Epigenetic variation in *Arabidopsis* disease resistance

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Plant pathogen resistance is mediated by a large repertoire of resistance (*R*) genes, which are often clustered in the genome and show a high degree of genetic variation. Here, we show that an *Arabidopsis thaliana R*-gene cluster is also subject to epigenetic variation. We describe a heritable but metastable epigenetic variant *bal* that overexpresses the *R*-like gene *At4g16890* from a gene cluster on Chromosome 4. The *bal* variant and *Arabidopsis* transgenics overexpressing the *At4g16890* gene are dwarfed and constitutively activate the salicylic acid (SA)-dependent defense response pathway. Overexpression of a related *R*-like gene also occurs in the *ssi1* (*suppressor of SA insensitivity 1*) background, suggesting that *ssi1* is mechanistically related to *bal*.

[*Key Words*: Epigenetics; cytosine methylation; resistance genes; *bal*; *DDM1*; *Arabidopsis*]

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Epigenetic variation is often overlooked as a source of phenotypic variation for natural or artificial selection. Variation in epigenetic information, encoded at the chromatin level rather than the nucleotide sequence level, is commonly thought to be transient and unlikely to underlie stable changes in phenotype. There is considerable evidence, however, that epigenetic changes, particularly those due to alterations in DNA methylation, can be inherited through meiosis and mimic traditional mutations.

Some of the earliest recognized heritable epigenetic alleles involve transposons whose differential DNA methylation and chromatin structure affect the expression of neighboring genes (McClintock 1951; Martienssen et al. 1990; Martienssen and Baron 1994; Fedoroff et al. 1995). Another classic example of epigenetic inheritance is paramutation, wherein interactions between alleles cause chromatin-mediated heritable changes in gene expression (Kermicle et al. 1995; Hollick et al. 1997; Walker 1998). Several groups have recently reported defects in flower development caused by heritable epigenetic alleles (epialleles) associated with abnormal DNA methylation. Hypermethylated epialleles of *AGAMOUS* (Jacobsen et al. 2000) and *SUPERMAN* (Jacobsen and Meyerowitz 1997; Jacobsen et al. 2000), which affect flower structure, and hypomethylated epialleles of *FWA* (Soppe et al. 2000), which delay flowering time, have been recovered from both mutagenized *Arabidopsis* populations and DNA hypomethylation lines such as *ddm1*, *met1*, and antisense-cytosine methyltransferase *MET1* (Finnegan et al. 1996; Ronemus et al. 1996). Plant epimutations associated with altered DNA methylation can also occur spontaneously. Heritable, differentially methylated *P1* alleles that condition altered kernel pigmentation were reported in maize (Das and Messing 1994). Bender and colleagues have shown that an endogenous inverted repeat can induce methylation and silencing of homologous gene sequences (*pai2*) at an unlinked site in the *Arabidopsis* genome (Bender and Fink 1995; Jeddeloh et al. 1998; Luff et al. 1999; Melquist et al. 1999). Recently, a naturally occurring floral variant of the plant *Linaria* was discovered to be caused by a hypermethylated, silenced *Lcyc* allele (Cubas et al. 1999). Fungi (Colot et al. 1996; Irelan and Selker 1997; Selker 1997) and animals (Michaud et al. 1994; Morgan et al. 1999) also show heritable epigenetic variation associated with differential DNA methylation.

We have been studying the effects of genomic hypomethylation using mutations in the *DDM1* (*DECREASE IN DNA METHYLATION 1*) gene of *Arabidopsis*. *ddm1* mutations lead to a reduction in cytosine methylation throughout the genome (Vongs et al. 1993). Repeated DNA loses methylation immediately in *ddm1* mutants, but low copy sequences gradually lose methylation as *ddm1* mutants are propagated through successive generations by self-pollination (Kakutani et al. 1996). The *DDM1* locus encodes a SWI2/SNF2-like protein that resembles chromatin remodeling engines in yeast, *Drosophila*, and humans (Jeddeloh et al. 1999).

ddm1 loss-of-function mutations lead to developmental abnormalities including late flowering, dwarfing, and altered floral structures (Kakutani et al. 1996; Jacobsen et al. 2000). Typically, these defects are only observed after self-pollinating *ddm1* mutants for several generations. *Arabidopsis* normally is a self-pollinator, but *ddm1* mu-

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tants display the inbreeding depression typical of many outcrossing species. The *ddm1*-induced developmental defects are caused by stable alterations at sites unlinked to the *ddm1* mutation (Kakutani et al. 1996). These alterations behave like classical Mendelian mutations and are stable when segregated from *ddm1*. Some of these heritable alterations are caused by insertion of transposons, which are activated in *ddm1* mutants (Miura et al. 2001; Singer et al. 2001). However, *ddm1* also causes inherited epigenetic changes, including hypermethylated *superman* (*sup*) and *agamous* (*ag*) epialleles and hypomethylated *fwa* epialleles (Jacobsen et al. 2000; Soppe et al. 2000).

Here, we report the molecular mechanism underlying a *ddm1*-induced pleiotropic defect *bal* that connects epigenetic regulation and plant pathogen defense responses. Several important molecular components involved in plant pathogen signaling have been identified, including plant resistance (*R*) genes, salicylic acid (SA), and pathogenesis-related (*PR*) proteins (Hammond-Kosack and Jones 1996; Glazebrook 1999). *R* genes encode proteins that recognize, either directly or indirectly, specific signals or gene products produced by pathogens, leading to the activation of defense signal transduction pathways (Ellis et al. 2000). The most common class of *R*-gene products consists of both a nucleotide-binding site (NBS) and multiple leucine-rich repeat (LRR) motifs (Hammond-Kosack and Jones 1997). There are ~165 NBS-LRR (nucleotide binding site–leucine-rich repeat) class *R*-like genes in the *Arabidopsis* genome (for details, see http://www.niblrrs.ucdavis.edu). One of the major induced pathways is dependent on salicylic acid (SA), a small aromatic compound related to aspirin (Durner et al. 1997). SA-dependent signaling induces *PR* gene expression, which is correlated with elevated resistance (Uknes et al. 1992; Maleck et al. 2000). Several modifiers of SA signaling have been identified by mutations causing either constitutive expression of *PR* genes (e.g., *cpr* [Bowling et al. 1994], *ssi* [Shah et al. 1999]) or nonexpression of *PR* genes (*npr1*; Cao et al. 1994, 1997; Ryals et al. 1997).

Our results indicate that *bal* is an epigenetic alteration mapping to a cluster of NBS-LRR-class disease-resistance genes. In the *bal* variant, overexpression of one gene in the cluster stimulates the disease response pathway and causes dwarfing and elevated disease resistance. Overexpression of a related NBS-LRR gene occurs in *ssi1* (*suppressor of SA insensitivity 1*) plants, which constitutively express *PR* genes (Shah et al. 1999). The overexpression phenotype and other genetic parallels suggest that the *ssi1* mutant is mechanistically related to the *bal* variant.

Results

bal *is a heritable* ddm1*-induced defect*

Inbred *Arabidopsis* lines deficient in DNA methylation accumulate a spectrum of phenotypes, including late flowering, floral structure defects, and dwarfing (Kakutani et al. 1996; Jacobsen et al. 2000). To understand the molecular basis of such developmental abnormalities, we focused on a dwarfing variant (*bal*) generated in an inbred *ddm1* hypomethylation mutant line (strain Columbia). The *bal* phenotype is characterized by twisted leaves, dwarfed stature, and reduced fecundity (Fig. 1; Kakutani et al. 1996). We crossed a phenotypic *bal ddm1* plant to wild-type Columbia and Landsberg erecta plants and generated segregating F_2 populations. In these F_2 families, the *bal* phenotype segregated as a single Mendelian trait independent of the *ddm1* mutation (Kakutani et al. 1996). The Columbia *bal* defect behaved as a semidominant allele relative to the wild-type Columbia *BAL* allele (Fig. 1A), but acted in a recessive manner relative to the wild-type Landsberg erecta *BAL* allele (Kakutani et al. 1996).

We followed the stability of the *bal* defect in four independent *bal DDM1* lines propagated through five generations of self-pollination, and we observed no phenotypic reversion when the plants were grown under normal conditions. We conducted all subsequent phenotypic and molecular characterization of the *bal* defect using *DDM1* wild-type backgrounds from Columbia and Landsberg erecta.

The bal *variant resembles constitutive pathogen defense mutants*

The morphology of the *bal* variant resembles *Arabidopsis* mutants that constitutively express pathogen defense

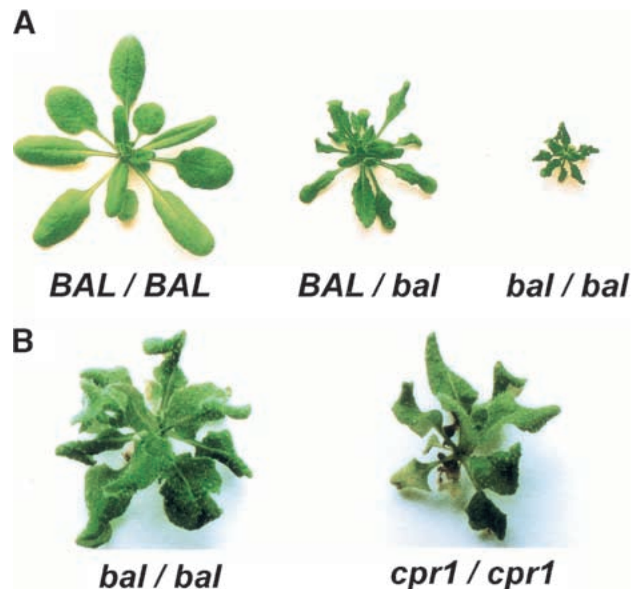

Figure 1. Phenotype of the semidominant *bal* defect. (*A*) Twenty-five-day-old individuals (Columbia strain, wild-type *DDM1*) from a population segregating the *bal* alteration. Homozygous *bal*/*bal* individuals show severe dwarfing and twisted leaves. Heterozygous individuals are intermediate in size and leaf twisting. (*B*) A higher-magnification view of *bal*/*bal* homozygotes compared with *cpr1-1*/*cpr1-1* homozygotes (Columbia strain, ~4 wk old).

genes (e.g., *cpr 1* [Bowling et al. 1994], *mpk4* [Petersen et al. 2000], and *ssi1* [Shah et al. 1999]). Figure 1B compares the morphology of *bal* homozygotes and plants homozygous for the *cpr1* mutation. *cpr1* was isolated from a chemical mutagenesis screen for plants that constitutively express *PR* genes (Bowling et al. 1994). The morphological similarities prompted us to determine whether the *bal* variant also constitutively expresses *PR* genes in the absence of pathogen. Figure 2A shows that *PR2* transcripts are expressed in uninfected *bal* and *cpr1* homozygotes, but are absent in wild-type siblings. *PR1* and *PR5* transcripts were also expressed in uninfected *bal* (Fig. 2B) and *cpr1* plants (data not shown.) *PR2* transcript is detectable in *BAL/bal* heterozygotes, but at significantly lower levels compared with *bal* homozygotes (Fig. 2B). Therefore, the *bal* variant is a constitutive expresser of *PR* genes.

Decreased pathogen growth and dwarf morphology in the bal *variant are dependent on salicylic acid signaling*

Because the *bal* variant constitutively expresses *PR* genes, we sought to determine if the *bal* alteration affects resistance to pathogens. We investigated the growth of the bacterial pathogen, *Pseudomonas syringae* (pv. tomato strain DC 3000), to which *Arabidopsis* strain Columbia is susceptible (Whalen et al. 1991). Figure 3A shows that this virulent *P. syringae* strain grows ~3 orders of magnitude on wild-type Columbia hosts by 4 d postinoculation. Bacterial growth on *bal* homozygotes is attenuated; bacterial populations in *bal* homozygous leaf tissue are reduced 10- to 100-fold (Fig. 3A; data not shown) relative to populations in wild-type leaves at 4 d postinoculation.

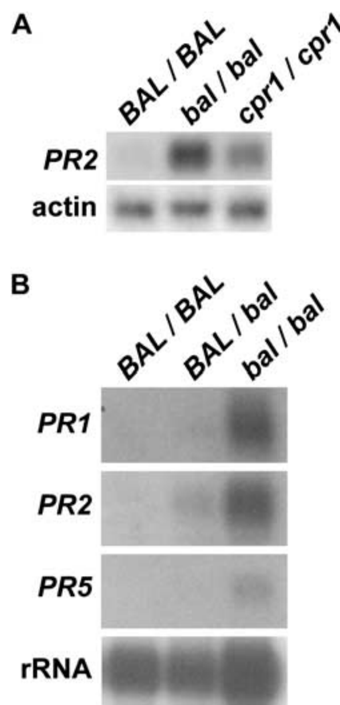

Figure 2. Constitutive expression of pathogenesis-related (*PR*) protein genes in the *bal* variant. (*A*) Northern blot analysis using RNA samples prepared from uninfected wild-type (*BAL/BAL*), *bal/bal*, and *cpr1-1/cpr1-1* plants (all in strain Columbia). The membrane was first hybridized with a *PR2* cDNA probe to show that the *bal* variant constitutively expresses *PR2*, as does the positive control *cpr1-1* mutant. The membrane was subsequently hybridized with an actin probe as a control for RNA loading. (*B*) Northern blot analysis examining expression of three *PR* genes in wild-type *BAL/BAL* plants, heterozygous *BAL/bal* and homozygous *bal/bal* siblings.

Many plant defense responses are mediated through salicylic acid (SA)-dependent signaling pathways. Therefore, we examined whether the morphological and pathogenesis defects shown by *bal* homozygotes could be suppressed by disrupting SA signaling. We crossed the *bal* variant (strain Columbia) to an *nahG* transgenic line (strain Landsberg erecta; Delaney et al. 1994) expressing a microbial enzyme that inactivates SA. *P. syringae* (DC 3000) grows to a higher density on *nahG* transgenic tissue relative to wild-type Columbia tissues (Fig. 3A) and Landsberg erecta (data not shown). Bacterial growth in *nahG bal* homozygous tissue is also significantly higher than growth in either *bal* or wild-type plants. As shown in Figure 3B, *nahG* also significantly suppresses *bal* morphological defects, although the *nahG bal* plants retain some characteristics of the *bal* variant, such as mildly twisted leaves and reduced stature. These findings indicate that the full phenotypic expression of the *bal* variant is dependent on SA signaling.

The bal *defect is metastable*

Attempts to induce suppressor/revertant mutations in a *bal DDM1* background resulted in the recovery of an unexpectedly high frequency of phenotypically normal plants or individuals with dramatically reduced *bal* phenotypes. We treated *bal DDM1* seeds (M1) with either the alkylating agent ethylmethylenesulfonate (EMS) or γ-irradiation (see Materials and Methods). We recovered >10% normal or phenotypically intermediate plants among the progeny (M2) of the plants grown from the EMS-treated seeds (Fig. 4, Table 1). In the γ-ray treated experiment, ~7% of the M2 plants showed a wild-type or weakened *bal* phenotype. The mock-treated M2 populations contained no phenotypic revertants. The observed phenotypic reversion of *bal* in both experiments is at least 20-fold greater than the expected recessive knockout frequency in a comparable *Arabidopsis* mutagenesis (~0.1% of the M2 population; Haughn and Somerville 1987). Five phenotypically normal M2 plants were outcrossed to wild-type Columbia plants, and no segregation of phenotypically *bal* plants was seen in F_2 generations (among a total of 2040 F_2 individuals examined). These results suggest that the restoration of the normal phenotype is caused by reversion of the *bal* allele rather than by the action of unlinked extragenic suppressors.

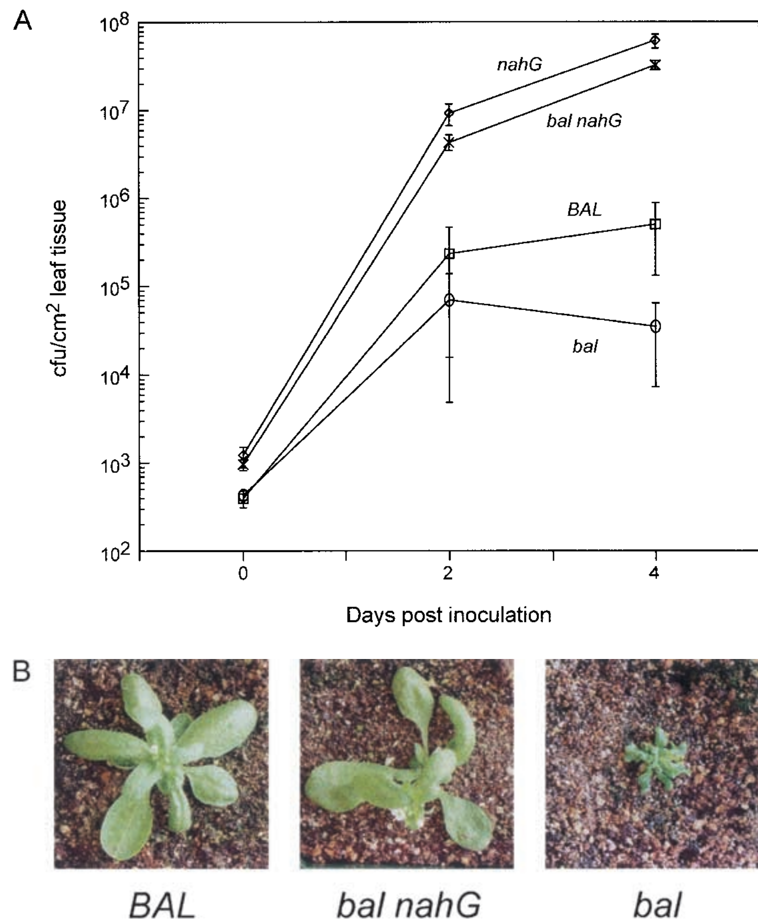

Figure 3. Pathogenesis and morphology phenotypes of the *bal* variant are salicylic acid (SA)-dependent. (*A*) Growth of *Pseudomonas syringae* pv. tomato strain DC3000 is reduced in *bal* homozygotes relative to wild-type *BAL* plants. *P. syringae* cultures (1×10^5 CFU/mL) were vacuum-infiltrated into whole plants at day 0. The concentration of bacteria in leaf samples was determined at 0, 2, and 4 d postinoculation. Each measurement, in colony-forming units per centimeter squared (CFU/cm^2) of leaf tissue, was done in triplicate; error bars on each data point indicate standard errors. Introduction of the *nahG* transgene suppresses the increased resistance phenotype of the *bal* defect (*nahG*, Landsberg erecta plants carrying a *nahG* transgene; *bal nahG*, homozygous individuals in a mixed Columbia and Landsberg erecta background). (*B*) The *nahG* transgene partially suppresses the *bal* morphological phenotypes. Representative plants are shown (*BAL*, wild-type Landsberg erecta; *bal nahG*, homozygous individual in a mixed Columbia Landsberg erecta background; *bal*, Columbia *bal* homozygote).

Characterization of the BAL *locus*

The high frequency of *bal* reversion is consistent with either hypermutable genetic mechanisms (e.g., transposition, DNA rearrangements) or metastable epigenetic alterations (e.g., chromatin-controlled gene expression states). To determine the basis of the *bal* variant, we first sought to molecularly identify the *BAL* locus. We mapped the *bal* alteration to a small genetic interval (<0.2 cM) on the lower arm of Chromosome 4 (see Fig. 5A). This genetic interval corresponds to a 152-kb window. The region has an ~4-fold increase in the ratio of physical to genetic distance, which in *Arabidopsis* normally averages ~200 kb/cM (Copenhaver et al. 1998). The observed recombination suppression is caused by strain-specific genomic rearrangements within the region (Parker et al. 1997; Noël et al. 1999).

A large portion of the physical window containing the *bal* alteration is composed of a 90-kb gene cluster that contains 10 genes or gene fragments encoding NBS-LRR-class disease-resistance proteins (Parker et al. 1997; Bevan et al. 1998). Jones and colleagues discovered that the functional *RPP5* (resistance to the downy mildew pathogen *Peronospora parasitica*) gene was embedded within this gene cluster in the Landsberg erecta strain (Parker et al. 1997). The Columbia version of the NBS-LRR gene cluster contains two apparently full-length, potentially functional disease-resistance genes. The eight remaining NBS-LRR genes are fragments, or are interrupted by mutations or retrotransposon insertions. The 152-kb window defined by our mapping contains 22 additional predicted genes (Bevan et al. 1998; http://mips.gsf.de).

Gene expression profile in the bal *variant*

We searched the 152-kb window for molecular changes correlated with the *bal* phenotype. We first screened for genomic alterations using Southern blots and hybridization probes tiling across the window. We found no DNA rearrangements in this comparison of *bal* and wild-type *BAL* samples. In particular, we did not detect structural polymorphisms involving the two retrotransposable elements inserted into the NBS-LRR gene cluster (data not shown).

We next examined the transcripts originating from the NBS-LRR gene cluster using Northern blot analysis. We detected no transcripts from the two retrotransposons in the NBS-LRR cluster in either wild-type Columbia or *bal* homozygotes. However, hybridization probes corresponding to exons of the NBS-LRR genes detected a 5-kb transcript in wild-type *BAL* Columbia plants. This transcript species was overexpressed fourfold in *bal* compared with *BAL* homozygotes (Fig. 6A).

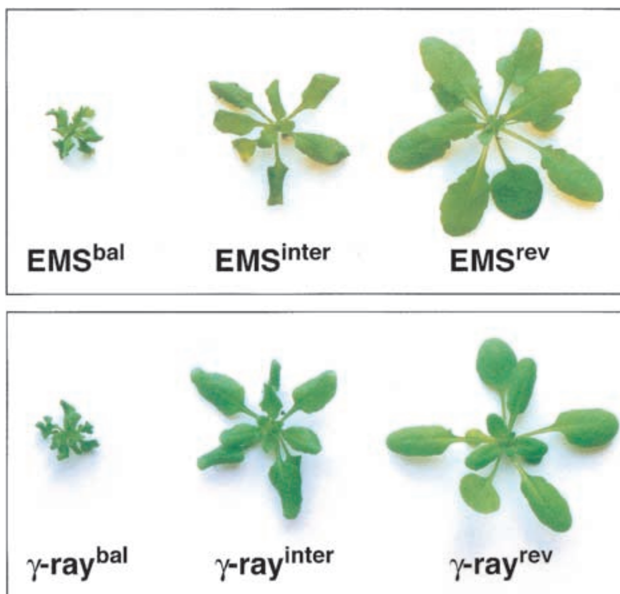

Figure 4. Representatives of the three phenotypic classes among M2 *bal DDM1* populations treated with EMS or γ-rays. The two panels show M2 sibling plants characteristic of the three phenotypic classes in Table 1. (*Top* panel) EMS-treated M2 population; (*bottom* panel) γ-ray-treated M2 population. Superscripts indicate phenotypic severity: (bal) phenotypic; (inter) intermediate; (rev) revertant.

We investigated the correlation between the abundance of the 5-kb transcript and the phenotypic severity of the *bal* defect by measuring transcript levels in EMS-induced revertants. As shown in Figure 6A, the abundance of the 5-kb transcript was decreased to approximately wild-type levels in the EMS-induced revertants lacking *bal* variant phenotypes. Similar results were seen for the γ-ray-induced phenotypic revertants (data not shown). *BAL/bal* heterozygotes, which displayed intermediate phenotypes, contained an intermediate level of the 5-kb transcript (Fig. 6B). The levels of the 5-kb transcript were not elevated in nonphenotypic *ddm1* plants (data not shown).

Expression of genes in the NBS-LRR gene cluster

Because the NBS-LRR coding sequences in the region share 90% or greater nucleotide identity, Northern analysis was unable to distinguish among the different genes in the region. We took four approaches to identify which genes are expressed from the NBS-LRR cluster in wild-type Columbia (see Fig. 5B). First, a search of EST databases identified cDNA clones originating from four genes in the region (*At4gxxxxx*): *16890*, *16900*, *16950*, and *16990*. In addition, five EST matches were found for the two retrotransposons inserted into the penultimate 3′ intron of *At4g16860* and *At4g16900*. We also screened a wild-type Columbia cDNA library (Kieber et al. 1993) and isolated six additional clones that matched three genes: *16890*, *16900*, and *16950*. As a supplementary approach, we designed RT-PCR primers to amplify products corresponding to transcripts from the most intact NBS-LRR coding sequences in the cluster. Expression from individual NBS-LRR genes in the cluster was detected by RFLP or sequence analysis of the RT-PCR-amplified products. Sequence analysis of nine RT-PCR clones indicated that the major transcript in wild-type Columbia originates from the *16890* gene (5 of 9 clones); we also found expression from *16900* (3 of 9 clones) and *16950* (1 of 9 clones). RFLP analysis of bulk RT-PCR products supported the conclusion that the major transcript source in wild-type Columbia is the *16890* gene (data not shown). A parallel RT-PCR RFLP profile indicated that *16890* is also the major source of transcript in *bal* variant plants (data not shown). These analyses indicate that the 5-kb transcript detected on Northern blots (hereafter, referred to as the *BAL* transcript) originates from the *16890* gene. The *16890* gene is one of the two genes in the cluster predicted to produce a full-length NBS-LRR-class protein.

The DNA sequence of the At4g16890 *gene is unchanged in the* bal *variant*

To see if the observed overexpression of the *16890* gene was caused by a mutation, we determined the nucleotide sequence of a 7-kb region from the *bal* variant encompassing the coding sequence of the gene and the entire upstream region up to the next annotated gene, *16900* (data not shown). No sequence changes were found. These data support the argument that the increased expression of the *16890* gene is not caused by a nucleotide sequence change.

Overexpression of the At4g16890 *gene phenocopies the* bal *defect*

We then asked if overexpression of the *16890* gene causes the *bal* phenotype. Using *Agrobacterium*-mediated T-DNA transformation, we constructed three independent transgenic lines (strain Columbia) containing

Table 1. *Chemical and physical mutageneses cause* bal *reversion at a high frequency*

Treatment	Phenotype			*n*
	Wild-type	Intermediate	*bal*	
0 mM EMS			100%	1725
30 mM EMS	2.5%	8.8%	88.7%	1102
0 kRad γ-ray			100%	525
120 kRad γ-ray	1.6%	5.6%	92.8%	625

bal/bal; DDM1/DDM1 seeds were treated with either ethylmethanesulfonate (EMS) or γ-rays, or mock-treated. The treated seeds (M1) were planted and M2 progeny were examined for the *bal* phenotype. *n* indicates the number of M2 progeny examined.

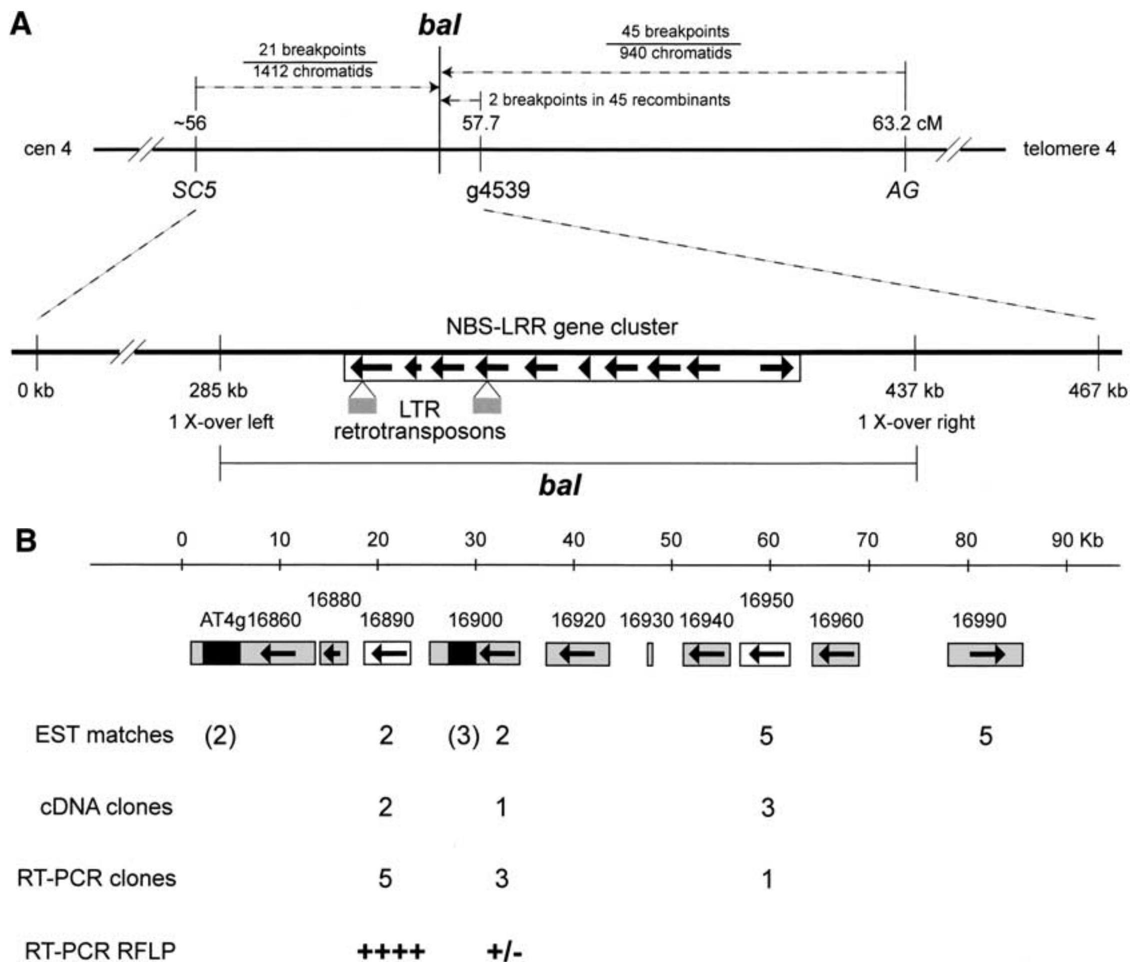

Figure 5. Map-based identification of the *BAL* locus. (*A*) (*top*) A genetic map of the region carrying the *bal* alteration. Distances are marked in centiMorgans (cM), and relevant genetic markers are shown beneath the genetic map. The dashed lines above the genetic map indicate the distribution of recombination breakpoints in the region. (*Bottom*) A physical map of the region with the minimal genetic window containing the *bal* alteration shown by the bracketed line. The 90-kb NBS-LRR gene cluster (*RPP5*-like) from strain Columbia is shown, as well as the positions of two LTR retrotransposons. (*B*) A higher resolution representation of the predicted *RPP5*-like genes (white and gray boxes) and LTR retrotransposons (filled boxes) in the 90-kb NBS-LRR gene cluster. Arrows indicate the predicted direction of transcription. Gray boxes indicate *RPP5*-like genes that are predicted to be nonfunctional because of point mutations and/or retrotransposon insertions. White boxes indicate genes predicted to encode functional NBS-LRR-class proteins. The results of our gene expression survey of the region are shown below the physical map. The number of matches in the public EST databases is indicated below each predicted coding sequence and the LTR retrotransposons (in parentheses). The number of matches to six newly isolated cDNA clones from the region is also shown. The distribution of sequenced RT-PCR clones and RFLP analysis of RT-PCR bulked products (data not shown) indicates that *At4g16890* is the predominantly expressed gene in the cluster. Noël et al. (1999) use an alternative nomenclature to describe this region: *At4g16890* = *RPP5* homolog *Col-B*.

the *16890* gene driven by the strong viral promoter, *P35S*. In the first generation (T_1), we recovered *35S::BAL* transgenic plants that phenocopied the dwarfing and twisted leaves of the *bal* variant. Control T_1 transformants containing the vector construct without the *16890* gene always displayed a wild-type phenotype. These results indicate that the *35S::BAL* transgene recapitulates the *bal* phenotype (see Fig. 7). In T_2 families generated by self-pollination of phenotypic T_1 *35S::BAL* transgenics, plants displaying the *bal* phenotype were recovered at a frequency of 55% (96 *bal*::79 wild-type phenotype). The T_2 segregation ratios are consistent with stochastic silencing of the *35S::BAL* transgene, which is frequently noted in 35S-driven transgenes in plants.

Next, we examined the expression of the *16890* gene using Northern analysis in the T_2 generation comparing individuals with wild-type or *bal* phenotypes. As shown in Figure 8, the level of the 5-kb *BAL* transcript was elevated in transgenic plants showing the *bal* phenotype. No increase in the abundance of the *BAL* transcript was noted in nonphenotypic segregants. Moreover, *PR1* gene expression was elevated in *35S::BAL* phenotypic individuals but was absent in nonphenotypic segregants. Based on progeny tests for the transgenic kanamycin-resistance marker, some of the nonphenotypic T_2 individuals seg-

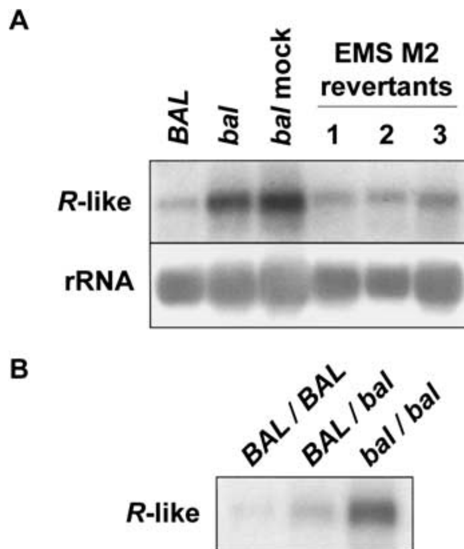

Figure 6. Overexpression of an NBS-LRR gene is associated with the *bal* phenotype. (*A*) Northern blot analysis showing overexpression of an *NBS-LRR*-like gene in phenotypic *bal* variants. (*BAL*) *BAL/BAL*; (*bal*) *bal/bal* variant; (*bal* mock) phenotypic plants from the mock-treated EMS M2 population; (1, 2, 3) independent nonphenotypic revertant plants from an EMS-treated M2 population. (*Bottom* panel) RNA loading control. (*B*) Northern blot analysis showing that the NBS-LRR like transcript level correlates with phenotypic severity in a population segregating the *bal* variant allele. The autoradiogram resulted from hybridization of the membrane used in Figure 2.

regated kanamycin-resistant seedlings in the T_3 generation, consistent with the hypothesis that silencing of the *35S::BAL* transgene occurs. However, all T_2 *bal* phenotypic plants contained the *35S::BAL* transgene and overexpressed the 5-kb *BAL* transcript.

The transgenic data support the hypothesis that overexpression of an NBS-LRR gene causes the *bal* phenotype. This result was corroborated by the phenotype of *bal eds1-2* double mutants (data not shown). *EDS1* distinguishes TIR (toll interleucin receptor) and LZ (leucine zipper) classes of NBS-LRR gene products; *eds1* mutations block signaling downstream of TIR, but not LZ, class *R*-genes (Aarts et al. 1998; Clarke et al. 2001). The *eds1-2* mutation suppressed the *bal* morphology and constitutive *PR* gene expression phenotype (data not shown), consistent with the *16890* gene being a TIR-class *R*-gene.

Overexpression of R-*gene coding sequence in* suppressor of SA insensitivity 1 *(*ssi1*) variant*

There are several parallels between the phenotype and genetics of the *bal* variant and the *ssi1* mutant. The *ssi1* mutation was isolated in the Nossen background as an extragenic suppressor of an *npr1* (*nonexpresser of pathogenesis-related genes*) mutation (Shah et al. 1999). Like the *bal* variant, the phenotype of the *ssi1* mutant is characterized by dwarfing, narrow leaves, and reduced fertility. The phenotypic effects of the *ssi1* mutation and the *bal* variant are not dependent on the *npr1* mutation (data not shown). The *ssi1* mutant shows constitutive *PR* gene expression and decreased growth of *P. syringae*. The *ssi1* dwarfing and constitutive *PR* gene expression are suppressed by the presence of the *nahG* transgene, indicating that these phenotypes are SA-dependent. The *ssi1* mutation is semidominant to the Nossen *SSI1* allele and maps to the lower arm of Chromosome 4 close to the *BAL* locus (Shah et al. 1999).

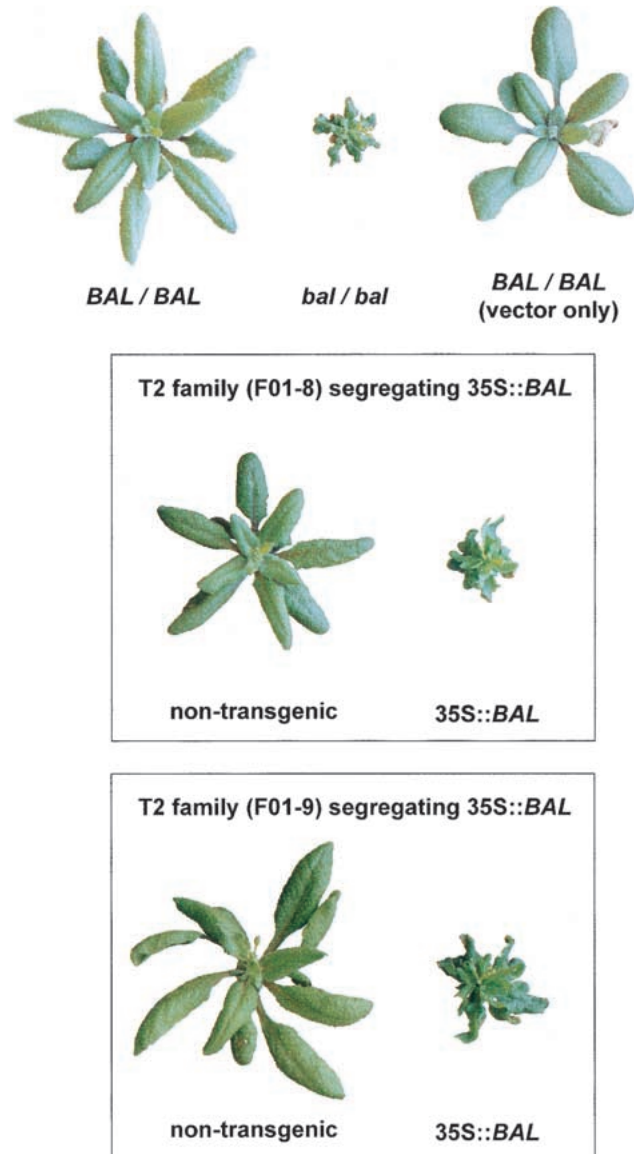

Figure 7. Overexpression of *At4g16890* in transgenic plants phenocopies the *bal* variant. (Top) Control plants: (*BAL/BAL*) *wild type; (bal/bal*) homozygous *bal* variant, (*BAL/BAL* vector only) wild type transformed with the T-DNA vector (pMD1). (*Middle*, *bottom*) Segregation of the dwarfing *bal* phenotype is shown by representative plants from two T_2 families segregating the *35S::BAL* transgene. All plants pictured (Columbia strain background) are 24-day-old plants and were grown in parallel in an environmental chamber.

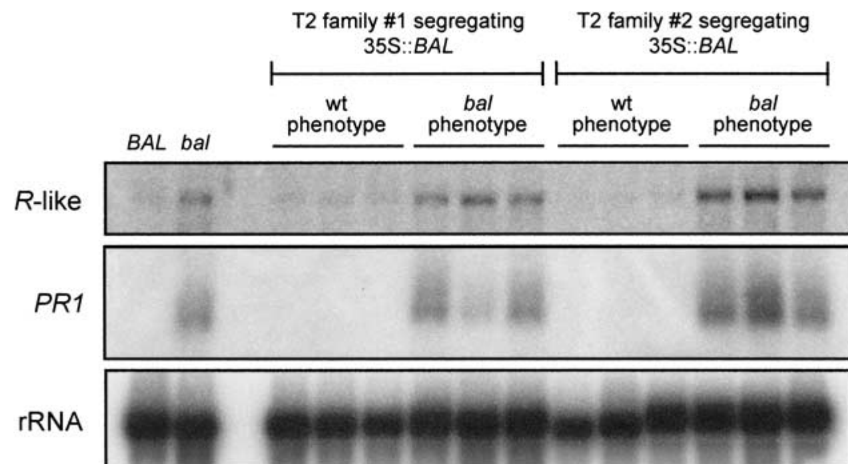

Figure 8. Transcript analysis of *35S::BAL* transgenics. (*Top* panel) Northern blot of total RNA hybridized with an *R*-like probe recognizing the ~5-kb *BAL* transcript. (*Middle* panel) The membrane was rehybridized with a probe recognizing the *PR1* transcripts. (*Bottom* panel) The membrane was rehybridized again with a 25S rRNA gene probe as an RNA loading control. Two control lanes are shown on the left: (*BAL*) wild type; (*bal*) *bal/bal DDM1/DDM1* variant. The remaining lanes contain total RNA prepared from six individuals (wild-type phenotype vs. *bal* phenotype) from two independent T_2 families segregating a *35S::BAL* transgene. All plants are in the Columbia background.

The genetic and phenotypic parallels between the *bal* and *ssi1* defects led us to investigate the *ssi1* variant further. First, we examined the stability of the *ssi1* phenotype in response to EMS mutagenesis. We found a high frequency of phenotypically normal or intermediate plants in the M1 generation: 14% (50 mM EMS) and 9% (120 krad of γ-irradiation) compared with 0% in mock-treated controls. Next, we investigated the expression within the Chromosome 4 NBS-LRR gene cluster in the *ssi1* variant and the appropriate wild-type background. Figure 9 shows that in the Nossen background, the level of the transcript was increased in the *ssi1* variant. The metastability of the *ssi1* variant and its association with the overexpression of an *R*-like gene further suggest that the *ssi1* and *bal* variants may be caused by the same underlying mechanism.

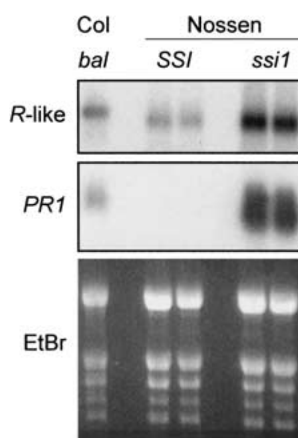

Figure 9. Overexpression of an *RPP5*-like gene in the *ssi1* (*suppressor of SA insensitivity 1*) mutant. Northern blot analysis using an *RPP5*-like hybridization probe detected the *BAL* transcript in the Columbia *bal* variant (*bal*) and recognized a smaller message in Nossen compared with Columbia. The abundance of the cross-hybridizing transcript is elevated in the *ssi1* mutant relative to a wild-type Nossen contrl. (*Bottom* panel) RNA loading control.

Discussion

Here, we show that a *ddm1* mutation induced a stable alteration, *bal*, which activates plant defense responses in the absence of pathogens. Several lines of evidence suggest that *bal* is an epigenetic variant. First, the *bal* alteration arose in an inbred *ddm1* hypomethylation background known to produce epigenetic variation (e.g., *sup* [Jacobsen et al. 2000], *ag* [Jacobsen et al. 2000], and *fwa* [Soppe et al. 2000] epialleles). Second, the *bal* alteration causes a change in gene expression that is not associated with large-scale genomic alterations or changes in nucleotide sequence of the implicated *At4g16890* gene. Third, the *bal* alteration can be destabilized in response to DNA-damaging agents, consistent with previous demonstrations that mutagens can alter DNA methylation and produce epigenetic alleles that are either hypomethylated or hypermethylated (Riggs and Jones 1983; Wilson and Jones 1983; Jacobsen and Meyerowitz 1997; Soppe et al. 2000). Furthermore, our preliminary results indicate that the Chromosome 4 *R*-like gene cluster and associated LTR retrotransposons are methylated in wild-type Columbia, and that this region is hypomethylated in *ddm1*-inbred backgrounds (T.L. Stokes and E.J. Richards, unpubl.). We hypothesize that overexpression of *At4g16890* in the *bal* variant is caused by heritable hypomethylation of critical sites in the region or is triggered by hypomethylation below a threshold level. Although we have found hypomethylated sites in the *R*-gene cluster of the *bal* variant, we have not yet identified methylation changes that are strictly correlated with *At4g16890* overexpression.

In our study, overexpression of the *At4g16890 R*-like gene in both the *bal* variant and transgenic *Arabidopsis* induces defense responses in the absence of pathogen. Previous studies show that overexpression of a non-LRR-class resistance gene *Pto* or an LRR-NBS resistance gene *Prf* induces defense responses and enhances pathogen resistance in transgenic tomato (Oldroyd and Staskawicz 1998; Tang et al. 1999). In contrast to the *bal* variant and the *35S::BAL Arabidopsis* transgenics, tomato plants overexpressing *Pto* or *Prf* fail to show dramatic whole-

plant defects. Recently, Xiao et al. (2001) reported that the overexpression of *RPW8*, a novel *R*-gene locus containing two genes, in *Arabidopsis* leads to broad-spectrum fungal resistance without obvious phenotypic abnormalities. The different phenotypic consequences of *R*-gene overexpression may reflect peculiarities of the different plant species and/or specific interactions of the different resistance genes. We note that some signaling mutants that affect steps downstream of *R*-genes show dwarfing and twisted leaf phenotypes (e.g., *cpr1* [Bowling et al. 1994], *mpk4* [Petersen et al. 2000]), which closely resemble the *bal* variant phenotype.

R-gene products act to recognize, either directly or indirectly, specific pathogen strains. After recognition, *R*-gene products signal through one or more defense pathways (Glazebrook 1999). The *BAL* gene is transcribed in the absence of pathogen, and the level of the transcript is not increased by pathogen attack (data not shown). Most characterized NBS-LRR *R*-genes are expressed at low constitutive levels (e.g., Meyers et al. 1999) similar to that seen for the *BAL* transcript. Mild developmental phenotypes were seen in *BAL/bal* heterozygotes that express ~2.5-fold higher levels of the *BAL* transcript. Severe morphological abnormalities resulted from a fourfold overexpression of the *BAL* transcript, levels similar to the highest seen in recovered *35S::BAL* transgenics. These results suggest that the expression of the *BAL* transcript may be constrained to an upper limit of expression.

Our results also address the mechanism of the *ssi1* variant, which was originally identified in the Nossen background as a suppressor of *npr1-5* (Shah et al. 1999). Several pieces of evidence indicate that *bal* and *ssi1* variants are caused by the same mechanism. First, both *bal* and *ssi1* are semidominant alterations that cause dwarfing and activation of defense pathways independent of *NPR1* function. Second, the *ssi1* mutation maps to the same genetic region (<5 cM) as the *BAL* locus. Third, both *bal* and *ssi1* overexpress an *R*-like gene from the cluster mapping to the bottom arm of Chromosome 4. Finally, both the *bal* and *ssi1* variants are destabilized by mutagens. Our results suggest that the *ssi1* phenotype is caused by overexpression of an LRR-NBS *R*-like gene, which may be tied to a metastable epigenetic alteration.

The semidominant nature of the *bal* and *ssi1* alterations is consistent with the proposed overexpression mechanism. *BAL/bal* heterozygotes expressed ~2.5 times the level of *BAL* transcript compared to wild-type *BAL/BAL* homozygotes in a Columbia background. Interestingly, the Columbia *bal* alteration behaved as a recessive allele when crossed into a wild-type Landsberg erecta background. The *RPP5* NBS-LRR haplotype in Landsberg erecta is rearranged relative to Columbia, and we have not detected a corresponding *BAL* transcript from the Landsberg locus (data not shown). Combination of a 4×-overexpressing Columbia *bal* allele with a null Landsberg allele is not sufficient to exceed a threshold for phenotypic expression. On the other hand, combination of a 4×-overexpressing Columbia *bal* allele with a 1×-expressing Columbia *BAL* allele exceeds this threshold, leading to mild phenotypic consequences. We note that the Columbia *bal* allele in combination with a wild-type Nossen allele acted semidominantly, consistent with the idea that the wild-type Nossen allele can specify some *R*-like gene function (data not shown). The overexpression model of *ssi1* is also consistent with recent findings that *SSI1/SSI1/ssi1* triploid progeny (tetraploid Columbia × Nossen *ssi1* diploid) fail to show morphological phenotypes (Greenberg 2000). In this case, the presence of the two wild-type Columbia loci in a triploid background would dilute the *R*-like gene function below the critical threshold.

Resistance genes are highly reiterated in plants, and our results point toward the importance of epigenetic regulation of these gene clusters prevalent in plant genomes. For example, the *Arabidopsis* genome, once believed to be relatively simple in structure, contains ~37% of the predicted protein coding sequences in families of five members or more, and 17% of all predicted *Arabidopsis* genes are multicopy and tandemly arrayed (The *Arabidopsis* Genome Initiative 2000). Our results suggest that epigenetic modification may play an important role in regulating gene clusters by cementing silent or intermediate expression states.

Recent results from several groups suggest that epigenetic alterations may play an important role in controlling phenotypic variation in both laboratory and natural populations. In *Arabidopsis* laboratory strains, epigenetic variation at several loci has been induced by both chemical mutagenesis and DNA hypomethylation (Jacobsen and Meyerowitz 1997; Jacobsen et al. 2000; Soppe et al. 2000). The induced epialleles described to date fall into two categories: metastable, silenced hypermethylated alleles (*ag*, *sup*, and *pai2*) and stable, hypomethylated epialleles associated with ectopic gene expression (*fwa*). The stability of the *bal* alteration is consistent with the hypothesis that DNA hypomethylation is involved. The recent discovery of hypermethylated silenced *Lcyc* alleles in *Linaria* shows that epigenetic variation can affect plant morphology and fitness in natural populations as well (Cubas et al. 1999). The induced genomic alterations characterized here (*bal* and *ssi1*) can modulate pathogen–plant interactions, raising the possibility that similar variants might arise and be selected in natural populations.

Materials and methods

Plant growth

Plants were grown in a mixture of Redi-Earth (Scotts):vermiculite (60%:40%) in environmental growth chambers (16 h of illumination [fluorescent + incandescent]/day, 70% relative humidity, 22°C) or under similar conditions in a greenhouse. Under short day length (8 h of illumination/day) and high humidity (95%), the morphological phenotypes of *bal* are attenuated. *bal* plants grown under short day length were used for the *P. syringae* inoculations (see Fig. 3).

Genetic mapping of the bal *defect*

The *bal* variant was generated from a *ddm1-2* line in the Columbia strain background, self-pollinated through six genera-

tions. We mapped *bal* in an F_2 segregating population generated from a cross between a *bal/bal; DDM1/DDM1* (Columbia) plant and a *BAL/BAL; DDM1/DDM1* (Landsberg erecta) individual (Kakutani et al. 1996). Phenotypic *bal/bal* F_2 plants were genotyped initially using the flanking CAPS markers, *AG* and *SC5* (Konieczny and Ausubel 1993; http://www.arabidopsis.org). We generated new CAPS markers from the available genomic sequence (Bevan et al. 1998) to establish a genetic and physical interval containing the *bal* alteration. This window is defined by a centromere-proximal marker: forward primer, 5′-AGACG CTGGAGTATCTTCACC-3′; reverse primer, 5′-CAGGAGGT GAGTTTCATCCTC-3′; polymorphic *Taq*I site and a telomere-proximal marker: forward primer, 5′-AATCATTGTCACCGAT CACC-3′; reverse primer, 5′-TGTACCGCCGTTCTGCTAC-3′; polymorphic *Hae*III site.

Nucleic acid isolation and analysis

Genomic DNA samples were purified using QIAGEN protocols and columns, or by the Urea Lysis miniprep protocol (Cocciolone and Cone 1993). Southern analyses were performed as described previously (Jeddeloh et al. 1998). Total RNA samples were isolated using either QIAGEN RNeasy or Bio-Rad AquaPure protocols. RNA was size-fractionated by electrophoresis through 1.5% agarose formaldehyde gels and blotted to GeneScreen (NEN DuPont) nylon membranes using capillary action and 50 mM sodium phosphate buffer (pH 6.5). All hybridizations were done following the protocol of Church and Gilbert (1984), and membranes were washed at 60°C in 0.2× SSC, 0.1% SDS. Hybridization probes were radiolabeled using the random priming protocol (Feinberg and Vogelstein 1983), and unincorporated radionucleotides were removed by size-filtration columns. We used the following hybridization probes: *Arabidopsis actin 2* gene (GenBank U41998); *PR1* (Uknes et al. 1992); *PR2* (Dong et al. 1991); *PR5* (Uknes et al. 1992); *R*-like gene (ColF; Parker et al. 1997); *25S rRNA* gene (pARR17; Kakutani et al. 1996). DNA sequencing was performed using linear double-stranded templates generated by genomic amplification and Big-Dye Terminator Cycle Sequencing (Perkin-Elmer) protocols/reagents.

bal *reversion analysis*

bal/bal; DDM1/DDM1 seeds were hydrated overnight in water and then treated with different concentrations of EMS (0, 10, 20, 30, 40, and 50 mM; Sigma M0880) for 8 h. Seeds were then washed overnight in water, dried, and sown on soil. The resulting M1 plants were allowed to grow to maturity, and self-pollinated M2 seeds were harvested in independent batches (~20 M1 plants per M2 pool). A similar protocol was followed for the γ-ray mutagenesis experiments except that dried seeds were treated by exposure to a γ-ray source (Department of Radiology, Washington University Medical School). The data shown in Table 1 represent the pooling of at least three M2 families.

BAL *transcript analysis*

A wild-type Columbia cDNA library (Kieber et al. 1993) was screened (7.5×10^5 plaques) using a probe that represented a conserved portion of the open reading frames in the gene cluster. RT-PCR analysis used poly(A)$^+$-selected (Dynabeads) RNA and the following primers: forward primer, 5′-AGAAATTGA TCGTGCAAAGTCCAAGGGTAATCC-3′; reverse primer, 5′-AGAGTTCCTTCCCACAGTTTCTCAAGCTTACT-3′; with Stratascript II (Stratagene) RT enzyme. The amplified products, corresponding to exon 2–intron 2–exon 3 from *AT4g16890*, were either sequenced using Big-Dye Terminator Cycle Sequencing (Perkin Elmer) protocols/reagents or were digested with *Hae*III and/or *Hph*I (New England Biolabs), which distinguished among *At4g16860*, *16890*, *16900*, *16920*, and *16950*.

Generation of transgenic plants

The genomic region encompassing the *At4g16890* gene was amplified using the following primers: forward primer, 5′-CTAG TCTAGACGGCAAATTGTTTCGTGGCCATCTTGTCA-3′; reverse primer, 5′-CGCGGATCCCAATGTGGAAAAGATAG ATGTATGGTAC-3′. The ends of the resulting 5-kb fragment were cut with *Xba*I and *Bam*HI (New England Biolabs), and the fragment was cloned into the pMD1 vector (a derivative of pBI121; Mark Dixon, unpubl.) downstream of a strong viral promoter, *P35S*. The amplified *35S::16890* gene encodes a full-length protein with one missense mutation at position 950 in the LRR domain (S 950 L). Only 8 of the 21 LRRs in RPP5 (the closest relative in the *Arabidopsis* genome) contain a leucine adjacent (toward the N terminus) to the altered position, which is occupied by either serine or isoleucine. The poor conservation at this position and the similar structure of leucine and isoleucine suggest that the change will not alter the function of the gene product. The construct was electroporated into *Agrobacterium tumefaciens* strain GV 3101 (Koncz and Schell 1986). Plants were transformed using the floral dip protocol (Clough and Bent 1998). Harvested seeds were ethanol- and bleach-sterilized, and transformants were selected on plates with 4.6% Murashige and Skoog salts (GIBCO-BRL), Gamborg's vitamins (Sigma), and 50 mg/L kanamycin (Sigma).

Characterization of pathogen defense response

Five-week-old *Arabidopsis* plants were inoculated by vacuum infiltration with *Pseudomonas syringae* p.v. tomato strain DC3000 (Whalen et al. 1991) at an initial density of 1×10^5 CFU/mL. The concentration of bacteria in the plant leaves was assayed after 0, 1, and 4 d as described previously (Whalen et al. 1991). Data points represent the means of three independent determinations ± standard error of the mean. *nahG bal* double mutants were identified by testing for kanamycin resistance (linked to the *nahG* transgene) and scoring a CAPS marker closely linked to *bal*: forward primer, 5′-AGACGCTGGAGT ATCTTCACC-3′; reverse primer, 5′-CAGGAGGTGAGTTTC ATCCTC-3′; polymorphic *Taq*I site. Northern blots were done on double mutants to verify that *At4g16890* and *nahG* were expressed at expected levels (data not shown). Crosses were done between *bal/bal* and *npr1-1/npr1-1* plants and double mutants in the F_2 generation were identified by first scoring for dwarfing and then genotyping at the *NPR1* locus using a molecular marker. A similar approach was used to generate *bal eds1-2* double mutants.

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