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Expression of the β-Glucuronidase Gene in Pollen of Lily (*Lilium longiflorum*), Tobacco (*Nicotiana tabacum*), *Nicotiana rustica*, and Peony (*Paeonia lactiflora*) by Particle Bombardment[1]

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A β-glucuronidase (GUS) gene that is under the control of the anther-specific LAT52 promoter of tomato (*Lycopersicon esculentum*) and the nopaline synthetase polyadenylation terminator was successfully expressed in pollen of *Lilium longiflorum*, *Nicotiana tabacum*, *Nicotiana rustica*, and *Paeonia lactiflora* using a pneumatic particle gun. The GUS gene in plasmid pBI221 was also expressed, to a lesser extent, in pollen of all of these species. The presence of methanol in the substrate solution for histochemical GUS assay and the incubation time in this solution influenced successful detection of GUS expression in bombarded pollen. Cytological analysis of GUS-expressing pollen of lily showed that introduced gold particles were seen in intracellular compartments of pollen, including the vegetative cytoplasm, vegetative nucleus, and generative cytoplasm.

It has been reported (Guerrero et al., 1990) that a pollen-specific promoter, Zm13, from monocotyledonous maize directs pollen-specific gene expression in transgenic tobacco plants. However, an anther-specific promoter, LAT52, from tomato has been reported to be silent in monocotyledonous lily pollen, based on bombardment-mediated transformation (Twell et al., 1991; Plegt et al., 1992).

In this study, we introduced the same plasmid used by the previous authors (Twell et al., 1991; Plegt et al., 1992), containing the GUS gene under the control of the LAT52 promoter, into pollen grains of lily (*Lilium longiflorum*), tobacco (*Nicotiana tabacum*), *Nicotiana rustica*, and peony (*Paeonia lactiflora*) by a pneumatic particle gun device (Iida et al., 1990), and have found that the GUS gene is successfully expressed in the grains of these plant species as determined by both histochemical and fluorometric assay methods. Possible reasons for the discrepancy between our present results and those of the previous authors (Twell et al., 1991; Plegt et al., 1992) are discussed.

MATERIALS AND METHODS

Plant Materials and Particle Bombardment

Lily (*Lilium longiflorum* cv Georgia) plants were grown in a greenhouse or in the field as reported elsewhere (Takegami et al., 1981). Lily pollen was collected from flower buds (160 mm long) that were fully matured but had not undergone anthesis. Pollen of tobacco (*Nicotiana tabacum* cv Samsun) was also collected from fully matured flower buds that had not undergone anthesis. Immature pollen of *Nicotiana rustica* cv Rustica was collected from flower buds whose corollas were approximately 7 to 9 mm long. Immature pollen of peony (*Paeonia lactiflora* cv Kumoinotsuru and cv Yamatoshu) was collected from flower buds containing uninucleate and early binucleate stage microspores.

Lily pollen was suspended in White's modified solution (White, 1963) containing 0.5 M Suc (pH 5.8) at a density of 2×10^4 cells/mL. Pollen of tobacco and *N. rustica* was suspended in medium C (Kyo and Harada, 1986) at a density of 5×10^4 cells/mL. Pollen of *P. lactiflora* was suspended in M4 medium (Ono and Harashima, 1981) at a density of 10^5 cells/mL.

By vacuum filtration, 2 mL of the suspension of pollen of each species was spread in a circle (35 mm in diameter) onto the surface of a piece of filter paper (ADVANTEC TOYO No. 2, 55 mm diameter). Pollen was bombarded once with plasmid DNA-coated or noncoated gold particles of 1.1 μm in diameter (Tokuriki Honten Co., Ltd., Tokyo, Japan) at an accelerating pressure of 200 kg/cm² by using a pneumatic particle gun device, as described elsewhere (Iida et al., 1990). After bombardment, the filter paper with pollen was transferred to a Petri dish (60 mm in diameter) containing two

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Abbreviations: CaMV 35S promoter, cauliflower mosaic virus 35S promoter; 4-MU, 4-methyl umbelliferone; GUS, β-glucuronidase; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

pieces of filter paper moistened with 2 mL of the appropriate culture medium mentioned above for each plant species. The pollen was incubated at 26°C in the dark, then assayed for GUS expression.

Plasmid DNAs

Plasmid pLAT52-7 (Twell et al., 1989a) and pBI221, which contain the GUS gene, were used for bombardment. The plasmid pLAT52-7 (provided by Dr. Sheila McCormick, U.S. Department of Agriculture-Agricultural Research Service/University of California-Berkeley, Albany, CA) contains a chimeric gene consisting of a promoter fragment (−492 to +110) of the anther-specific LAT52 gene from tomato (Twell et al., 1989b) fused to the GUS gene along with the nopaline synthetase polyadenylation terminator.

The plasmid DNA pBI221 (Clontech, Palo Alto, CA), which has the GUS gene under the control of the CaMV 35S promoter and nopaline synthetase polyadenylation region, was also used.

Histochemical and Fluorometric GUS Assays

Expression of the GUS gene in bombarded pollen was assayed histochemically according to the procedure of Jefferson et al. (1987) as modified by Kosugi et al. (1990). The filter paper with bombarded pollen was placed on 500 μL of a solution for GUS assay and incubated at 37°C for 10 h for lily pollen and 12 h for pollen of the other species. The GUS assay solution contained 1.9 mM X-Gluc, 20% methanol, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% Triton X-100 in 0.1 M sodium phosphate buffer (pH 7.0) (X-Gluc solution). The blue-colored, GUS-expressing pollen grains were counted under a binocular microscope. The activity of GUS enzyme in bombarded pollen was determined fluorometrically according to Jefferson et al. (1987).

Treatment for Removal of Exine and Starch Granules from GUS-Expressing Pollen of Lily

Bombarded lily pollen was stained with X-Gluc solution for 10 h as described above and fixed with ethanol:acetic acid (3:1, v/v). It was then hydrolyzed in 1 N HCl at 60°C for 10 min to remove both the sculptured cell wall of exine and dense starch granules (Tanaka and Ito, 1980; Takegami et al., 1981) and stained with 1% propionic orcein. Intracellular location of gold particles in pollen was determined microscopically.

RESULTS

When lily pollen that had been bombarded with noncoated gold particles was incubated at 37°C for 10 h with the X-Gluc solution lacking both 20% methanol and 0.1 M sodium phosphate, all of the cells exhibited intense blue color (Fig. 1A). Similar results were obtained with pollen of other species studied here (data not shown). Lily pollen had the highest endogenous GUS activity of pollen of the four species studied here; when nonbombarded pollen was incubated in the same X-Gluc solution and at the same temperature, it took 15 min for pale blue color to appear in the cells for lily and about 12 h for other species.

This background blue color due to the endogenous GUS activity was almost completely diminished when the bom-

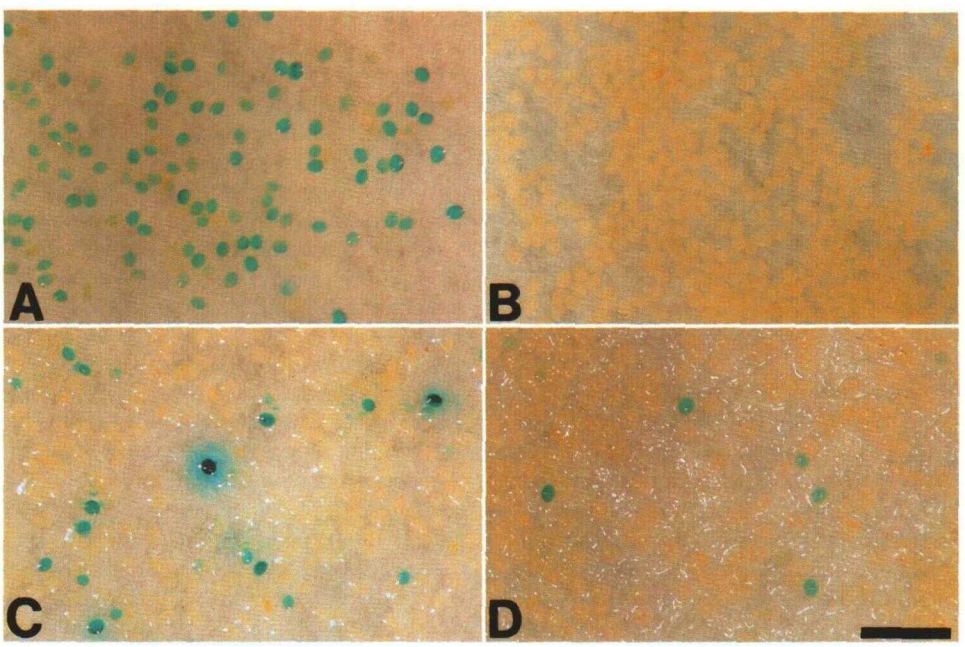

Figure 1. Results of histochemical assay for GUS expression in lily pollen. Mature pollen was bombarded with noncoated (A and B), pLAT52-7-coated (C), and pBI221-coated (D) gold particles, after which it was cultured at 26°C for 24 h and incubated at 37°C for 10 h in the X-Gluc solution containing (B–D) or not containing (A) 20% methanol and 0.1 M sodium phosphate. Bar = 0.5 mm.

barded lily pollen was incubated at 37°C for 10 h with the X-Gluc solution containing 20% methanol and 0.1 M sodium phosphate (Fig. 1B). Similar results were obtained with pollen of the other species studied (data not shown). Both the presence of methanol and the incubation time seemed to be essential (at least for lily pollen) for elimination of the background blue color. For example, incubation of lily pollen grains for 5 h in the X-Gluc solution lacking 20% methanol but containing 0.1 M sodium phosphate resulted in the appearance of blue color in the cells. Also, prolonged incubation (for more than 12 h) of nonbombarded lily pollen in the X-Gluc solution containing methanol and sodium phosphate produced a faint blue color in the cells. Thus, in the following part of this study the X-Gluc solution contained both methanol and sodium phosphate and the incubation time of bombarded pollen of all species was kept at less than 12 h (see "Materials and Methods").

A number of blue-colored, GUS-expressing cells were clearly observed in lily pollen bombarded with pLAT52-7 (Fig. 1C). Also, GUS expression was detected in those cells bombarded with pBI221, although the blue color was less intense (Fig. 1D; also, see below). Similar results with both plasmids were obtained with pollen of tobacco, *N. rustica*, and *P. lactiflora* (Table I).

The frequency of GUS-expressing pollen grains in five taxa after bombardment with pLAT52-7 and pBI221 is summarized in Table I. The frequency in lily pollen bombarded with pLAT52-7 was at most 0.92%, which was close to that in tobacco (0.7%) and even higher than the values in *N. rustica* (0.36%) and *P. lactiflora* (0.32%). These values are close to those obtained with suspension-cultured cells of tobacco BY-2 using our present particle gun device (Yamashita et al., 1991), indicating that the frequency of gene delivery into pollen grains and cultured cells (using this device) is similar.

The frequency of GUS-expressing pollen grains in lily pollen bombarded with pLAT52-7 (see Table I) is about 9-fold higher than that in tobacco pollen (Twell et al., 1989a) and one-third of that obtained with the same construct in pollen of *Nicotiana glutinosa* (Plegt et al., 1992). In general, the values in our present study are 1 order of magnitude higher than those reported with a pollen-specific PA2 promoter in tobacco (Stöger et al., 1992) and *N. glutinosa* (Plegt et al., 1992) pollen.

It has been reported that transient expression of the GUS gene driven by the CaMV 35S promoter is not detected histochemically in pollen grains of lily (Plegt et al., 1992) and *N. glutinosa* (Plegt et al., 1992) and is detected only rarely in those of tobacco (Twell et al., 1989a; Stöger et al., 1992) and *Tradescantia* (Hamilton et al., 1992). Also, in pollen of various transgenic plants including tobacco (Guerrero et al., 1990; Plegt et al., 1992), tomato (Plegt et al., 1992), and petunia (Mascarenhas and Hamilton, 1992), the expression of the GUS gene driven by the CaMV 35S promoter has not been detected histochemically. However, our present results clearly indicate that the CaMV 35S promoter does induce expression of the GUS gene in all of the five taxa studied, although the frequency of GUS-expressing pollen grains with pBI221 was two to six times lower than that with pLAT52-7, depending on plant species (see Table I). We think that this low frequency with pBI221 is somehow related to the fact that the activity of the GUS enzyme was lower in the grains bombarded with pBI221 than with pLAT52-7 (see below).

Table II. *GUS activity in pollen bombarded with pLAT52-7 and pBI221*

Species	GUS Activity	
	pLAT52-7	pBI221
	pmol 4-MU h^{-1} μg^{-1} protein	
L. longiflorum	2.05 ± 0.16[a]	0.25 ± 0.03
N. tabacum	563.5 ± 343.9	0.76 ± 0.67

[a] Average ± SD of three experiments.

Table II shows GUS activity determined fluorometrically in pollen of lily and tobacco that was bombarded with pLAT52-7 and pBI221. GUS activity in pollen grains of tobacco bombarded with pLAT52-7 was more than 270-fold higher than in those of lily. This indicates that LAT52 promoter derived from dicotyledonous tomato is more active in pollen grains of tobacco than in those of lily.

Both lily and tobacco pollen grains bombarded with pBI221 exhibited GUS activity although to a lesser extent than in the case of pLAT52-7 (see Table II). This agrees with the results obtained by the histochemical assay as described above. Note that the observed GUS activity with the CaMV 35S promoter did not differ greatly between lily and tobacco pollen. These values of GUS activity with the CaMV 35S promoter were smaller than those (1.4–1.9 pmol 4-MU h^{-1} μg^{-1} protein) reported for pollen grains of tobacco (Stöger et al., 1992) and *Tradescantia* (Hamilton et al., 1992).

Table I. *Number of GUS-expressing pollen grains observed in pollen of five taxa bombarded with pLAT52-7 and pBI221*

Pollen		Time of Incubation with X-Gluc	No. of GUS-Expressing Pollen Grains	
Species	Stage		pLAT52-7	pBI221
		h		
L. longiflorum	Mature	10	297 ± 73[a] (0.74 ± 0.18)[b]	65 ± 17 (0.16 ± 0.04)
N. tabacum	Mature	12	649 ± 54 (0.65 ± 0.05)	99 ± 79 (0.10 ± 0.08)
N. rustica	Immature	12	292 ± 70 (0.29 ± 0.07)	122 ± 28 (0.12 ± 0.03)
P. lactiflora	Immature	12		
cv Kumoinotsuru			148 ± 74 (0.07 ± 0.04)	39 ± 26 (0.02 ± 0.01)
cv Yamato-shu			494 ± 142 (0.25 ± 0.07)	259 ± 98 (0.13 ± 0.05)

[a] Average ± SD of three to four experiments. [b] Values in parentheses are percent of total.

GUS activity in tobacco pollen observed here with the LAT52 promoter is about one-tenth of that observed with this promoter in pollen of transgenic tobacco plants (Twell et al., 1989a). A similar difference between transient and stable expression of the GUS gene has been reported with the pollen-specific promoter PA2 in tobacco pollen; GUS activity (approximately 18 pmol 4-MU h^{-1} μg^{-1} protein) detected by a transient expression assay in pollen into which the gene driven by the PA2 promoter was introduced (Stöger et al., 1992) is 5 to 10 times lower than the values (90–190 pmol 4-MU h^{-1} μg^{-1} protein) in pollen of transgenic plants having the same construct (Plegt et al., 1992; Stöger et al., 1992). On the other hand, in the case of the CaMV 35S promoter the GUS activities in tobacco pollen as determined by transient assay (Stöger et al., 1992) and in transgenic plants (Twell et al., 1989a; Plegt et al., 1992; Stöger et al., 1992) were similar (1.5–4.2 pmol 4-MU h^{-1} μg^{-1} protein).

As reported previously (Yamashita et al., 1991), intracellular localization of gold particles introduced by bombardment is directly detected in cultured tobacco cells. In general, pollen has a thick exine and starch granules that hampered direct observation of gold particles introduced into the cells. But when bombarded pollen grains of lily, after being assayed for GUS expression, were fixed with acetic ethanol, hydrolyzed with 1 N HCl to remove the exine and starch granules, and stained with orcein (see "Materials and Methods"), introduced gold particles became microscopically visible as black dots in the cells (Fig. 2). In GUS-expressing pollen into which pLAT52-7 had been introduced, gold particles were seen in the vegetative cytoplasm (arrowheads in B and D), vegetative nucleus (arrows in B, C, and D), and generative cytoplasm (double arrow in B). It should be noted, however, that the treatment for removal of the exine and starch granules employed here (hydrolysis with 1 N HCl at 60°C for 10 min) may have caused displacement of introduced particles from their original sites of localization. Note that no blue color was detected in pollen bombarded with noncoated gold particles (Fig. 2A).

The number of gold particles detected in each GUS-expressing pollen grain did not seem to influence the intensity of blue color; the number of particles was 11, 1, and more than 6 in Figure 2, B, C, and D, respectively. GUS-expressing pollen grains that contained more than 100 gold particles were also observed (data not shown), but the intensity of blue color in these cells was similar to that of Figure 2B or Figure 2D. The reason(s) for this is not fully understood yet.

DISCUSSION

Our present result that the LAT52 promoter of tomato successfully directed expression of the GUS gene in lily pollen does not agree with previous results indicating that this promoter is silent in lily pollen (Twell et al., 1991; Plegt et al., 1992).

We examined possible factors, such as culture conditions and composition of the X-Gluc solution, that may affect the

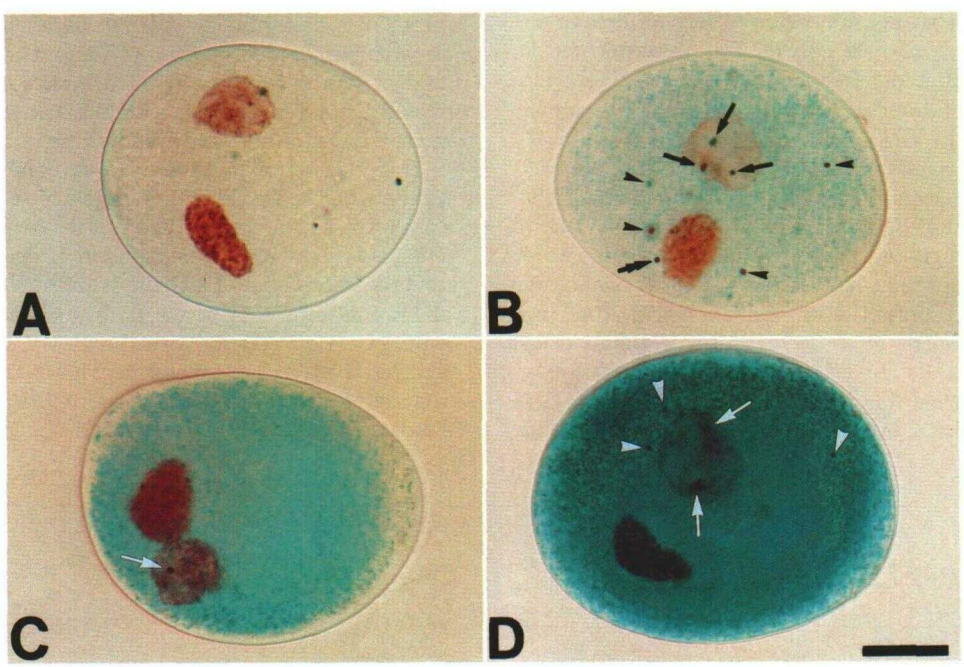

Figure 2. Typical photomicrographs for cytological observation of gold particles in GUS-expressing lily pollen. Mature pollen was bombarded with noncoated (A) and pLAT52-7-coated (B–D) gold particles, after which it was cultured at 26°C for 24 h and incubated at 37°C for 10 h in the X-Gluc solution containing 20% methanol and 0.1 M sodium phosphate. They were then fixed with acetic ethanol, treated for removal of exine and starch granules, and stained with propionic orcein as described in "Materials and Methods." Gold particles were seen as black dots in the vegetative cytoplasm (arrowheads in B and D), vegetative nucleus (arrows in B, C, and D), and generative cytoplasm (double arrow in B). Bar = 30 μm.

efficiency of GUS expression in bombarded lily pollen. The culture medium for the bombarded pollen was changed from White's modified medium (present study) to Murashige and Skoog medium (Twell et al., 1991), but the number of blue spots, indicating GUS-expressing pollen grains, was the same within experimental errors (data not shown). Similar results were obtained when the period of culture of bombarded pollen before addition of X-Gluc solution was changed from 24 h (present study) to 16 h (Twell et al., 1991; Plegt et al., 1992).

In light of the facts that lily had the highest endogenous GUS activity of pollen among four species studied, and that this activity could not be completely eliminated when the X-Gluc solution lacked 20% methanol (see above), it is most likely that the presence (present study) or absence (Twell et al., 1991; Plegt et al., 1992) of 20% methanol in the X-Gluc solution for the assay of GUS expression in bombarded pollen of lily is a reason for the contradiction between our results and previous results. The effects of other factors such as plant variety and the use of tungsten particles will be the subjects of future study.

As reported with cultured tobacco cells (Yamashita et al., 1991), the minimum requirement for the expression of foreign genes in particle-bombardment-mediated transformation is successful introduction of DNA-coated particles into the nucleus of the target cells. Whether or not there is a strict correlation in lily pollen between the localization of bombarded DNA-coated gold particles in the nucleus or cytoplasm and GUS activity is a subject for future study.

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