Salicylic Acid-Mediated Cell Death in the Arabidopsis len3 Mutant

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Received December 19, 2005; Accepted February 8, 2006; Online Publication, June 23, 2006
[doi:10.1271/bbb.50683]

The Arabidopsis lesion initiation 3 (len3) mutant develops lesions on leaves without pathogen attack. len3 plants exhibit stunted growth, constitutively express pathogenesis-related (PR) genes, PR-1, PR-2, and PR-5, and accumulate elevated levels of salicylic acid (SA). Furthermore, len3 is a semidominant, male gametophytic lethal mutation with partial defects in female gametophytic development. To determine the signaling pathway activated in len3 plants, we crossed the len3 plants with nahG, npr1-1, and pad4-1 plants and analyzed the phenotypes of the double mutants. The len3-conferred phenotypes, including cell death and PR-1 expressions, were suppressed in the double mutants. Thus SA, NPR1, and PAD4 are required for the phenotypes. However, none of these double mutants could completely suppress the len3-conferred stunted growth. This result suggests that an SA-, NPR1-, and PAD4-independent pathway is also involved in the phenotype. Treatment with BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid), an SA analog, induced cell death in len3 nahG plants but not in len3 npr1 or len3 pad4 plants, suggesting the involvement of the PAD4-dependent but SA-independent second signal pathway in cell death in len3 plants.

Key words: Arabidopsis; lesion; salicylic acid; cell death

Plants defend themselves from attack by microbial pathogens by activating a battery of defense responses after infection. In the last decade, studies on the genes controlling expression of defense responses in plants have focused on identification of mutants, followed by isolation and ordering of the genes within branches of the signal transduction networks by double-mutant analysis.1) Gene-for-gene resistance is a particularly strong form of plant disease resistance. Plants carry specific resistance (R) genes that are able to recognize pathogens carrying corresponding avirulence (avr) genes. This reaction triggers a rapid defense response that generally includes programmed cell death, hypersensitive response (HR). Activation of the HR triggers systemic acquired resistance (SAR). This response includes the accumulation of a signal molecule, salicylic acid (SA), throughout the plant and the consequent expression of a characteristic set of defense genes, including pathogenesis-related protein-1 (PR-1). Other defense responses are also activated by signal transduction networks that require jasmonic acid and ethylene as signal molecules.1–3)

The transgene nahG encodes a bacterial salicylate hydroxylase that destroys SA by converting it to catechol. Transgenic plants expressing nahG were important for providing the first demonstration that SA is required for expression of defense effector genes such as PR-1, and for the phenomenon of SAR.4) Two genes, PAD4 and EDS1, are required to activate SA accumulation in response to some, but not all, SA-inducing stimuli. These genes encode proteins similar to triacylglycerol lipases that interact with each other, but so far there is no evidence that lipase activity is required for the function of either protein.5) NPR1 acts downstream from SA. When SA levels are low, NPR1 exists in an oligomeric form in the cytoplasm. When SA levels increase, the NPR1 oligomers dissociate into monomers owing to reduction of disulfide linkages holding the monomers together.6,6) The monomers then enter the
nucleus, where they interact with TGA-type transcription factors. These factors are required for the activation of PR-1 expression by SA. Some SA-dependent defense responses are independent of NPR1, so there must be another branch of the SA signaling pathway.\(^{3,6}\) Ordering of events in SA signaling is complicated by the fact that the pathway includes several feedback loops.\(^1\) Cell death promotes SA production, but SA production promotes cell death. PAD4 and EDS1 are required for SA production under some conditions, and expression of these genes is enhanced by SA.\(^5\) SA levels are higher in infected \(npr1\) mutants than in infected wild-type plants, suggesting that NPR1 functions in controlling SA levels as well as in responding to elevated SA.\(^{3,6}\)

Many mutants with constitutively high levels of SA, defense gene expression, and disease resistance also display a lesion-mimic phenotype. They undergo spontaneous cell death in the absence of pathogen attack. Some of the genes defined by these mutations might function as regulators of cell death.\(^7\) Among these mutants, clear evidence pointing to a role of SA in cell death comes from analysis of \(Arabidopsis\) accelerated cell death (\(acd\)) and lesion simulating disease (\(lsd\)) mutants that spontaneously activate cell death and defense pathways.\(^7\)\(^{10}\) In many of these mutants, including \(acd6,\)\(^{10}\) \(acd11,\)\(^{11}\) and \(lsd6,\)\(^{12}\) \(nahG\) transgene expression suppresses the cell death phenotype, while this can be restored by application of an SA analog such as benzo(1,2,3)thiadiazole-7-carboxylic acid (BTH).

In this paper, we describe characteristics of the \(len3\) mutant, an \(Arabidopsis\) lesion initiation mutant, which shows cell death phenotype. The \(len3\) mutation appears to activate at least three signaling pathways: an SA-dependent and NPR1-dependent pathway, required for \(PR-1\) expression, a PAD4-dependent and SA-independent one, required for cell death, and an SA-, NPR1-, and PAD4-independent one, required for stunted growth. All of these pathways appear to be involved in the \(len3\)-conferred phenotypes.

**Materials and Methods**

*Plants and growth conditions.* Plants were grown on MS medium or soil at 22 °C under short-day conditions (9 h light/15 h dark) at 100 \(\mu\)mol m\(^{-2}\) sec\(^{-1}\) fluorescent illumination. Wild-type plants (\(Arabidopsis\) thaliana ecotype Columbia: Col) were used for experiments. Seeds of \(npr1-1,\) \(pad4-1,\) Col, Landsberg erecta (Ler), Keswick (Ksk), and Nossen (Nos) were obtained from \(Arabidopsis\) Biological Resource Center (Ohio State University, Columbus, OH). The \(nahG\) line was a gift of Novartis (Research Triangle Park, NC). All of the mutant lines are in Col background. For benzo(1,2,3)-thiadiazole-7-carboxylic acid (BTH) treatment, plants were sprayed with BTH at 100 \(\mu\)M. Infection and growth determination of \(Pseudomonas\) syringae pv maculicola-ES4326 were done as described previously.\(^1\)

**Genotyping of double mutants.** The mutant alleles used for construction of double mutants with \(len3\) were \(nahG,\) \(npr1-1,\) and \(pad4-1.\) We generated the double mutants using a \(len3\) plant as pollen recipient. The presence of the \(nahG\) transgene was confirmed by PCR amplification of the \(nahG\) gene. Lines homozygous for \(nahG\) loci were identified by screening \(F_2\) populations derived from individual \(F_1\) lines. The \(F_2\) lines that showed 100% resistance to kanamycin in the \(F_3\) population were considered homozygous for \(nahG\) loci. The \(len3\) mutation was tightly linked to a T-DNA, as well as to a functional kanamycin resistance gene (see “Results”). Thus the genotype of \(len3\) plants was confirmed by PCR amplification of the T-DNA border flanking sequence. The genotypes of the \(npr1-1\) and \(pad4-1\) loci were determined by cleaved amplified polymorphic sequence (CAPS) analysis, as described previously.\(^1\)

**Histochmeisty.** Autofluorescence and callose examination, staining with trypan blue, and dianminobenzidine (DAB) were all described previously.\(^1\)

**RNA extraction and Northern analysis.** Total RNA was prepared from leaves using the Concert Plant RNA Reagent according to the manufacture’s instructions (Invitrogen, Tokyo). The RNA concentration was determined spectrophotometrically. Equal amounts of total RNA (20 \(\mu\)g) were loaded on a denaturing 1.2% (w/v) agarose gel containing 6% formaldehyde, fractionated by electrophoresis, and transferred to Zeta-probe GT (Nihon Bio-Rad, Tokyo). Equal loading of RNA was verified by staining with ethidium bromide and comparison of the amounts of ribosomal RNAs in each sample before transfer to the membrane. The blots were hybridized to a \(^{32}\)P-labeled fragment of the probe.

**Extraction and analysis of SA.** Leaves of 7-week-old plants were harvested and the salicylic acid (SA) and SA glucoside (SAG) levels were measured as described previously.\(^1\)

**Results**

**Characterization of \(len3\) mutant**

To identify genes involved in defense signaling in \(Arabidopsis,\) approximately 3,300 T-DNA tagged \(Arabidopsis\) lines were screened for individuals that developed lesions.\(^1\)\(^{13}\) We identified one plant and designated it \(len3.\) All of the following experiments were performed with a mutant line that was backcrossed two times to wild-type plants (Col). The \(len3\) plants were smaller than the wild-type plants (Fig. 1A), and showed a few punctuating cell death patches on the leaves of 7-week-old plants (Fig. 1A, B). The lesion parts and neighboring regions in the leaves were stained with dianminobenzidine (DAB), a histochemical regent for \(H_2O_2,\) and with trypan blue, a regent for cell death. UV microscopy also
revealed considerable autofluorescence and callose deposits in areas corresponding to the lesions in the len3 plants (Fig. 2). These results show that the phenotype of len3 plants resembles hypersensitive responses.

When wild-type plants (Col) were crossed with pollen from len3 plants, no F₁ plants showed cell death. F₂ progeny from this cross also showed no cell death (Table 1). On the other hand, when len3 plants were crossed with pollen from Col, a segregation ratio of 15 cell death⁻/Col: 6 cell death⁻⁺ was obtained. This result suggests that len3 is a putative dominant gametophytic mutant and cannot transmit through male gametes. Gametophytic dominant lethal mutations that are penetrant in one sex, but are not through the other, can be recovered only as heterozygotes. Thus the len3 plants must be heterozygous for the mutation. Therefore, the genotype of len3 should be len3/LEN3. In that case, if the len5 mutation affected a gene essential for male gametophytic development, the ratio of cell death⁻: cell death⁻⁺ in self-progeny would be 1:1 rather than the expected 1:3, but F₂ progeny from cell death and stunted F₁ plants of this cross showed a segregation ratio of 465 cell death⁻/Col: 367 cell death⁻⁺ ($\chi^2 = 11.54; P < 0.005$), indicating that the ratio was 1:< 1. This result suggests the possibility that the LEN3 gene is also partially required for female gametophytic development.

Judging from these results, len3 appears to be a heterozygous, semidominant, male gametophytic lethal mutation that also partially affects female gametophytic development.

**PR genes are constitutively expressed in len3 plants**

Since several lesion mimic mutants express the genes encoding PR proteins, northern analysis was performed to analyze the expression of PR genes in len3 plants. The len3 plants exhibited constitutive expression of PR-1, PR-2, and PR-5 genes (Fig. 3).

**Endogenous levels of free SA and SAG are elevated in len3 plants**

The high levels of PR gene expression in len3 plants raised the possibility that this mutation might affect endogenous SA levels. To assess this possibility, endogenous levels of free SA and SA glucoside (SAG) were monitored. The len3 plants were found to contain a 13-fold higher basal level of free SA and a 23-fold higher level of total SA (free SA + SAG) than the respective ones detected in wild-type plants (Fig. 4).

**Growth of PsmES4326 in len3 plants**

To determine whether len3 plants exhibit enhanced disease resistance, we analyzed the growth of Pseudomonas syringae pv. maculicola ES4326 in len3 plants.
Seven-week-old plants were infected with the strain and bacterial growth was determined 3 d post inoculation. The growth of \textit{Psm} \textit{ES4326} in \textit{len3} plants was not different from that seen in wild-type plants in most experiments (data not shown).

SA is required for \textit{len3}-conferred phenotypes

Because of the central role of SA in regulating defense responses, we tested its involvement in the \textit{len3}-conferred phenotypes. We removed SA from \textit{len3} plants by crossing \textit{len3} plants with a plant having a \textit{nahG} transgene. The \textit{len3 nahG} plants were intermediate in size between \textit{len3} plants and wild-type plants and lacked cell death patches (Fig. 1). We did not detect the presence of autofluorescent phenolic compounds, callose deposition, staining of dead cells by trypan blue, or staining of \(H_2O_2\) by DAB (Fig. 2). The expression of SAR-associated \textit{PR} genes was also reduced in \textit{len3 nahG} plants (Fig. 3). Consequently, we propose that \textit{len3} activates two signaling pathways; one is an SA-dependent pathway that leads to cell death, expression of \textit{PR} genes, and reduced stature, and the other, an SA-independent one required for the reduced stature of \textit{len3} plants.

\textbf{BTH treatment induces cell death in \textit{len3 nahG} plants}

To determine whether the effects of the \textit{nahG} transgene resulted from blocking the SA signaling pathway specifically, we applied a synthetic SA analog, BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid), to the \textit{len3 nahG} plants, and examined cell death. Application of BTH induced cell death in small pathches on the leaves within 5 d of treatment. The cell death phenotype was more striking in BTH-treated \textit{len3 nahG} plants than in intact \textit{len3} plants (Fig. 5). The induction of the cell death phenotype of \textit{len3 nahG} plants by BTH treatment indicates that a component of the SA signaling pathway is required for the \textit{len3}-conferred phenotype.

\textbf{Phenotype of double mutants}

The suppression of \textit{len3}-conferred phenotypes by the \textit{nahG} transgene prompted us to examine the possible role of \textit{NPR1} and \textit{PAD4}, which are required for SA signal transduction. \textit{PAD4} encodes a lipase-like protein that might function in an SA-amplification loop.\textsuperscript{5} \textit{NPR1} encodes an ankyrin repeat protein, which acts downstream of SA.\textsuperscript{3,6} The \textit{len3 pad4} and \textit{len3 npr1} plants were of the same size as the \textit{len3 nahG} plants (data not shown), and exhibited no cell death phenotype (Fig. 5). This result suggests that \textit{PAD4} and \textit{NPR1} are required for \textit{len3}-conferred phenotypes.

\textbf{Expression of \textit{PR} genes in double mutants}

Northern analysis was performed to determine how \textit{npr1} and \textit{pad4} affect the expression of the \textit{PR} genes in the \textit{len3} mutants. As shown in Fig. 3, \textit{PR-1} expression decreased markedly in \textit{len3 npr1} and \textit{len3 pad4} plants. In contrast, the expression of \textit{PR-2} and \textit{PR-5} genes was only partly suppressed in both double mutant plants. We suggest that \textit{NPR1} and \textit{PAD4} are involved in \textit{PR-1}
expression in len3 plants, but that the expression of PR-2 and PR-5 genes is mediated by both NPR1-dependent and NPR1-independent SA signaling pathways in len3 plants.

SA accumulation in double mutants
To assess whether the cell death phenotype and the levels of PR transcripts in double mutants were associated with the endogenous levels of SA, we determined the levels of SA and SAG in these plants (Fig. 4). The increased accumulation of SA in len3 plants was suppressed in len3 nahG plants. We also observed that pad4 suppressed the accumulation of SA. In contrast to this, len3 npr1 plants accumulated SA at an intermediate level between the levels in len3 plants and len3 pad4 plants. Hence we propose that PAD4 and NPR1 are involved in the accumulation of SA, as positive regulators, in len3 plants and that the contribution of PAD4 is larger than that of NPR1.

BTH does not induce cell death in len3 npr1 or len3 pad4 plants
The len3-conferred cell death phenotype was not observed in len3 npr1 or len3 pad4 plants as it was not in len3 nahG plants. In contrast to len3 nahG plants, BTH application did not induce lesion formation in len3 pad4 or len3 npr1 plants (Fig. 5). This result suggests that PAD4 and NPR1 are required for BTH-induced cell death.

Mapping of len3
So far, more than 1,000 kanamycin-resistant (T-DNA associated) F2 plants derived from len3 F1 plants have shown len3-conferred lesion-forming phenotypes (data not shown). This result suggests that the close linkage of a single-locus T-DNA insertion to the LEN3 locus and len3 segregated as a single Mendelian locus. Genomic DNA flanking the T-DNA left border was isolated by TAIL-PCR (data not shown). We designed primers from the flanking genomic sequence of this T-DNA and used them to genotype len3 plants. The kanamycin-resistant plants had the T-DNA insertion in the same locus and showed len3-conferred lesion-forming phenotypes (data not shown). We also tried to place LEN3 on a genetic map. We crossed len3 plants (Columbia ecotype [Col]) to Landsberg erecta (Ler) plants. F2 plants from this cross were scored for the len phenotype. Unlike the case of backcrosses to Col, no clear segregation was observed. len3 was also crossed with Nossen (Nos) and Keswick (Ksk) plants. Like F2 plants from the Ler cross, F2 plants from these crosses displayed no clear segregation for lesion formation. This suggests not only the possibility of reduced penetrance of the mutant phenotype or the presence of a modifying gene(s) in the ecotypes except for Col, but also another possibility that a downstream factor of LEN3 is absent or its function is compromised in Ler, Ksk, and Nos. Hence efforts to determine a map position for len3 were unsuccessful.

Discussion
In this study, we have characterized the Arabidopsis len3 mutant, a lesion initiation mutant. The mutant exhibited the cell death phenotype and stunted growth. The len3 plants constitutively express the PR-1, PR-2, and PR-5 genes and accumulate elevated levels of SA. Furthermore, len3 is a semidominant, male gametophytic lethal mutation and might partially affect female gametophytic development. Although many lesion mimic mutants have been isolated, the above phenotypes of len3 plants are unique. Hence len3 is a novel mutant.

To determine the signaling pathway activated in len3 plants, we crossed the len3 plants with nahG, npr1-1, and pad4-1 plants and analyzed the phenotypes of the double mutants. The len3-conferred phenotypes, including cell death (Fig. 5) and PR expression (Fig. 3), were suppressed in these double mutants. Hence SA, NPR1, and PAD4 are thought to be required for the phenotypes. But none of these double mutants completely suppressed the len3-conferred stunted growth, indicating that an SA-, NPR1-, and PAD4-independent pathway is also involved in the phenotype (Fig. 6). It has been suggested that the jasmonic acid/ethylene signaling pathway is the SA-independent pathway in many lesion mimic mutants,1,7 but this remains to be determined for len3 plants.

The induction of cell death in len3 nahG plants by BTH treatment indicates that a component of the SA signaling pathway is required for the cell death phenotype of len3 plants. Since BTH treatment did not cause cell death in wild-type plants, we propose that a
second signal works together with SA to activate the cell death pathway in len3 plants. Furthermore, because len3 nahG plants treated with BTH showed an enhanced cell death phenotype, we would propose that the second signal is negatively regulated by SA although it works together with SA. The existence of the second signal required for cell death was previously proposed by Rate et al. together with SA. The existence of the second signal is negatively regulated by SA although it works together with SA. Therefore, we would propose that the second signal works together with SA to activate the cell death pathway in len3 plants. Furthermore, because len3 nahG plants treated with BTH showed an enhanced cell death phenotype, we would propose that the second signal is negatively regulated by SA although it works together with SA. The existence of the second signal was previously proposed by Rate et al. for acd6. Analogous to that model, the len3 nahG mutation leads to SA accumulation, which activates the NPR1-dependent pathway for PR-1 expression. The len3 mutation also causes generation of the proposed second signal, which works with SA to induce cell death. The requirement for SA and the second signal to activate cell death also explains why BTH treatment induces cell death in len3 nahG plants but not in nahG plants. The len3 nahG plants accumulate the second signal but lack SA, and this deficit is remedied by BTH treatment. In contrast, nahG plants do not accumulate the second signal, and thus BTH does not induce cell death.

PAD4 and NPR1 are involved in the accumulation of SA as positive regulators in len3 plants, and the contribution of PAD4 is larger than that of NPR1 (Fig. 4). The results fit well the model of the SA-dependent signaling pathway, in which PAD4 is located upstream of SA accumulation and NPR1 is located downstream of SA and acts as an SA signal transducer. In contrast to this, Clarke et al. reported that SA concentration was higher in the cpr npr1 double mutants than in the cpr single mutants, suggesting that npr1 is defective in feedback regulation of SA accumulation. In this case, NPR1 is a negative regulator of SA accumulation. The role of NPR1 in SA accumulation might be complicated. Further study is necessary to understand the precise mechanism of the function of NPR1 in SA accumulation.

BTH does not induce cell death in len3 npr1 or len3 pad4 plants. Since BTH functions as an SA analog through NPR1, BTH application does not produce cell death in len3 npr1 plants (Fig. 6). On the other hand, a question arises as to how the pad4 mutation affects the cell death signaling. Two mechanisms may be proposed to explain the effect. First, the len3 mutation might activate a pathway upstream of PAD4, because our epistatic analyses show that len3 phenotypes depend on SA accumulation as well as on functional PAD4. The induction of cell death by treatment with an SA-analog, BTH, occurs in SA-deficient len3 nahG plants but not in len3 pad4 plants. This result suggests that PAD4 not only functions in SA accumulation but also controls other signals that work in conjunction with SA to induce cell death. Thus the pad4 mutation leads to suppression of the second signal. PAD4 encodes a lipase-like protein, and thus if the second signal is a lipid-derived molecule, the level of the second signal might be reduced in len3 pad4 plants, which would lead to suppression of cell death (Fig. 6). Second, the len3 pad4 plants accumulated slightly higher SA than did the len3 nahG plants (Fig. 4). As described above, the second signal required for cell death in len3 plants is negatively regulated by SA. If the SA level of len3 pad4 plants is enough to suppress the second signal pathway, cell death is suppressed in len3 pad4 plants. Further study is required to understand the molecular mechanism.

Although our results suggest that len3 is a single Mendelian locus linked to T-DNA, we can not rule out the possibility that two mutations are required for the len3 phenotype. Even in that case, since no plants with the T-DNA showed the wild-type phenotype in segregation analysis, two mutations must be linked tightly to the T-DNA. The T-DNA border flanking sequence was located on the upper arm of chromosome 1 (data not shown). None of the previously published Arabidopsis mutants that develop lesions map near that position. Hence len3 represents a novel locus. Because len3 is a semidominant mutant, it is difficult to elucidate the precise function of LEN3 through studies on len3 phenotypes, but our results suggest that LEN3 might be an important factor that functions in gametophyte development and resistance response. The isolation of a loss-of-function len3 allele and the cloning of the LEN3 gene should clarify these issues in the future.

Acknowledgments

We thank Novartis (Research Triangle Park, NC) for its gift of the nahG line, and Dr. Tadashi Asahi for...
critical review of this manuscript. This work was partially supported by a grant-in-aid for scientific research at Fukui Prefectural University to A.I. from Fukui Prefecture, Japan.

References