J. Plant Biol. (2011) 54:172–179
DOI 10.1007/s12374-011-9153-z

ORIGINAL RESEARCH

Overexpression of the *Activated Disease Resistance 1-like1* (*ADR1-L1*) Gene Results in a Dwarf Phenotype and Activation of Defense-Related Gene Expression in *Arabidopsis thaliana*

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Received: 9 November 2010 / Revised: 21 January 2011 / Accepted: 24 January 2011 / Published online: 8 February 2011

Abstract The protein encoded by the *activated disease resistance 1-like1* (*ADR1-L1*) gene (locus name, At4g33300) belongs to the activated disease resistance 1 (ADR1) family of coiled-coil nucleotide-binding site leucine-rich repeat-type disease resistance proteins. This family contains four proteins and they have specific features in their amino acid sequences. It has been reported that ADR1 protein belongs to the ADR1 family, which is related to not only defense response but also drought tolerance. We found that transgenic plants over-expressing the *ADR1-L1* gene showed a dwarf phenotype and morphological change in leaves. The expression levels of defense-related genes and the resistance to *Pseudomonas syringae* pv. *tomato* DC3000 were increased in transgenic plants. However, enhancement of drought tolerance and activation of abiotic response genes were not observed. When the growth temperature was changed from 22°C to 28°C, the expression of defense-related genes and the enhancement of resistance to a bacterial pathogen were suppressed and the dwarf phenotype and morphological change of leaves recovered.

Keywords ADR1 family · *Arabidopsis* · Disease resistance · Dwarf phenotype · *R* gene · SA signaling

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Introduction

Disease resistance (*R*) genes are specificity determinants of plant immune response. When R proteins recognize *avirulence* gene (*avr*) products by direct or indirect interaction, a defense response is triggered. R proteins contain some common motifs, which mainly include a nucleotide-binding site (NBS), leucine-rich repeat (LRR), Toll/interleukin-1 receptor (TIR) domain, a coiled-coil (CC) structure, protein kinase domain, and transmembrane domain. According to these features, several classes are distinguished among most *R* genes: intracellular protein kinases, receptor-like protein kinases with an extracellular LRR domain, membrane-bound LRR proteins, membrane-bound CC proteins, and intracellular LRR proteins with an NBS (Martin 1999; McDowell and Woffenden 2003; Liu et al. 2007). Intracellular LRR proteins with an NBS are a major class. R proteins in this class have been divided into two groups according to their N-terminal structures. One group contains a CC structure and is called CC-NBS-LRR type, and the other group contains a TIR structure and is called TIR-NBS-LRR type (Pan et al. 2000; Meyers et al. 1999, 2003).

Fifty-one CC-NBS-LRR proteins were found in the *Arabidopsis thaliana* Col-0 genome and they were divided into four groups, designated as CNL clades A, B, C, and D (Meyers et al. 2003). CNL-A is the smallest clade containing only six genes. These six genes were divided to two subgroups and one subgroup was called activated disease resistant 1 (ADR1) family. Four genes, *ADR1*, *ADR1-like1* (*ADR-L1*), *ADR1-L2*, and *ADR1-L3* genes, belong to the ADR1 family (Chini and Loake 2005). The proteins encoded by these genes have several conserved features in their amino acid sequences. The RNBS-D and MHDV motifs in the NBS domain showed sequence specificities unique for only this family. A specific motif, named TVS motif, was also found in the NBS domain of only ADR1 family members. The amino acid sequences of LRR and NBS domains in the four proteins of the ADR1 family showed 83% and 75% homology, respectively. The LRR domain is more highly conserved than the NBS domain in ADR family proteins. This is a feature observed only in ADR1 family proteins in *Arabidopsis* NBS-LRR proteins (Chini and Loake 2005). These specific characters found in the ADR1 family arouse interest in the functions of ADR1 family proteins. However, only the *ADR1* gene has been analyzed (Grant et al. 2003; Chini et al. 2004).

An activation-tagging mutant that activated expression of the *ADR1* gene showed constitutive expression of defense-related genes and accumulation of salicylic acid (SA) and caused a defect of growth and curling of leaves (Grant et al. 2003). Furthermore, this activation-tagging mutant and transgenic plants overexpressing the *ADR1* gene showed enhancement of drought tolerance and activation of *DREB2A* gene expression (Chini et al. 2004). Although it has often been observed that activation of a defense-related gene caused a defect of plant growth, R proteins in general have not been shown to be related to drought tolerance. We were therefore interested in the roles of proteins belonging to the ADR1 family and we attempted to analyze other members belonging to this family.

In this study, we analyzed the *ADR1-L1* gene (locus name, At4g33300) belonging to the ADR1 family. We found that plants overexpressing the *ADR1-L1* gene showed a defect of plant growth and morphological changes of leaves and that they activated the expression of defense-related genes and had enhanced resistance to *Pseudomonas syringae* pv. *tomato* DC3000. However, the transgenic plants did not show enhancement of drought tolerance or activation of abiotic-response genes. The results suggested that the role, as played by the ADR1 protein, in both biotic response and abiotic response is not common to proteins of the ADR1 family. Activation of the expression of defense-related genes and enhancement of resistance to *P. syringae* were suppressed and the defect of plant growth and morphological changes of leaves were recovered when plants overexpressing the *ADR1-L1* gene were grown under a high temperature condition.

Materials and Methods

Plant Materials and Growth Conditions

A. thaliana (L.) Heynh. ecotype Columbia was used as a wild-type plant in this study. Plants were grown on rock wool and vermiculite at 22°C or 28°C under continuous light.

Plasmid Construction and Plant Transformation

The coding region including introns of the *At4g33300* gene was amplified using MA40-OE5 and MA40-OE3 primers (Table 1). Polymerase chain reaction (PCR) was carried out with PfuUltra High-Fidelity DNA polymerase (Strategene, USA). The amplification profile was an initial denaturation at 94°C for 2 min, followed by 24 cycles of denaturation at 96°C for 20 s, annealing at 56°C for 30 s and extension at 68°C for 3 min, and final extension at 72°C for 10 min.

Table 1 Primer sequences for PCR

Primer name	Sequence
MA40-OE5	gcccgGGTATCCAATGGCCATCACCG
MA40-OE3	gcccgggTTATTCGTCAAGCCAGTCTAG
MA40-5	GTATCCAATGGCCATCACCG
MA40-3	TGCAACCAGGAAACTTAAACG
EF1α-5	CTCCTTTCAGATTCTTACTTGC
EF1α-3	TCATTTGGCACCCTTCTTCAC
PR1-5	GTAGGTGCTCTTGTTCTTCCC
PR1-3	CACATAATTCCCACGAGGATC
BGL2-5	GAGCTACAGAGATGGTGTCAG
BGL2-3	ATGTAGCTGAAGTAAGGGTAG
PDF1.2A-5	TCATGGCTAAGTTTGCTTCCA
PDF1.2A-3	AAATACACACGATTTAGCACC
Thi2.1-5	GTAGAAGCAAAGATCTGCTGC
Thi2.1-3	AGTTCTGGAGAGTGTTCATG
PAD4-5	TCATGGACGATTGTCGATTCG
PAD4-3	CTACTAAGTCTCCATTGCGTC
SID2-5	TTTCTGCAATGGCTTCACTTC
SID2-3	CAACATTGCTTTCTTATTGTGAG
WRKY70-5	CTAACAAATGGATACTAATAAAGC
WRKY70-3	ACTCAAGATAGATTCGAACATG
DREB2A-5	GAGTACCTCAAGTCAGTCTGAG
DREB2A-3	CTCGGATAGAGAATCAACAGTC

Small letters indicate non-corresponding sequences to the template for adding restriction enzyme sites

PCR products were separated by agarose gel electrophoresis and extracted from the gel piece containing the amplified DNA. After adding A residues at their 3′ sites using rTaq polymerase (Takara, Japan) and dATP, this fragment was ligated to the *Xcm*I site in the p3T vector (Molecular Biotechnology, Germany). After nucleotide accuracies had been confirmed by nucleotide sequencing, the resultant plasmid was used to construct a plasmid for transformation in plants. The *β-glucuronidase* gene in the pBI121 vector was removed with *Bam*HI and *Sac*I digestion and self-ligation using the *Bam*HI-*Sma*I-*Sac*I adoptor sequence. The resultant plasmid was named p35S-NOST vector. The PCR product cloned in the p3T vector was ligated to the *Sma*I site in the p35S-NOST vector. After orientation of inserted DNAs had been confirmed, the resultant plasmid was used as a *35S::ADR1-L1* construct.

The plasmid was introduced into *Agrobacterium tumefaciens* strain C58 by the freeze–thaw method (Nishiguchi et al. 1987) and was used to transform the wild-type plant by vacuum infiltration (Bechtold et al. 1993). T1 transformants were selected by resistance to kanamycin. We checked the introduced DNA fragments in transformants by PCR analysis of genomic DNA. Transformants were named *35S::ADR1-L1* plants.

Expression Analysis

Total RNA was prepared from rosette leaves and inflorescence stems of 4-day-old plants after anthesis of the first flower by a method using guanidium thiocyanate (Chirgwin et al. 1979). After total RNA had been digested with RQ1 DNase (Promega, USA), reverse transcription was performed using a PrimeScript II 1st strand cDNA synthesis kit (Takara, Japan). The cDNA was amplified using Ex-Taq (Takara, Japan) with various primers (Table 1). The amplification profile was an initial denaturation at 94°C for 2 min followed by various cycles of denaturation at 96°C for 20 s, annealing at 56°C for 30 s and extension at 68°C for 3 min, and final extension at 72°C for 10 min. Reverse transcription (RT)-PCR products were examined by agarose gel electrophoresis.

Pathogen Resistance Assay

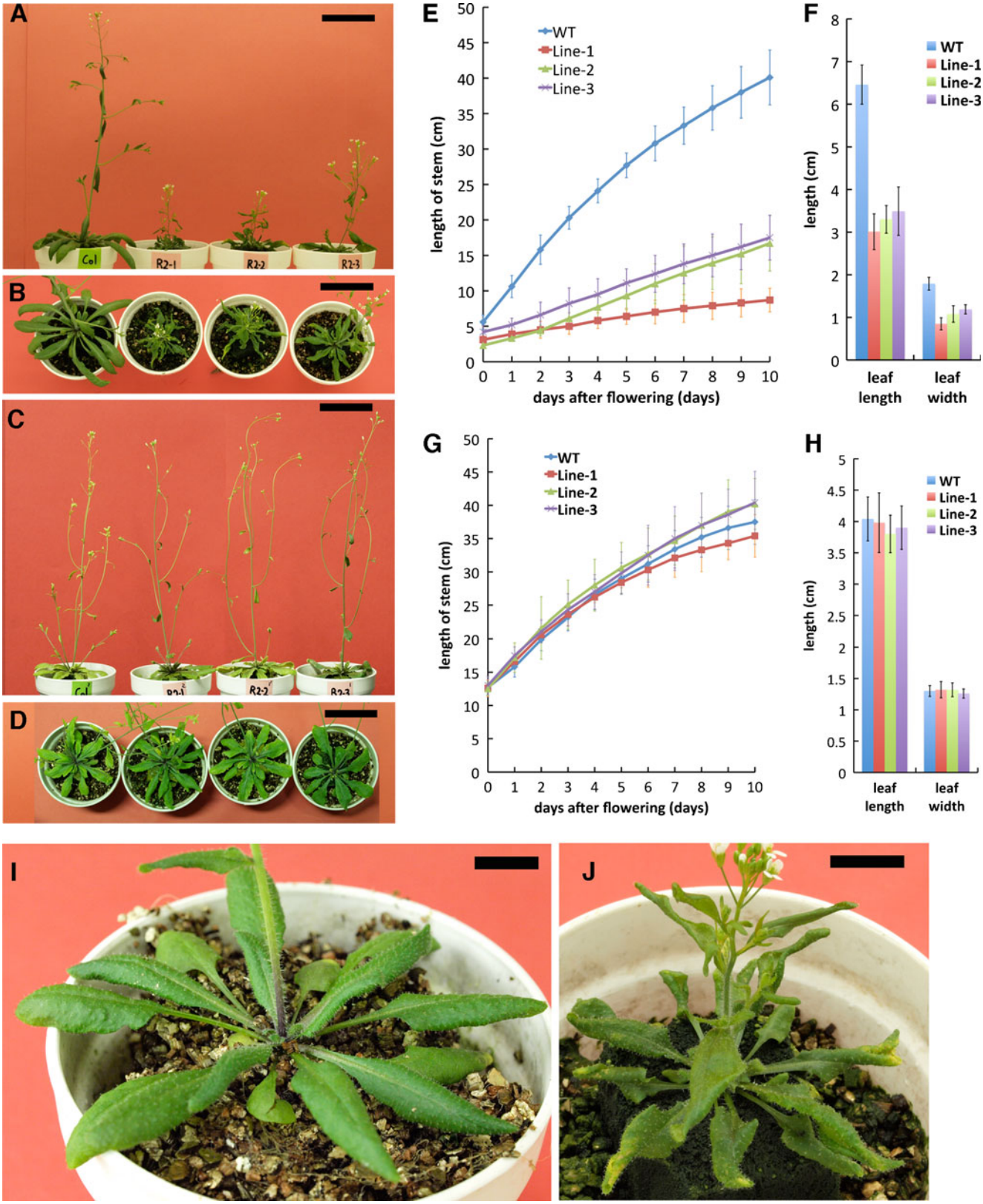
P. syringae pv. *tomato* DC3000 was grown for 6 h at 27°C in King's medium B [2% (*w*/*v*) Bacto proteose peptone No 3, 0.15% (*w*/*v*) K_2HPO_4, 0.15% (*w*/*v*) $MgSO_4$ $7H_2O$, 1% (*v*/*v*) glycerol] containing 100 μg mL^{-1} rifampicin and 25 μg mL^{-1} kanamycin. Bacterial cells were collected by centrifugation and resuspended at 10^5 colony-forming units (cfu) mL^{-1} in a solution of 10 mM $MgCl_2$ and 0.02% (*v*/*v*) Silwet L-77. Plants used for *P. syringae* infection were grown at 22°C for 4 weeks or at 28°C for 3 weeks. Since the growth of plants occurred first under a high temperature condition, both plants grown at 22°C for 4 weeks and at 28°C for 3 weeks were just before the first anthesis. Inoculation was performed by vacuum infiltration of the bacterial suspension. The amount of bacteria in plants was analyzed immediately after inoculation and 1 and 2 days after inoculation. For each sample, five leaf disks were pooled five times per data point. Leaf disks were bored from the infiltrated area, ground in 10 mM $MgCl_2$, and serially diluted to measure the cfu (Tsutsui et al. 2008).

Results

Dwarf Phenotype and Morphological Changes of Leaves Induced by Overexpression of the *ADR1-L1* Gene

The *ADR1-L1* gene encodes a protein of 816 amino acids that possesses an N-terminal CC structure, NBS, and C-terminal LRR domains. The ADR1-L1 protein is one of four proteins belonging to the ADR1 family. In order to characterize the function of the ADR-L1 protein, we generated transgenic plants overexpressing the *ADR1-L1* gene under control of the cauliflower mosaic virus 35S promoter (*35S::ADR1-L1* plants). We grew plants under continuous light. Since *A. thaliana* ecotype Columbia is a long-day plant, continuous light may have no special effects on plant growth. Twelve lines of *35S::ADR1-L1* plants were obtained and they showed a semidwarf phenotype at 22°C. We performed further analyses of three independent lines (Fig. 1). RT-PCR analysis revealed that expression of the *ADR1-L1* gene was increased in *35S::ADR1-L1* plants at both 22°C and 28°C (Fig. 2).

Figures 1a and b show that the size of *35S::ADR1-L1* plants was reduced at 22°C. Successive measurements of stem lengths of primary inflorescence of these transgenic lines after anthesis of the first flower showed that shortening of the primary inflorescence stem occurred constantly in each stage of bolting in *35S::ADR1-L1* plants (Fig. 1e). Ten days after anthesis of the first flower, the stem lengths of primary inflorescence of *35S::ADR1-L1* lines 1, 2, and 3 plants were approximately 22%, 42%, and 44% of those of wild-type plants, respectively. Measurements of the sizes of the largest rosette leaf in each individual at 4 days after anthesis of the first flower showed reduction in the sizes of leaf length and leaf width in *35S::ADR1-L1* plants. Leaf lengths and leaf widths of the largest rosette leaf of *35S::ADR1-L1* plants were 46–54% and 47–66% of those of wild-type plants, respectively (Fig. 1f). Furthermore, *35S::ADR1-L1* plants showed morphological change of leaves as curled and twisted shapes. These changes were observed not only in rosette leaves but also in


A
Col
R2-1
R2-2
R2-3
B
C
D
E
WT
Line-1
Line-2
Line-3
length of stem (cm)
days after flowering (days)
F
length (cm)
leaf length
leaf width
G
H
I
J

◀**Fig. 1** Morphological phenotype of *35S::ADR1-L1* plants. **a–d** Wild-type and lines 1, 2, and 3 of *35S::ADR1-L1* plants are arranged from *left to right*. Plants were grown at 22°C (**a** and **b**) and at 28°C (**c** and **d**). *Scale bar* 5 cm. **e** and **g** Measurements of stem length of primary inflorescence of wild-type (n=8) and *35S::ADR1-L1* line 1 (n=8), line 2 (n=8), and line 3 (n=8) plants grown at 22°C (**e**) and at 28°C (**g**). Data were obtained each day after anthesis of the first flower. Each point represents the average with standard deviation. **f** and **h** Measurements of leaf sizes of wild-type (n=8) and *35S::ADR1-L1* line 1 (n=8), line 2 (n=8), and line 3 (n=8) plants grown at 22°C (**f**) and at 28°C (**h**). Leaf length and leaf width of the largest leaf were measured. The leaves were harvested 4 days after anthesis of the first flower. *Color bar and black line* indicate the average and standard deviation, respectively. **i** and **j** Enlargement photos of wild-type (**i**) and *35S::ADR1-L1* (**j**) plants grown at 22°C. *Scale bar* 1 cm

cauline leaves (Fig. 1i and j). Figure 1c and d show *35S::ADR1-L1* plants grown at 28°C. The defect of plant growth and morphological change of leaves were recovered. Measurements of stem length and leaf size revealed that there were no differences between *35S::ADR1-L1* plants and wild-type plants when they were grown at 28°C (Fig. 1g and h).

It has been reported that activation of *ADR1* gene expression causes an enhancement of drought tolerance, and we therefore examined the drought tolerance of *35S::ADR1-L1* plants according to the method described by Chini et al. (2004). However, we failed to observe a difference in drought tolerance between *35S::ADR1-L1* plants and wild-type plants (data not shown).

Analysis of Defense-Related Gene Expression

We performed RT-PCR to analyze defense-related gene expression in *35S::ADR1-L1* plants (Fig. 2). We used the *elongation factor 1a* gene as an internal control. *Pathogenesis-related gene 1* (*PR1*) and *beta-1,2-glucanase* (*BGL2*) genes were used as marker genes for SA-dependent defense response, and *plant defensin 1.2A* (*PDF1.2A*) and *Thionin 2.1* (*Thi2.1*) genes were used as jasmonic acid (JA)/ethylene (ET)-dependent pathogen-responsive genes. The expression levels of *PR1* and *BGL2* genes in *35S::ADR1-L1* plants were higher than those in wild-type plants at 22°C. Remarkably, transcripts of *PR1* and *BGL2* genes were scarcely detected in *35S::ADR1-L1* and wild-type plants at 28°C. The expression levels of *PDF1.2A* and *Thi2.1* genes in *35S::ADR1-L1* plants were almost the same as those in wild-type plants at 22°C and at 28°C.

Phytoalexin deficient 4 (*PAD4*) and *salicylic acid induction deficient 2* (*SID2*) genes are involved in the process of SA accumulation by a pathogen-activated signal. The expression levels of both genes in *35S::ADR1-L1* plants were slightly higher than those in wild-type plants at 22°C. The expression levels of these genes in *35S::ADR1-L1* plants and wild-type plants at 28°C were lower than

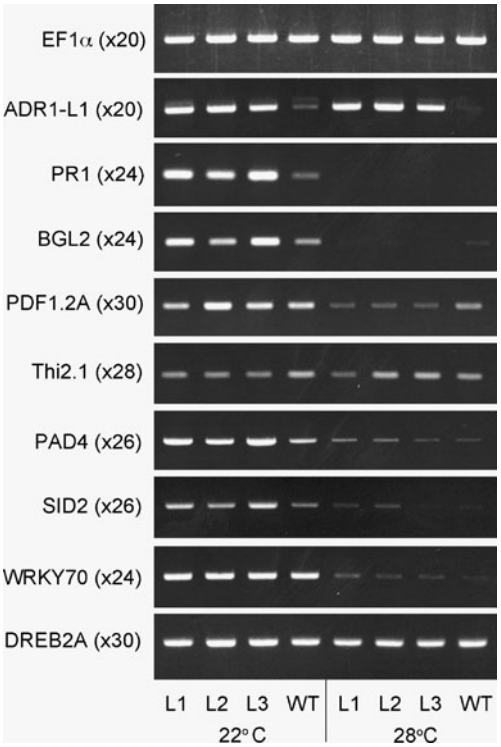


Fig. 2 RT-PCR analysis of the *ADR1-L1* gene, several defense-related genes and drought-responsive gene in *35S::ADR1-L1* line 1 (*L1*), line 2 (*L2*), and line 3 (*L3*) and wild-type (*WT*) plants grown at 22°C and at 28°C. Cycle numbers of PCR are shown in *parentheses*. Locus names are as follows: *EF1a* At5g60390, *ADR1-L1* At4g33300, *PR1* At2g14610, *BGL2* At3g57260, *PDF1.2A* At5g44420, *Thi2.1* At1g72260, *PAD4* At3g52430, *SID2* At1g74710, *WRKY70* At3g56400, *DREB2A* At5g05410

those at 22°C, and a difference between RNA accumulation in *35S::ADR1-L1* plants and that in wild-type plants at 28°C was not observed. *WRKY70 DNA-binding protein* (*WRKY70*) gene is involved in the process by which the expression of defense genes is activated by the SA signal. The expression level of this gene in *35S::ADR1-L1* plants was almost the same as that in wild-type plants at 22°C. The expression of this gene was suppressed at 28°C in both *35S::ADR1-L1* plants and wild-type plants.

It has been reported that plants overexpressing the *ADR1* gene showed accumulation of *DREB2A* transcripts (Chini et al. 2004). As shown in Fig. 2, there was no difference between the expression levels of *DREB2A* gene in *35S::ADR1-L1* plants and wild-type plants.

Enhancement of Resistance to Bacterial Pathogen Infection by Overexpression of the *ADR1-L1* Gene

Expression analysis of defense-related genes showed that *PR1* and *BGL2* genes were activated in *35S::ADR1-L1* plants at 22°C. The possibility therefore existed that a basal resistance to pathogens is continuously activated in *35S::*

ADR1-L1 plants. We examined the growth of the virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000 in *35S::ADR1-L1* and wild-type plants. As shown in Fig. 3, bacterial numbers in *35S::ADR1-L1* plants were 10 times less than those in wild-type plants at 1 and 2 days after inoculation at 22°C. In contrast with the results at 22°C, there was no difference in bacterial numbers between *35S::ADR1-L1* plants and wild-type plants at 28°C. These results indicated that overexpression of the *ADR1-L1* gene enhances resistance to bacterial pathogen infection at 22°C but this enhancement is suppressed at 28°C.

Discussion

The *ADR1-L1* gene belongs to the ADR1 family, which is the smallest group of CC-NBS-LRR-type *R* genes in *A. thaliana*. We found that transgenic plants overexpressing the *ADR1-L1* gene showed a semi-dwarf phenotype and morphological changes of leaves at 22°C. These phenotypes were observed in plants activating expression of the *ADR1* gene (Grant et al. 2003). It has usually been observed that constitutive activation of SA signaling causes a defect of plant growth (Clarke et al. 1998; Shah et al. 2001; Yoshioka et al. 2001; Shirano et al. 2002; Gil et al. 2005; Gou et al. 2009; Miura et al. 2010). Curled leaves have often been observed in plants activating SA-dependent defense response (Shah et al. 2001; Yoshioka et al. 2001; Gil et al. 2005; Gou et al. 2009). The expression levels of *PR1* and *BGL2* genes in *35S::ADR1-L1* plants, the expression of the genes being activated by SA signaling (Uknes et al. 1992), were higher than those in wild-type plants. The expression of *PAD4* and *SID2* genes, which encode proteins regulating SA accumulation (Shah 2003), was also activated in *35S::ADR1-L1* plants. Furthermore, overexpression of the *ADR1-L1* gene induced resistance to bacterial pathogen infection. These results suggest that the ADR1-L1 protein is involved in the SA-dependent plant defense system.

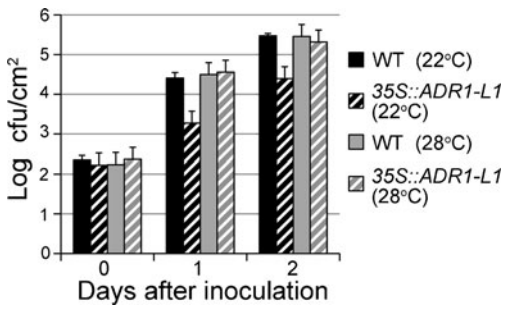


Fig. 3 Growth of *P. syringae* pv. *tomato* DC3000 in wild-type and *35S::ADR1-L1* (line 1) plants at 22°C and 28°C. Four-week-old plants at 22°C and 3-week-old plants at 28°C were inoculated. *Bar and line* indicate the average and standard deviation, respectively

The expression levels of defense-related genes other than *PDF1.2A* and *Thi2.1* genes were decreased at 28°C compared with the levels at 22°C in both *35S::ADR1-L1* plants and wild-type plants. The expression of *PR1* and *BGL2* genes was strongly suppressed at 28°C. The enhancement of defense responses in *35S::ADR1-L1* plants was also suppressed at 28°C. The dwarf phenotype and the morphological changes of leaves were recovered in *35S::ADR1-L1* plants at 28°C. These results suggest that the SA-dependent plant defense system mediated by ADR1-L1 protein was suppressed under a high temperature condition. It has often been reported that activation of defense response by a mutation or by overexpression of the *R* gene was suppressed in high temperature conditions. Transgenic plants overexpressing the *RPW8* gene by their own promoter showed spontaneous development of hypersensitive response-like lesions in the absence of a pathogen, but the development of these lesions was suppressed under high temperature conditions (Xiao et al. 2003). The dwarf phenotype and high expression levels of defense-related genes were observed at 22°C but were suppressed at 28°C in the *snc1* mutant, a mutant that constitutively activates SNC1 function by a gain-of-function mutation (Li et al. 2007), and in the *bon1* mutant, which causes loss of the negative regulation of SNC1 expression (Yang and Hua 2004). Defective growth was observed in the *cpr30* mutant at 22°C, but it was recovered at 28°C (Gou et al. 2009). It was reported that resistance to *P. syringae* pv. *tomato* DC3000 mediated by three *R* genes belonging to different classes, TIR-NB-LRR class, CC-NB-LRR class and LRR-TM class, was inhibited by a moderate elevation in temperature (Wang et al. 2009). It is highly probable that temperature modulation of defense responses to biotrophic pathogens is a general phenomenon. The strong suppression of expressions of *PR1* and *BGL2* genes at 28°C in both wild-type and *35S::ADR1-L1* plants suggests that the inhibition of disease responses at a high temperature was caused by the decrease of their expression levels. The regulation of disease responses by temperature may be controlled downstream of SA signaling, although the molecular mechanisms of the regulation are unknown.

The expression levels of *PDF1.2A* and *Thi2.1* genes, which are activated by JA/ET signaling (Epple et al. 1995; Penninckx et al. 1996), in *ADR1-L1* plants did not change from those in wild-type plants. Plants overexpressing the *ADR1* gene exhibit activation of not only *PR1* gene expression but also *PDF1.2* gene expression (Grant et al. 2003). It has been reported that overexpression of the *ADR1* gene did not result in accumulation of JA and ET, and it was suggested that an alternative cue engages *PDF1.2* gene expression in plants overexpressing the *ADR1* gene. Overexpression of the *ADR1-L1* gene may not trigger this cue to engage *PDF1.2A* expression. This point shows a difference between the roles of ADR1 and ADR-L1

proteins in plants. We did not observe enhancement of drought tolerance and activation of *DREB2A* gene expression in *35S::ADR1-L1* plants, although constitutive expression of the *ADR1* gene enhanced drought tolerance and activated expression of the *DREB2A* gene (Chini et al. 2004). This point also shows a difference between the roles of ADR1 and ADR-L1 proteins in plants.

The role of the ADR1 protein, which is related to both disease resistance and drought tolerance, is not a character conserved among members of the ADR1 family, although members of the ADR1 family have common features that are not observed in other NBS-LRR R proteins. The amino acid sequences are highly conserved in the NBS domain, the linker domain between NBS and LRR domains, and the LRR domain. However, the homology of amino acid sequences in the N-terminal region containing the CC structure is relatively low (Chini and Loake 2005). The variation of amino acid sequences in CC structures may lead to the difference in signaling pathways activated by R proteins.

Plants overexpressing the *ADR1-L1* gene showed typical phenotypes that were observed in plants activating SA-dependent defense response. On the other hand, some of the phenotypes in plants overexpressing the *ADR1-L1* gene were different from those in plants overexpressing the *ADR1* gene, although the *ADR1-L1* gene is closely related to the *ADR1* gene. It is possible that the ADR1-L1 protein is related to abiotic stresses other than drought stress. Further analyses of various R proteins and various abiotic stresses should provide insights into the interplay between biotic and abiotic stress signaling as well as mechanisms of the plant defense system.

Acknowledgments We are grateful to Dr. Junji Yamaguchi at Hokkaido University for permission to use the facilities for pathogen resistance assay and Mr. Kuniaki Katsumata and Ms. Rikako Tanaka at Hokkaido University for instruction of pathogen resistance assay. A part of this work was supported by grants-in-aid for scientific research from the Japanese Ministry of Education, Science and Culture.

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