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Autoimmunity in Arabidopsis acd11 Is Mediated by Epigenetic Regulation of an Immune Receptor

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Abstract

Certain pathogens deliver effectors into plant cells to modify host protein targets and thereby suppress immunity. These target modifications can be detected by intracellular immune receptors, or Resistance (R) proteins, that trigger strong immune responses including localized host cell death. The accelerated cell death 11 (acd11) “lesion mimic” mutant of Arabidopsis thaliana exhibits autoimmune phenotypes such as constitutive defense responses and cell death without pathogen perception. ACD11 encodes a putative sphingosine transfer protein, but its precise role during these processes is unknown. In a screen for lazarus (laz) mutants that suppress acd11 death we identified two genes, LAZ2 and LAZ5. LAZ2 encodes the histone lysine methyltransferase SDG8, previously shown to epigenetically regulate flowering time via modification of histone 3 (H3). LAZ5 encodes an RPS4-like R-protein, defined by several dominant negative alleles. Microarray and chromatin immunoprecipitation analyses showed that LAZ2/SDG8 is required for LAZ5 expression and H3 lysine 36 trimethylation at LAZ5 chromatin to maintain a transcriptionally active state. We hypothesize that LAZ5 triggers cell death in the absence of ACD11, and that cell death in other lesion mimic mutants may also be caused by inappropriate activation of R genes. Moreover, SDG8 is required for basal and R protein-mediated pathogen resistance in Arabidopsis, revealing the importance of chromatin remodeling as a key process in plant innate immunity.

Introduction

Unlike vertebrates, plants lack a somatic, adaptive immune system and immunological memory [1]. Therefore, plants rely on a large repertoire of pre-existing immune receptors, encoded by hypervariable Resistance (R) genes, which recognize specific pathogens and activate strong defense responses. These responses include the programmed cell death (PCD) of host cells at infection sites to restrict pathogen access in a process called the hypersensitive response (HR). R proteins are triggered by pathogen-specific effector proteins that have evolved to perturb or disrupt host processes to facilitate infection. While some pathogen effectors are recognized extracellularly, the majority are targeted to various intracellular compartments of the plant host and identified there. In most cases, R proteins are activated by detecting modifications to host proteins targeted by pathogen effectors. This model, known as the “guard hypothesis” [2,3], has been supported in numerous instances. For example RIN4, a host protein with key roles in basal defense, is under surveillance by multiple R proteins, and at the same time is the target of multiple pathogen effectors [4]. Most R proteins have been classified as NB-LRRs, named after their central nucleotide-binding (NB) and C-terminal leucine-rich repeat (LRR) domains, although various exceptions exist [5]. The N-terminal domains of NB-LRR R proteins fall into two broad categories: those with homology to Drosophila Toll and mammalian Interleukin-1 Receptor (TIR), and those with predicted coiled-coil (CC) regions [6]. Members of the animal NOD-like receptor (NLR) family exhibit similar domain architecture to plant NB-LRRs, and NLRs are likewise involved in immunity [7,8]. Like NB-LRR proteins, NLRs have several types of amino-termini including protein–protein interaction domains associated with proteins involved in programmed cell death and inflammation. Several autoimmune diseases in humans have been associated with mutations in NLRs [9].

In plants, there are numerous examples of mutants with autoimmune-related phenotypes. These so-called “lesion-mimics” are, in many cases, caused by mutations in genes hypothesized to be negative regulators of the HR [10]. Other examples include point mutations in NB-LRR R proteins [11,12]. Since R proteins have the potential to trigger host PCD, their activity is tightly regulated. R genes are typically constitutively expressed at low levels and some are up-regulated in response to pathogen-derived...
**Author Summary**

Plants defend themselves against pathogens via immune receptors that trigger responses including the suicide of infected cells to limit pathogen growth. The accelerated cell death 11 (acd11) knockout mutant of the model plant *Arabidopsis thaliana* kills itself in the absence of invading pathogens. By screening for secondary mutations that rescue acd11, we discovered two LAZARUS (LAZ) genes required for death. The first, LAZ2, encodes an enzyme that methylates histones, the major protein component of chromatin. This particular histone modification is generally involved in epigenetic remodeling of chromatin to a more permissive state for transcription of associated DNA. We show that expression of the second gene, LAZ3, is dependent on LAZ2 activity, suggesting that LAZ5 is a direct target of LAZ2. LAZ5 is a member of an immune receptor class involved in detection of specific pathogens and subsequent cell death. We propose that acd11, and other suicidal mutants, result from autoimmunity triggered by immune receptors controlled by chromosomal modifications. Interestingly, we found that defects in LAZ2 result in enhanced susceptibility to bacterial pathogens, suggesting that it controls other genes involved in innate immunity.

peptides or to the accumulation of the phytohormone salicylic acid (SA) [13,14]. Little is known about the transcriptional control of R genes. Intriguingly, members of a cluster of related *Arabidopsis* R genes are endogenously suppressed at the post-transcriptional level by RNA silencing, suggesting that pathogens that interfere with the silencing machinery unwittingly up-regulate steady-state R protein levels [15]. At the protein level, inappropriate activation is likely prevented by autoinhibition, high rates of turnover, and alternatively spliced products. Recently, it has become clear that hybrid necrosis, a deleterious genetic incompatibility observed in many intra- and interspecific plant hybrids, is associated with autoimmunity [16]. One example of this type of autoimmune response in *Arabidopsis* was shown to be dependent on an NB-LRR R protein, suggesting that these immune receptors have a broad mandate over PCD that extends beyond pathogen defense [17].

The lethal, recessive accelerated cell death 11 (acd11) mutant of *Arabidopsis* is characterized by constitutive activation of immune responses and PCD in the absence of pathogen attack [18]. ACD11 encodes a putative sphingosine transfer protein with homology to HET-C2 of the fungus *Podostipa arenicola*. Allelic variants of het-c determine compatibility during fusion of hyphae from different strains, causing PCD in combination with specific alleles at other het loci [19]. acd11 mutants develop normally until the 2-4 leaf stage, and PCD involves the phytohormone SA such that expression of a bacterial SA hydroxylase (NahG) strongly suppresses cell death. Application of SA agonists, such as benzothiadiazol-3-methyl ester (BTH), restores autoimmunity in acd11. Interestingly, the genetic requirements for acd11 cell death are similar to those for the HR triggered by TIR-NB-LRR immune receptors [18,20].

We report here that cell death in acd11 is suppressed by mutations in genes encoding a histone methyltransferase and a TIR-NB-LRR R protein. In addition, the expression of the R gene is dependent on the activity of the histone modifying enzyme. We propose that the TIR-NB-LRR is triggered by the absence of ACD11, implying that ACD11 (or a complex containing ACD11) may be a guarded pathogen effector target. Alternatively, since ACD11 may be involved in production of a lipid signal, the absence of this signal may induce LAZ5 expression in an SA-dependent manner. Our study provides strong evidence that a specific type of histone modification is directly involved in chromatin remodeling and transcriptional control of a subset of R genes including LAZ5.

**Results**

**laz2 suppresses cell death in acd11**

To isolate genes required for cell death in acd11, Landsberg erecta (Ler) ecotype acd11-1 plants harboring the NahG transgene were mutagenized with ethyl-methanesulfonate (EMS), diepox-ybutane (DEB) or γ-irradiation. ~200 suppressors of acd11 were subsequently identified as plants that survived following BTH treatment. Genetic analyses of 43 such suppressors grouped them into 12 recessive and 2 dominant loci referred to as lazarus (laz) mutants, after the biblical resurrection. One of the laz mutants found in the suppressor screen, laz2, abolished cell death in response to BTH in the acd11 NahG background, and exhibited similar levels of cellular ion leakage as wild type (Fig. 1, A and B). laz2-1 acd11-1 NahG plants also exhibited abnormal development (e.g. early flowering, increased shoot branching) that, along with acd11 suppression, was inherited recessively (data not shown). Two other laz2 alleles with similar morphology, laz2-2 and laz2-3, were confirmed by complementation tests (Fig. S1A). Global transcript profiles of laz2-1 acd11-1 NahG, Ler wild-type, NahG, and acd11-1 NahG plants were acquired by hybridizing total mRNA, isolated before and 72 h after BTH treatment, to Affymetrix ATH1 GeneChip arrays. laz2-1 exhibited dramatic suppression of the top 500 most significantly regulated genes in acd11-1 after 72 h BTH (Fig. S2A) In addition, a strong negative Pearson correlation of −0.87 was obtained for global expression fold change between laz2-1 acd11-1 and acd11-1, indicating that gene expression in acd11-1 was strongly affected by the laz2-1 mutation (Fig. S2B).

The LAZ2 locus was identified using a map-based approach. Briefly, Ler laz2-1 acd11 NahG was crossed to Columbia ecotype (Col-0) acd11 NahG to generate a segregating F2 mapping population after BTH treatment. Ecotype-specific linkage markers were used to map laz2-1 to a ~150 kb region at the bottom of chromosome 1 (Fig. S3). Candidate genes were selected and sequenced based on annotated mutant phenotypes at The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org), revealing an irradiation-induced 28-bp deletion in the third exon of the gene At1g177300 (Fig. 2A). This locus was also sequenced in laz2-2 acd11-1 NahG, revealing an EMS-induced G to A transition converting tryptophan 1536 to a premature stop.

**LAZ2 encodes the histone methyltransferase SDG8**

Sequence analysis revealed that LAZ2 encodes the histone lysine methyltransferase (HKMT) SET (Su(var)3-9, E(z) and Trithorax-conserved) DOMAIN GROUP 8 (SDG8), otherwise known as EARLY FLOWERING IN SHORT DAYS (EFS) and CAROTENOID CHLOROPLAST REGULATORY 1 (CCR1) [21,22]. The mutation in laz2-1 causes a frame-shift just upstream of the sequence encoding the conserved SET associated cysteine-rich domains, while that in laz2-2 introduces a stop codon upstream of a motif conserved within the RPBI subunits of RNA polymerase II [23]. SDG8 is homologous to yeast SET2, which is associated with methylations at histone 3 lysine 36 (H3K36). Another yeast HKMT, SET1, modifies H3K4. Both H3K4 and H3K36 methylations marks are typically associated with active transcription [24]. While *Arabidopsis* has 43 annotated SDG proteins, SDG8 groups with H3K36-specific HKMTs in fungi and animals along with 4 other *Arabidopsis* proteins [25]. During transcription in
yeast, SET1 and SET2 are recruited to active chromatin by the RNA polymerase II-associated PAF1 complex, where they promote gene expression by facilitating chromatin opening, thus enhancing transcription initiation and elongation, respectively [26]. A similar mechanism seems to be conserved in Arabidopsis based on studies of sdg mutants. SDG8 was first identified as a gene that controlled flowering time via its activity on the transcription of the key floral repressor FLOWERING LOCUS C (FLC), an epigenetically regulated MADS box transcription factor (TF) [27,28]. Expression of the FLC paralog MADS AFFECTING FLOWERING 1 (MAF1) is also dependent on SDG8, which is required for di- and trimethylation of H3K36 [25]. In addition to flowering time, SDG8 regulates carotenoid composition and shoot branching via modification of chromatin at specific loci [22,29]. Our microarray expression analysis revealed that MAF1 and CRTISO, both recently confirmed as direct targets of SDG8 [22,25], exhibited very low expression levels in the absence of LAZ2 (Fig. S4). Deficient expression of these and similar genes likely contributes to the developmental phenotypes observed in laz2. Furthermore, the loss-of-function mutant sdb2-2 (SALK_026642) shared laz2 morphology (Fig. S1B) and suppressed adl-1-2, an ACD11 knockout in the Col-0 ecotype (Fig. 2B).

Cell death in adl-1 is dependent on the R gene LAZARUS 5

Transcriptome analysis of genes normally induced in adl-1 NahG after BTH treatment showed that one of the most affected genes in laz2-1 was At5g44870, annotated as an NB-LRR R gene (Fig. 3A). This agrees with data from a previous study showing that At5g44870 is severely down-regulated in ccr1-1 (sdb2) leaf tissues [22]. A number of adl-1 suppressors found in the same screen as laz2 were dominant. One of these, laz5 Dominant 1 (laz5-D1), was mapped to a region close to this R gene (Fig. S5). Sequencing of At5g44870 in laz5-D1 revealed a G to A transition at the splice donor site (+1 position) of intron 4 likely resulting in deletion of exon 5 (Fig. 3B). To confirm that this mutation resulted in suppression of adl-1, two allelic dominant suppressors, laz5-D2 and laz5-D3, were sequenced: both had lesions in At5g44870 (below), hereafter referred to as LAZ5.

LAZ5 encodes a TIR-class NB-LRR of unknown pathogen specificity with sequence similarity to Pseudomonas syringae expressing the effector AvrRPS4 [30]. The DEB-induced laz5-D2 mutation is a T to A transversion changing isoleucine 287 to asparagine (I287N). This mutation is within the P-loop motif of the NB domain essential for coordination of bound nucleoside triphosphates [5]. The EMS-induced point mutation in laz5-D3 (G811E) lies in the LRR domain, which has been implicated in R protein activation [31]. Accelerated cell death in adl-1-1 was suppressed by laz5-D1 and laz5-D2 (Fig. 3C), and laz5-D alleles suppressed adl-1 cell death irrespective of BTH induction or the presence of NahG (Fig. 3D). Furthermore, over-expression of laz5-D2 or laz5-D3 (35S:laz5-D2 or 3) suppressed adl-1 death after induction, confirming that dominant negative mutations in LAZ5 are responsible for suppression of the adl-1-dependent autoimmune response (Fig. S7).

Transgenic plants over-expressing R-genes can exhibit spontaneous cell death and/or constitutive defense responses [32]. In agreement with these observations and the phenotype associated with deletion of ACD11, over-expression of wild-type LAZ5...
(35S:LAZ5) in the Col-0 background resulted in 30 out of 38 transgenic plants exhibiting acd11-like cell death which did not survive to set seed (Fig. S8). Since LAZ5 transcription is likely dependent on SDG8 HKMT activity, and the suppression of acd11 by laz2/sdg8 is recessive, we predicted that a loss-of-function mutation in LAZ5 would suppress acd11 in a recessive manner. As expected, a null T-DNA insertion mutant of At5g44870 (SALK_087262; here termed laz5-1) suppressed acd11-2 cell death recessively in plants without NahG (Fig. 4A). A second T-DNA insertion mutant allele of LAZ5 (SAIL_874-D10) also suppressed cell death in acd11-2 (data not shown). Expression of LAZ5 was assayed by real-time PCR in wild-type, laz5-1, and sdg8-2 plants 24 hours after syringe inoculation with the virulent bacterial pathogen Pseudomonas syringae tomato (P.s.t.) DC3000 or with 10 mM MgCl2 (mock control). While pathogen treatment induced LAZ5 expression in wild type, transcript levels in sdg8-2 were comparable to that in the laz5-1 null mutant (Fig. S9A). This confirms the microarray expression data shown in Fig. 3A. The apparent lack of LAZ5 expression in sdg8-2 was seen in several independent experiments with plants at different stages and/or treated with other pathogen strains (data not shown). Moreover, ACD11 expression was unaffected in laz5-1 and sdg8-2 (Fig. S9B), and transcript accumulation of several TIR-NB-LRR-encoding genes homologous to LAZ5 was seemingly unaffected in 3-week old sdg8-2 plants compared to wild-type control with the possible exception of At5g43230 (Fig. S10).

An important question is whether LAZ5 is the relevant target of SDG8 required for acd11 cell death. To help answer this question, we transformed laz2-1 acd11-1 NahG plants with a genomic construct of LAZ5 under control of a constitutive promoter and monitored cell death by ion leakage after BTH treatment compared to relevant controls (Fig. S11). LAZ5 over-expression restored cell death in leaf discs between 3 and 8 days after induction, indicating that lack of LAZ5 expression in sdg8 is a major cause of the suppression of acd11 cell death. However, it cannot be excluded that other targets of SDG8 histone methyltransferase activity also contribute to BTH-induced cell death in acd11.

SDG8 directly modifies chromatin at the LAZ5 locus

To test whether laz2 directly affects histone methylation at the LAZ5 locus, chromatin immunoprecipitation (ChIP) was conducted using antibodies against specifically modified histones. In laz2-1 acd11-1 NahG, trimethylated (me3) H3K36 levels were reduced in chromatin associated with the 5' coding regions of MAF1 (control) and LAZ5, when compared to the acd11-1 NahG control (Fig. 4B). Enrichment of H3K36me3 in LAZ5 chromatin was not influenced by BTH treatment or acd11 homozygosity (Fig. S12A). This suggests that activation of cell death in acd11 does not result in hyper-trimethylation at H3K36, but rather that this histone modification is required for proper LAZ5 expression. There was no effect of genotype on levels of total H3 (Fig. 4C). H3K36me3 is not a general mark for genes up-regulated in acd11, since we found no enrichment at FMO1 chromatin 72 h after BTH induction (Fig. S12B, C). Moreover, absence of LAZ2/SDG8 had no effect on H3K36me3 levels at the constitutively expressed ACTIN locus (Fig. 4C) or the MAP KINASE KINASE 4 (MKK4) locus (Fig. S12D).

To elucidate H3K36 methylation status irrespective of acd11 and NahG, we also conducted ChIP assays on sdg8-2 single mutant
and Col-0 wild-type seedlings. It was previously shown that loss of SDG8 resulted in both a decrease in global H3K36me3 levels and a coincident increase in global monomethylated (me1) H3K36, a mark associated with transcriptional repression in Arabidopsis [25].

In wild-type plants, MAF1 and LAZ5 chromatin was enriched for H3K36me3, whereas the level of H3K36me3 was diminished in sdg8-2 (Fig. 4D). Conversely, H3K36me1 levels at these loci were higher in sdg8-2 and reduced in wild type. Treatment of seedlings for 3 hours with an HR-inducing bacterial pathogen had no effect on the methylation status of H3K36 (data not shown). Also, H3 trimethylation of LAZ5 chromatin at other lysine residues (K4, K9, K27), was not affected by loss of SDG8 (Fig. S12E).

**Figure 3.** *acd11* autoimmunity requires LAZ5, a TIR-NB-LRR immune receptor. A, Expression of *At5g44870* (LAZ5) in Ler WT, *acd11-1* NahG and *laz2-1* *acd11-1* NahG before and 72 h after treatment with 100 μM BTH relative to Ler WT at time 0 (log2 scale). Microarrays were performed on duplicates or triplicates of independent RNA preparations from aerial parts of 4-week-old plants before and 72 h after BTH treatment. LAZ5 is significantly differentially expressed between the genotypes (p = 7e-7) and over time (p = 0.005) as determined by Two-Way ANOVA. B, LAZ5 with locations of mutations in 3 *laz5-D* alleles and the 2 T-DNA insertions *laz5-1* (SALK_087262) and *laz5-2* (SAIL_B74-D10). Boxes and lines represent exons and introns. Domains encoded by exons are marked TIR (A), NB (B), LRR (C), C-terminal extension (D). Asterisk marks the region amplified for ChIP and short arrows represent flanking primers. 500 bp = base pairs. C, Ion leakage death assay of leaf discs from 5-week-old Ler WT, *acd11-1* NahG, *laz5-D1* *acd11-1* NahG, and *laz5-D2* *acd11-1* NahG after treatment with 100 μM BTH. Means ± s.d. were calculated from 4 discs per treatment with 4 replicates in an experiment. D, Ler WT, *acd11-1* and *laz5-D1* *acd11-1* plants 3 weeks after germination. doi:10.1371/journal.ppat.1001137.g003
**SDG8 is required for pathogen resistance in Arabidopsis**

To determine whether SDG8 and/or LAZ5 are required for basal resistance to virulent pathogens, leaves of 4-week-old *sdg8-2*, *laz5-1*, wild-type and an allele of enhanced disease susceptibility 1 (*eds1-2* introgressed into Col-0) mutants were syringe-inoculated with *P.s.t.* DC3000 and growth was assayed after 4 days. Bacteria grew to ∼9-fold higher titers in *sdg8-2* than in wild-type or *laz5-1*, while titers in *eds1* were yet another order of magnitude higher (Fig. 5A). Growth of another strain of bacterial pathogen, *Pseudomonas syringae maculicola* (*P.s.m.* ES4326, tested on *sdg8-2*, *laz5-1*, wild-type and *eds1* with similar results (Fig. 5B). We did not observe elevated bacterial growth in *sdg8-2* when we used *P.s.t.* DC3000 expressing *AvrRpt2* (Fig. S13A), a non-pathogenic mutant defective in delivery of effectors to host cells [33]. These data indicate that SDG8, but not LAZ5, is required for full resistance to virulent pathogens. Furthermore, we found that SDG8 is involved in resistance to avirulent pathogens mediated by other R proteins, for example RPM1. Plants were syringe-inoculated with *P.s.t.* DC3000 expressing HR-inducing *AvrRpm1*, *AvrRpt2*, *AvrRps4* or *AvrPphB* and growth was assayed after 3 or 4 days. Bacterial titers were ∼15-fold higher in *sdg8-2* than in wild-type or *laz5-1* for *P.s.t.* expressing *AvrRpm1* (Fig. 5C). This suggested that *RPM1*-mediated resistance is defective in *sdg8-2*. To confirm this, growth of *P.s.m.* ES4326 expressing *AvrB* was assayed after 3 days: *AvrB* is also recognized by RPM1, and resistance to this avirulent pathogen was affected in *sdg8-2* to a similar level as *P.s.t.* with *AvrRpm1* (Fig. 5D). In both cases, bacterial titers were comparable to the *rpm1-3* null mutant [34]. Defects in SDG8 had a consistent, yet statistically insignificant effect on growth of *P.s.t.* DC3000 expressing *AvrPphB* (Fig. S13B) resistance to which is dependent on the *R* gene *RPS5* [35]. In addition, *sdg8-2* did not affect RPS2- or RPS4-mediated resistance to *AvrRpt2* [36,37] (Fig. 5E) and *AvrRps4* [30] (Fig. 5F). Corroborating the pathogen growth assay, transcript levels of *RPM1* and *RPS5* were low or absent in 4-week-old *sdg8-2* compared to wild-type, whereas expression of *RPS2* and *RPS4* in *sdg8-2* was similar to that in wild-type (Fig. 5G and S13C). Defects in *LAZ2* did not have a detectable effect on transcript accumulation of *RPM1*, *RPS5*, *RPS2* or *RPS4* (data not shown). As with *LAZ2*, we conducted ChiP assays at the *RPM1* locus in untreated seedling tissue from *laz2-1 acd11-1 NahG* versus *acd11-1 NahG* (in Ler) and *sdg8-2* versus wild-type (in Col-0). We observed lower H3K36me3 and higher H3K36me1 levels at *RPM1* chromatin in the absence of functional *LAZ2*/SDG8, indicating that *RPM1* is an example of another *R* gene that is regulated by histone methylation (Fig. S14). These results indicate that SDG8 targets a subset of *R* genes and other genes involved in more general aspects of basal defense.
Figure 5. SDG8 is required for plant innate immunity. A, Growth of virulent *P.s.t.* DC3000 in Col-0 WT, *sdg8-2, laz5-1* and *eds1* 0 and 4 days after infiltration with bacteria at OD$_{600} = 0.0001$. B, Growth of virulent *P.s.m.* ES4326 in 3-week old Col-0 WT, *sdg8-2, laz5-1* and *eds1* plants 0 and 4 days after infiltration with bacteria at OD$_{600} = 0.0001$. C, Growth of avirulent *P.s.t.* DC3000 expressing *AvrRp1* in Col-0 WT, *sdg8-2, laz5-1* and rpm1-3 plants 0 and 3 days after infiltration with bacteria at OD$_{600} = 0.001$. D, Growth of avirulent *P.s.m.* ES4326 expressing *AvrB* in Col-0 WT, *sdg8-2, laz5-1* and rpm1-3 plants 0 and 3 days after infiltration with bacteria at OD$_{600} = 0.001$. E, Growth of avirulent *P.s.t.* DC3000 expressing *AvrRpt2* in Col-0 WT, *sdg8-2, laz5-1* and *ndr1* 0 and 3 days after infiltration with bacteria at OD$_{600} = 0.001$. F, Growth of avirulent *P.s.t.* DC3000 expressing *AvrRps4* in Col-0 WT, *sdg8-2, laz5-1* and *eds1* plants 0 and 4 days after infiltration with bacteria at OD$_{600} = 0.00005$. Log-transformed values are means $\pm$ s.d. (n = 6). Asterisks indicate statistical significance (p < 0.0001) determined by unpaired Student’s t-test. The experiments were repeated twice with similar results. cfu = colony forming units. G, Transcript accumulation of *RPM1, RPS2*, and *RPS4* in 3-week-old Col-0 wild-type and *sdg8-2* plants, as determined by qRT-PCR. Data is normalized to *ACT1* (ACT1) and presented as relative expression compared to Col-0 = 1.0, mean $\pm$ s.d. (n = 3).

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Discussion

Chromatin remodeling has emerged as a complex regulator of transcription and an epigenetic mechanism to maintain lasting changes in gene activity states. Dynamic post-translational modifications of various residues of histones tails, including methylation, phosphorylation, acetylation, and ubiquitination, play important roles in both promoting and repressing gene expression by recruiting histone binding proteins and chromatin remodeling enzymes [38]. The combinatorial nature of histone modifications results in a complex “histone code” that adds an important level of control to fine-tune gene-specific responses to broader transcriptional inputs [39]. Changes in chromatin state may therefore modulate gene expression in a context-dependent manner to maintain a flexible response to pathogen attack. In plants, this process has been proposed as a mechanism for priming SA-responsive loci during systemic acquired resistance to pathogens [40].

So far, relatively few studies directly address epigenetic processes related to chromatin modification to plant innate immunity and/or PCD. Defects in *HISTONE DEACETYLASE 19* (HDA19) and *HISTONE MONOUBIQUITINATION 1* (HUB1) increase susceptibility to necrotrophic fungal pathogens in *Arabidopsis* [41,42]. Furthermore, defects in genes involved in histone variant replacement, and the variant H2AZ itself, result in increased resistance to virulent bacterial pathogens, some spontaneous cell death, and up-regulation of defense genes [43]. More commonly, the “memory” of chromatin remodeling activity is observed as increased levels of open chromatin marks (H3Ac, H3K4me2, etc) at the promoters of many SA-responsive genes, such as *PATHOGENESIS-RELATED 1* (PR-1) and WRKY TFs [40,44,45]. The clearest example of immune response at the level of chromatin comes from Alvarez-Venegas and colleagues, who showed that the HKMT ARABI-DOPSIS TRITHORAX 1 (ATX1, also known as SDG27) controls expression of WRKY70, a TF involved in pathogen response [46]. ATX1-dependent H3K4me3 signatures at the promoter of WRKY70, a TF involved in pathogen response [46]. ATX1-dependent H3K4me3 signatures at the promoter of WRKY70 correlated with WRKY70 transcriptional up-regulation. Intriguingly, although ATX1 regulates expression of a large set of genes, a high proportion of immunity-related genes exhibited reduced expression in the knockout mutant, including various TIR-NB-LRR R genes [47]. Numerous examples exist of microbes and viruses manipulating host chromatin remodeling machinery or histones directly in animals [48,49]. Strikingly, toxins from unrelated bacterial pathogens of animals have evolved to modify host histones, reducing transcriptional activity of key immunity genes [50]. The only clear instance of related phenomena identified among plant pathogens is the case of the Crown Gall disease-causing bacterium *Agrobacterium tumefaciens* which selectively modulates the expression of host variant histone genes to allow genomic integration of its T-DNA [51,52].

There is conflicting data on whether loss of *sdg8* influences H3K4 methylation, H3K36 methylation, or both [22,23,25,28]. We detected a dramatic effect of *laz2*/*sdg8* on H3K36 methylation status of chromatin at various loci and no difference in H3K4me3 levels at *LAZ5*, although the H3K4 methylation status of chromatin at other loci in *laz2* backgrounds remains to be investigated. In addition, our data suggest that monomethylation of H3K36 at *MAFI* and *LAZ5* chromatin relies on HKMTs other than SDG8. One of these, SDG26, was previously shown to act antagonistically to SDG8 by repressing FLC expression, although global H3K36me1 levels were unaffected in the *sdg26* mutant [25]. The significance of H3K36me1 enrichment in *sdg8-2* remains unknown. One hypothesis is that H3K36 methylation proceeds in a stepwise fashion, with the accumulation of H3K36me1 (due to activity of an unknown HKMT) being a consequence of a block in further di- and trimethylation at this residue normally mediated by SDG8. Alternatively, monomethylation of H3K36 may represent a transcriptionally repressive mark that accumulates only in the absence of di- and trimethylation due to disruption of the balance between antagonistic chromatin modifiers. For example, the SET-domain containing *Arabidopsis* proteins TRITHORAX-RELATED-ED PROTEIN 5 (*ATRX5*, also known as SDG15) and ATX6/R- SDG34 are H3K27-specific monomethyltransferases essential for transcriptional repression in heterochromatin [53]. Further studies should examine if other predicted H3K36-specific HKMTs, namely SDG4, SDG7, SDG24 and SDG26, have any role in H3K36 monomethylation, trimethylation and/or antagonistic control of expression of *LAZ5* and other genes with roles in immunity or are required for cell death in *add1*. Moreover, further work is required to determine the mechanisms by which SDG8-dependent changes in H3 methylation regulates the expression of specific genes.

A clue to the function of LAZ5 activation comes from the isolation in our screen of dominant alleles. This indicates that the mutant form (laz5-D) of the R protein likely interferes with activity of the wild-type copy since plants heterozygous for the laz5 null mutation do not suppress *add1*, indicating haploinsufficiency of *LAZ5*. Dominant negative activity has been described for mutations in the *R* gene *N* from tobacco, and indeed for a point mutation (G216E) in the P-loop motif of N [54]. N was later found to oligomerize in the presence of a Tobacco mosaic virus elicitor, likely through interaction of TIR domains [55]. This oligomerization was an early event in pathogen perception and was independent of mutations that have an effect on HR induction. Therefore, it is possible that laz5-D mutants form inactive oligomers with wild-type LAZ5 and/or accessory proteins. An example of this scenario from animal innate immunity comes from NOD2, an NLR involved in recognition of bacterial cell wall components: an endogenously truncated form, NOD2-S, interacts with full-length NOD2 to potentiate signaling [56]. In plants, there are examples of truncated R proteins, generated by alternative splicing, playing a key role in signaling [57,58]. At present, it is an open question whether LAZ5 oligomerizes and how this relates to cell death activation. It should be noted that, while all the laz5 alleles isolated thus far in the *add1* suppressor screen were dominant negative, only 43 of the ~200 unknown recessive
mutants were placed into complementation groups, and even fewer were mapped. Therefore, a recessive lac5 knockout allele may exist among our unmapped suppressors.

In this study we have identified the chromatin modifying enzyme SDG8, and its specific target LAZ5, as regulators of autoimmune cell death in acl11. Furthermore, sdg8 mutants exhibit enhanced susceptibility to virulent and avirulent pathogens, whereas lac5 mutants do not, suggesting that other targets of SDG8 are important for general resistance. We also show that transcription of a subset of R genes, including LAZ5 and RPM1, is likely to be directly or indirectly dependent on LAZ2 activity. One scenario that may account for the enhanced susceptibility of sdg8 mutants to virulent pathogens could be the consequence of SDG8 action on multiple NB-LRR loci. If so, the effectors delivered by *Pseudomonas* triggers a weak R gene response, in sdg8 a subset of these do not accumulate and thus are no longer available to signal for defense against the invading pathogen. Intriguingly, SDG8 is not expressed until 8 days after germination [23], a stage preceding the initiation of cell death in acl11. SDG8 may therefore developmentally regulate targets such as LAZ5 and may exemplify a key difference in the programmed defenses required during seed maturation and the inducible defenses used during plant growth.

Lesion mimic mutants such as acl11 are useful tools in the genetic dissection of innate immunity in plants [10]. Whereas several of these mutants have putative roles in ceramic signaling or synthesis [59,60] or auto-activate R proteins [11], the majority of lesion mimic mutants represent proteins with no straightforward connection to PCD. Milder autoimmune, associated with constitutive activation of defense responses and dwarf morphology without coincident HR, can similarly be the result of point mutations in immune receptors (Zhang et al., 2005), or deletion of signaling intermediates such as MAP kinases [61]. Knockout mutants that eliminate host guardees mimic the effects of pathogen effectors, and have been found to exhibit R-gene-dependent lethality [62]. Therefore, it is possible that many lesion mimic/autoimmune mutants may correspond to gene functions that are guarded by NB-LRRs. If so, the diverse functions of these genes may be “red herrings” not directly related to PCD but only implicated in this process due to their targeting by pathogen effectors. Such may be the case for acl11, although we have been unable to detect any interaction between full-length or truncated LAZ5 and ACD11 in yeast or in planta (data not shown). Previously, we reported the identification of ACD11-interacting proteins [63], which we are testing for interaction with LAZ5. Two predictions about wild-type products of autoimmune mutants emerge from this model. First, suppressor screens should identify R genes. Second, pathogen effectors should target them either directly or indirectly via interacting partners or products of their activities. We currently have no evidence that ACD11 is targeted by pathogenic effectors, or that ACD11 contributes to disease resistance in the absence of LAZ5. While future work may strengthen this hypothesis, an alternative model is that ACD11 is involved in negatively regulating SA-dependent expression of LAZ5 (or a subset of R genes) perhaps via some lipid signal.

Materials and Methods

**Plant material and growth conditions**

*A. thaliana* plants were grown on soil or MS-agar plates at 21°C with an 8 h or 12 h photoperiod. *sdg8-2* (*SALK_026642*) and *lac5-1* (*SALK_008262*) T-DNA insertion lines, both previously described as null mutants [23,64], were generated by SIGnAL [65] and obtained from the Nottingham Arabidopsis Stock Centre (NASC; Nottingham, UK). Homozygous genotyping primers were 5'-TAAAGAGGTTCTGATCATAGG-3' with 5'-CAGTGTCCAGTAAAGCTGTC-3' for *sdg8-2* and 5'-TATGTTTTCCTCCAGTGGCCAG-3' with 5'-ATCATGCTCTCAAATGGAG-3' for *lac5-1*. Sequences of primers used to detect acl11-1, acl11-2, and NahG are available upon request.

**Suppressor screen**

Three lots of 920–950 mg Ler *acl11-1 NahG* seeds were incubated for 4 h in either 0.74% (w/v) EMS (Sigma-Aldrich, St Louis, MO, USA) prepared in 0.1M sodium phosphate buffer, pH 5, with 5% DMSO, or 10 mM DEB (Sigma-Aldrich) in water, followed by rinsing. γ-irradiation of 300 mg *acl11-1 NahG* seeds was performed at the Risø Reference Laboratory (Denmark) with 500 Gy from a Cobalt-60 source. M1 plants were grown in families of 125 individuals, 3500 M2 plants per family were screened for BTH-resistant suppressors. ~3 million M2 plants from 845 M1 pools or ~100,000 M1 plants were scored. Putative mutants were genotyped to be homozygous for acl11-1 by PCR.

**Ion leakage assay**

Conductivity assays were conducted essentially as previously described [66].

**Microarray hybridization**

Total RNA was isolated from three independent biological replicates of relevant genotypes at 0 and 72 hr after BTH treatment. RNA was labeled and amplified according to the MessageAmp Biotin-enhanced kit (Ambion) protocol and hybridized to 51 ATH1 GeneChips after Affymetrix protocols.

**Chromatin immunoprecipitation and real-time PCR**

ChIP antibodies purchased from Abcam (Cambridge, UK) included anti-H3 (ab1791), anti-H3K36me1 (ab9048), anti-H3K36me3 (ab9050) and anti-H3K27me3 (ab6002). ChIP antibodies against H3K4me3 (pAb-056-050) and H3K9me3 (pAb-003-050) were purchased from Diagenode (Liège, Belgium). Quantitative PCR primers for ChIP analysis were LAZ5 5'-GAGTCGTGGCAGTTGTTCAT-3' with 5'-GAAGATGGACAGTGGAGATTCAC-3' and 5'-CTATATTCTGAGCATAAGGAA-3' with 5'-CCCTATCGGAGATTGGAACG-3', MAff: 5'-CCCTATCGGAGATTGGAACG-3' with 5'-GAAGATGGACAGTGGAGATTCAC-3'. Conductivity assays were conducted essentially as previously described [67], using 1 μg of each antibody. Real-time PCR to quantify the immunoprecipitated DNA was performed using Brilliant II SYBR Green qPCR kit (Stratagene), and reactions were run on an iCycler IQ (Bio-Rad, Hercules, CA, USA). In all cases, ChIP values were calculated using the Delta-Delta Ct (ddCt) algorithm to determine relative gene expression utilizing the ‘percent input method’. Briefly, signals obtained from the ChIP were divided by signals obtained from an input sample representing the amount of chromatin used in the ChIP. The ‘% input’ value shows what proportion of this starting material is found in the eluate after IP with appropriate Ab.

For expression analyses, RNA was extracted from relevant genotypes using the Qiagen RNasy RNA extraction kit followed by DNase treatment as per the manufacturer’s instructions. Equal amounts of RNA were subjected to one-step real-time PCR using the same kit as described for ChIP except with reverse transcriptase included. For all sample/primer combinations, a control without reverse transcriptase was included to exclude genomic DNA contamination.
Cloning and generation of transgenic plants

3.9-kb fragments of laz2-1 D alleles were amplified from genomic DNA (laz5-D1 acl11-2 NahG, laz5-D2 acl11-2 NahG, laz5-D3 acl11-2 NahG) and cloned into modified pCAMBIA-3300 as described [68], using a uracil-excision based cloning technique (USER, New England Biolabs). Cloning primers were 5’-gtgttaaATGCGG-GCATCTTCGGAAATAC-3’ and 5’-gtgttaaatttaaatTTACAAATAAA-CCCAAATGATAATTTAG-3’. A 3.9-kb fragment of LAZ2 was amplified from genomic DNA (wild type Ler), cloned into pENTR/D-TOPO (Invitrogen) and transferred to Gateway-compatible constitutive expression vectors pGWB502 or pGWB521 [69] by LR recombination reaction (Invitrogen). Cloning primers used were 5’-CAGATTGCGACGTCTCCGAATAC-3’ and 5’-TTAGGCTTTAATGATAATTTAG-3’. The final constructs were verified by sequencing, electroporated into Agrobacterium tumefaciens strain GV3101 and used to transform acl11-1 NahG or wild type plants by floral dip method [70]. Transgenic plants were selected on soil with glufosinate (20mg/L) hygromycin B followed by transplanting to soil (35S:laz5-D alleles) or on MS-agar plate with (20mg/L) hygromycin B and 59 ganti-H3K36-me3 antibody, or (a) 35S:laz5-D2 or (b) 35S:laz5-D3, 10 d after treatment with 100 μM BTH. Found at: doi:10.1371/journal.ppat.1001137.s008 (2.43 MB TIF)

Accession numbers


Supporting Information

Figure S1 laz2 alleles and sdg8 share morphological phenotypes, such as early flowering. A. 16-day-old Ler acl11-1 NahG plants homozygous for 3 different laz2 alleles. B. 21-day-old Col-0 WT plants homozygous for sdg8-2. Found at: doi:10.1371/journal.ppat.1001137.s001 (3.77 MB TIF)

Figure S2 Transcriptome analysis of laz2-1 suppression of the BTH-induced response in acl11-1 A. The effect of laz2-1 on 355 significantly over-expressed genes among the top 500 differentially expressed genes in response to BTH treatment in acl11-1 NahG plants. B. Scatterplot of global expression fold change comparison between acl11-1 NahG versus NahG (y-axis) and laz2-1 acl11-1 NahG versus acl11-1 NahG (x-axis) 72 h after BTH induction. Found at: doi:10.1371/journal.ppat.1001137.s002 (0.41 MB TIF)

Figure S3 Ecotype-specific markers used to map the LAZ2 locus to ~120 kb on the bottom of chromosome 1. Left is centromere, right is telomere. Relative positions of markers are indicated, as are numbers of recombinants remaining at each marker position. Figure shows a rough (~1 megabase) and fine (~150 kb) map of the laz2-1 locus and detail of genomic region between final recombinants, with associated genes and BAC clones. A star marks the LAZ2 gene with the defect determined by sequencing. Found at: doi:10.1371/journal.ppat.1001137.s003 (0.19 MB TIF)

Figure S4 Expression of A) CRTISO (At1g06820) and B) MAF1 (At1g77070) in Ler WT, NahG, acl11-1 NahG and lac2-1 acl11-1 NahG before and 72 h after treatment with 100 μM BTH relative to WT at time point 0 (log2 scale). Found at: doi:10.1371/journal.ppat.1001137.s004 (0.12 MB TIF)

Figure S5 Ecotype-specific markers were used to map the LAZ5 locus to ~80 kb on the bottom of chromosome 5. Left is centromere, right is telomere. Relative positions of mapping markers and numbers of recombinants are indicated. Figure shows a map of the laz5-D1 locus and the genomic region between final recombinants, with associated genes. Asterisk marks the LAZ5 gene with the defect determined by sequencing. Found at: doi:10.1371/journal.ppat.1001137.s005 (0.24 MB TIF)

Figure S6 Alignment of LAZ5 and the five most similar Arabidopsis TIR-NB-LRR R proteins, as determined by The Functional and Comparative Genomics of Disease Resistance Gene Homologs Project [http://niblbrs.ucdavis.edu/TN_TNL_phylogeny.html]. Sequences include RPS4 (Atig54250), CSA1 (Atig17880), Atig36150, Atig45200, and Atig45230. Mutated residues in laz5-D2 and laz5-D3 are highlighted. Asterisks indicate amino acids predicted to be absent due to the splice site mutation in laz5-D1. Found at: doi:10.1371/journal.ppat.1001137.s006 (1.35 MB TIF)

Figure S7 Over-expression of dominant negative laz5-D alleles suppresses acl11. Figure shows acl11-2 NahG (in Col-0) control and representative transgenic lines of acl11-2 NahG stably transformed with A) 35S:laz5-D2 or B) 35S:laz5-D3, 10 d after treatment with 100 μM BTH. Found at: doi:10.1371/journal.ppat.1001137.s007 (1.70 MB TIF)

Figure S8 Over-expression of the wild-type LAZ5 R gene results in cell death. Figure shows Col-0 wild-type control and two representative transgenic lines of Col-0 stably transformed with a construct over-expressing genomic LAZ5 (35S:LAZ5). Found at: doi:10.1371/journal.ppat.1001137.s008 (2.43 MB TIF)

Figure S9 Expression of A) LAZ5 and B) ACD11 in 3-week-old Col-0 wild-type, laz5-1 and sdg8-2 mutant plants 24 h after infiltration with P. s.l. DC3000 at OD600 = 0.01 or 10mM MgCl2, mock control, as determined by qRT-PCR. Data is normalized to ACTIN1 (ACT1) and presented as relative expression (fold) compared to Col-0 mock = 1.0 (dashed line), mean ± s.d. (n = 3).

Figure S10 Transcript accumulation of LAZ5 homologs in 3-week-old Col-0 wild-type and sdg8-2 plants, as determined by qRT-PCR. Data is normalized to ACT1 and presented as relative expression compared to Col-0, mean ± s.d. (n = 3). Found at: doi:10.1371/journal.ppat.1001137.s010 (0.23 MB TIF)

Figure S11 Ion leakage cell death assay of leaf discs from 3-week-old WT, laz2-1 acl11-1 NahG, acl11-1 NahG and laz2-1 acl11-1 NahG over-expressing LAZ5 plants after BTH treatment. The former were selected segregating T2 plants from a transgenic line of genomic LAZ5 in expression vector pGWB521, and confirmed by qRT-PCR. Data is presented as fold change in conductivity (μS cm⁻¹) relative to initial value at Day 3. Means ± s.d. were calculated from 6 discs per treatment with 4 replicates within an experiment. Found at: doi:10.1371/journal.ppat.1001137.s011 (0.13 MB TIF)

Figure S12 A) H3K36me3 at LAZ5 chromatin is independent of acl11. ChiP analysis of LAZ5 with 1 μg anti-H3K36me3 antibody (IP) or no Ab (mock) expressed as % input. Tissue was from 3-week-old NahG, acl11-1 NahG and laz2-1 acl11-1 NahG seedlings (Ler background) before and 24 h after treatment with 100 μM BTH. B. H3K36me3 is not a general mark for genes up-regulated in acl11. Expression of FMO1 (Atig19259) in Ler WT, acl11-1 NahG and laz2-1 acl11-1 NahG before and 72 h after treatment with 100 μM BTH relative to wild-type at time point 0 (log2 scale). C. ChiP analysis of FMO1 with 1 μg anti-H3K36me3 antibody
Data is normalized to and 4 days after infiltration with bacteria at OD600 = 0.001. Log-
H3K36-me3 antibody or 1 expression compared to Col-0 = 1.0, mean forming units.
were repeated once or twice with similar results. cfu = colony product (34 cycles).
AvrPphB expressing bacteria at OD600 = 0.001. mutant in Col-0 WT and
transposon affected by 
EtBR-stained PCR product (34 cycles).
Figure S13 A similar results.

Found from: doi:10.1371/journal.ppat.1001137.s013 (0.16 MB TIF)

**References**