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Selection and orientation of adjacent genes influences DAM-mediated male sterility in transformed maize

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Abstract

Anther-targeted expression of *E. coli* DNA (Adenosine-N6-)-Methyltransferase (DAM) in maize was tested as a means to produce male-sterile plants. A high frequency of male-sterile plants with reduced anther size was observed when DAM was regulated by the maize anther-specific promoter 5126 (5126:DAM) and placed upstream of the herbicide resistance gene, *pat*, regulated by the cauliflower mosaic virus (CaMV) 35S promoter (35S:PAT). In contrast, placement of 5126:DAM upstream of a *pat* gene regulated by either the maize ubiquitin (UBI:PAT) or rice actin (rACTIN:PAT) promoters resulted in male-fertile plants. Based on these observed differences, DAM-mediated sterility was used as a phenotypic marker to assess the contribution of factors affecting gene expression such as orientation of the transcription units, choice of regulatory sequences mediating expression of adjacent genes, and effects of varying the anther-specific promoter regulating DAM. Constructs that place a portion of the CaMV 35S promoter, including the native AS-1 sequences, between 5126:DAM and UBI:PAT yielded a high frequency of male-sterile plants with reduced anther size. Significant differences in the frequency of male-sterile events and the associated anther size were also observed when the position of 35S:PAT was changed relative to 5126:DAM. These data provide evidence that gene expression in transformed maize plants can be impacted by simply altering the order, orientation or regulatory sequences of adjacent genes.

Introduction

The spatial separation of the male and females flowers in maize is an important agronomic tool that affords a means to control pollen release for the generation of hybrid corn. Conventional hybrid production utilizes mechanical removal of the terminal tassel prior to pollen shed or genetic conversion of the female inbred parent line to a C- or S-type male sterile cytoplasm to effect cross-pollination by the male inbred parent planted in adjacent rows (Wych, 1988). The identification of tissue-specific promoters, together with the improvements in maize transformation capabilities, has allowed the development of dominant sterility systems as an additional approach to generate male-sterile plants for use in hybrid production (Greenland et al., 1998). Practical implementation of this type of dominant pollen control requires linkage of the male-sterile phenotype to a selectable trait such as herbicide resistance. Constitutive expression of the phosphinothricin *N*-acetyltransferase gene (*pat*) would confer herbicide resistance to the regenerated male-sterile plants and could also be used as a selectable marker for maize transformation (Gordon-Kamm et al., 1990).

Several studies in plants have demonstrated that different promoter:gene combinations can be effective in perturbing normal function in male-reproductive tissues resulting in dominant male-sterile plants (Koltunow et al., 1990; Mariani et al., 1990; Thorness et al., 1991; Worrall et al., 1992; Goldberg et al., 1993; Kriete et al., 1996; Custers et al., 1997; Beals & Goldberg, 1997; De Block et al., 1997; Hernould et al., 1998). Recent work in tobacco (Van Blokland et al., 1998) and maize suspension cells (Brouwer, 1998) have found that constitutive expression of the *E. coli* DNA (Adenosine-N^6-)-Methyltransferase (DAM) gene (Brooks et al., 1983) results in developmental abnormalities and inhibition of cell growth,

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respectively. Moreover, adenine methylation has been reported to influence the transient expression of reporter genes tested in barley aleurones and protoplasts (Graham & Larkin, 1995; Rogers & Rogers, 1995). Although the mechanisms by which the DAM enzyme influences cell growth and/or gene function in the studies described are unknown, these observations suggested that anther directed expression of DAM could also be a viable means to render maize male sterile. Toward this end, the *E. coli dam* gene was first placed under the transcriptional control of maize anther specific promoters and upstream of the various constitutively expressed *pat* genes on a T-DNA vector and transformed into maize. Interestingly, a high frequency of male-fertile plants were recovered when anther-directed expression of *dam* was linked to herbicide selection systems that employed a maize ubiquitin or a rice actin promoter to transciptionally regulate *pat* expression. This was in contrast to the high frequency of male-sterile plants recovered when anther-directed expression of *dam* was placed upsteam of a 35S:PAT gene. Based on these observations, anther specific expression of DAM and the resultant fertility phenotype was developed as an *in vivo* assay to systematically test and evaluate factors (e.g., gene order, orientation, enhancer elements) within transformation cassettes that modulate gene expression in maize. Maize is particularly well suited for using fertility as a phenotypic marker since the male (tassel) and female (ear) reproductive structures are spatially separated. In addition, using male-fertility as an indirect measurement to assess gene expression provides an alternative to quantitative reporter gene assays as these are often labor intensive, require accurate developmental timing when measuring reporter gene activity and often do not correlate well with biological manifestation of a given trait or phenotype. The observations made using this phenotypic assay may be relevant to other studies that test promoter or gene function as they report the impact gene order and orientation of transcription units within a T-DNA can have on gene expression in transformed plants.

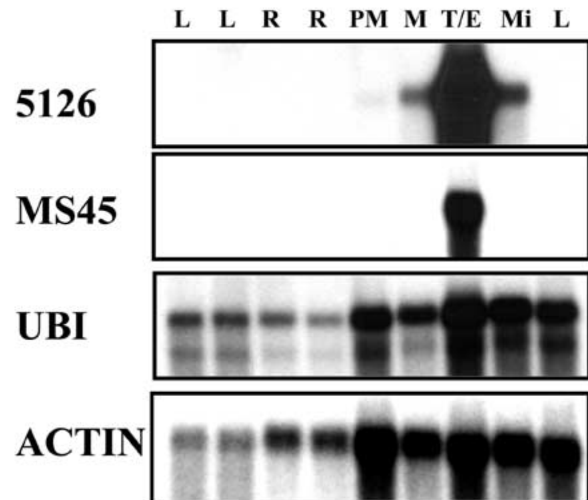

Figure 1. RNA hybridization analysis indicates anther expression of the maize 5126 and MS45 mRNA. RNA was isolated from 2-week-old soil-grown maize seedlings (A188) using 200 mg frozen pulverized leaf (L), or root (R). Anther RNA was isolated by harvesting 100 anthers from a region on a developing tassel identified microscopically to be at the following developmental stages of microsporogenesis: premeiotic (PM), early meiosis (M), tetrads and early vacuolate microspores (T/E), mid-vacuolate microspores (Mi), and late (vacuolated) microspores (L). Poly(A)$^{+}$ enriched RNA (ca.0.5 μg) was loaded into each lane and the filters were hybridized with DNA probes as described in the Methods. Exposure to film for all of the probes were similar (2–4 h) with the exception of actin, which required a 9 h exposure.

Results

Anther-directed expression of E. coli *DNA Adenosine methyltransferase renders maize male sterile and results in abnormal tapetal cells and microspores*

In order to test the ability of *E. coli* DAM to render maize plants male sterile when expressed in a tissue specific manner, two candidate anther-specific promoters were identified based on expression patterns of their corresponding cDNAs. 5126 originated from a maize tassel cDNA library (described in Cigan & Albertsen, 1997) while MS45 was isolated through genetic and molecular analysis of a nuclear recessive male-sterile maize mutant (Albertsen et al., 1993). RNA hybridization analysis presented in Figure 1 is consistent with the prediction that these cDNAs encode gene products found in male-reproductive tissues. Transcripts encoding 5126 and MS45 were readily detected in developing anthers at approximately the tetrad to early vacuolate stage of microspore development (Chang & Neuffer, 1989), while root and leaf tissues did not reveal the presence of these transcripts (Figure 1). As described in Figure 2, promoter: *dam* fusions using either 5126 or MS45 were placed on a T-DNA vector adjacent to a *pat* gene, regulated by different plant constitutive promoters. The constructs were introduced into HI-type II embryos via *Agrobacterium*-mediated transformation. Transformed callus was selected on bialophos-containing media, plants were regenerated and the male-fertility

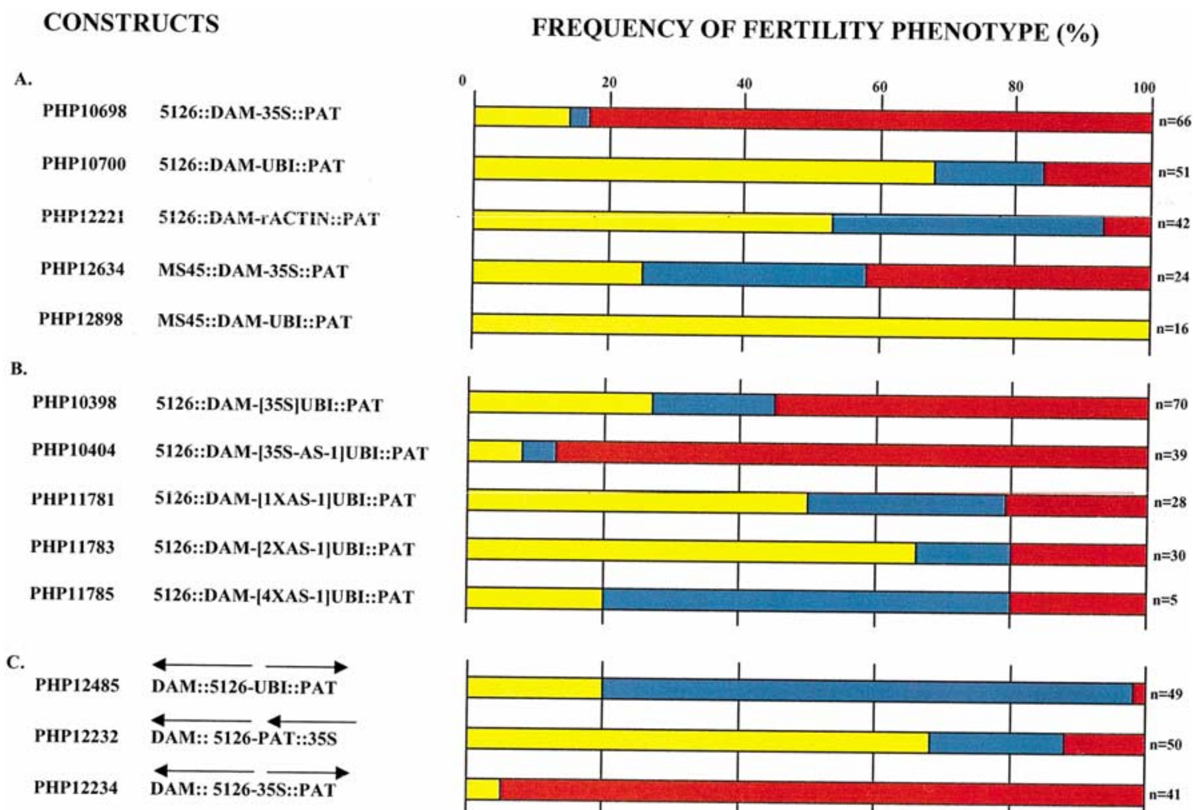

Figure 2. Frequency of generating male-sterile maize plants is impacted by the promoter choice and gene orientation relative to the selectable marker. The percentage of the three classes of male-fertility phenotype observed for each construct is represented by the bar graph. The size of the red, blue and yellow bars correspond to the number of events (expressed as a percentage of the total number of events) classified as male-sterile, shedder and male-fertile, respectively. The total number of independent events (*n* =) for each construct is listed to the right of each bar graph. An event represents transformed plants that are identical as determined by DNA hybridization analysis of the inserted T-DNA after plant regeneration. The constructs are identified numerically and descriptively at the left of the figure. Arrows above the construct descriptions in Section C depicts the direction of transcription of each gene.

phenotype evaluated. DNA hybridization analysis of genomic DNA from individual regenerated plants was used to identify independent transformation events and to establish transformed vector copy-number. As has been previously reported for *Agrobacterium*-mediated transformation of maize (Ishida et al., 1996), 70% of the regenerated plants in these experiments contained one to two copies of the T-DNA inserts integrated with no significant rearrangement detected (data not shown).

Generally, three different male-fertility phenotypes were observed in the regenerated plants (T0 generation): Male-sterile plants in which anthers were not extruded from the glumes and did not contain pollen, 'shedders', which are a class of male-sterile plants that extruded a few anthers containing small amounts of pollen, and male-fertile plants that extruded anthers filled with pollen. When 5126:DAM was present upstream of the 35S:PAT selectable marker in PHP10698 (5126:DAM-35S:PAT), a high-frequency of male-sterile maize plants was observed in the T0 generation; 83% of the events were male sterile, 3% were 'shedders' and 14% were male fertile (Figure 2A). Male-sterile plants were morphologically normal in stature and development and ears produced seed when pollinated by wild-type inbred maize lines. As shown in Figure 3A, sterile anthers from plants transformed with 5126:DAM-35S:PAT were consistently one-third to one-half the size of fully-developed fertile anthers and did not contain microspores beyond the early vacuolate stage of development. The male-sterile and herbicide resistant phenotypes conferred by 5126:DAM-35S:PAT were stable and heritable in subsequent generations, with the majority of the events

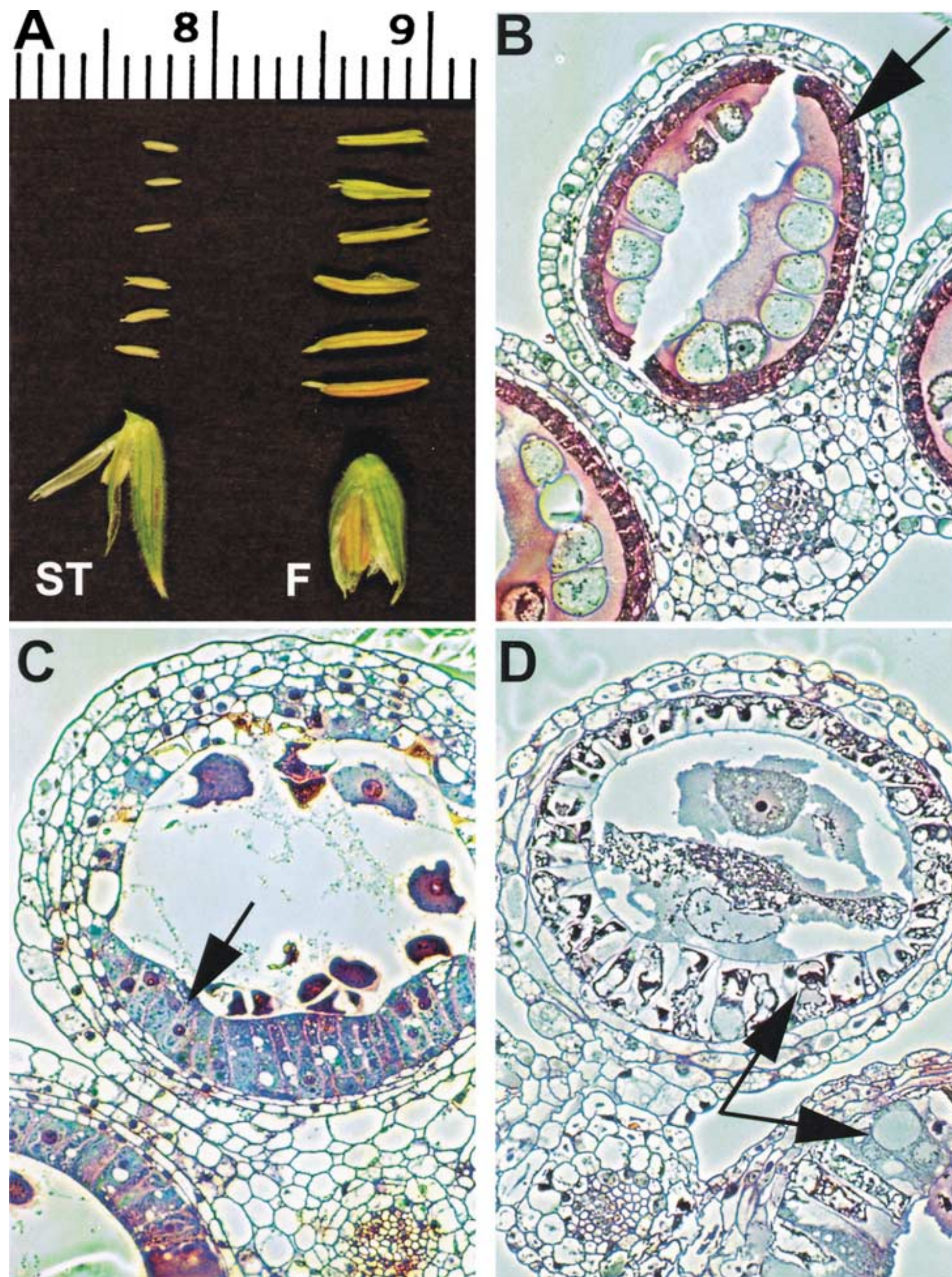

Figure 3. Anthers containing 5126:DAM-35S:PAT are small in size and exhibit an abnormal tapetum. A. Anthers and glumes from male-sterile (ST) 5126:DAM-35S:PAT containing plants and wild-type male-fertile (F) plants harvested at maturity. B. Cross-section of wild-type anthers harvested at tetrad stage of microspore development. C and D are cross-sections of male-sterile anthers from two independent events containing 5126:DAM-35S:PAT harvested at two days after the identification of microspore mother cells containing condensed chromatin representative of nuclear events early in meiosis. In wild-type anthers from similarly grown plants, this time point typically corresponds to the early vacuolate stage of microsporogenesis. Arrows point to the tapetum.

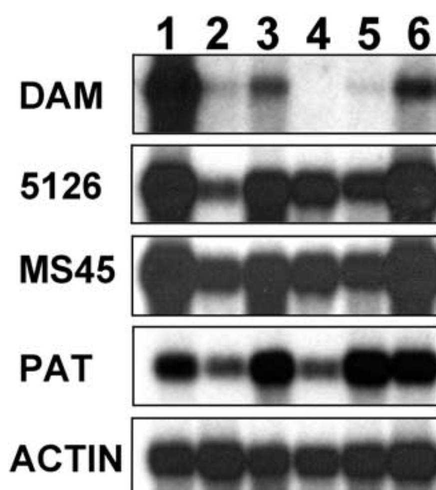

Figure 4. Plants containing 5126:DAM frame-shift express normal levels of 5126 and MS45 transcripts in anthers. Poly(A)$^+$ RNA was isolated from 100 anthers at tetrad to early vacuolate stage of microspore development. Lanes 1–6 represent 0.5 μg of anther RNA from independent events transformed with 5126:DAM f/s-35S:PAT (PHP8146) using microprojectile bombardment of immature maize embryos (Tomes et al., 1995). The filter was hybridized sequentially with the probes described to the left of the panel. Exposure to film for all of the probes were similar (2–4 h) with the exception of DAM, which required 5 days.

Figure 5. Endogenous 5126 and MS45 RNA transcripts are absent in anthers from male-sterile 5126:DAM-35S:PAT plants. Poly(A)$^+$ RNA was isolated from fertile (F) and male-sterile 5126:DAM-35S:PAT (S) anthers as described in legend of Figure 1. Fertile anthers were harvested at early vacuolate stage of microsporogenesis, while the male-sterile anthers were collected at an equivalent developmental stage (see Figure 3 legend). Filters were sequentially hybridized with the probes described to the left of the panel.

exhibiting an expected Mendelian segregation ratio (1:1) for single insertions of the linked traits. Therefore, it is not likely that the male-sterile phenotype was due to the process of transformation or plant regeneration. It is also unlikely that the observed male-sterile phenotype was a result of an indirect mechanism affecting the expression of the endogenous 5126 gene. Transformation of maize with a version of the *dam* coding region containing a 1 base pair frame-shift mutation (5126:DAM f/s) yielded only male-fertile plants. As shown in Figure 4, hybridization analysis of anther RNA from plants containing this frame-shifted *dam* gene indicated detectable levels of the *dam* transcript as well as normal levels of endogenous anther specific 5126 mRNA found in untransformed plants (Figure 1).

In contrast to the readily detected DAMf/s, 5126 and MS45 transcripts in anthers from 5126:DAM f/s containing plants (Figure 4), RNA hybridization analysis revealed that endogenous 5126 and MS45 mRNAs were reduced or undetectable in anthers from male-sterile PHP10698 plants (Figure 5). The *dam* transcript was only weakly detected in a few male-sterile events following 5–7 day exposure to film (data not shown). The steady-state levels of the ubiquitin and actin mRNAs were not significantly altered suggesting 5126 and MS45 genes may be expressed in a cell-layer specific manner within the anther. To examine the tissue layer specificity of 5126:DAM mediated sterility, maize anthers harvested at the meiosis II to early vacuolate microspore stage of development were prepared from untransformed fertile plants and compared to similarly staged anthers from male-sterile plants containing different single-copy insertions of PHP10698 (5126:DAM-35S:PAT). The cross-sections in Figure 3C and D are representative of anthers from male-sterile plants containing 5126:DAM-35S:PAT. The microspores were arrested during late meiosis II to tetrad formation, while tapetal cells from these male-sterile plants were highly vacuolated and not uniform in size as compared to fertile anthers from untransformed plants (Figure 3B) and to maize anther sections in the literature (Kiesselbach, 1999). The timing of DAM-mediated breakdown of the tapetum and microspores was consistent with transcriptional expression pattern of the 5126 mRNA, which expressed maximally during tetrad to early vacuolate stages of microsporogenesis (Figure 1). Together these results suggested that the male-sterility phenotype associated with 5126:DAM-35S:PAT was the result of anther specific expression of the *E. coli dam* gene.

The frequency and stability of obtaining DAM mediated male-sterility is reduced when the linked pat *gene is transcribed by ubiquitin or actin promoters*

In contrast to the results described above, the frequency and stability of the male-sterile phenotype of 5126:DAM transformed maize plants was reduced when promoters other than CaMV 35S transcribed the adjacent herbicide resistance marker, *pat* (Figure 2A). In maize plants transformed with PHP10700 (5126:DAM-UBI:PAT), only 16% of the events were male-sterile. The difference between PHP10700 and PHP10698 (5126:DAM-35S:PAT), is that in PHP10700, the maize ubiquitin promoter replaced the CaMV 35S promoter for expression of the *pat* gene. As shown in Table 1, among the 36 single copy PHP10700 events, only three were male sterile. This was in contrast to the plants containing PHP10698 where 37 of the 46 single-copy insertion events yielded male-sterile plants. Moreover, the size of male-sterile anthers from PHP10700 events correlated with the number of PHP10700 inserts. Events having more than two T-DNA inserts exhibited small anthers (one-third to one-half the size of fully developed fertile anthers), while plants containing one T-DNA insert had anthers that were nearly wild-type in size. These observations suggested that DAM functioned in PHP10700, but the ability to recover male-sterile plants was dependent upon the number of inserted T-DNAs. The majority of events generated with PHP10700 were classified as either male-fertile or shedders (Figure 2A and Table 1). In progeny of single-copy sterile events containing PHP10700, only male-fertile herbicide resistant plants were recovered despite the observation that intact 5126:DAM-UBI:PAT inserts were inherited at the expected frequency as determined by DNA hybridization analysis (data not shown). Similar observations were made when the rice actin promoter was used to express the herbicide resistance gene, *pat*. Seven percent of the events transformed with 5126:DAM-rACTIN:PAT (PHP12221) were classified as male-sterile, 40% shedders and 53% male-fertile in the T0 generation (Table 1). Advancement of the single-copy shedder class of PHP12221 events yielded only male-fertile herbicide resistant plants in the T1 generation.

Table 1. Male-sterility associated with 5126:DAM/35S:PAT is independent of insert copy number

CONSTRUCT	COPY	MALE-FERTILITY PHENOTYPE FERTILE	SHEDDER	STERILE	
5126::DAM/35S-PAT PHP10698	1	7	2	37	
	2	0	0	0	
	>2	2	0	18	
		9	2	55	n=66
		13%	3%	83%	
5126::DAM/Ubi-PAT PHP10700	1	28	5	3	
	2	0	0	1	
	>2	7	3	4	
		35	8	8	n=51
		68%	16%	16%	
5126::DAM/rACTIN-PAT PHP12221	1	18	8	1	
	2	3	6	0	
	>2	1	3	2	
		22	17	3	n=42
		53%	40%	7%	

For the first three constructs described in Figure 2A, the number of events containing single, two or greater than two T-DNA inserts is shown for each class of male-fertility phenotype. The number of *dam* and *pat* inserts was determined for each event by DNA hybridization analysis (Southern, 1975). The percentages shown in this table correspond to the frequencies depicted as red, blue or yellow bars in Figure 2A.

The reduced frequency and instability of the male-sterile phenotype associated with replacement of the CaMV35S promoter in 5126:DAM-35S:PAT(PHP10698) was also observed when a different anther specific promoter, MS45, directed the expression of *dam*. In contrast to the high frequency of male-sterile events observed with PHP10698, only 42% events recovered with MS45:DAM-35S:PAT (PHP12634) were male-sterile (Figure 2A). Within the male-sterile population though, four events contained single-copy insertions of PHP12634 and had anther sizes similar to that observed with 5126:DAM-35S:PAT. The reduced frequency of male-sterile events associated with MS45:DAM-35S:PAT when compared to 5126:DAM-35S:PAT may be due weaker transcriptional activity of the MS45 promoter as the steady-state level of the MS45 mRNA was lower than that of 5126 (Figure 1). This was consistent with the observation that when MS45:DAM was linked 5′ to the UBI:PAT selectable marker, no male-sterile events were recovered (Figure 2A). Together these results indicate that the ability of 5126:DAM or MS45:DAM to confer a male-sterile phenotype was dependent upon the choice and placement of the selectable marker.

Frequency of male sterile maize increases when 35S sequences are placed downstream of 5126:DAM

As shown above, placement of either UBI:PAT or rACTIN:PAT 3′ to the 5126:DAM gene resulted in a low-frequency of male-sterile plants, while a similar

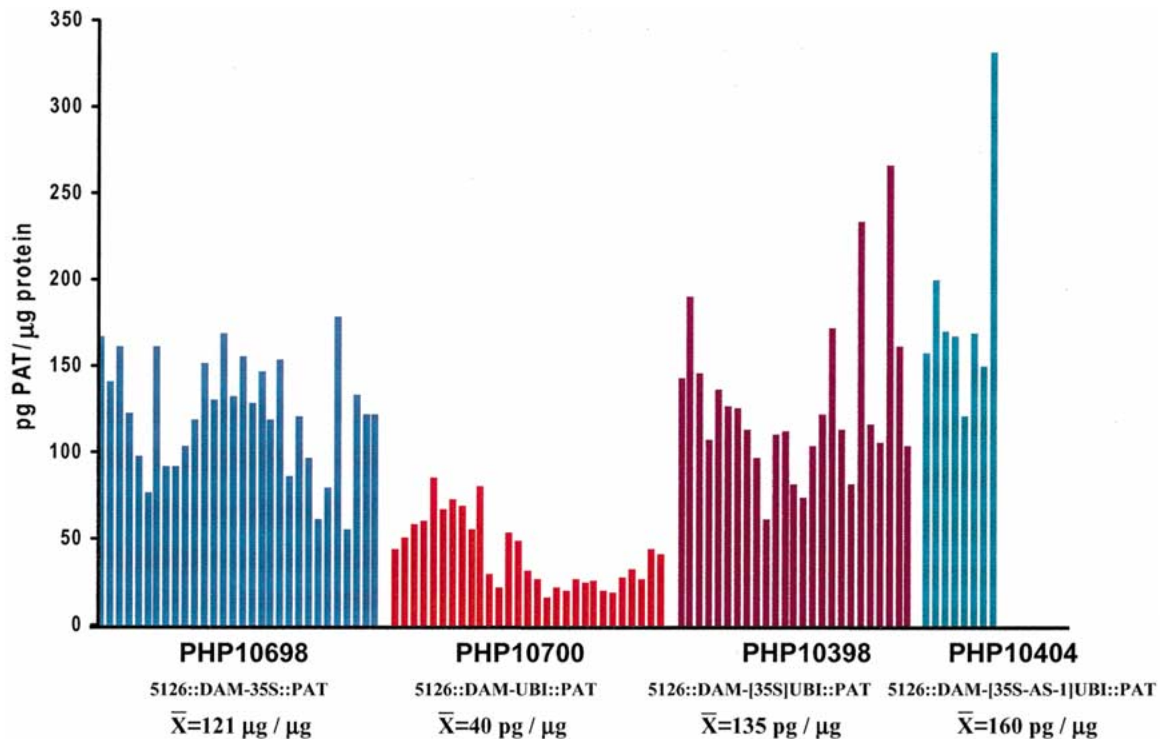

Figure 6. High levels of PAT expression in leaf correlates with the presence of 35S sequences. PAT protein levels were determined using an ELISA assay and are expressed as pg PAT detected per microgram total protein. Each bar represents the PAT protein value and is an average of duplicate samples assayed for each low-copy event. The corresponding construct and the mean PAT protein level are described along the *x*-axis.

placement of 35S:PAT resulted in a high frequency of male-sterility. To determine which regions of the CaMV 35S promoter contribute to the enhanced phenotypic expression of 5126:DAM, sequences of the 35S promoter were placed into PHP10700, in between 5126:DAM and UBI:PAT. A high-frequency of male-sterile plants was observed in events transformed with PHP10404 (5126:DAM[35SAS-1]UBI:PAT), a construct that contains a 376 bp fragment from the CaMV 35S promoter that includes the AS-1 element, an auxin-responsive element identified in tobacco (Lam et al., 1989; Liu & Lam, 1994) (Figure 2B). Several single-copy PHP10404 events were male sterile with the small anther phenotype similar to that observed with PHP10698 (5126:DAM- 35S:PAT). Furthermore, the small anther size and the frequency of male-sterile events associated with the regenerated plants were maintained in subsequent generations. Upon removal of the AS-1 element (PHP10398; 5126:DAM[35S]UBI:PAT), a reduction in the frequency of male-sterile events was observed as compared to PHP10404 (55% and 83%, respectively). In contrast to PHP10404, several of the single-copy male-sterile PHP10398 plants displayed anthers that were nearly wild-type in size and the sterility phenotype was not inherited to the next generation.

In addition to the observed increase in the sterility phenotype frequency when the CaMV35S sequences were inserted into these constructs, *pat* gene expression also increased. The effects of the inserted CaMV35S sequences on expression of the 3′ UBI:PAT gene were examined using ELISA assays. Leaf PAT protein amounts (pg/µg total protein) from plants containing PHP10698, PHP10700, PHP10398, and PHP10404 are shown in Figure 6. Placement of the 35S sequences with or without the native AS-1 element upstream of UBI:PAT resulted in an average three-fold increase in PAT protein over the average UBI:PAT value (PHP10398, PHP10404 and PHP10700 averaged 135 pg/µg, 160 pg/µg and 40 pg/µg, respectively). PAT levels in PHP10398 and PHP10404 plants were equal to or slightly higher than the PHP10698 plants (121 pg/µg), respectively.

To examine the possibility that the AS-1 elements were responsible for the phenotypic effects observed with PHP10398 or PHP10404, single or multiple cop-

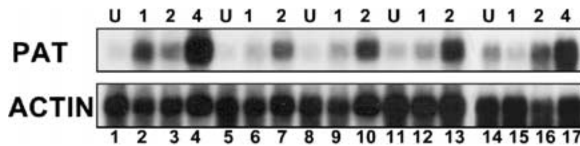

Figure 7. Multiple AS-1 elements incrementally increase *pat* RNA in maize callus. PolyA$^+$ RNA was harvested from callus grown on maintenance media that contained 1.5 mg/l 2,4-D. Lanes 1–17 represent 0.5 μg RNA prepared from independent low-copy events containing (1) 5126:DAM[1XAS-1]-UBI:PAT: PHP11781, (2) 5126:DAM [2XAS-1]-UBI:PAT: PHP11783, (4) 5126:DAM [4XAS-1]-UBI:PAT:PHP11785 and a UBI:PAT (U) containing construct. The filters were hybridized with *pat* and Actin DNA probes.

ies of the AS-1 element were placed into PHP10700. Introduction of one, two and four copies of AS-1 (PHP11781, 11783 and 11785, respectively) that were placed 3′ to 5126:DAM and 5′ to UBI:PAT did not significantly increase the frequency of male-sterile plants recovered as compared to PHP10700 (Figure 2B). However, these multimerized elements did have an effect on the expression of the downstream UBI:PAT gene in maize callus. RNA hybridization analysis shown in Figure 7 indicates that as the number of inserted AS-1 elements increased from none (UBI:PAT) to 4X (PHP11785) an incremental increase in *pat* steady-state transcript levels was observed. Thus, despite the absence of an observable effect on the upstream 5126:DAM gene, the data indicate that these 52 nucleotides can function to increase downstream *pat* expression in maize callus grown in the presence of 2,4-D.

Orientation of 5126:DAM relative to the juxtaposed selectable marker impacts the male-sterility phenotype

In the previously described constructs, the promoter transcribing the *pat* gene was immediately 3′ of 5126:DAM. To determine whether relative gene orientation influences the frequency of recovering male-sterile plants, PHP10700 and PHP10698 were altered to place the 5126 promoter proximal to the Ubiquitin promoter in PHP12485 (DAM:5126-UBI:PAT) and the CaMV35S promoter in PHP12234 (DAM:5126-35S:PAT). In PHP12232, the transcription termination region of 35S:PAT was placed proximal to the 5126 promoter (DAM:5126-PAT:35S). Although inverting the orientation of 5126:DAM relative to the Ubiquitin promoter (PHP12485) increased the total number of events displaying an intermediate shedder phenotype in the T0 generation, this construct did not significantly increase the observed frequency of male-sterile events when compared to PHP10700. In contrast, altering the orientation of the components comprising PHP10698 resulted in two different observations. Placement of 5126:DAM downstream of 35S:PAT (PHP12232), resulted in a significant reduction in the frequency of male-sterile events (Figure 2C). Of the male-sterile events recovered with PHP12232, all contained multiple copy insertions of the vector and had nearly wild-type size anthers. Conversely, when the 5126 promoter was placed proximal to the 5′ region of the 35S:PAT gene (PHP12234), the frequency of male-sterile events increased to 95%. Interestingly, sterile anthers from single-copy insertions of PHP12234 were slightly smaller when compared to sterile anthers from PHP10698 single-copy events. The frequency of recovering male-sterile events and the associated small anther size of PHP12234-containing plants was inherited in subsequent generations. Together these observations suggested that the orientation of 5126:DAM relative to 35S:PAT had a strong influence on the frequency of recovering male-sterile maize plants.

Discussion

In this report, the *E. coli* DNA adenine methyltransferase gene has been used to generate male-sterile corn plants when placed under the transcriptional control of the anther specific maize promoters 5126 and MS45. Aside from being male sterile, plants containing these constructs were otherwise morphologically normal. Cytological examination of the anthers from 5126:DAM-35S:PAT male sterile plants revealed that the tapetal cell-layer was abnormal and is likely dysfunctional, resulting in cessation of microspore development. It is unlikely that DAM-mediated sterility occurred via a co-suppression mechanism (for review see, Fagard & Vaucheret, 2000), as introduction of a non-functional *dam* (5126:DAM f/s) yielded male-fertile plants with detectable DAM(f/s) transcripts and normal levels of endogenous 5126 mRNA. It is more likely that DAM-mediated male sterility was a direct consequence of *dam* expression during tassel development as the efficiency of recovering male sterile plants was directly related to the relative strength of the anther specific promoter (5126 vs. MS45). Previous studies have shown that constitutive expression of *E. coli dam* in tobacco was correlated with increased adenine methylation of genomic DNA and an abnormal plant morphology (Van Blokland et al.,

1998). It is unclear whether expression of 5126:DAM in maize anthers functions in a similar fashion to alter normal cell processes as examination of anthers from male sterile plants has not revealed increases in adenine DNA methylation (unpublished observation). This may be expected as the tapetum is a single cell layer within the anther that is morphologically abnormal in 5126:DAM-35S:PAT male-sterile plants.

As described above, the frequency of recovering male sterile maize plants was dependent upon the choice of anther specific promoters directing the transcription of DAM. Surprisingly, factors other than the anther specific promoter also greatly impacted the proportion of male sterile plants generated by a specific construct. Varying the constitutive promoters expressing the juxtaposed *pat* gene resulted in dramatic differences in the frequency of DAM-mediated sterility. In contrast to the frequency observed with plants transformed with 5126:DAM-35S:PAT, when 5126:DAM was placed upstream of either the maize Ubiquitin- or rice Actin-*pat* gene, the frequency of recovering male-sterile events was low and stable inheritance of this phenotype was dependent on the presence of multiple inserts of 5126:DAM. Placement of portions of 35S sequences with or without an AS-1 element downstream of 5126:DAM in the constructs PHP10404 (5126:DAM-[35S-AS1]-UBI:PAT) or PHP 10398 (5126:DAM- [35S]-UBI:PAT), respectively, increased both *pat* expression and to varying degrees increased the frequency of recovering male-sterile plants. The stronger effects on the DAM-mediated phenotype require CaMV 35S sequences from –428 to –52 inclusive of the auxin responsive AS-1 region (PHP10404). These observations were independent of the transformation method employed. When constructs equivalent to PHP10698, PHP10700, PHP10398, and PHP10404 were introduced into maize embryos using microprojectile bombardment, similar frequencies of DAM-mediated male-sterility to those reported here were obtained (unpublished observations). In addition to having a strong influence when placed downstream of 5126:DAM, juxtaposition of 35S:PAT in an orientation such that these genes are divergently transcribed (PHP12234; DAM:5126-35S:PAT), resulted in plants with anthers having the most severe manifestation of the male-sterile phenotype.

One interpretation of these observed differences would be that inhibitory *cis*-acting sequence elements within the Ubiquitin and Actin promoters reduce transcriptional expression of 5126:DAM and this negative effect was alleviated by increasing the distance through placement of regions of CaMV 35S promoter downstream of 5126:DAM and upstream of UBI:PAT. Alternatively, 35S sequences may contribute to DAM-mediated male sterility directly by increasing transcription of 5126:DAM. As the system described in this report is a phenotypic assay that is the result of the ablation of the single cell layer where DAM is likely expressed, direct quantitative analysis of gene transcription relative to the placement of 35S sequences was not possible. The observations presented here are, however, consistent with the idea that sequences within the 35S promoter alter adjacent gene activity in plants by functioning as a transcriptional enhancer to increase expression of closely associated promoters in a position and orientation independent manner (Kay et al., 1987; Fang et al., 1989) and these effects are potentiated when the region to −55 is included (Odell et al., 1988). Other studies have also reported changes in gene activity as a consequence of the introduction of *cis*-acting elements thought to alter the local chromatin environment (S/MARs) (for review see, Spiker & Thompson, 1996; Gallie, 1998). Whether the CaMV 35S sequences used in these experiments function by stabilizing chromatin structure, and/or by acting as a tissue-specific transcriptional enhancer by recruiting factors that increase adjacent promoter activity is not known. Neither a reduction in transformation frequency nor abnormal phenotypes associated with constitutive DAM expression in tobacco (Van Blockland et al., 1998) was observed among the various 5126:DAM constructs introduced into maize. This would support the idea that the CaMV 35S sequences do not override the tissue-specific expression pattern of the 5126 promoter in maize. Although the general applicability of these observations to other tissue-specific gene combinations has yet to be systematically tested, recent studies using activation tagging in *Arabidopsis* report examples of enhanced endogenous gene expression rather than constitutive ectopic expression that is generally associated when 35S elements are placed immediately adjacent to genes (Neff et al., 1999; Weigel et al., 2000).

The positive influence of a closely associated CaMV 35S promoter however does not always impart single-copy male sterility. Although three constructs contained identical gene components, plants containing PHP12232 (DAM:5126-PAT:35S) demonstrated a reduced frequency of male-sterile plants when compared with either PHP12234 (DAM:5126-35S:PAT) or

PHP10698 (5126:DAM-35S:PAT). This difference observed with PHP12232 may be the result of transcriptional interference of 5126:DAM due to inefficient termination by the upstream 35S:PAT gene. Negative effects of transcriptional interference on the expression of downstream genes have been documented for naturally occurring adjacent promoters in yeast where intergenic regions are minimal (Greger et al., 2000; Springer, et al., 1997). Similar observations have been reported for adjacent genes in expression cassettes introduced into mammalian cells (Greger, et al., 1998). The results presented in this report show that in plants, positioning of adjacent genes also confer contrasting phenotypic effects. The observed differences using this dominant male-sterility system may be pertinent to studies that introduce genes into plants by either biolistic or *Agrobacterium* mediated transformation as a means to study gene function or develop crops with new or modified traits or phenotypes.

Methods

Construction of maize transformation vectors

The *E. coli* DNA (Adenosine-N^{6}) methyltransferase coding-region (nucleotides 195–1132 from Brooks et al., 1983) was modified by site-directed mutagenesis (Su & El-Gewley, 1988) and a *Sma*I site introduced at nucleotide 186, nine nucleotides 5′ to the initiating codon ATG of the *dam* gene. The *Nco*I site of a 528 bp *Hin*dIII-*Nco*I fragment that contained the maize 5126 promoter (nucleotides 985–1490, see accession number I75204) was filled in with dNTPs using T4 DNA polymerase and ligated to the *Sma*I site contained at the 5′ end of the *dam* gene using standard DNA techniques as in Sambrook et al. (1989). The 5126-*dam* junction resulted in the addition of three amino acids, MET-GLY-THR, to the *dam* open-reading-frame encoded by the following sequence, 5′-CCATGGGGACAATG-3′, with the initiation codon for the MET of the native *dam* gene underlined and corresponds to nucleotides 195–197 in Brooks et al. (1983). Transcription of this gene was terminated by the addition of the 3′ sequences from the potato proteinase inhibitor II gene (PinII) (nucleotides 2–310, from An et al., 1989). To construct the maize transformation vector PHP10698, the 1.7 kb chimeric gene containing the 5126 promoter, *E. coli* DNA methyltransferase gene and PinII 3′ non-translated region (5126:DAM) was cloned as a *Hin*dIII-*Nco*I fragment upstream of the 35S:PAT gene in the vector pSB11 (pSB31 from Ishida et al., 1996 lacking the *Eco*RI fragment insert carrying the 35SGUS and 35SBAR genes). The 35S:PAT gene encoding the enzyme phopsphinothricin acetyltransferase (PAT) from *Streptomyces viridochomagenes* (nucleotides 6–557 from Strauch et al., 1988, accession number A02774) under the transcriptional regulation of the cauliflower mosaic virus (CaMV) 35S promoter and terminator regions (nucleotides 6906–7439, and 7439–7632, respectively from Franck et al., 1980) was contained on an *Nco*I-*Kpn*I fragment as described in the expression cassette pDH51 (Pietrzak et al., 1986). The orientation and position of the 5126:DAM gene relative to the selectable marker gene, 35S:PAT, was such that the termination region of 5126:DAM was proximal to the 35S promoter. PHP10700 was similarly constructed with the exception that the 35S:PAT gene was replaced with a DNA fragment containing the UBI:PAT gene. UBI:PAT contained the same *pat* coding region described above under the regulation of the maize Ubiquitin-1 promoter including the first intron (−899 to +1092 from Christensen et al., 1992) and the Nopaline synthase (nos) terminator (678–420 described in Bevan et al., 1983). In the construct PHP12221, the rice Actin promoter and intron (nucleotides −834 to +395 from McElroy et al., 1990) replaced the Ubiquitin promoter and intron in PHP10700.

Construct PHP12234 was identical to PHP10698 with the exception that the orientation of the 1.7-kb fragment containing the 5126:DAM gene was reversed, placing the 5126 promoter adjacent to the 35S promoter. Similarly, the orientation of 5126:DAM was inverted in PHP10700 to generate PHP12485. In PHP12232, the orientation of the selectable marker gene 35S:PAT was reversed compared to PHP12234, which placed the selectable marker upstream of the 5126:DAM gene with the termination region of 35S:PAT gene proximal to the 5126 promoter. PHP10404 was identical to PHP10700 with the exception that a 376-bp fragment of the CaMV 35S promoter (6906–7382 from Franck et al., 1980) was inserted downstream of the 5126:DAM termination region. The sequence of this 35S promoter region was altered by site-directed mutagenesis to introduce a *Hin*dIII site at nt7382 (Franck et al., 1980; −52 relative to the transcription start site described as 7435 in Pietrzak et al., (1986)) for cloning purposes. PHP10398 was identical to PHP10404 with the exception that the region from the inserted 35S promoter lacked nucleotides 7341–7382, a region reported to contain an

auxin response element in dicots (described in Liu & Lam (1994) as −90 to −52, relative to transcription start site). The 40 bp *Eco*RV-*Hin*dIII fragment from PHP10404 containing the −90 to −52 region from CAMV 35S was subcloned into the *Eco*RV-*Hin*dIII site of Bluescript (Stratagene), and this plasmid was used to construct 1, 2, or 4 repeats of this region. The 40 bp (1X), 85 bp (2X), and 175 bp (4X) elements were inserted as *Not*I-*Hin*dIII fragments downstream of the 5126:DAM gene in PHP10700 to generate plasmids, PHP11781, PHP11783, and PHP11785, respectively. To generate a non-functional DAM protein, the *dam* gene was linearized at position 500 (Brooks et al., 1983) by digestion with *Bam*HI, the ends were filled in with dNTPs and T4 polymerase, and ligated together. This resulted in an addition of four nucleotides to the coding sequence, shifting the open reading frame. This frame-shifted *dam* gene (DAM f/s) replaced the *dam* gene in PHP10698 with pUC8 (New England Biolabs) replacing pSB11 plasmid sequences to generate PHP8146. To construct plasmids that placed the *dam* gene under the transcriptional control of another maize anther preferred promoter, a 1.4 kb *Hin*dIII-*Nco*I fragment containing the maize MS45 promoter found on plasmid PHP6054 (Albertsen et al., 2000) replaced the 5125 promoter in plasmids PHP10698 and PHP10700, to generate and MS45:DAM-35SPAT(PHP12634) and MS45DAM:UBI:PAT (PHP12898).

Agrobacterium strain construction

The plasmids described above were introduced into *Agrobacterium* LBA4404 using standard methods of triparental bacterial mating (Ditta et al., 1980). The resultant *Agrobacterium* strains carrying the co-integrate from the constructed plasmid and pSB1 (Ishida et al., 1996) were selected and used for maize transformation experiments.

Maize transformation

Immature embryos (1.0–1.2 mm) from Hi-type II hybrid maize (Armstrong et al., 1994) were used in *Agrobacterium*-mediated transformation experiments following the 'optimized Hi-II protocol' as described in example 4 of Zhao et al. (1999) with the following modifications: the resting phase was eliminated, and a seven day co-cultivation period was followed directly by bialophos selection.

Cytology

Maize anthers were isolated from developing tassels and fixed overnight at 4°C in 3% paraformaldehyde/1% gluteraldehyde/0.1 M sodium phosphate, pH 7.2. Anthers were washed in cold 0.1 M sodium phosphate, pH 7.2, three times for 10 min. The samples were dehydrated in a graded ethanol series over 3 h. Ethanol was used as the transitional solvent for infiltration with LR-White resin (EMS, Fort Washington, PA.). Infiltration resulting in 100% resin was accomplished in 5 days at room temperature on a rotating wheel. The samples were transferred to blocks and baked for 2 days at 58°C. One micron sections were cut with glass knives using a Leica ultramicrotome, and triple-stained (Chamberlin et al., 1993).

RNA isolation and hybridization analysis

Poly(A)$^+$ RNA was isolated using a modification of Chomczynski and Sacchi (1987) developed for use with the QuickPrep mRNA purification system (Amersham Pharmacia Biotech). PolyA$^+$ RNA (ca. 0.5–1 μg) was suspended in a loading buffer (0.72 ml formamide, 0.16 ml 10X MOPS, 0.26 ml formaldehyde (37%), 0.18 ml water, 0.1 ml glycerol, 0.05 ml saturated bromophenol blue solution), electrophoresed in 1% formaldehyde-agarose gels in MOPS buffer (1 × MOPS is 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA), transferred to Hybond Nytran F/P membrane (Amersham Pharmacia Biotech) with 20X SSC (3.0 M sodium chloride, 1.5 M sodium citrate, pH 7.5), UV-crosslinked and hybridized with DNA probes at 65°C in an ExpressHyb solution (Clontech, Palo Alto, CA). Filters were washed at 65°C with four changes of 250 ml wash buffer (0.5XSSC, 0.1%SDS) and exposed to XAR film (Kodak) with intensifying screens at −70°C. DNA probes were removed from the filters for rehybridization with additional DNA probes by washing the membranes at 90°C with four changes of 250 ml of 0.1% SDS.

DNA isolation and hybridization analysis

DNA was isolated from maize leaf tissue according to a modified procedure of Murray and Thompson (1980). Nine millilitres of CTAB extraction buffer (100 mM Tris, [pH 7.5], 1% Hexadecyltrimethylammonium bromide (CTAB), 0.7 M N sodium chloride, 10 mM EDTA, 1% 2-mercaptoethanol) was added to 300 mg of lyophilized leaf tissue, vortexed and

incubated at 65°C for 1 h. Five millilitres of a chloroform/octanol (24:1) solution was added and mixed for 5 min. Extracts were centrifuged for 40 min at 3500 × g. The top layer was removed, to which 11 ml of CTAB precipitation buffer (100 mM Tris [pH 7.5], 1% CTAB, 10 mM EDTA) was added, mixed by inversion, and allowed to stand for 30 min and then centrifuged for 10 min at 3500 × g. Pellet was resuspended in 2 ml of high salt buffer (100 mM Tris [pH 7.5], 10 mM EDTA, 0.7 M sodium chloride) containing 10 μl of RNaseA (10 mg/ml) then incubated at 37°C for 1 h. Five millilitres of cold 100% ETOH was added and mixed gently. The DNA was hooked using a bent 9-inch Pasteur pipette and washed in 0.2 M sodium acetate/76% ETOH and 0.2 M ammonium acetate/76% ETOH for 10 min and 1 min, respectively. Genomic DNA was resuspended in 500 microlitre of TE (10 mM Tris [pH 7.5], 1 mM EDTA). Five micrograms of DNA was digested with various restriction enzymes, followed by electophoresis in 0.8% Tris-acetate agarose gels, and transferred to Hybond Nytran F/P membrane (Amersham) with 25 mM sodium phosphate (pH 6.5) according to the procedure of Southern (1975). Filters were incubated in ExpressHyb solution (Clontech) with different DNA probes at 65°C overnight, washed with four changes of 500 ml 0.1X SCP/0.1% SDS (20X SCP is 2 M sodium chloride, 0.6 M sodium phosphate dibasic, 20 mM EDTA, pH 7.2) at 65°C and exposed to XAR film (Kodak) with intensifying screens at −70°C. DNA probes were removed by washing the filters at room temperature for 20 min in 0.1 M sodium hydroxide/0.2% SDS, 20 min in 0.1 M Tris pH 7.5/0.2% SDS then at 65°C for 20 min in 0.1X SCP/0.1%SDS.

Probes for RNA and DNA Hybridization

The DNA hybridization probe for the 5126 gene was derived from a full-length 5126 cDNA and is contained on a 568 bp *Alw*NI DNA restriction fragment (nucleotides 376–944, accession number I75226). The hybridization probe for the *dam* gene consists of a 635 bp *Bam*HI DNA restriction fragment (nucleotides 490–1134, Brooks et al., 1983). The *pat* gene hybridization probe was a 319bp *Bam*HI DNA restriction fragment containing nucleotides 1–316 (accession number A02774). A DNA hybridization probe for the MS45 gene was a 485 bp *Xho*I-*Not*I restriction fragment including nucleotides 390–810 from a full-length MS45 cDNA (accession number A80078). For normalization of RNA extractions, a maize actin cDNA contained on a 1.3 kb *Eco*RI/*Xho*I DNA restriction fragment was used as a hybridization probe. The UBI probe contained nucleotides 1-467 Accession number S94464. For use as probes, the DNA fragments were radiolabeled with [α-^{32}P] dCTP using a random-primer kit from Amersham.

PAT ELISA assays

Corn leaves were sampled using a standard-size paper hole punch. Four leaf punches per plant were harvested in duplicate and ground in 500 μl PBST (0.13 M sodium chloride, 0.008 M sodium phosphate (dibasic), 0.001 M potassium phosphate (monobasic), 0.003 M potassium chloride, 0.05% Tween-20, pH 7.4). The extracts were cleared by centrifugation and protein concentrations determined following the Bradford method as described (BioRad Laboratories). Fifty microlitres of sample (0.1–10 μg protein) was added in duplicate to wells of an ELISA plate pre-coated and stabilized with purified PAT antibodies (PAT antibodies a gift from E. Kulisek, Pioneer Hi-Bred). Fifty microlitres of biotinylated anti-PAT diluted 1:250 in PBSTO (PBST, 0.5% ovalbumin, 0.01% thimerisol) was added to each well, mixed thoroughly and incubated for 2 h at 37°C. The plates were washed with 2.0 ml PBST with 5, 400 μl changes, draining the excess liquid off. Streptavidin-alkaline phosphatase (Gibco-BRL) was diluted 1:4000 in a PBSTO solution made by diluting equal volumes PBSTO with PBST. One hundred microlitres of this solution was added to each well and incubated for 1 h at 37°C. Substrate tablets (Sigma 104–105, pNPP 5 mg tablets) were prepared following the manufacturers directions and 100 μl substrate solution was added to each well and the plate incubated for 30 min at 37°C. Reactions were stopped by the addition of 50 μl 1.0 M sodium hydroxide and the O.D. values were determined at 405 nm. The quantity of PAT protein in each sample was determined by comparison to a standard curve generated on each plate with purified PAT protein concentrations ranging from 0 to 10 μg/ml. The values are expressed as pg PAT/μg protein sample.

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