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Comparison of transient and stable expression by a pollen-specific promoter: the transformation results do not always agree

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Abstract In the analysis of 5' sequence elements necessary for promoter activity in pollen various promoter-reporter gene constructs usually show similar patterns of expression in pollen when assayed by either transient transformations or after stable transformations in transgenic plants. Out of several fragment lengths of the *Zm13* pollen-specific promoter linked to GUS, we found that the largest fragment (–1001 to +61) showed approximately eightfold higher expression in pollen of transgenic plants than transient transformations by microprojectile bombardment. No other promoter fragment tested showed this difference. Titration of a limited number of transcription factors by the large number of potential binding sites in this promoter fragment along with the large number of promoter copies introduced during transient transformation are possible explanations for this observation.

Key words *Zm13* pollen-specific promoter · Transient transformation · Transgenic plants · Transcription factor competition

Introduction

Transient transformation of plant cells via microparticle bombardment is often utilized to assess the efficiency of a transformed promoter-marker gene construct (Ow et al. 1986; Ye et al. 1990; Twell et al. 1991; Hamilton et al.1992). In most of the published work on promoter analysis of pollen-expressed genes, transient transformation assays have given the same results as subsequent stable analyses (e.g. Bate and Twell 1998; Hamilton et al. 1998).

Zm13 is a pollen-specific gene whose promoter has been isolated and utilized in a number of studies and has been shown to be very active in both monocot and dicot pollen (Guerrero et al. 1990; Myoshi et al. 1995; Hamilton et al. 1998). Deletion analysis of the promoter region and subsequent transient transformation of promoter-GUS constructs into *Tradescantia paludosa* pollen by microparticle bombardment showed that promoter fragments with 5' ends beginning at –261 bp prior to the start of transcription produce GUS expression levels essentially equal to those produced by any smaller promoter fragments down to and including –119 bp (Hamilton et al. 1998). Further deletion to –101 bp severely limits marker-gene expression in this system (Hamilton et al. 1992, 1998). The –1001 bp promoter construct showed much less expression in transient transformation assays than the –260 bp construct, suggesting that a negative element might be present in the region between –260 and –1001 bp (Hamilton et al. 1992). We further analyzed the pollen-specific *Zm13* promoter and report here a discrepancy between the level of pollen expression shown by the –1001 bp promoter construct in transient transformations and the level in stably transformed plants. Potential explanations for this discrepancy are proposed.

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Materials and methods

Promoter constructs

Deletion mutants of the *Zm13* promoter were fused to the β-glucuronidase (GUS) reporter gene joined to the nopaline synthase 3' terminator (Jefferson et al. 1987) as previously described (Hamilton et al. 1992). The four promoter fragments used in this study had 5' ends at bases –1001, –261, –119 and –101. Most of these

fragments have 3' ends at +61; others with 3' ends at +118 have shown no difference in any assay (data not shown). A control plasmid was also used which consisted of the *Zm13* –261 to +61 promoter fragment driving the luciferase gene/nos terminator from the pGEM-*luc* plasmid (Promega). A standard quantity of this –261/LUC/NOS plasmid was co-bombarded with the *Zm13* constructs for reference, and the extract was assayed for luciferase activity using an Aminco Chem Glow luminometer model J4-7441, as previously described (Hamilton et al. 1998).

Agrobacterium-mediated transformation of *Arabidopsis*

Plasmid constructs for *Agrobacterium*-mediated transformation into *Arabidopsis thaliana*, ecotype Landsberg erecta, were made in the binary vectors pCGN1557 or pCGN1559 (kindly provided by Calgene) (McBride and Summerfelt 1990). *Agrobacterium tumefaciens* strains EHA101 or EHA105 (kindly provided by Elizabeth E. Hood) (Hood et al. 1986) were used for the transformations. Transgenic plants were obtained as described in Hamilton et al. (1998). Plants from the T2 or later generations that were homozygous for the transgene were used for the GUS assays. At least six independent transformants of each type were assayed.

Pollen transformation and fluorometric detection of GUS activity

Pollen from greenhouse-grown *Tradescantia paludosa* plants was used as the source of pollen for the transient transformation assays. Pollen was harvested and stored at –20°C before use. Germination was routinely about 80%. Pollen was spread and germinated on sterile plastic petri plates (60 mm × 15 mm) containing 3 ml of pollen medium, and bombardment conditions were as previously described (Hamilton et al. 1992, 1998). To facilitate complete recovery of pollen from the surface of the germination plates, cut cellophane circles (boiled with three changes of water) were placed on top of the solid medium, and 4 mg of pollen was spread over the surface with an additional 250 µl of liquid medium. The cellophane layer permitted normal pollen germination and tube growth while allowing the bombarded and germinated pollen to be easily scraped off the plates for homogenization. After removal from the plate, the germinated pollen was collected in a microcentrifuge tube to which was added 100 µl of 2× ice cold cell culture lysis reagent (1×CCLR: 25 mM Tris-phosphate, pH 7.8; 2 mM dithiothreitol; 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; 1% Triton X-100: see Promega Protocols and Applications Guide under Luciferase Assay System) containing a small amount of 25-µm glass beads to aid in grinding. The pollen was ground for 3 min directly in the microfuge tube with a close–fitting teflon pestle driven by an electric motor. The resulting homogenate was frozen at –80°C until use.

For the assay, the homogenate was centrifuged at 10,000×*g* for 10 min at 4°C to pellet debris, and 50 µl of the supernatant was removed for analysis. This 50 µl was added to 200 µl of 1 mM 4-methylumbelliferyl-β-D-glucuronide (Sigma) in 1×CCLR, and incubated at 37°C. At timed intervals 50-µl aliquots were removed and added to plastic cuvettes containing 1.6 ml of 0.2 M Na_2CO_3. Concentration of the methylumbelliferone product was determined using a Perkin Elmer model MPF-3L fluorescence spectrophotometer as described (Hamilton et al. 1992). Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as the standard. GUS activity was calculated as nmol of 4-methylumbelliferone per min per mg protein. The experiments were done three times with three replicates each time.

For assaying GUS activity of the transgenic *Arabidopsis* plants, ten anthers taken from two newly opening flowers of the same plant were ground in 50 µl of 1×CCLR buffer and then processed similar to pollen. Intact anthers were used because of the difficulty of isolating sufficient pollen for these assays; however, no GUS activity was found in the anther walls (data not presented). Experiments were repeated at least three times with five replicates for each independent transformant. GUS activity was measured and calculated as above.

Table 1 Results of transient and stable assays of promoter fragment activities. Values reflect percent of GUS activity relative to the –261 promoter fragment±standard error

Promoter fragment	Transient assay	Stable assay
–1001	101±16	780±171 (*n*=6)
–261	100	100
–119	86±13	101±7 (*n*=6)
–101	6.5±1.4	6.5±1.0 (*n*=11)

Results and discussion

Identical *Zm13* promoter/GUS constructs were either bombarded into *Tradescantia* pollen or transformed into *Arabidopsis* plants, and then analyzed for relative amounts of GUS expression. In both cases GUS activity was calculated as nmol of 4-methylumbelliferone per min per mg protein, and the expression level produced by the –261 bp promoter was used as the 100% level for both assays. As shown in Table 1, in both the transient and the stable assays the promoter fragments with 5' ends at –261, -119 and –101 bp produced levels of expression in pollen which were comparable in the two assays. However, the –1001 bp promoter fragment produced an unexpectedly high level of expression in the six stable transformants tested, roughly eightfold greater than transient levels. One transformant showed approximately 17-fold increase in expression; without this outlying point the average expression of the –1001 fragment was 600±56. Histochemical staining of anthers from transgenic plants shows the difference between the –1001 and –261 bp constructs more strikingly (Fig. 1).

The transient assay results for the –1001 bp promoter (Table 1) are similar to those obtained with the –261 bp promoter. In a previous study (Hamilton et al. 1992), however, the –1001 bp promoter was found to have about half the activity of the –261 bp promoter in transient assays. This is probably because in the earlier work (Hamilton et al. 1992) tungsten particles were used as the DNA carrier, and an early model gene gun was used for transformation. The more recent data were obtained using gold particles and a fast-acting helium release gun resulting in roughly 12-fold greater transformation efficiency (data not shown). The present data thus do not support the earlier suggestion that a negative element might reside upstream of –261. In fact, there must be additional cis-elements in this region that increase the activity of the minimal promotor.

The large difference between the activity of the –1001 bp promoter in transient transformations and activity of stably transformed plants could be due to several reasons. Since equal quantities in µg of DNA were used in all transient bombardment treatments, a construct containing a larger promoter, as in the –1001 bp construct, would actually result in fewer molecules of the construct being introduced into a pollen grain. A calculation shows that bombardments with the –1001 bp pro-

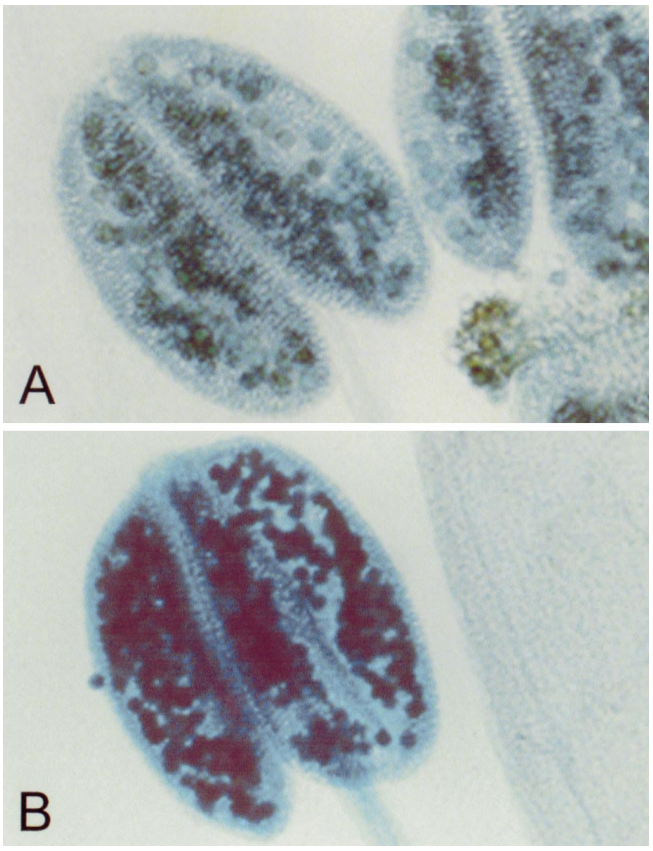

Fig. 1A, B Anthers of transgenic plants stained for GUS activity as described by Hamilton et al. (1992). The anthers were stained under identical conditions. **A**: –261 bp construct; **B**: –1001 bp construct

moter would contain 12% fewer copies of the DNA than in the case of the –261 bp promoter. However, this is not sufficient to account for the sixfold to eightfold difference observed.

Another possibility for the difference in activity might be the heterologous system used. In the transient transformation assays, the maize (monocot) promoter was introduced into *Tradescantia* (monocot) pollen, but in the stable transformations the maize promoter was introduced into a dicot plant, *Arabidopsis*. However, this is also not likely to be an explanation since the *Zm13* promoter seems to be equally active in both monocots and dicots (Guerrero et al. 1990; Myoshi et al. 1995; Hamilton et al. 1998), and because comparisons of transient and stable transformation with other *Zm13* promoter fragments have not shown this extreme result.

A more likely reason for these observations rests on the large numbers of copies of the promoter construct that are introduced into each pollen grain during microprojectile bombardment relative to the limited number of individual transcription factors that are probably present.

Eyal et al. (1995) have provided evidence for a relatively small pool of transcription factors in tobacco pollen. When twelve copies of the pollen-specificity element of the *LAT52* promoter were cointroduced into pollen by transient transformation along with a LAT52 promoter-reporter gene construct, there was a fourfold reduction in *LAT52* promoter activity, indicating limiting levels of transcription factors in tobacco pollen.

Assuming that a single gold particle needs to penetrate a pollen grain for it to be transformed and that 10% of the DNA added was originally adsorbed to the particles, we calculate that there would be 1042 copies of the plasmid on a single gold particle. The presence of such a large number of promoter molecules of this transgene could result in apparently lower promoter activity because of competition for transcription factors, but this is not seen with the –261 bp construct and so is unlikely to be the only factor involved. An analysis of the promoter sequence between –261 bp and –1001 bp shows that there are eight regions (two forward and six reverse) that have identical sequence to five out of six bases of the 6 bp quantitative element (Q-element), AGGTCA, identified in the promoter and located between –107 to –102 bp (Hamilton et al. 1998). In the region between –1 and –261 bp there are only two sectors, one forward and one reverse with five of six matches. In addition, between –261 and –1001 there are 63 regions with four of six identical matches with the Q-element that could potentially titre out transcription factors. Removal of the Q-element by base substitution results in a tenfold reduction of promoter activity in pollen (Hamilton et al. 1998). Some support for the hypothesis of limiting transcription factors in pollen was obtained by introducing multiple copies of the Q-element into pollen by microprojectile bombardment. When a construct containing an additional copy of the Q-element upstream of the –119 or –261 bp promoters was introduced into pollen the activity of the promoters was increased; however, with 4 direct repeats of the Q-element there was a decrease in the activity of the promoter relative to the –119 and –261 bp normal promoters

In wild-type maize plants there is a single copy of the *Zm13* gene in the genome (Stinson et al. 1987). In the *Zm13* promoter/GUS transgenic *Arabidopsis* plants, Southern analysis has shown that, with the exception of one transgenic plant which contained four copies of the promoter construct, all independently transformed transgenic plants used for this analysis had just a single copy of the –1001 bp promoter-GUS construct (data not shown). One might thus expect no more than four copies of the transgene in the vegetative cell nucleus of the pollen grain. A large number of copies of the –1001 bp promoter is introduced into a single pollen grain in the transient transformations. This, coupled with at least eight additional sites in each copy, creates a total of about 8000 sites to which transcription factors could potentially bind. This very likely titers out the transcription factors present in the pollen grain, resulting in a reduction of the level of transgene expression.

Quantitation of transient gene expression may be a problem if limited numbers of transcription factors are

available in the transformed tissue. Serial dilutions might be required to determine the optimum transgene concentration that would provide the maximum expression level. This might, however, not be a problem for minimal promoters with limited numbers of binding sites, but should be taken into account for larger fragments which could potentially contain many such sites.

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