

Epigenetic Control of an Endogenous Gene Family Is Revealed by a Novel Blue Fluorescent Mutant of *Arabidopsis*

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Summary

The Wassilewskija strain of *Arabidopsis* has four genes encoding the tryptophan enzyme phosphoribosylanthranilate isomerase (*PAI*) located at three unlinked sites. These four *PAI* genes are methylated over their regions of DNA homology. When *PAI* copy number is reduced by deletion of two tandemly arrayed genes (*MePAI1-PAI4*), a mutant with fluorescent, tryptophan-deficient phenotypes results, because the two remaining methylated *PAI* genes (*MePAI2* and *MePAI3*) supply insufficient *PAI* activity. These two methylated genes can be inherited through meiosis, even when they are segregated away from each other in crosses to a strain with unmethylated *PAI* genes. However, the mutant phenotypes conferred by the methylated *PAI* genes are unstable, and mutant plants yield occasional revertant somatic sectors and progeny. Revertant lines display coordinately reduced methylation of both *PAI2* and *PAI3*, implying that this hypomethylation acts in a concerted manner across the genome rather than at individual sites.

Introduction

Meiotically and mitotically heritable changes in gene expression that cannot be accounted for by changes in DNA sequence are referred to as epigenetic phenomena. In several eukaryotic organisms, epigenetic control or imprinting of gene expression is correlated with changes in cytosine methylation of the affected locus. In mammals, methylated and silenced imprinted genes are inherited on either the maternal or paternal chromosome, whereas the genes inherited on the homologous chromosome are hypomethylated and expressed, creating functionally haploid regions of the genome (Peterson and Sapienza, 1993; Razin and Cedar, 1994). For example, the mouse *H19* gene is methylated over an 8 kb region on the paternally inherited chromosome, but not on the maternally inherited chromosome, and only the maternally inherited gene is expressed (Bartolomei et al., 1993). In contrast, the mouse *Igf2r* gene shows imprinting where methylation of the gene has a positive effect on its expression (Stöger et al., 1993). In this case, expression of the *Igf2r* gene has been correlated with the presence of methylation in an intron sequence exclusively on the maternally inherited chromosome.

In a number of fungal systems, methylation is correlated with gene duplication resulting from transformation. For example, in transformed premeiotic cells of *Neurospora crassa* containing an integrated transgene and an identical resident gene, both copies of the repeated DNA are specifically methylated and riddled with point mutations, a phenomenon called repeat-induced point mutation (RIP) (Selker, 1990; Selker et al., 1993). In transformed premeiotic cells of *Ascobolus immersus* (Barry et al., 1993; Rhounim et al., 1992), both copies of the repeat are specifically methylated and silenced, but not mutated, a phenomenon called methylation induced premeiotically (MIP). In these fungal systems, once RIP or MIP methylation of a region has been established, it is heritable even when the two gene copies that stimulated the process are segregated away from each other.

In plants, some of the best characterized examples of epigenetic phenomena include regulation of the maize transposable elements *Ac* (Brutnell and Dellaporta, 1994), *Spm* (Banks et al., 1988), and *Mutator* (Martienssen and Baron, 1994) by cytosine methylation correlated with loss of transposon gene expression. In these cases, methylation and transposon activity can vary in different tissues of the plant. Therefore, regulation of repetitive elements such as transposons by reversible epigenetic mechanisms might serve as a way of maintaining the balance between proliferation of the element and damage to the host organism under changing conditions.

It is likely that all methylation-correlated epigenetic phenomena, including genomic imprinting in mammals, repeat-induced methylation in fungi, and regulation of transposons in maize, arose from a common ancestral mechanism. However, in no system is it understood how particular genes or regions of the genome are targeted for silencing, what factors control epigenetic expression states, how cytosine methylation is related to alteration of gene expression, or how epigenetic patterns are inherited (Martienssen and Richards, 1995).

In many plant species, including *Arabidopsis thaliana*, gene silencing phenomena have been observed as a consequence of transforming the plant with transgenic DNA constructs designed to overexpress the transgene ectopically (Matzke and Matzke, 1995). When the transgene contains endogenous plant sequences, transgenic lines can develop with reduced expression of both the transgene sequences and the resident genomic sequences, a phenomenon called cosuppression. In some examples of this transgene-induced silencing, loss of gene expression correlates with increased copy number and cytosine methylation of the DNA at silenced loci, particularly in cases where the transformed homologous segment is a promoter sequence (Assaad et al., 1993; Kilby et al., 1992). However, in other cases there is no detectable methylation of inactivated sequences. These discrepancies suggest that plants may inactivate transgene expression by more than one mechanism (Matzke and Matzke, 1995). In particular,

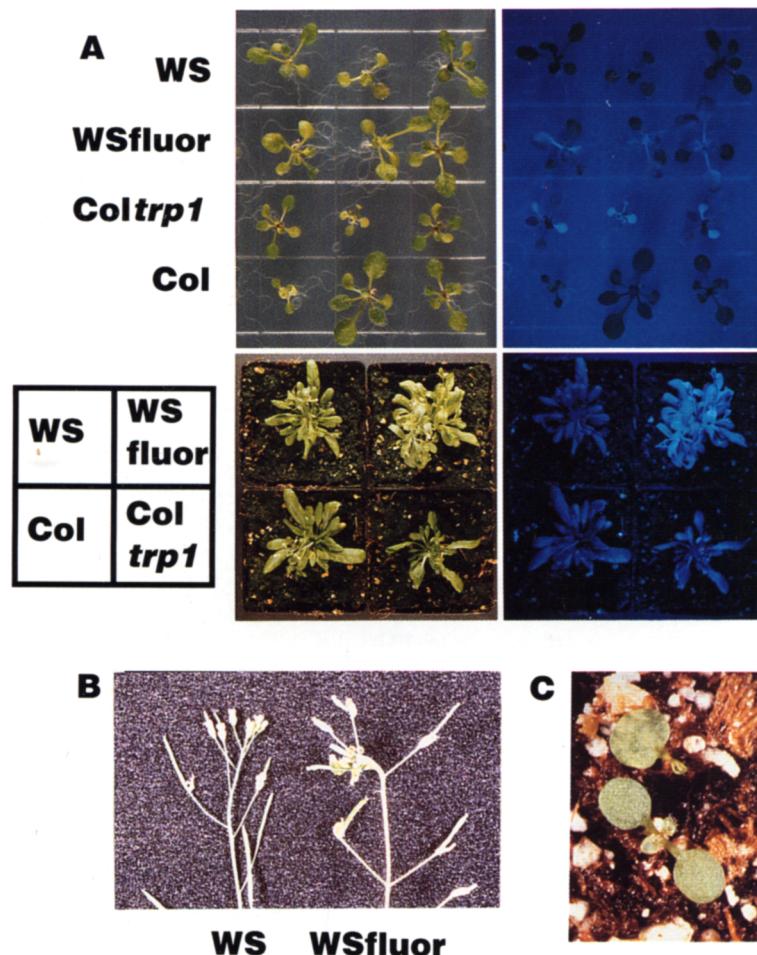


Figure 1. Tryptophan and Indole-3-Acetic Acid Deficiency Phenotypes of the WS Fluorescent *pai* Mutant

(A) WS, the WS fluorescent *pai* mutant (WSfluor), the Col *trp1-100 gl1-1* mutant (Col*trp1*), and Col are shown (top) as 2-week-old seedlings grown on plant nutrient sucrose agar medium and (bottom) as 4-week-old plants grown in soil, photographed under visible (left) and UV light (right).

(B) Shown are 8-week-old inflorescence shoots of WS and the WS fluorescent *pai* mutant (WSfluor).

(C) Shown are 1-week-old seedlings of the WS fluorescent *pai* mutant segregating a plant with fused cotyledons.

examples of transgene inactivation that show no detectable methylation have been proposed to be down-regulated by a feedback-triggered posttranscriptional mechanism such as selective degradation of overproduced transgene transcripts (Dehio and Schell, 1994).

We describe a mutant of *Arabidopsis* that reveals a copy number- and methylation-correlated loss of gene expression for a gene family encoding the third step of the tryptophan biosynthetic pathway, phosphoribosylanthranilate isomerase (PAI). The modulation of PAI gene expression by methylation provides the first example of an endogenous gene family regulated by epigenetic mechanisms in *Arabidopsis*. This system displays a number of features in common with epigenetic phenomena described in mammalian, fungal, and maize systems and appears distinct from examples of overexpression-induced cosuppression in transgenic plants.

Results

Mutant Phenotypes

We recovered five isolates of a novel blue fluorescent *Arabidopsis* mutant from the ecotype Wassilewskija (WS) (see Experimental Procedures). The five isolates are allelic, yielding fluorescent F1 progeny when crossed to each

other in pairwise combinations. However, the five isolates are not allelic to the ecotype Columbia (Col) *trp1-100* fluorescent mutant (Rose et al., 1992) because crosses between the two types of fluorescent mutants yield nonfluorescent F1 progeny.

Enzyme assays show that the WS fluorescent mutant is deficient in the tryptophan enzyme PAI relative to WS and Col (data not shown). This *pai* mutant displays a number of phenotypes consistent with both tryptophan and indole-3-acetic acid deficiencies. The mutant accumulates the same anthranilate derivatives as the fluorescent *trp1-100* mutant (Last and Fink, 1988; Rose et al., 1992), but it becomes most fluorescent in the adult portions of the plant, in contrast to *trp1-100*, which is most fluorescent in the cotyledons (Figure 1). The mutant also displays a number of striking morphological defects (Figure 1). Adult plants are bushy with yellow-green leaf tissue, and late-developing mutant inflorescence shoots display abnormal structures. Approximately 2% of mutant seedlings develop with abnormal fused cotyledons, compared with fewer than 0.1% in WS. Both the inflorescence shoot and cotyledon phenotypes are similar to those observed for the *pin1-1* mutant, a mutant with approximately 10-fold decreased levels of the plant growth regulator indole-3-acetic acid (Liu et al., 1993; Okada et al., 1991). All mor-

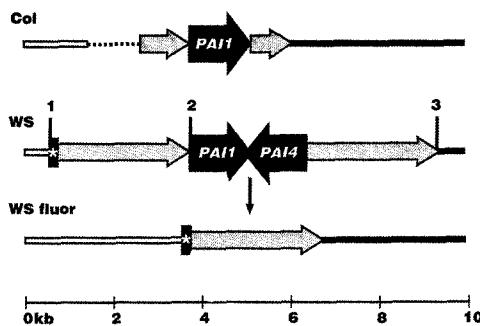


Figure 2. The WS *PAI1-PAI4* Duplication Is Deleted by Homologous Recombination between Flanking Direct Repeats to Yield the WS Fluorescent *pai* Mutant

The genomic structures at the *PAI1* locus for Col, WS, and the WS fluorescent *pai* mutant (WSfluor) are diagrammed. *PAI* coding sequences are represented by closed arrows with white lettering, and the partial *pai4* 5' duplication at the left border of the region in WS and WSfluor is represented by a closed box marked with a white asterisk. Flanking direct repeat sequences are shown as stippled arrows. The Col *PAI1* gene is flanked by truncated direct repeat sequences, also shown as stippled arrows (Li et al., 1995; J. B., unpublished data); on the right, approximately 700 bp of the direct repeat remain, and on the left the dotted line indicates a region uncharacterized by sequence or Southern blot analysis, so that the length of the direct repeat on this side is unknown. Col lacks the *pai4* 5' sequence. The GenBank accession numbers for the sequences indicated by positions 1, 2, and 3 are U34758, U34757, and U34762, respectively.

phenological phenotypes cosegregate with blue fluorescence of the WS *pai* mutant through two backcrosses to WS and one outcross to Col.

WS Has a Novel Arrangement of *PAI* Genes

Because enzyme assays showed that the WS fluorescent *pai* mutant was deficient in *PAI* activity, we cloned the *PAI* genes from parental WS and the mutant to determine whether these genes contain defects in the mutant strain. We found that WS has a different arrangement of *PAI* genes than the previously characterized standard genetic ecotype Col: Col carries three unlinked *PAI* genes (Li et al., 1995), whereas WS carries an unusual duplication and rearrangement of *PAI* genes at one of the three *PAI* loci. Furthermore, as previously described for the three Col *PAI* genes, the four WS *PAI* genes are highly homologous to each other, with greater than 90% identity at the nucleic acid sequence level, extending even through introns and 5' and 3' noncoding sequences over a region of approximately 2 kb for each gene.

We characterized three classes of *PAI* clones from a WS genomic library (see Experimental Procedures) that correspond by map position to the *PAI1*, *PAI2*, and *PAI3* classes previously described for the Col ecotype (see below). This analysis reveals a striking difference between Col and WS at the *PAI1* locus: in Col this locus carries a single *PAI1* gene, but in WS this locus carries two almost perfectly duplicated *PAI* genes in a tail-to-tail inverted repeat (*PAI1-PAI4*) separated by 276 bp of intervening sequences (Figure 2). Both of these genes are over 98% identical to the Col *PAI1* gene throughout their coding sequences and introns, but the WS *PAI4* protein has a 3

amino acid deletion at the 3' end of the gene near the border of a conserved region (residues 252–254) and therefore might not encode a functional polypeptide.

The *PAI1-PAI4* inverted repeat coding sequences are flanked by approximately 3.1 kb of direct repeat sequences. In addition, the direct repeat sequence adjacent to the *PAI1* gene extends into a partial duplication (up to base pair +381 from the ATG start codon) of the *PAI4* gene, designated *pai4* 5' (Figure 2). The 3.1 kb direct repeat sequence is not present elsewhere in the WS or Col genomes, as determined by Southern blot analysis.

The WS *PAI2* and *PAI3* loci are structurally similar to the equivalent loci in Col, based on restriction maps of these regions. The WS *PAI2* coding and intron sequence is over 99% identical to the Col *PAI2* sequence. The predicted amino acid sequence of WS *PAI2* differs from that of the Col *PAI2* protein by one conservative change.

We mapped the WS *PAI1-PAI4*, *PAI2*, and *PAI3* loci by identifying polymorphisms between the WS and Col ecotypes at these loci and following their segregation on a mapping population of random F₂ plants from a cross between WS and Col (Figure 3). The *PAI1-PAI4* polymorphism maps at approximately 9 cM on chromosome 1, the *PAI2* polymorphism is completely linked to a standard mapping marker at approximately 15 cM on chromosome 5, and the *PAI3* polymorphism segregates at approximately 70 cM on chromosome 1. These map positions agree with those previously assigned for the Col *PAI* genes (Li et al., 1995).

The *pai* Mutant Has Deleted *PAI1-PAI4*

To characterize the WS fluorescent *pai* mutant at the molecular level, we first analyzed the arrangement of *PAI* genes in the mutant and the parental WS strain by Southern blot using a *PAI1* cDNA as a probe. DNA fragments corresponding to the *PAI1-PAI4* genes are deleted in the mutant relative to WS, while the structures of the *PAI2* and *PAI3* loci remain intact between the two. We isolated the deleted *PAI1-PAI4* locus from a genomic library made from one of our five fluorescent mutant isolates to determine the precise structure of the deletion. Our analysis reveals that the mutant has lost the *PAI1-PAI4* inverted repeat and one copy of the long flanking direct repeat sequence, leaving behind only the partial *pai4* 5' duplication and the second long flanking direct repeat sequence (*Apai1-pai4*; Figure 2). This deletion structure is most simply explained by a homologous recombination event between the two long direct repeats to loop out the *PAI1-PAI4* duplication. Homologous recombination also accounts for the apparently spontaneous nature of the deletion. Characterization of the *Apai1-pai4* region from the four other fluorescent mutant isolates by Southern blot and sequence analysis shows that they all have the same deletion structure.

WS Carries an Endogenous Defect at *PAI2*

A key insight into the connection between the *PAI* genes and the fluorescence phenotype in our mutant emerged from analyses of outcrosses of the WS fluorescent *pai*

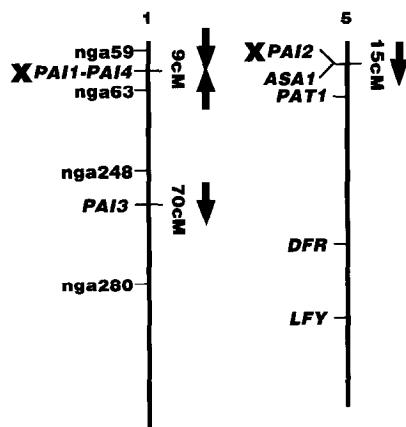


Figure 3. Map Positions of WS PAI Genes and the Two WS Fluorescent *pai* Mutant Defects Coincide

Map positions for both genes and mutants were determined relative to the standard mapping markers shown (see Experimental Procedures). Homologous *PAI* gene segments are represented as closed arrows. Blue fluorescence defect positions are indicated (bold X). *PAI* gene positions are based on the assignments from the Dean and Lister recombinant inbred (RI) mapping lines (Lister and Dean, 1993) and updated map positions as of June, 1995. The *PAT1* marker maps 8 cM South of *PAI2/ASA1*, placing it at approximately 24 cM on this map.

mutant to the Col ecotype. We found that while only a single recessive trait segregates in a backcross to WS (Table 1, line 1), two unlinked recessive traits segregate in an outcross to Col to give the fluorescence phenotype (Table 1, line 3). These results suggest that the second fluorescence defect is endogenous to the parental WS strain, so that only the Δ *pai1-pai4* mutation is required in this strain to yield the fluorescence phenotype. In contrast, the Col strain lacks the endogenous fluorescence defect and requires two independently segregating defects for a fluorescent *pai* phenotype.

We mapped the positions of the two fluorescence defects by preparing DNA from fluorescent F2 progeny of a cross between the WS fluorescent *pai* mutant and Col and scoring segregation of polymorphic markers along each chromosome to find regions linked to the phenotype (Figure 3; see Experimental Procedures). We found complete linkage for one mutant locus with the Δ *pai1-pai4* mutation

and complete linkage for a second mutant locus with *PAI2*. *PAI3* is not linked to the fluorescence phenotype. This genetic evidence that *PAI3* does not contribute to the mutant phenotype is consistent with the previous determination in Col that *PAI3* has significantly lower expression levels than *PAI1* or *PAI2* (Li et al., 1995).

We isolated and sequenced the *PAI2* locus from parental WS and the *pai* mutant to determine the molecular nature of the endogenous WS *PAI2*-linked defect. The parental WS and fluorescent mutant lines have identical *PAI2* coding and intron sequences with no significant differences from the Col *PAI2* sequence. These results indicate that the WS *PAI2*-linked defect is not a mutation in the *PAI2* structural gene.

pai Mutant Phenotypes Are Not Stable

A critical insight into the nature of the WS *PAI2*-linked defect came from our observation that the phenotypes of the WS fluorescent *pai* mutant are not completely stable. Approximately one in every five adult fluorescent plants displays nonfluorescent leaf somatic sectors, and 1%–5% of the progeny of self-pollinated fluorescent mutant plants are nonfluorescent and morphologically wild type (Figure 4). Revertant nonfluorescent somatic sectors tend to follow boundaries of the leaf vasculature, particularly the midrib. Sectors appear most often on the late-developing cauline leaves along the inflorescence shoot. The relatively sharp sectoring boundaries suggest that the product of the *PAI* enzyme, 1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate, is cell autonomous or only weakly diffusible. Because some revertant sectors are confined to a small patch of cells in a single tissue layer, while others appear to extend through multiple tissue layers, the reversion events are likely to be both clonal and nonclonal in origin.

Eight independent nonfluorescent revertant progeny of the WS fluorescent *pai* mutant (R0 generation) all yielded mixed fluorescent and nonfluorescent progeny in the next generation (R1), suggesting that the revertants are either heterozygous or chimeric for the reversion event. We isolated true-breeding nonfluorescent lines from the progeny of four of these revertants (R2 generation). These lines have not segregated any fluorescent progeny out of thou-

Table 1. Segregation of *PAI* Phenotypes in Reciprocal Test Crosses

Female Parent		Male Parent	Fluorescent F1 (%)	
			Expected	Observed
WSfluor ^a	×	WSfluor	50	44 (137 of 310)
WSfluor		WS		
WSfluor	×	WSfluor	50	17 (66 of 387)
WS		WSfluor		
WSfluor	×	WSfluor	25	23 (85 of 371)
WSfluor		Col		
WSfluor	×	WSfluor	25	7 (30 of 448)
Col		WSfluor		

^aWSfluor, WS fluorescent *pai* mutant.

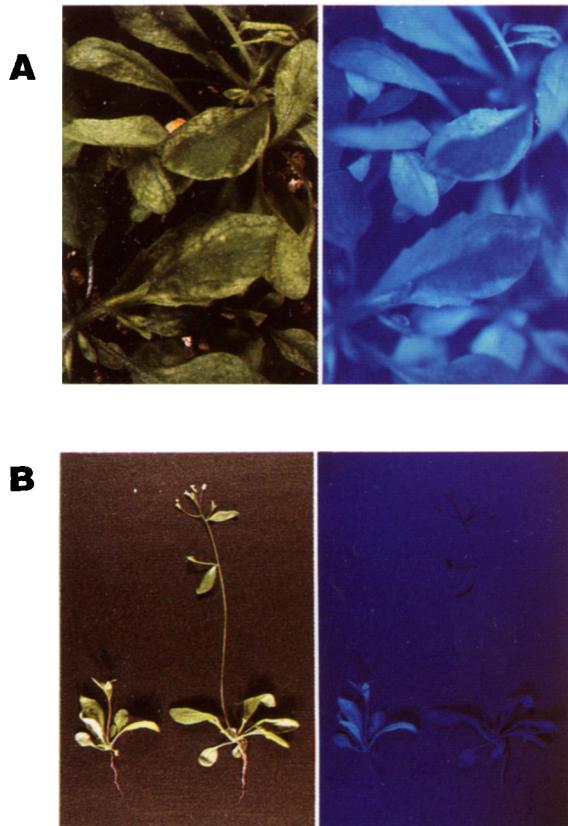


Figure 4. WS Fluorescent *pai* Mutant Phenotypes Are Unstable
(A) Somatic revertant sectors on the leaves of the WS fluorescent *pai* mutant are shown photographed under visible (left) and UV (right) light.
(B) Two isogenic strains, the WS fluorescent *pai* mutant (left) and an R3 generation germinal revertant line (right) are shown as 4-week-old plants, photographed under visible (left) and UV (right) light.

sands of progeny tested in the next generation (R3), suggesting that reversion is irreversible or only very rarely reversible.

Several lines of evidence pointed to the *PAI/2* locus as the unstable element in the *pai* mutant. First, genetic analysis shows that a defect at this locus is required for the mutant phenotype; however, sequence analysis shows that the defect is not a mutation in the coding sequence of the gene. Second, the other locus required for the mutant phenotype, *PAI/1-PAI/4*, has incurred a nonrevertible deletion mutation. Third, the *PAI/3* locus is not genetically linked to the phenotype and has relatively low expression levels, indicating that it does not contribute significantly to the levels of *PAI* activity in the plant.

The WS *PAI/2*-Linked Defect Corresponds to *PAI/2* Cytosine Methylation

We hypothesized that instability of the WS *PAI/2*-linked defect could provide a molecular explanation for the phenotypic instability of the fluorescent *pai* mutant. A possible model for this instability was that the defect might represent a spontaneously reversible epigenetic silencing of

PAI/2 gene expression. This model proposes that in the silenced state, *PAI/2* expression is low, resulting in plants with a fluorescent phenotype, and in the unsilenced state *PAI/2* expression is increased, yielding nonfluorescent sectors and progeny. In many other cases of gene silencing observed in mammals, fungi, and plants, silenced loci are cytosine methylated and unsilenced loci are hypomethylated (Martienssen and Richards, 1995). Consistent with this silencing model, Southern blot analysis shows that methylation-sensitive restriction enzymes are strongly inhibited from cleaving both *PAI/2* and *PAI/3* sequences in DNA prepared from the fluorescent mutant, but only weakly inhibited in DNA prepared from the revertant lines.

Digests diagnostic for DNA cytosine methylation, particularly *Hpa*II, which is blocked from cleaving both *M^{ec}CCGG* and *C^{Me}CGG*, and *Msp*I, which is blocked from cleaving *M^{ec}CCGG*, display cleavage patterns on our DNA samples indicative of heavy internal and lighter external methylation at the single *CCGG* sites in the *PAI/2* and *PAI/3* sequences of the fluorescent mutant (Figure 5). In addition, heavy *PAI* gene methylation is a preexisting state in WS, where all four *PAI* genes display an even higher degree of methylation than the fluorescent mutant strain. In contrast, DNA prepared from the Col ecotype displays little or no methylation at any of three *PAI* genes. This striking difference in *PAI* gene methylation between the two ecotypes correlates with the genetic observation that WS carries an endogenous blue fluorescence defect linked to *PAI/2* (*MePAI/2*), but Col does not (*PAI/2*).

In WS and the fluorescent mutant, we found methylation of restriction enzyme sites across the *PAI* coding sequences, but not at nearby flanking sites, suggesting that *PAI* gene methylation is localized to the repetitive portions of the *PAI* sequences. In particular, an *Msp*I site at -386 bp and a *Hinc*II site at -482 bp from the ATG start of the *PAI/4* gene (just outside the region of shared *PAI* homology) show no detectable methylation, while an *Msp*I site at +427 bp and a *Hinc*II site at +3 bp internal to the ATG of *PAI/4* are heavily methylated (Figure 5; data not shown).

Alternative models for the instability of the WS *PAI/2*-linked defect that involve changes in DNA structure or sequence at the *PAI/2* locus are extremely unlikely because we detected no differences between the fluorescent mutant and revertant lines with Southern blot analysis using probes spanning 20 kb of the locus or with sequence analysis of the *PAI/2* gene (data not shown).

Two Unlinked *PAI* Genes Are Coordinately Hypomethylated in Revertants

Analysis of four independent revertant lines derived from the fluorescent mutant indicates that cytosine methylation in all of these revertants is reduced at both the *PAI/2* and *PAI/3* genes (Figure 5). To test explicitly whether hypomethylation of both *PAI* genes is required for the nonfluorescent phenotype, we isolated hybrid progeny from crosses between the WS fluorescent *pai* mutant and Col with different combinations of *PAI/2* and *PAI/3* genes and analyzed them for both phenotype and *PAI* gene methyla-

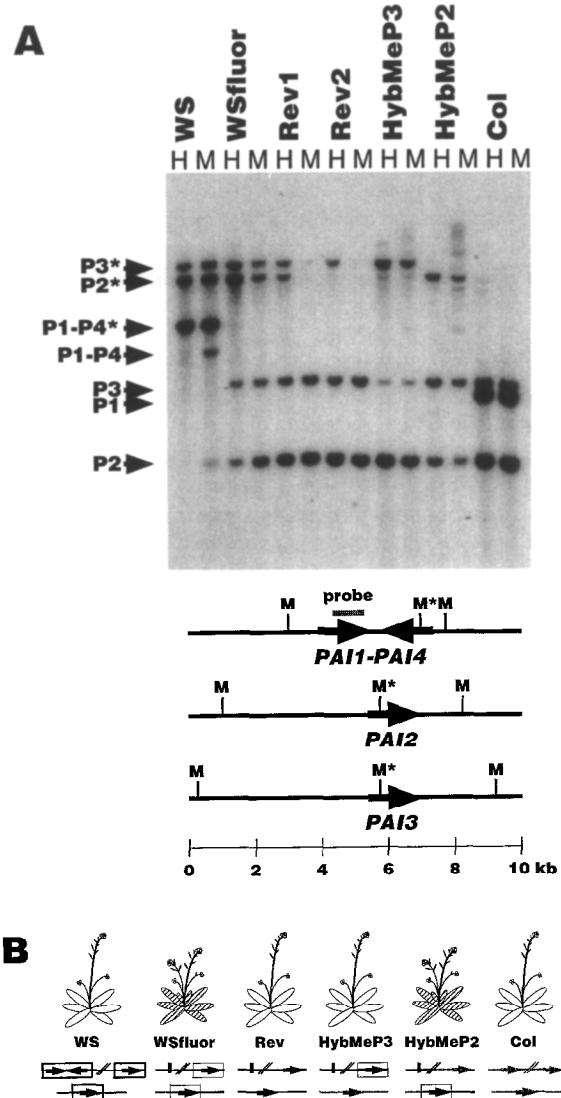


Figure 5. *PAI* Genes Are Cytosine Methylated in the WS Fluorescent *pai* Mutant

(A) Shown is a genomic Southern blot of DNA prepared from whole 4-week-old plants of WS, the WS fluorescent *pai* mutant (WSfluor), two independent true-breeding nonfluorescent revertant R3 generation lines of the WS fluorescent *pai* mutant (Rev1 and Rev2), a P2 MeP3 hybrid F4 line (HybMeP3), a MeP2 P3 hybrid F4 line (HybMeP2), and Col, loaded in pairs cut with isoschizomers HpaII (H) or MspI (M) and probed with an internal 0.7 kb PstI fragment of a *PAI1* cDNA. MspI restriction maps of the WS *PAI1-PAI4*, *PAI2*, and *PAI3* loci are diagrammed, with methylated sites indicated by an asterisk. Twice as much Col DNA was used as in the other samples to determine with more sensitivity whether there is residual *PAI* methylation in this strain. (B) Summary of *PAI* gene methylation and corresponding plant phenotypes for the samples tested in (A). Hatching indicates fluorescence. The upper chromosome corresponds to chromosome 1 carrying the *PAI1-PAI4* genes or *Δpai1-pai4* deletion and the unlinked *PAI3* gene. The lower chromosome corresponds to chromosome 5 carrying the *PAI2* gene. Stippling indicates Col DNA; bold indicates WS DNA.

tion. Two important points emerge from these analyses. First, only loss of methylation from the *PAI2* gene (*MePAI2*) is required for a revertant nonfluorescent phenotype, whereas the methylation state of the weakly expressed *PAI3* gene is irrelevant. Second, hybrid lines can stably maintain a pattern in which only one of two *PAI* genes (either *MePAI2* or *MePAI3*) is methylated, suggesting that there is no transfer of the methylation information between these two unlinked homologs. Therefore, coordinate hypomethylation of both *PAI* genes in all the revertant lines we have tested suggests that these spontaneous events involve a concerted rather than a gene-specific alteration of epigenetic information.

We isolated two classes of hybrid progeny from an outcross of the WS fluorescent *pai* mutant to Col: those that have inherited the *Δpai1-pai4* mutation and *MePAI3* from the WS mutant parent, but have inherited unmethylated *PAI2* from the Col parent (*Δpai1-pai4/Δpai1-pai4 PAI2/PAI2 MePAI3/MePAI3*, designated P2 MeP3 hybrids), and those that have inherited the *Δpai1-pai4* mutation and *MePAI2* from the WS mutant parent, but have inherited unmethylated *PAI3* from the Col parent (*Δpai1-pai4/Δpai1-pai4 MePAI2/MePAI2 PAI3/PAI3*, designated MeP2 P3 hybrids). Three independent P2 MeP3 segregants isolated are all nonfluorescent and morphologically wild type, whereas three independent MeP2 P3 segregants isolated all display full fluorescent mutant phenotypes. These lines have maintained their inherited methylation patterns over two generations of self-pollination (Figure 5). Thus, *PAI* methylation segregates in outcrosses as if it were a genetic trait.

PAI Hypomethylation Correlates with Increased Gene Expression

To examine correlations between loss of *PAI* gene methylation and loss of the fluorescent phenotype in our mutant lines, we analyzed steady-state levels of *PAI* transcripts in RNA samples prepared from the fluorescent mutant and revertant lines (Figure 6). We found that the hypomethylation in nonfluorescent revertant lines correlates with increased *PAI* gene expression relative to the isogenic fluorescent mutant. Northern blot analysis shows that the fluorescent *pai* mutant and MeP2 P3 hybrid lines have very low residual levels of *PAI* transcripts, whereas hypomethylated revertant lines or the P2 MeP3 hybrid lines have approximately 3-fold more *PAI* transcripts. Surprisingly, WS displays the highest level of *PAI* transcripts, even though it also displays heavy methylation of all four of its *PAI* genes. WS also uniquely displays a high molecular weight species that might represent a readthrough transcript of the *PAI1-PAI4* duplication.

To test whether chemically induced hypomethylation of the WS fluorescent *pai* mutant relieves *PAI* gene silencing, we grew the mutant on agar medium containing the methyltransferase inhibitor 5-azacytidine (5-AC) (Jones, 1985). We found that 5-AC concentrations as low as 1 µg/ml cause a complete loss of fluorescence in the epigenetic *pai* mutant at 2 weeks; in contrast, the genetic *trp1-100* mutant is fully fluorescent on this medium (data not

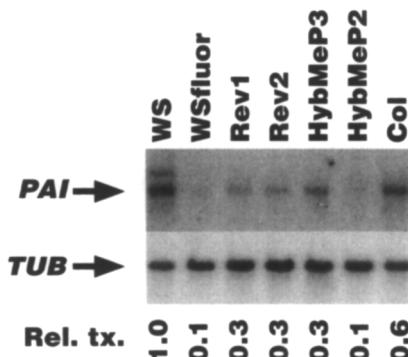


Figure 6. Hypomethylated Revertants Have Increased *PAI* Gene Expression Relative to the WS Fluorescent *pai* Mutant

Northern blot analysis of whole plant RNA prepared from 4-week-old plants of the same samples shown in Figure 5A. Duplicate blots were probed with an internal 0.7 kb PstI fragment of a *PAI*1 cDNA (*PAI*; exposed for 3 weeks) or an α -tubulin probe (*TUB*; exposed for 24 hr) to normalize loading of samples. Rel. tx., *PAI* transcript levels normalized to the major WS *PAI* band.

shown). These results suggest that methylation plays a direct role in loss of *PAI* gene expression.

***MePAI2*-Correlated Silencing Is Transmitted through Meiosis**

The *pai* mutant can segregate in crosses at close to the predicted Mendelian ratios if the female parent in the cross is homozygous mutant (Table 1, lines 1 and 3). However, when the female parent is heterozygous for the *pai* mutation, then the mutant phenotype is transmitted at approximately 3-fold less than the predicted Mendelian ratios (Table 1, lines 2 and 4). An analogous female gametogenesis defect has been previously described for the *trp4-1 trp1-100* double mutant (Niyogi et al., 1993). This defect, presumably caused by a deficiency in a product of the tryptophan pathway, prevents mutant gametes from competing successfully with wild-type gametes in a heterozygous female parent, so that they are underrepresented in the progeny population. We discovered our gametogenesis defect in standard backcrosses and outcrosses with the *pai* mutant: in a backcross to WS, only 6% (36 of 654) rather than the expected 25% of the F2 progeny are fluorescent, and in an outcross to Col only 2% (12 of 644) rather than the expected 6.25% of the F2 progeny are fluorescent. The reciprocal test crosses shown in Table 1 demonstrate that the low transmission of the mutant phenotype is specific to crosses through a heterozygous female parent.

Because one of the defects segregating in the Table 1 crosses is semistable and does not correspond to a DNA sequence mutation (*MePAI2*), we wanted to determine whether the low transmission of this defect is satisfactorily accounted for by the female gametogenesis deficiency or whether there is also a selective erasure of the *MePAI2* silencing program in outcrosses through a heterozygous female parent. We therefore analyzed the F1 progeny of

the test cross from line 4 of Table 1 not only for phenotype, but also for genotype at the *PAI*1 and *PAI*2 loci using polymorphic markers that distinguish Col from WS (see Experimental Procedures). In this outcross to the nonmethylated Col strain, fewer than 1% of the nonfluorescent progeny have erased the silencing program at the WS *MePAI2* locus, consistent with the 1%–5% reversion frequency observed in self-pollinating fluorescent mutant plants.

Discussion

A Natural Epigenetic Phenomenon in Arabidopsis

We have described the methylation of an endogenous gene family in the WS strain of *Arabidopsis* that is analogous to epigenetic phenomena described for a number of other animal, fungal, and plant systems. This epigenetic program is revealed by a deletion mutation that reduces the copy number of methylated WS *PAI* genes to confer a mutant phenotype dependent on the methylation and silencing of the remaining genes. Methylated and silenced loci from the deletion mutant can be meiotically transmitted through self-pollination and outcrosses, but they are no longer completely stable, so that in every generation a few percent of phenotypically wild-type germinal revertant plants arise. These revertant lines have coordinately hypomethylated their *PAI* genes. Once established, revertant lines remain in a stably hypomethylated and expressed state in subsequent generations. Our system thus demonstrates that methylation correlates with silencing of at least one *PAI* gene, that establishment of methylation and silencing are correlated with a high copy number of the affected genes, that methylation and silencing can be maintained for many generations after the copy number has been reduced, and that the signal that reverses this epigenetic program acts across the genome at unlinked sites rather than at individual loci.

Previous reports of gene silencing in *Arabidopsis* involve phenomena generated in transgenic strains. Some examples appear similar to the endogenous gene silencing described here in that they correlate with increased copy number and methylation of the affected sequences; however, interpretation of these examples is complicated by technical aspects of plant transformation. In particular, since plant transformation occurs by insertion of the transgene into random sites in the genome, each insert is influenced by different constraints on expression determined by the chromosome structure in the region of the insertion. Because transgenic lines do not form an isogenic series, it is difficult to compare how the copy number and arrangement of transgenes correlates with silencing across different lines. In contrast, the *PAI* genes in the methylated ecotype WS, the deleted *pai* mutant, and the unmethylated ecotype Col provide a natural isogenic series, with copy numbers of two, one, and no genes at the *PAI*1 locus and combinations of methylated or unmethylated genes at the other *PAI* loci that allow analysis of establishment, maintenance, and reversion of methylated genes at linked and unlinked sites.

Duplication of *PAI* Genes

A structural comparison of the *PAI* loci in WS and Col suggests that the *PAI* gene organization in these two strains was generated by a transposable element. The *PAI* locus in Col carries a single-copy *PAI*1 gene, whereas the *PAI* locus in WS contains duplicated *PAI* genes, *PAI*1-*PAI*4, plus a partial duplication, *pai*4 5', embedded in a complex structure of inverted and direct repeats (Figure 2). This type of structure is similar to transposon-generated rearrangements in maize and *Antirrhinum* biosynthetic genes (Bollmann et al., 1991; Stinard et al., 1993), suggesting that a progenitor *PAI* sequence might have been associated with a transposable element that moved copies of the sequence to unlinked sites in the *Arabidopsis* genome to create the *PAI*1, *PAI*2, and *PAI*3 duplications and to engender in addition a series of local rearrangement events at the *PAI*1 locus to create the *PAI*1-*PAI*4 duplication in WS but not in Col. The DNA sequence homologies of the *PAI* genes are also consistent with horizontal transfer of *PAI* genes by a transposable element.

Establishment of *PAI* Gene Methylation

Both the WS and Col ecotypes show the standard pattern of methylation concentrated mostly at repetitive rDNA and centromeric sequences, but not at single-copy or small gene family sequences (Pruitt and Meyerowitz, 1986; Vongs et al., 1993; J. B., unpublished data). However, WS carries an extra *PAI* gene (*PAI*4) relative to Col and, further, has the *PAI*1-*PAI*4 genes arrayed in an inverted repeat structure. A number of different models could account for how *PAI* gene methylation is signaled by this increased copy number.

For example, methylation could be triggered by a threshold level of ectopic pairing among the four WS *PAI* homologs, but not the three Col *PAI* homologs. Because ectopic pairing can interfere with chromosome segregation and lead to deleterious ectopic recombination events, repetitive regions of the genome could be singled out for methylation and association with factors that block ectopic pairing, as has been proposed for MIP and RIP in fungi (Selker, 1990). The *PAI* genes might be targeted for methylation by a pairing mechanism because these genes are homologous to each other over approximately 2 kb, including intron sequences. In this regard, introns might have evolved as a mechanism for breaking up stretches of homology between gene family members to prevent ectopic pairing.

Inverted repeats have been proposed to stimulate gene silencing by forming hairpins or other aberrant pairing structures. For example, silencing of a *mini-white* transgene in *Drosophila* is much more severe for inverted repeat transgenes than for direct repeat arrays (Henikoff and Dorer, 1994). In addition, plant transposon-generated inverted repeats in *Antirrhinum* (Bollmann et al., 1991) and maize (Stinard et al., 1993) can produce alleles that have dominant-negative effects on expression of the wild-type locus in a heterozygote, presumably through aberrant pairing interactions. In the case of *PAI* gene silencing, we are currently testing whether the WS *PAI*1-*PAI*4 inverted

repeat can communicate methylation to the unmethylated *PAI*2 and *PAI*3 genes from Col in hybrid plants. Transformation of our *Arabidopsis* strains with various segments and arrangements of *PAI* genes will also allow us to define better the nature of the signal that establishes silencing.

An alternative to the DNA pairing model for silencing is the RNA-directed DNA methylation model. In this model, methylation and silencing could be triggered by overexpression of *PAI* transcripts beyond a threshold level to feedback inhibit their own expression. For example, RNA-directed DNA methylation has been proposed in a case where replication-proficient plant RNA viroids in transgenic tobacco stimulate specific methylation of the transgene viroid DNA, while replication-defective viroid constructs do not (Wassenegger et al., 1994). However, in our system, *PAI* expression levels are relatively low even in the unmethylated strain Col (Figure 6), whereas the transgenic constructs described have extremely high expression levels.

Maintenance and Reversion of *PAI* Gene Methylation

The methylation of *PAI* genes established in WS has remained stably imprinted for more than ten generations of self-pollination and has also persisted in the *pai* mutant for at least six generations of self-pollination. Furthermore, methylated *PAI* genes can be transmitted from the *pai* mutant through crosses to the unmethylated ecotype Col in a Mendelian fashion. These observations suggest that methylation can be maintained and inherited even in the absence of an establishing stimulus.

Methylation-correlated gene silencing in mammalian (Jones, 1985), fungal (Goyon and Faugeron, 1989), and plant systems (Kilby et al., 1992) can be relieved by culturing silenced cells in the presence of the methyltransferase inhibitor 5-AC. We have found that when *pai* mutant plants are grown on medium containing 5-AC they are nonfluorescent, suggesting that methylation plays a direct role in the loss of *PAI* gene expression. However, methylation of the four *PAI* genes in parental WS does not cause a loss of total *PAI* transcripts. Because our genetic data demonstrate a loss of expression only at the WS *PAI*2 locus, it is possible that this is the only *PAI* locus where methylation correlates with silencing. A more detailed examination of the methylation patterns of the *PAI* genes using genomic sequencing methods might reveal different patterns for the different genes diagnostic of different expression states.

An important observation from our analysis of spontaneous nonfluorescent revertant plants is that these plants become coordinately hypomethylated at two unlinked *PAI* loci. This coordinate hypomethylation is not due to loss of methylation at one locus potentiating loss from the second locus, because hybrid plants that have a methylated WS *MePAI*2 gene but an unmethylated Col *PAI*3 gene, or vice versa, can stably maintain these methylation patterns. Instead, coordinate loss of methylation in revertant lines implies a concerted reprogramming of the unlinked *PAI*2 and *PAI*3 genes. This result is consistent with the finding that two independent maize genes, each under negative control by an epigenetically regulated *Mutator* element, coor-

dinately revert in leaf tissue sectors and coordinately increase *Mutator* methylation in these sectors (Martienssen and Baron, 1994).

Revertant cells might represent populations within the plant that are susceptible to a reversion signal because they have undergone a particular developmental transition. For example, reversion of the *Mutator*-regulated leaf mutations has been proposed to occur in cells with a prolonged cell cycle, affording extra time for a genome-wide resetting of epigenetic control (Martienssen and Baron, 1994). Consistent with this model, we observe the highest frequency of *pai* mutant somatic sectoring on late-developing leaves. Furthermore, epigenetic reprogramming in germinal cells allows these plants to create phenotypic diversity in a genetically homogeneous population.

Usefulness of the *PAI* System

Among examples of epigenetic phenomena from systems as diverse as imprinting of genes in mammals (Peterson and Sapienza, 1993), repeat-induced methylation in *Aspergillus* and *Neurospora* (Selker, 1990), regulation of transposable elements in maize (Martienssen and Richards, 1995), and the *Arabidopsis PAI* gene silencing described here, there are common mechanistic themes: changes in gene expression correlate with cytosine methylation of the affected locus, and, once established, the affected locus can be chromosomally inherited. In addition, most of these examples correlate with an increased copy number of the affected locus. Thus, these phenomena are likely to have evolved from a common ancestral mechanism.

The epigenetic regulation of the *Arabidopsis PAI* genes provides a number of unique advantages for dissecting the mechanism of epigenetic control with a molecular-genetic approach. For example, the ability to score gain and loss of *PAI* gene silencing with a visual screen for gain and loss of blue fluorescence makes it possible to use this system as the basis for mutant screens for *trans*-acting loci that either destabilize or hyperactivate *PAI* gene silencing. A further advantage of the *Arabidopsis* system is that it is likely to be tolerant of defects in epigenetic regulation, because the *Arabidopsis* methylation regulatory mutant *ddm1*, which has 70% reduced cytosine methylation, is viable and fertile (Kakutani et al., 1995; Vongs et al., 1993). In contrast, mouse mutants deficient in a methyltransferase gene are only approximately 30% hypomethylated, but die during embryogenesis (Li et al., 1992). Therefore, our observation that the simple, genetically tractable plant *Arabidopsis* has maintained epigenetic controls analogous to those found in more complex eukaryotes provides a valuable system for the study of these mechanisms.

Experimental Procedures

Mutant Isolation

T-DNA-mutagenized pools of WS (Errampalli et al., 1991) were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University and grown on plant nutrient sucrose medium (Haughn and Somerville, 1986) for 3 weeks. Seedlings were screened for blue fluorescence under short wavelength ultraviolet (UV) illumination, and five independent mutants were isolated. In none of the five mutant isolates

was the fluorescence phenotype linked to the kanamycin-resistant T-DNA tag. One kanS line was selected for further analysis.

Protein and DNA Methods

Tryptophan enzymes were assayed as described (Last and Fink, 1988; Niyogi et al., 1993). Standard techniques for cloning, sequencing, and hybridizations were performed as described previously (Ausubel et al., 1989).

Mapping Methods

DNA was prepared from 72 fluorescent F₂ plants from a cross of the WS fluorescent mutant to Col to map the chromosomal locations of the two defects that confer fluorescence. Standard CAPS (Konieczny and Ausubel, 1993) and SSLP (Bell and Ecker, 1994) PCR-based mapping was performed as described previously. One defect mapped between SSLP markers nga59 and nga63 and was completely linked to a *PAI1* CAPS marker. The second defect was completely linked to the standard ASA1 CAPS marker and to a *PAI2* CAPS marker. Information about primers, PCR conditions, and enzymes for the *PAI1*, *PAI1*, *PAI2*, and *PAI3* CAPS markers reported here is available upon request. The map positions of the WS *PAI1*, *PAI2*, *PAI3*, and *PAT1* markers were determined on a WS × Col mapping population (gift of Dr. B. Bartel).

Analysis of Test Cross Progeny

To analyze the genotypes of the progeny of the test cross in line 4 of Table 1, we used our *PAI* gene-specific markers to determine whether *PAI1* and *PAI2* loci were inherited from the WS or from the heterozygous parent. Mendelian segregation predicts that, at any locus, 50% of the progeny should be homozygous WS (W) and 50% should be heterozygous WS/Col (H). Thus, the unlinked *PAI1* (P1) and *PAI2* (P2) genes are predicted to segregate at 25% P1W P2W:25% P1W P2H:25% P1C P2H:25% P1H P2H. As shown in Table 1, line 4, experimentally only 7% of the progeny of this cross are fluorescent. We found that all (30 of 30) of these fluorescent plants have the P1W P2W genotype. We also analyzed 192 randomly selected nonfluorescent F₁ plants as representative of the other 93% of progeny from the cross. We found that 46 of 192 × 93% (22%) are P1W P2H; 53 of 192 × 93% (25.5%) are P1H P2W; 92 of 192 × 93% (48%) are P1H P2H; and 1 of 192 × 93% (0.5%) is P1W P2W. If only the P1W P2W double *pai* defect genotype is selected against during female gametogenesis, equal proportions of the other three classes of progeny would be expected ($33\% \times 93\% = 31\%$ each). In fact, the P1W P2H class (22%/31% = 70% of expected) and the P1H P2W class (25.5%/31% = 82% of expected) are also underrepresented in this cross. At an unlinked marker, nga172, on chromosome 3 (Bell and Ecker, 1994), 42 of 87 (48%) plants from the nonfluorescent F₁ population were homozygous WS, and 45 of 87 (52%) were heterozygous, showing that an unlinked marker can segregate at close to expected proportions (50% W and 50% H) in this population.

Cloning WS *PAI* Genes

A Col ecotype *PAI1* cDNA (*PAI1c* is B2869) was isolated from the λYES *Arabidopsis* cDNA library (Elledge et al., 1991) by complementation of an *Escherichia coli* strain defective in *PAI* activity. A 0.7 kb internal PstI fragment from this cDNA clone was routinely used as a hybridization probe for Northern and Southern blot analyses of *PAI* genes. *PAI* genes were cloned from WS by screening a WS genomic library constructed by Sau3AI partial digestion of WS DNA ligated into BamHI-cut λDASH arms (Stratagene) with the *PAI1c* probe. *PAI* genes were also cloned from the fluorescent WS mutant by constructing a library from mutant DNA as described for the WS library and screening with the *PAI1c* probe. Inserts from these λ isolates were cloned as NotI fragments into pBluescript KS(II+) (Stratagene) for restriction mapping and sequence analysis (WS *PAI1-PAI4g-1* and *PAI1-PAI4g-2* are B3177 and B3178, respectively; WS *PAI2g-1* and *PAI2g-2* are B3179 and B3180, respectively; WS *PAI3g-1* and *PAI3g-2* are B3181 and B3182, respectively; and WS *Δpai1-pai4g* is B3183).

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