds16-1*, revealed that the *nsl1* lesions and growth retardation are dependent upon SA. The *nsl1* phenotypes are not suppressed under either the *rar1-10* or *sgt1b-1* genetic background. *NSL1* encodes a novel 612aa protein which contains a *m*embrane-*a*ttack *c*omplex/*p*erforin (MACPF) domain, which is conserved in bacteria, fungi, mammals and plants. The possible modes of action of NSL1 protein in negative regulation of cell death programs and defense responses are discussed.

Keywords Cell death · Defense responses · Salicylic acid · MACPF domain

Abbreviations

Avr	avirulence
CNGC	cyclic nucleotide-gated cation channel
Ds	*Dissociation* transposon
H_2O_2	hydrogen peroxide
HR	hypersensitive response
LRR	leucine rich repeat
MACPF	membrane attack complex and perforin
NahG	salicylate hydroxylase
MAP kinase	mitogen-activated protein kinase
NBS	nucleotide binding site
PCD	programmed cell death
PCR	polymerase chain reaction
PR genes	pathogenesis-related genes
R gene	disease resistance gene
ROS	reactive oxygen species
SA	salicylic acid

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SAR	systemic acquired resistance
TIR	Toll/interleukin-1 receptor

Introduction

Plants contain resistance (R) proteins that enable them to detect invading pathogens that express corresponding avirulence (Avr) proteins. In response to attempted infection by avirulent pathogens, plants induce defense gene expression and a rapid collapse of cells in the area of pathogen challenge; a phenomenon which is called hypersensitive response (HR) cell death. It is thought that the HR serves to restrict the spread of the pathogen by retaining it at the site of attempted infection. HR cell death also triggers local and systemic signaling for the activation of the various defenses in non-infected tissues, a response which is known as systemic acquired resistance (SAR) (Goodman and Novacky 1994). These defenses include the strengthening of cell walls and the accumulation of pathogenesis-related (PR) proteins and antimicrobial phytoalexins.

The HR is a form of programmed cell death (PCD) that involves genetically defined signaling pathways which function to tightly control the process (Greenberg 1997). The HR cell death is initiated by the specific perception of a pathogen Avirulence (Avr) protein or its product by corresponding plant R proteins (Dangl and Jones 2001). During pathogen infection, levels of the plant hormone salicylic acid (SA) rise and several lines of evidence indicate that this induction of SA synthesis is crucial in establishing resistance against a variety of pathogens (Alvarez 2000). Exogenous application of SA activates defense genes and enhances resistance to various pathogens (Ryals et al. 1996). Contrasting this, transgenic Arabidopsis expressing the bacterial SA hydroxylase gene *NahG* are depleted for SA and unable to mount an *R* gene-mediated HR (Delaney et al. 1994). Furthermore, SA biosynthesis deficient *Arabidopsis* mutants, such as *eds16*/*sid2* and *eds5*/*sid1*, accumulate very low levels of SA during pathogen infection and exhibit disease susceptibility (Nawrath et al. 2002; Nawrath and Metraux 1999; Wildermuth et al. 2001). Moreover, SA potentiates the induction of hydrogen peroxide (H_2O_2) accumulation, HR cell death and defense gene expressions (Shirasu et al. 1997). SA treatment also induces the expression of several TIR-NBS-LRR type *R* genes, suggesting that SA is involved in a positive feedback loop that regulates the expression of defense associated genes (Shirano et al. 2002). SA also accumulates in uninfected systemic tissues (Ryals et al. 1996) and plants that cannot accumulate or perceive increased levels of SA in systemic tissue do not develop SAR (Cao et al. 1994; Gaffney et al. 1993; Nawrath and Metraux 1999).

Plant mutants displaying misregulated cell death may be employed to uncover the regulatory mechanism of PCD. These mutants are often called lesion mimics because their phenotypes commonly resemble pathogen-inducible HR cell death lesions. A large number of plant mutants which exhibit spontaneous lesions in the absence of pathogen infection have been identified in maize (*Zea mays*) (Hoisington et al. 1982), *Arabidopsis thaliana* (Lorrain et al. 2003), barley (*Hordeum vulgare*) (Wolter et al. 1993), and rice (*Oryza sativa*) (Takahashi et al. 1999; Yin et al. 2000). These mutant lines often show hallmarks of the HR, such as the expression of pathogenesis-related genes and/or enhanced resistance to pathogen infection (Lorrain et al. 2003), thereby indicating the strong connection between PCD and disease resistance in plants. The analysis of Arabidopsis lesion mimic mutants, *ssi4*, *snc1*, *bal1* and *slh1*, revealed that inappropriately regulated R proteins may cause the constitutive activation of disease resistance response (Belkhadir et al. 2004b). In addition, the disruption of cellular metabolic homeostasis also causes the activation of HR cell death. For example, mutation of genes involved in synthesis or metabolism of cellular compounds such as porphyrin, fatty acid/lipid, phenolics, amino acid, sphingosine and sphingolipid, causes the activation of PCD (Lorrain et al. 2003). Several lines of evidence indicate that calcium binding and transport processes are important for regulating the signaling pathway leading to HR cell death. For example, several calcium ion transport mutants (*dnd1* (AtCNGC2); *hlm1* (AtCNGC4), (Lorrain et al. 2003) and calcium binding mutants (*mlo* (membrane-anchored calmodulin-binding protein), (Kim et al. 2002); *cpn1*/*bon1* (calcium-dependent, phospholipid binding protein), (Lorrain et al. 2003)) are also lesion mimics.

Intriguingly, resistance responses and cell death which both occur in lesion mimic mutants are also modulated by environmental conditions such as humidity (Jambunathan et al. 2001; Noutoshi et al. 2005; Xiao et al. 2003; Yoshioka et al. 2001), light (Greenberg and Ausubel 1993), day length (Dietrich et al. 1994), and temperature (Noutoshi et al. 2005; Xiao et al. 2003). Although the mechanisms underlying how these environmental conditions impact on R protein-mediated resistance are unclear, these

observations suggest the existence of shared mechanisms in response to biotic and abiotic stress in plants. These findings highlight the complexity of regulatory mechanisms and signaling networks in PCD.

Here, we report the isolation and characterization of the Arabidopsis lesion mimic mutant *nsl1* for *necrotic spotted lesions 1* (*nsl1*). In the absence of pathogens, *nsl1* spontaneously exhibits characteristic necrotic lesions scattered throughout the rosette and cauline leaves. Trypan blue staining revealed that lesions of *nsl1* consist of a mosaic of both dead and living cells. The accumulation of callose, autofluorescent compounds and SA was detected in *nsl1*. Furthermore, *nsl1* constitutively expresses marker genes that are commonly activated during HR and SAR. Crosses between *nsl1* and mutants deficient in SA biosynthesis revealed that the *nsl1* phenotypes are dependent upon SA. These results indicate that *nsl1* exhibits SA-dependent defense associated phenotypes. The *NSL1* gene encodes a novel 612aa protein which contains a MACPF domain. The MACPF protein family is found in Arabidopsis and rice and phylogenetic analysis suggests that these proteins arose or diverged prior to the monocot–dicot divergence. In support of our findings, it was recently reported that the knockout of a MACPF family gene, *CAD1*, also exhibits similar phenotypes to *nsl1* (Morita-Yamamuro et al. 2005), suggesting that these homologous genes independently regulate the defense signaling pathway.

Materials and methods

Plant materials and growth conditions

Ds-tagged *Arabidopsis* lines were described previously (Kuromori et al. 2004). *Ds15-0660-1* and *Ds15-2075-1* was designated as *nsl1-1* and *nsl1-2*, respectively. The *Ds* transposons in *nsl1-1* and *nsl1-2* are inserted just after a thymine base at 1968 bp and a guanine base at 127 bp from initiation codon, respectively. Plants were germinated and grown on germination medium (Valvekens et al. 1988) with 3% sucrose and 0.8% agar in a growth chamber or on soil at 22 °C under 16-h-light/8-h-dark cycles. For the examination of the temperature dependency of the *nsl1* phenotype, plants were grown in a growth chamber on soil at 22, 24 or 26 °C under 16-h-light/8-h-dark cycles.

Histochemistry

Trypan blue staining was performed as previously described (Bowling et al. 1997). Callose and autofluorescence detections were performed using the methods of Dietrich et al. (1994).

RNA analyses

Total RNA was prepared for RNA gel blot hybridizations using TRIZOL Reagent (Invitrogen Life Technologies, Carlsberd, CA, USA). Aliquots (10 μg) of total RNA were separated on a 1.25% agarose gel containing formaldehyde and blotted onto Hybond-N + nylon membranes (Amersham Biosciences Corp., Piscataway, NJ, USA). The probes were labeled with [α-^{32}P]dCTP using the BcaBEST Labelling Kit (TAKARA BIO INC., Otsu, Japan). Hybridizations were carried out at 65 °C, then the filters were washed once with 1× SSC/0.1% SDS and twice with 0.1× SSC/0.1% SDS at 65 °C for 15 min and filters were subsequently autoradiographed. The probe templates were prepared from RIKEN Arabidopsis full length cDNA clones by *Sfi*I treatment. The clones were: *PR1*(At2g14610, RAFL06-68-J19), *PR2*(At3g57260, RAFL09-66-C10), *PR5*(At1g75040, RAFL04-13-G17), *PRXc*(At3g49120, RAFL09-07-G15), *GST*(At1g02930, RAFL05-16-O07), *SAG13*(At2g29350, RAFL06-10-N05), *EDS1*(At3g48090, RAFL05-08-A14), *PAD4*(At3g52430, RAFL07-12-D22), *PDF1.2*(At5g44420, RAFL06-82-G15) and *EF1*α(At1g07920, RAFL08-18-F01).

Expression analysis

For RT-PCR analysis, the first strand cDNAs were synthesized from the total RNA of wild type and *nsl1* plants using Superscript III reverse transcriptase (Invitrogen). For the PCR amplification of *NSL1*, the primers were 5′-ACTAAGCCACAGTCTAAGATTG-3′ and 5′-CCAGTGAGTCTTCCGATATG-3′. For β-tubulin gene, the primers were 5′-ATCCCACCGGACGTTACAAC-3′ and 5′-TTCGTTGTCGAGGACCATGC-3′.

In silico expression analysis was performed at https://www.genevestigator.ethz.ch/.

SA and SA glucoside measurement

SA and SA glucoside were extracted and measured from 0.4 to 0.5 g (fresh weight) of plant shoot tissue as described previously (Nakashita et al. 2002).

Genetic analyses

For construction of the *nsl1-1*/*eds16-1* double mutant, *nsl1-1* was crossed with *eds16-1* (Wildermuth et al. 2001). For *nsl1-1*/*rar1-10* and *nsl1-1*/*sgt1b-1* double

mutants, *rar1-10* (Muskett et al. 2002) and *sgt1b-1* (Austin et al. 2002) mutants were crossed into *nsl1-1*. Genotypes were confirmed by PCR and sequencing.

Results

Isolation and characterization of nsl1 mutants

We generated stable transposon-tagged *Arabidopsis thaliana* lines with a modified maize *Ds* element which serves as an effective tool for comprehensive genetic screens (Kuromori et al. 2004). In the course of the comparative phenome analysis of this mutant pool with wild type plants, we isolated several mutants which exhibited dwarf phenotypes as one of the visible morphological phenotypic categories. Our *Ds* flanking sequence database was used for the identification of the *Ds*-insertion position of these mutants (RARGE, http://rarge.gsc.riken.go.jp/index.html) and we found that two independent lines, *Ds15-0660-1* and *Ds15-2075-1*, contain the *Ds* insertion in the same gene, *At1g28380* (Fig. 1A). Both of these independent mutant lines exhibited a dwarf phenotype when grown on soil (Fig. 1C). In both mutant lines, characteristic brown spots were observed on rosette leaves of 2 to 3-week-old plants (Fig. 1D). It is important to note that the necrotic-like lesions emerged on leaves of *nsl1* plants that were grown in agar medium plates under sterile conditions. These observations clearly indicate that these phenotypes occur independently of interaction with biotic agents. Because these lesions developed in the absence of pathogens, we named this mutant *nsl1* and we henceforth refer to *At1g28380* as *NSL1*. In addition to the spotted necrotic lesions, some leaves in *nsl1* showed chlorosis at the 2 to 3-week-old growth stage (Fig. 1D). In spite of the growth defect phenotype, most of the *nsl1* plants developed reproductive tissues and set seeds (data not shown). The brown spots were also observed on cauline leaves (data not shown).

In both mutant lines, the *nsl1* phenotype was segregated recessively and linked to the hygromycin resistance trait that was conferred by *Ds* (Table 1). The *Ds* transposons of *nsl1-1* and *nsl1-2* were inserted into the sixth and first exon of the *NSL1* gene, respectively (Fig. 1A). According to RT-PCR analysis, both *nsl1-1* and *nsl1-2* displayed reduced expression of *NSL1* (Fig. 1B). RNA gel blot analysis revealed that the expression level of *NSL1* in wild type plants is low and in *nsl1-1* the *NSL1* mRNA could not be detected (Fig. 5B). These results indicate that the loss or substantial decrease of *NSL1* mRNA is the cause of the *nsl1* phenotypes.

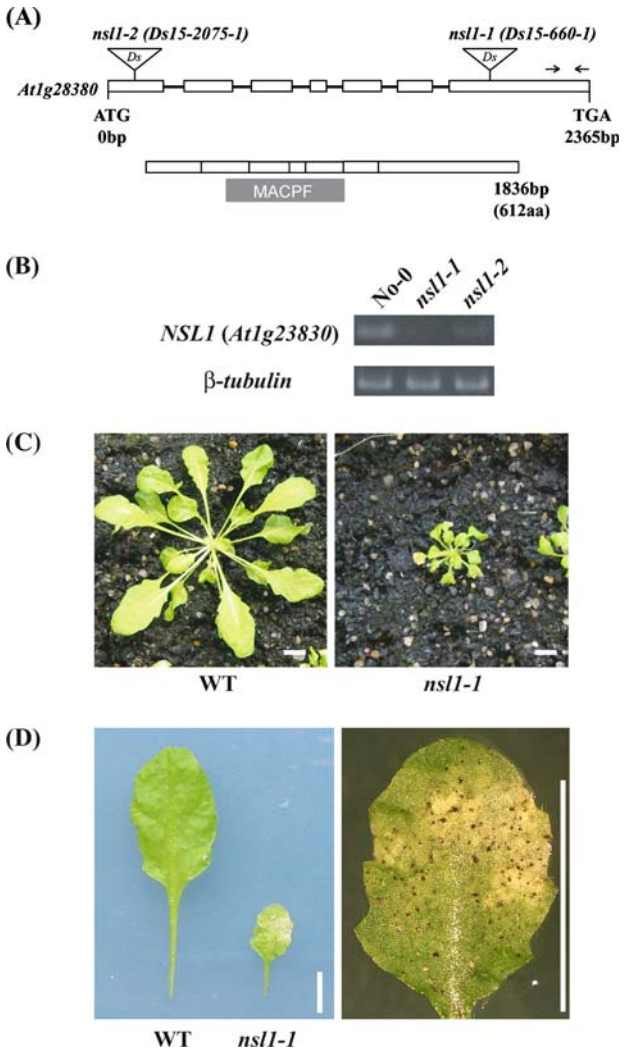


Fig. 1 Features of *NSL1*. (**A**) Molecular structures of *NSL1* genomic sequence (upper) and cDNA (lower). Exons and introns are indicated by boxes and horizontal lines, respectively. The position of MACPF domain is indicated below the cDNA structure by a shaded box. The positions of the *Ds* transposon in *nsl1* are represented as inverse triangles. The positions of primers that were utilized for RT-PCR are indicated by arrows. (**B**) RT-PCR analyses for *NSL1* expression in the *Ds* insertional mutants. The *β*-tubulin gene was used as a constitutive control. (**C**) Phenotype of *nsl1-1* and wild type (WT) plants grown on soil for three weeks. Bar = 5 mm. (**D**) The leaf phenotype of *nsl1-1* and WT. The enlarged photograph of *nsl1* rosette leaf is shown on the right side. Bar = 5 mm

The spontaneous lesion phenotype of nsl1 mutants

In the *nsl1* plants, the characteristic necrotic lesions appeared in 2 to 3-week-old seedlings (Fig. 1D). When leaves from the *nsl1* mutant were stained with trypan blue, spotted necrotic lesions were detected throughout the leaves (Fig. 2A, B) and these lesions were observed on both adaxial and abaxial surfaces (data not shown). Direct observation of lesions at high

Table 1 Segregation of hygromycin resistance and cell death phenotype in *nsl1*

Generation	Total of plants	Hygromycin resistance	Hygromycin sensitive	Null Hypothesis	χ^{2a}	*P*
F_3	331	236	95	3:1	2.038	0.153
Generation	Total of plants[b]	Normal Morphology	Lesions[c] Morphology	Null Hypothesis	χ^{2a}	*P*
F_3	226	154	72	2:1	0.163	0.686

[a]One degree of freedom

[b]Hygromycin resistance F_3 plants segregated from F_2 generation

[c]All plants showed typical *nsl1* phenotype

magnification revealed that they are composed of a mosaic of collapsed dead cells interspersed between living cells within a distinct localized area (Fig. 2C, D).

NSL1 encodes a novel protein which contains MACPF domain

The full-length cDNA clone RAFL07-09-K08 corresponding to the *NSL1* was isolated and we could refer the sequence from database. *NSL1* encodes a novel 612aa protein that contains a particular motif which shows significant similarity to the membrane-attack complex/perforin (MACPF) domain. The MACPF domain is defined as 197 residues that are conserved in complement components, C6, C7, C8-alpha, C8-beta and C9, and perforin of the mammalian immune system. In response to pathogen infection, a sequential and highly specific interaction between the constituent elements occurs to form transmembrane channels which are known as the membrane-attack complex (MAC). These pores are integrated into the phospholipid bilayer of target cells and facilitate the free passage of ions through it, a process which ultimately leads to osmotic cell lysis and cell death (Liu et al. 1995; Peitsch and Tschopp 1991). The MACPF domain was suggested to be important for the heterodimerization of MACPF proteins and integration of MAC into the cell membrane. The NSL1 protein sequence contains a MACPF domain but does not contain any of the other domains that are found in mammalian complement components.

We found that four and seven loci of *NSL1* homologous genes exist in the Arabidopsis and rice genome, respectively. One of the Arabidopsis MACPF genes, *CAD1*(*CONSTITUTIVELY ACTIVATED CELL DEATH1*, *At1g29690*), was recently characterized and the knockout of this gene triggers lesion mimic phenotypes that are similar to those of *nsl1* (Morita-Yamamuro et al. 2005). In order to examine the structural similarity of plant MACPF domain proteins, we generated a multiple sequence alignment using the deduced protein sequences of these eleven genes. With the exception of *At4g24290*, the deduced amino acid sequences are all supported by full-length cDNA clones. Since the identified cDNA clones corresponding to the *At4g24290* gene on the GenBank database appear to be immature splicing products, we used the amino acid sequence which was annotated by

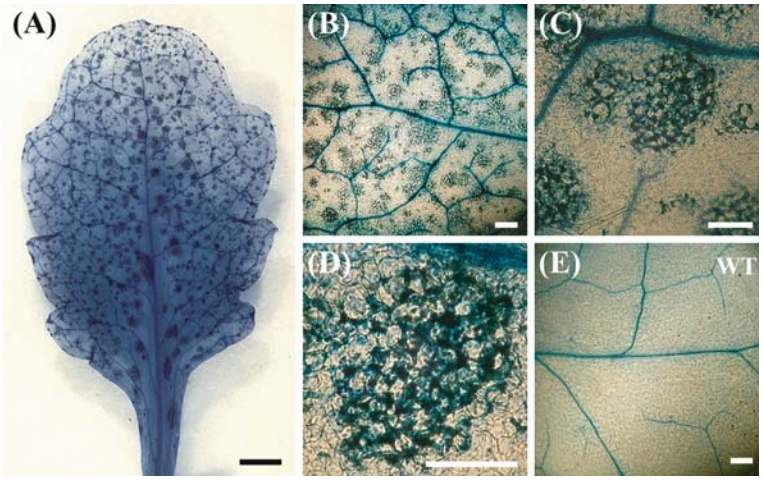


Fig. 2 The cell death phenotype of *nsl1*. The trypan blue staining of *nsl1-1* and wild type (WT) rosette leaves. Plants were grown on soil for three weeks. (**A**) Whole leaf image of the *nsl1* rosette leaf. Bar = 5 mm. (**B**, **C** and **D**) Enlarged photographs of the *nsl1* lesions. Bar = 1 mm. (**E**) WT rosette leaf. Bar = 1 mm

AGI (Arabidopsis Genome Initiative). In rice, two cDNA clones, P0401G10.6 and P0401G10.7, were isolated as alternative splicing products, and three differential transcripts corresponding to the P0419C03.10 locus were also found in the GenBank database. We selected the longest protein sequences from these alternatively spliced products as representative clones and used them for further comparative analysis. Multiple sequence alignment with the ClustalW program revealed that these plant MACPF domain proteins showed similarity not only within the MACPF domain but also throughout their overall putative amino acid sequences (Fig. 3). Overall, the sequence identity between NSL1 and the other MACPF homologues from Arabidopsis and rice ranges from 37% to 53%. As a means to examine the evolutionary relationships among these MACPF family proteins from different plant species, we estimated the phylogeny using the alignment result shown in Fig. 3. Interestingly, the phylogenetic tree analysis revealed that each protein of this family in Arabidopsis has one or two corresponding proteins in rice (Fig. 4). In addition, the rice P0419C03.10-1 protein seems to form an apparently independent clade. On an evolutionary level, NSL1 appears to be most closely related to the rice protein that is deduced from the cDNA OJ1214E03.15 and they share 53% sequence identity. These data suggest that the duplication and diversifications of members of this gene family occurred prior to the divergence of monocots and dicots. In rice, it is hypothesized that two sets of members from this gene family, P0431E05.15 with P0487D09.21 and P0039H02.131 with P0401G10.7, were likely further duplicated and diverged subsequent to the monocot–dicot split. Given the similarity of these proteins in Arabidopsis and rice, it is reasonable to consider that the homologues in rice may also regulate defense responses.

nsl1 mutants exhibit several defense associated phenotypes

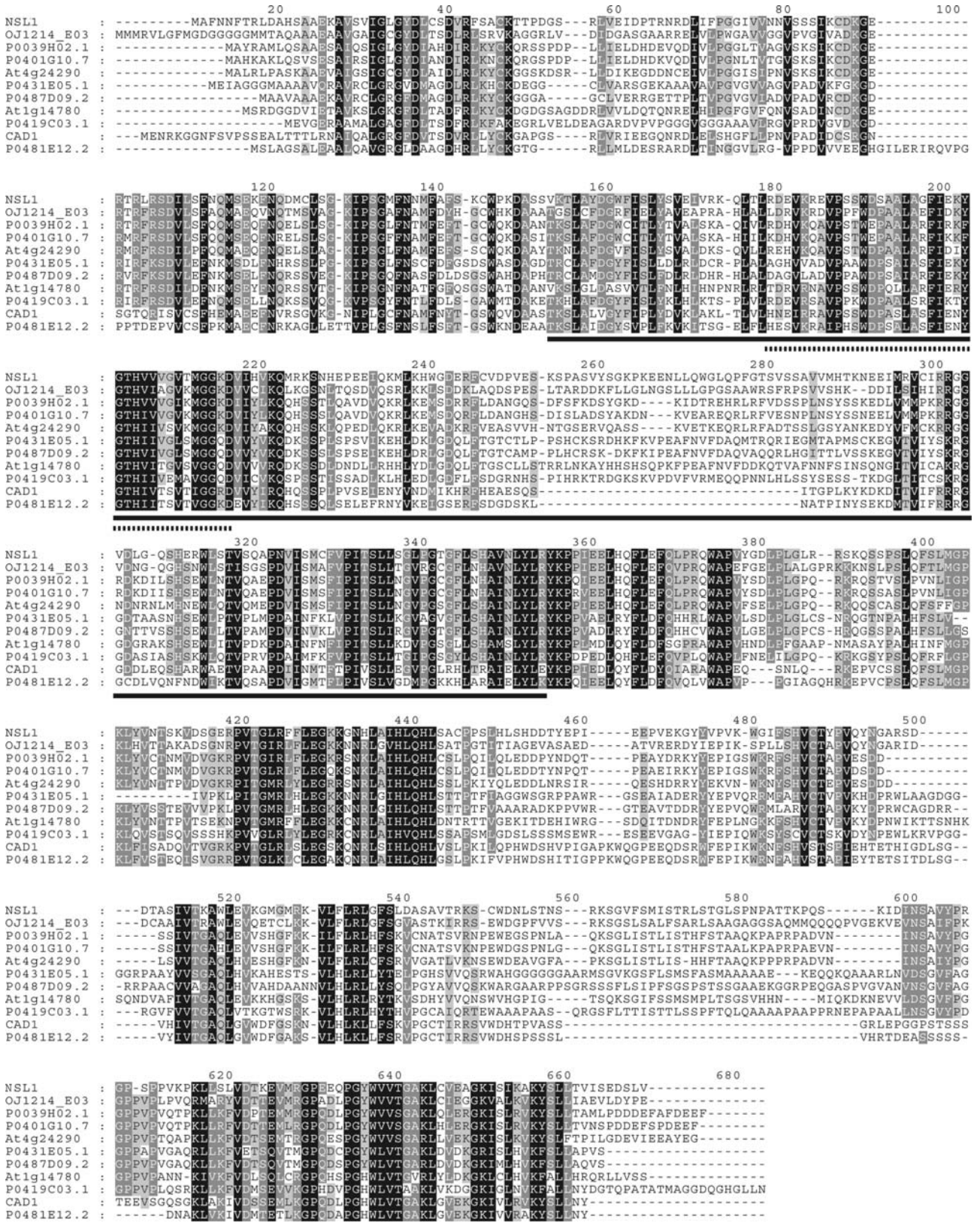
During the resistance response to pathogens in plants, callose and autofluorescent compounds accumulate proximal to the infection sites We were interested whether the *nsl1* mutant expresses defense responses in the absence of pathogens. Aniline blue staining was conducted for the detection of callose and we examined *nsl1* rosette leaves under ultraviolet light to monitor the accumulation of autofluorescent compounds. Compared with wild type plants, increased autofluorescence was detected in the *nsl1* rosette leaves (Fig. 5A, upper panel) and the *nsl1* mutants also displayed massive callose deposition (Fig. 5A, lower panel). Interestingly, the callose and autofluorescent compounds were localized inside and adjacent to the necrotic lesions.

nsl1 mutants constitutively express genes associated with HR and SAR

To further investigate the *nsl1* phenotype, we isolated RNA from *nsl1* plants and performed RNA gel blot analysis of several marker genes that are known to be induced during HR or SAR (Fig. 5B). RNA was extracted from shoot tissues of *nsl1* and wild type plants that were grown on soil for three weeks. The pathogenesis-related (*PR*) genes, *PR1*, *PR2*, and *PR5*, whose expression is tightly correlated with the HR, were all found to be constitutively expressed in *nsl1* plants. In addition, the transcripts of peroxidase C (PRXc) and a glutathione-*S*-transferase (GST), enzymes that are involved in oxygen metabolism and cellular detoxification, respectively, were also elevated in *nsl1*. Our findings correlate with previous observations that report constitutive expression of these genes in lesion mimic mutants (Brodersen et al. 2002; Greenberg and Ausubel 1993; Jabs et al. 1996). *EDS1* and *PAD4* are signaling components that act upstream of SA in *R* gene mediated defense pathways and their encoded proteins are involved in the SA amplification loop (Shirano et al. 2002). The mRNA levels of both *EDS1* and *PAD4* were higher in the *nsl1* mutants suggesting that this mutant may accumulate SA to a greater extent than wild type plants. The mRNA of *SAG13*, a gene which encodes a short chain alcohol dehydrogenase, is known to accumulate during senescence and in several lesion mimic mutants (Brodersen et al. 2002; Noutoshi

▶

Fig. 3 Comparison of the MACPF domain-containing protein sequences in Arabidopsis and rice. Alignment of the deduced amino acid sequence of *NSL1* and three paralogues from Arabidopsis and those of seven homologues from rice. With the exception of *At4g24290*, all deduced protein sequences were based on the full length cDNA clones obtained from GenBank. The conserved residues among the compared sequences are boxed in black or light gray based upon the degree of conservation. The position corresponding to MACPF domain is underlined and the position of the helix-loop-helix motif is indicated by broken line. The amino acid sequences were aligned using the ClustalW program. Gaps, which were introduced to maximize alignment, are indicated by dashes. At1g14780(GI:20466444), At1g28380(GI:16604599), At1g29690 (GI:18650618) and At4g24290(GI:30686484) from *A. thaliana* and P0039H02.131(GI:33146806), P0401G10.7(GI:57900281), P0419C03.10-1(GI:47848124), P0431E05.15(GI:52076970), P0481E12.26 (GI:34909512), P0487D09.21(GI:46390438) and OJ1214E03.15(GI:49328015) from rice


NSL1
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P0039H02.1
P0401G10.7
At4g24290
P0431E05.1
P0487D09.2
At1g14780
P0419C03.1
CAD1
P0481E12.2

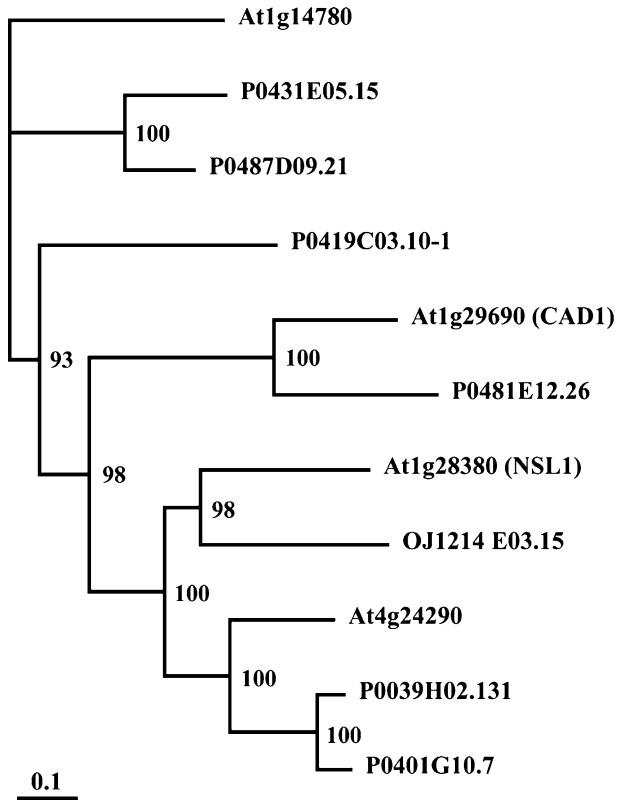

Fig. 4 Phylogenetic relationship between NSL1 and MACPF domain proteins in Arabidopsis and rice. The phylogenies that were generated with the ClustalW program were based on the neighbor-joining algorithm and bootstrap values are indicated for distance analysis. The scale bar indicates the distance corresponding to 0.1 amino acid substitutions per site

et al. 2005). Indeed, *SAG13* expression was confirmed to be upregulated in *nsl1*. Furthermore, we conducted microarray analysis using Agilent microarray 2 chip and these results were in direct agreement with our RNA gel blot analyses (data not shown). Collectively, our results support the notion that the *nsl1* plants exhibit defense responses that are associated with HR cell death.

SA is required for the *nsl1* phenotype

RNA gel blot analysis revealed that defense associated genes known to be controlled by SA were highly upregulated in *nsl1*. We therefore determined the SA content in *nsl1* and confirmed that SA levels in *nsl1* were higher than those in wild type plants (Fig. 6A). Free SA- and total SA- (free SA + SA glucoside (SAG)) levels were 4.5- and 8.5-fold higher, respectively indicating that SA synthesis is constitutively activated in the *nsl1* plants. In order to determine the importance of SA in the development of the *nsl1* phenotype, a reciprocal cross of *nsl1* with *eds16-1* was performed. In the resultant progeny, the dwarfing and spotted lesion phenotypes were partially suppressed by the *eds16-1* mutant background, thereby indicating that SA is important for the development of the *nsl1* phenotypes (Fig. 6B).

The growth retardation of nsl1 is affected by high temperature conditions

It is known that the defense associated phenotypes of many lesion mimic mutants are dependent on environmental conditions. To determine whether the *nsl1* phenotype is dependent upon temperature, *nsl1* plants were grown on soil in growth chambers maintained under different temperature conditions (22, 24 or 26 °C). The *nsl1* growth retardation phenotype was

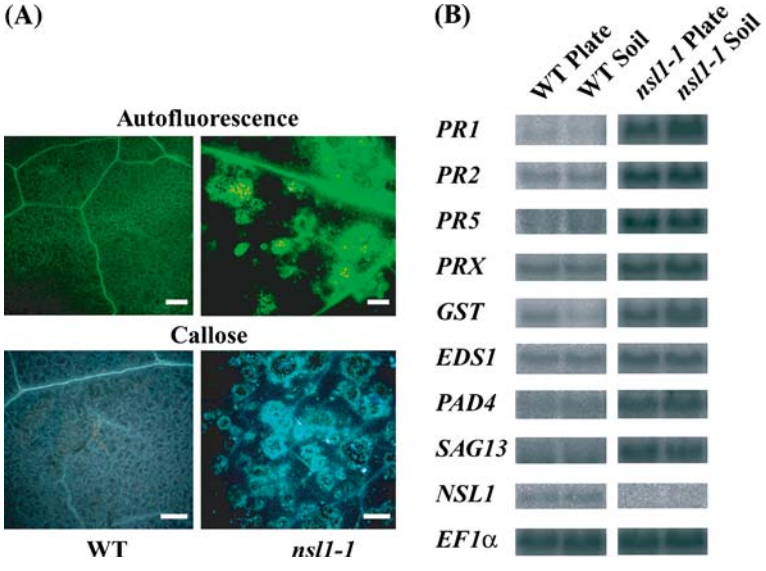

Fig. 5 Defense associated phenotypes in *nsl1*. (**A**) Detection of autofluorescence (top) and callose (bottom) in wild type (WT) and *nsl1-1* rosette leaves. Bar = 100 μm. (**B**) RNA gel blot analysis of the marker genes for hypersensitive response and disease resistance responses in WT and *nsl1* plants. RNAs were extracted from plants that were grown on plate or soil for 3 weeks, respectively

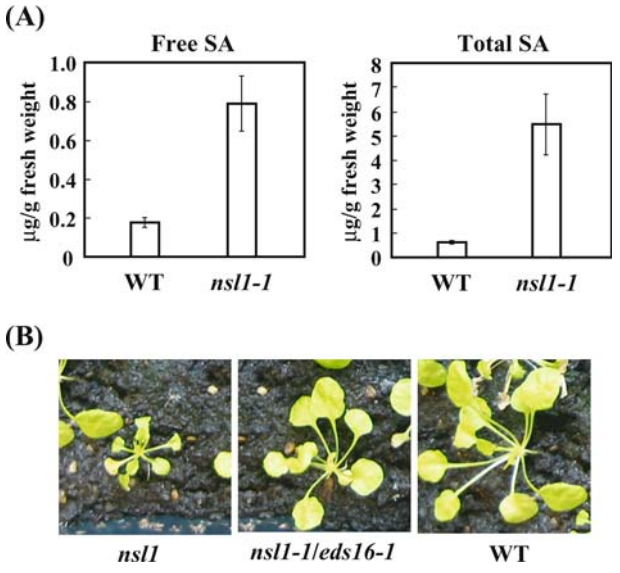

Fig. 6 SA level measurement and epistatic analysis of the *nsl1* mutant. (**A**) Free SA and total SA (free SA + SAG) levels in the *nsl1* mutant. For SA extraction, shoot tissue was harvested from wild type (WT) and *nsl1-1* plants that were grown on soil for 3 weeks (mean ± SD, $n = 3$). (**B**) Phenotypes of *nsl1-1*, *nsl1-1/eds16-1* and WT. Plants were grown on soil for three weeks and were subsequently photographed

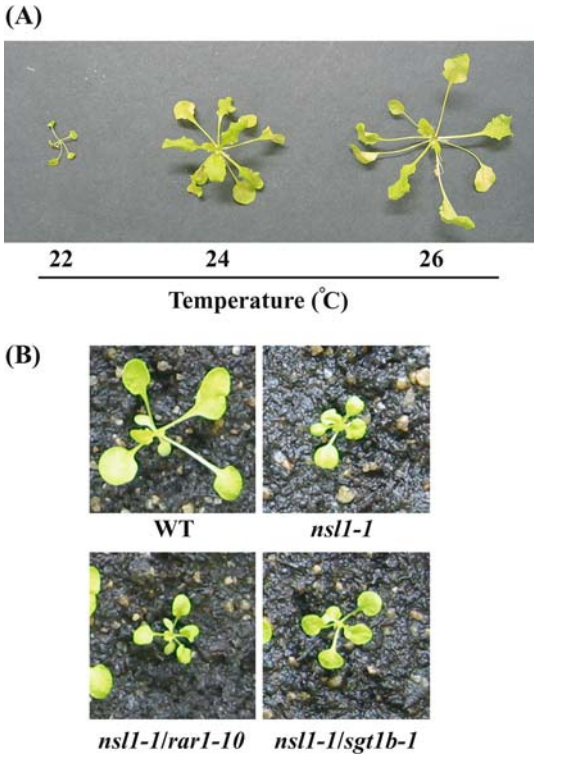

Fig. 7 Temperature dependency of the *nsl1* growth retardation phenotype. (**A**) The *nsl1-1* plants were grown on soil for three weeks in 22, 24 and 26 °C, respectively. All plants exhibited the characteristic brown spots on their rosette leaves. (**B**) Phenotype of WT, *nsl1-1/rar1-10* and *nsl1-1/sgt1b-1*. Plants were grown on soil for 3 weeks and were subsequently photographed

partially suppressed at 24 °C and completely suppressed at 26 °C (Fig. 7A). Interestingly, the cell death phenotype of *nsl1* was not suppressed at either 24 °C or 26 °C. These data indicate that the growth arrest and lesion formation of *nsl1* may be regulated by different signaling pathways.

We observed the HR-like lesions on rosette leaves of *nsl1* plants that were grown on agar plates (data not shown). Consistent with this phenomenon, RNA gel blot analysis which used RNA prepared from plant materials that were grown for three weeks on agar media revealed that marker genes expression for HR and SAR were not suppressed on plate grown *nsl1* plants (Fig. 5B). This result indicates that the cell death phenotype in *nsl1* is unlikely to be affected by high humidity conditions.

nsl1 signaling occurs independently of RAR1 and SGT1b

To investigate the *nsl1* signaling cascade, we crossed *nsl1* with *rar1-10* or *sgt1b-1*. Arabidopsis *RAR1* and *SGT1b* are known to be required for the expression of defense responses activated by several R proteins (Austin et al. 2002; Muskett et al. 2002; Tor et al. 2002; Tornero et al. 2002). We found that the *nsl1* phenotypes were not suppressed in either the *rar1-10* or *sgt1b-1* mutant backgrounds (Fig. 7B). These results indicate that NSL1 may not function in the R protein activated signaling pathways that recruit either RAR1 or SGT1b. Alternatively, NSL1 may function downstream of these resistance components.

Expression patterns of Arabidopsis MACPF family genes in stress responses

To further understand the function of *NSL1*, we used publicly available microarray data (https://www.genevestigator.ethz.ch/) to investigate the *NSL1* expression profile in response to various stress conditions (Zimmermann et al. 2004). There were no significant changes in the expression levels of *NSL1* and other MACPF family genes in response to various biotic stress agents, including avirulent, virulent and non-host *Pseudomonas syringae* strains (data not shown). We also noted that the expression of *NSL1* was not altered during senescence or in response to phytohormone treatments (data not shown). However, *NSL1* gene expression was dramatically upregulated by cycloheximide (CHX) treatment (Fig. 8). *NSL1* expression was also upregulated in roots in response to sodium chloride (Fig. 8). *NSL1* induction was detected as early as 30 min after

this salt stress and expression levels remained elevated until at least 24 h after treatment, peaking at around 6 h (Fig. 8). Interestingly, *NSL1* gene induction was not observed in microarray experiments which utilized shoot tissues (Fig. 8). There was a 17.7-fold increase in *NSL1* expression after CHX treatment and a 12.8-fold increase 6 h after salt stress. Surprisingly, among the MACPF family genes in Arabidopsis, only *NSL1* and *CAD1* exhibited highly similar expression profiles with respect to CHX and salt treatments (Fig. 8). These observations provide further evidence for the functional analogy between *NSL1* and *CAD1*. Because CHX blocks protein synthesis, it is plausible that transcriptions of *NSL1* and *CAD1* are negatively regulated by a repressor with a rapid turnover rate. Alternatively, *NSL1* and *CAD1* may be transcriptionally induced by reactive oxygen species (ROS) as CHX induces the oxidative burst in plants (Tenhaken and Rubel 1998). Production of ROS and of H_2O_2 has also been observed in abiotic conditions such as the photo-oxidative process that are commonly induced by salt stress (Foyer and Noctor 2000). It is therefore possible that *NSL1* and *CAD1* contribute to controlling the redox status in response to environmental conditions and collectively function to prevent excess cellular ROS which can lead to abnormal cell death.

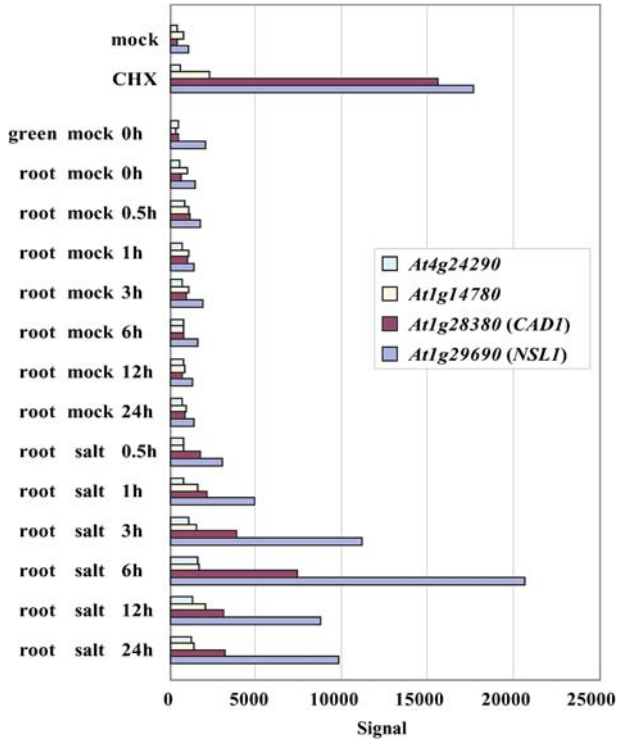

Fig. 8 In silico expression analysis of MACPF family genes in Arabidopsis. Microarray data and the creation of Digital Northerns were obtained from AtGenExpress at the Genevestigator site (https://www.genevestigator.ethz.ch/). Signal intensities were mean value for two replicates. Probe sets were At4g24290(254169_at), At1g14780(262887_at), At1g29690(259792_at) and At1g28380(261445_at) and note that 262887_at corresponds to At1g14780 and At1g14790. For CHX treatment, experiment No.113 was referred and 7-days old whole Col-0 plants (seedlings) incubated with or without 10 uM cycloheximide for 3 h were used as a source for RNA extraction. For salt stress, data on No120 and 123 experiments were used. Plant materials were shoot or root parts that were harvested from 18-days old Col-0 plants subsequent to their placement on 150 mM NaCl for the indicated time

Discussion

MACPF proteins in plants

NSL1 encodes a novel protein containing a MACPF domain (Fig. 1A) and is one of four MACPF family members in *Arabidopsis* (Fig. 4). In this study, we demonstrate that *nsl1* knockout mutants display spontaneous cell death and constitutive defense responses, suggesting that *NSL1* may negatively regulate disease resistance responses in plants. Since there are no available T-DNA insertion lines of corresponding gene in Col-0, we are not able to tell this mutation effect is peculiar to No-0. Interestingly it was recently demonstrated that the knockout of another MACPF family member, *CAD1*, also exhibits lesion mimic phenotypes (Fig. 4) (Morita-Yamamuro et al. 2005). The *cad1* phenotypes are similar to those we observed in *nsl1* and include the characteristic spotted necrotic lesions. Because of these similarities, *nsl1* would be expected to exhibit increased resistance to pathogens as shown in *cad1*. These two genes exhibit similar expression profiles in response to abiotic stresses (Fig. 8), suggesting that *NSL1* and *CAD1* may regulate the same signaling pathway. This idea is supported by the observation that these proteins are very similar, sharing 40% sequence identity. Since NSL1 and CAD1 are members of the same protein family and share similar sequence, it is possible that these proteins may be functionally redundant. However, contrasting this idea, the loss of either of these proteins activates defense signaling. It may therefore be that *NSL1* and *CAD1* expression is differentially controlled at the tissue level. Alternatively, differences in temporal expression patterns may differentiate the function of these genes. A further possibility is that NSL1 and CAD1 may physically interact to form heterodimers that are required for negative regulation of the cell death program. This hypothesis is supported by the biochemical analyses of MACPF domain proteins in mammalian complement components.

In mammals, the MACPF domain is required for the intramolecular interaction between homologous complement components C8α and C8β that form part of a trans-membrane pore (Musingarimi et al. 2002; Plumb et al. 1999). The amphipathic helix-loop-helix (HLH) motif within the MACPF domain, a putative membrane-spanning motif, has been suggested to be important for integration of the final pore structure into the plasma membrane (Peitsch et al. 1990). It was recently demonstrated that two MACPF-related proteins in a malarial parasite are required for membrane rupture which facilitates parasite invasion into host organs. These malarial MACPF-related proteins are thought to be involved in pore formation on the host cell membrane (Ishino et al. 2005; Kadota et al. 2004). The HLH motif is highly conserved among plant MACPF proteins (Fig. 3) and the NSL1 HLH is also highly similar to that of non-plant MACPF proteins. For example, the NSL1 HLH motif shares 35% identity to that of the mouse complement component C9. These data suggest that plant MACPF proteins may be localized at the cell and/or organelle membrane to form an incorporated pore which facilitates the transport of metabolic compounds. The metabolic imbalance induced by the absence of these pores in *nsl1* mutants may be the primary cause of the lesion mimic phenotypes. Further biochemical and cytological analyses of NSL1 and CAD1 are warranted and would clarify the molecular function of MACPF proteins in plant defense.

Possible mechanisms of misregulated triggering of defense responses resulted from loss of NSL1

Disease resistance responses that often culminate in the HR are activated by R proteins during incompatible interactions between plants and avirulent pathogens. Recent studies have revealed how R proteins may activate the resistance mechanism. The knockout of *CPN1*/*BON1* activates the R protein SNC1 leading to constitutive defense responses (Yang and Hua 2004). Similarly, the loss of RIN4 results in constitutive activation of the R proteins RPM1 and RPS2 (Belkhadir et al. 2004a). Because RIN4 directly interacts with RPM1, the guard hypothesis was proposed in which RPM1 guards RIN4 (Belkhadir et al. 2004b). According to this hypothesis, RPM1 senses pathogen infection by monitoring the status of RIN4 which is targeted by pathogen effector proteins during the infection process. In light of the guard hypothesis, NSL1 may interact with one or more R proteins and the loss of NSL1 may activate these R proteins to signal defense responses. In this scenario, the non-redundancy of NSL1 and CAD1 may indicate that these proteins are guarded by distinct R proteins. We note that the *nsl1* phenotypes are independent of *RAR1* or *SGT1b* whose functions are required for many R proteins (Shirasu and Schulze-Lefert 2003). These results indicate that NSL1 might control R proteins that do not require RAR1 or SGT1b for function. Alternatively NSL1 may function in R protein signaling downstream of RAR1 and SGT1b. It may also be that NSL1 controls signaling cascades leading to cell death and defense responses that are independent of R proteins.

Microarray data showed that the expression of *NSL1* and *CAD1* is induced by abiotic stresses such as CHX and sodium chloride but not biotic stimuli. These data suggest that *NSL1* functions in plant abiotic stress responses and the constitutive defense responses triggered by loss of *nsl1* may be a secondary effect but not reflection of primary function of *NSL1*. This idea is supported by the various lesion mimic mutants analyzed so far (Lorrain et al. 2003). Disruption of genes involved in cellular metabolism such as photosynthesis and respiration, which are the potential sources of cellular ROS, can cause spontaneous defense responses and these results suggest that imbalances of cellular ROS mediate inappropriate induction of defense responses. Indeed, plant disease resistance responses are hallmarked by a massive production of ROS referred to as the oxidative burst (Torres et al. 2002) and H_2O_2 or enhanced levels of ROS in mitochondria can induce defense activation and cell death in plants (Lam et al. 2001). In contrast, pre-exposure to oxidative stress or overproduction of catalase decreased resistance to pathogen infection (Mittler et al. 1999; Polidoros et al. 2001). On the other hand, ROS gene network is also known to be involved in plant abiotic stress responses (Mittler et al. 2004). Hence, we hypothesized that the knockout of *NSL1* may cause disturbances in ROS levels, leading to inappropriate activation of HR-like cell death and defense responses. This idea is also supported by the result that the *nsl1* dwarfism was suppressed in high temperature condition which may affect cellular ROS level. To clarify this hypothesis, it is also helpful to know that whether *nsl1* phenotype is sensitive to other abiotic stimuli such as various light conditions as demonstrated in other lesion mimics (Lorrain et al. 2003). Although it is unknown whether NSL1 directly regulate cellular ROS in response to abiotic stimuli, the analysis of cellular localizations of NSL1 and CAD1 would provide information about the precise function of these proteins, since mechanisms for the production and scavenging of ROS are known to exist in various cellular compartments (Foyer and Noctor 2005; Mittler et al. 2004).

Intriguingly, plants undergoing RNAi of AtMPK6 display similar symptoms to those of *nsl1* when

exposed to ozone (Miles et al. 2005). Ozone is an inducer of ROS in plants, and it may be that AtMPK6 is required for correct redox status control. Indeed, ROS are common signal mediators for both biotic and abiotic stresses and several lines of evidence implicate MAP kinase cascades in ROS signaling (Mittler 2002). For example, OMTK1, a MAP triple kinase from alfalfa, is specifically activated by H_2O_2 to signal cell death through the downstream action of the MMK3 MAP kinase (Nakagami et al. 2004). AtMPK6 is activated by both biotic and abiotic stresses, including H_2O_2 treatment (Ichimura et al. 2000; Yuasa et al. 2001). Another Arabidopsis MAP kinase, AtMPK4, is also activated by environmental stress conditions and negatively regulates SA dependent signaling and positively regulates JA signaling (Ichimura et al. 2000; Petersen et al. 2000). Furthermore, suppression of rice OsMPK5 induced constitutive expression of *PR* genes and resistance to blast fungus (Xiong and Yang 2003). Taken together, these data indicate that the ROS imbalance in *nsl1* mutants may lead to misregulated MAP kinase cascades that signal HR cell death.

We demonstrated that the *nsl1* growth arrest phenotype but not cell death was affected by high temperature conditions. It is known that many R protein mediated responses are modulated by environmental conditions including high temperature (Hwang et al. 2000; Noutoshi et al. 2005; Xiao et al. 2003; Yang and Hua 2004). This temperature sensitivity suggests that the mechanism leading to dwarfism could be shared in *nsl1* and R protein misregulated mutants. Although it remains unclear whether other abiotic stresses compromise the *nsl1* cell death phenotype, the identification of a temperature sensitive element may help to dissect the signaling pathway controlled by *NSL1*.

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