STUDIES ON BOMBARDMENT-MEDIATED TRANSFORMATION OF POLLEN CELLS IN ANGIOSPERMS

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CONTENTS

ABBREVIATIONS

bp	Base pair
CaMV	Cauliflower mosaic virus
GUS	β-glucuronidase
kb	Kilobase
MS	Murashige and Skoog
4-MU	4-methylumbelliferone
NOS	Nopaline synthase
NPTII	Neomycin phosphotransferase II
PEG	Polyethylene glycol
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

INTRODUCTION

There have been reports of various techniques for gene delivery into angiosperm pollen or microspores, including imbibition of pollen with DNA (Hess 1980), *Agrobacterium*-mediated transformation (Hess 1987; Pechan 1989), electroporation of pollen (Matthews et al. 1990; Fennell and Hauptmann 1992; Jardinaud et al. 1993), and polyethylene glycol-mediated transformation (Fennell and Hauptmann 1992).

However, all these techniques are problematic in their applicability or reproducibility. Electroporation has been reported to be effective to introduce foreign DNA into germinating pollen (Matthews et al. 1990), but it is not applicable to plant species in which *in vitro* pollen germination is difficult. Stoger et al. (1992) did not succeed in transformation of tobacco pollen by the imbibition of DNA solutions or co-cultivation with *Agrobacterium tumefaciens*. Hydrated pollen has been reported to have high nuclease activity, which is considered to be inhibitory to the introduction of exogenous DNA into pollen cells (Matousek and Tupy 1983; Booy et al. 1989). Isolation of pollen protoplasts is difficult except for some plant species including members of Liliaceae (Tanaka et al. 1987, 1989; Zhou 1988). *Agrobacterium* is generally thought not to infect pollen cells. Thus, studies on pollen transformation are far behind from those on transformation of somatic cells, and hence more reproducible and reliable techniques for gene transfer into pollen are needed.

Since the pioneering study of Klein et al. (1987), particle

bombardment has been shown to be a vital method for gene delivery into various types of intact plant cells and tissues. Initially transformation of pollen grains by this method was thought to be difficult because these cells have a thick and rigid exine except for the germ pore region. Twell et al. (1989b) were the first to show that pollen grains of tomato and tobacco can be transiently transformed by particle bombardment. Since then successful transient transformation by this method has been reported for pollen of various plant species, including dicotyledonous species e.g., tobacco (Twell et al. 1989b, 1991a; McCormick et al. 1991; Stoger et al. 1992), tomato (Twell et al. 1989b), *Nicotiana glutinosa* (Plegt et al. 1992) and monocotyledonous species e.g., maize, *Tradescantia* (Hamilton et al. 1992) and lily (Plegt et al. 1992).

I describe here my studies on gene transfer and expression in angiosperm pollen by particle bombardment. Using this system, transgenic haploid plants were regenerated via *in vitro* culture of bombarded pollen.

In chapter I, I first describe on my results on transient transformation of pollen cells by particle bombardment. In section 1, I show successful transient expression of the *gus* gene in pollen of various plant species. Optimization of bombardment conditions for pollen was also established mainly using lily pollen as described in section 2. In section 3, I compared the activities of promoters of pollen-expressed genes in pollen of mono- and dicot plants and found that dicot LAT52 promoter (monocot Zm13 promoter) has higher activity in dicot pollen than

monocot ones (in monocot pollen than in dicot ones). In section 4, I observed GUS-expressing lily pollen cytologically and found that introduced gold particles were seen in intracellular compartments of pollen, including the vegetative cytoplasm, vegetative nucleus, and generative cytoplasm.

In chapter II, I describe my studies on successful production of transgenic haploid plants via *in vitro* androgenesis of bombarded immature pollen. In section 1, I describe regeneration and cytological analysis of the transgenic haploid plants. Southern blot analyses and enzyme assays of them confirmed that these are true transgenic plants as described in section 2.

CHAPTER I

GENE DELIVERY AND EXPRESSION IN POLLEN CELLS BY PARTICLE BOMBARDMENT

Section 1. Transient expression of the β-glucuronidase gene in pollen by particle bombardment

A β-glucuronidase (GUS) gene is a useful reporter gene for studies of gene expression in higher plants because of its low endogenous activity in most types of plant cells (Jefferson et al. 1987). However, pollen cells of various plant species in general have a strong endogenous GUS activity which interferes with detection of the expression of the introduced foreign genes (Plegt and Bino 1989; Alwen et al. 1992).

Thus, the *gus* gene driven by an anther-specific LAT52 (Twell et al. 1989a) promoter from tomato was reported to be expressed in pollen grains of tobacco and tomato but not of lily (Twell et al. 1989b, 1991b). Plegt et al. (1992) also reported similar results that this promoter is silent in monocotyledonous lily pollen based on bombardment-mediated transformation. By changing the composition of the X-Gluc solution, however, I demonstrated that the β-glucuronidase gene under the control of the LAT52 promoter and the nopaline synthetase polyadenylation terminator was successfully expressed in pollen of lily(*Lilium longiflorum*), *Nicotiana tabacum*, *Nicotiana rustica* and *Paeonia lactiflora* by a pneumatic particle gun device (Iida et al. 1990b).

Firstly, 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). And yet lily had the strongest endogenous GUS activity of pollen among all the species studied here; when nonbombarded pollen was incubated in the same X-Gluc solution and at the same temperature, it took 15 min for lily (ca. 12 h for other species) before pale blue color appeared in the cells.

This background blue color due to the endogenous GUS activity was almost completely diminished when the bombarded 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 here. Both the presence of methanol and period of 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 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 faint blue color in the cells.

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). Similar results with both plasmids were obtained with pollen of tobacco, *N. rustica* and *P. lactiflora* (see Table 1).

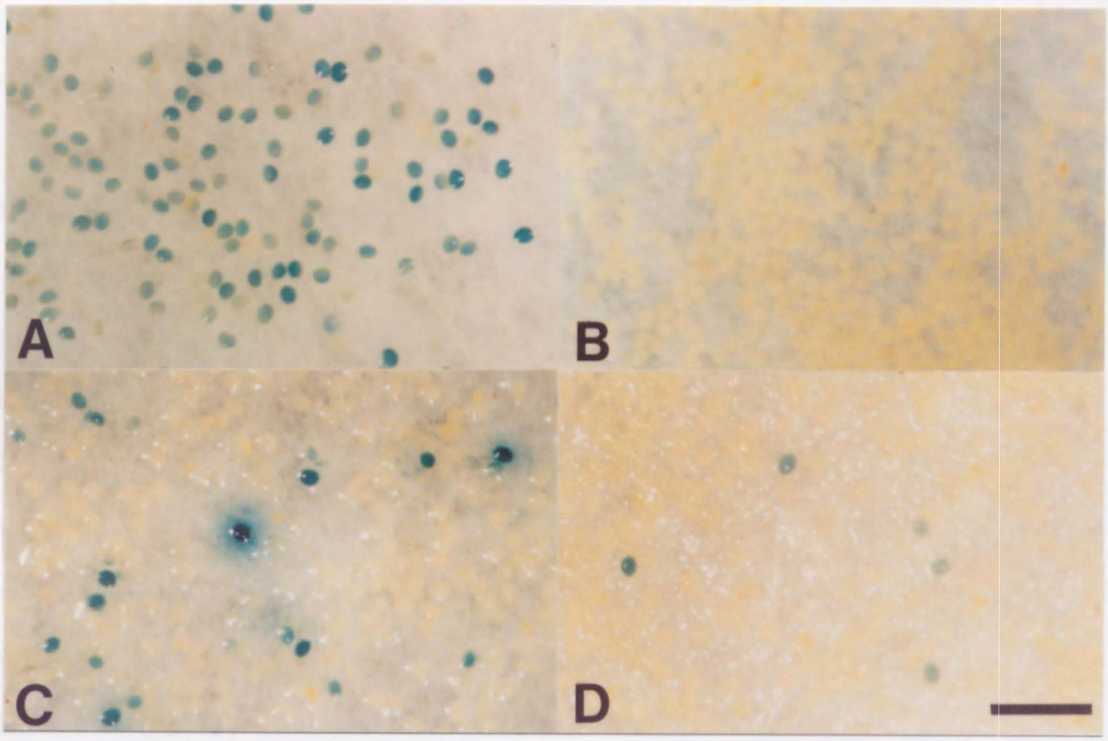

Figure 1. Results of histochemical assay for GUS expression in lily pollen. Mature pollen was bombarded with noncoated (A and B), pLAT52-7-coated (C) and pBI221-coated (D) gold particles, after which they were cultured at 26 °C for 24 h and incubated at 37 °C for 10 h in the X-Gluc solution containing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

The frequency of GUS-expressing pollen grains of five taxa after bombardment of pLAT52-7 and pBI221 is summarized in Table 1. The frequency in lily pollen with pLAT52-7 was at most 0.92%, which was close to that with tobacco (0.7%) or even higher than the values with *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 the same particle gun device (Yamashita et al. 1991).

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

It has been reported that transient expression of the *gus* gene driven by CaMV35S promoter is not detected histochemically in pollen grains of lily (Plegt et al. 1992) and *N. glutinosa* (Plegt et al. 1992) or only rarely detected in those of tobacco (Twell et al. 1989b; Stoger 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 CaMV35S promoter has not been detected histochemically. However, my present result clearly indicates that CaMV35S promoter does

Table 1. Number of GUS-expressing pollen observed in pollen of 5 taxa bombarded with pLAT52-7 and pBI221

Pollen		Time of incubation with X-Gluc (h)	No. of GUS-expressing pollen	
Species	Stage		pLAT52-7	pBI221
L. longiflorum	mature	10	297±73[a](0.74±0.18)[b]	65±17[a](0.16±0.04)[b]
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 of 3 to 4 experiments ± standard deviation.

[b] Figures in parentheses are percent of total.

induce expression of the *gus* gene in all of the five taxa studied although the frequency of GUS-expressing pollen with pBI221 was 2 to 6 times lower than that with pLAT52-7 depending on plant species and taxa (see Table 1). I think that this low frequency with pBI221 is somewhat related to the fact that the activity of the GUS enzyme was lower in the grains bombarded with pBI221 than with pLAT52-7 (see also chapter I, section 3).

In conclusion, I have presented evidence to show that pollen grains including those of lily have in general strong endogenous GUS activity, and that this background activity hinders the expression of LAT52-driven GUS activity in lily pollen. This background activity of GUS in these pollen cells can successfully and almost completely be suppressed by incubating bombarded pollen sample for less than 12 h in an enzyme assay solution in the presence of 20% methanol and phosphate buffer of pH 7 as shown in Fig. 1. Prior to this study, previous authors have shown that the addition of 20% methanol (Kosugi et al. 1990) and use of neutral pH solution (Alwen et al. 1992) are vital to suppress endogenous GUS activities in various plant cells.

The LAT52 promoter also has been used successfully to drive the expression of foreign genes in pollen of various species including tobacco (Twell et al. 1989b, 1991a; McCormick et al. 1991), *Nicotiana glutinosa* (Plegt et al. 1992). Two other types of anther-specific promoters from tomato (LAT56 and LAT59) and a pollen-specific PA2 promoter from petunia (Van Tunen et al. 1990) have also been shown to work in pollen of tobacco (McCormick et

al. 1991; Twell et al. 1991a; Stoger et al. 1992). The Zm13 promoter from maize (Hamilton et al. 1989; Hanson et al. 1989) has also been shown to work in pollen of maize, *Tradescantia* (Hamilton et al. 1992) and in lily and tulip (see chapter I, section 3).

Section 2. Optimization of bombardment conditions for pollen cells

1. Physical factors that influence gene delivery efficiency in pollen

The efficiency of gene delivery and expression in bombardment-mediated transformation in somatic cells is largely influenced by physical and cell physiological factors. Physical parameters such as type of metal particles (size, shape), accelerating pressure, amount of metal particles per projectile, and the number of shots affects transient expression frequency of the introduced *gus* gene (Klein et al. 1988a). In the case of lily pollen, increase of accelerating pressure from 84 to 200 kg/cm^2 gave rise to ca. 9-fold higher GUS expression frequency as shown in Table 1. Bombardment with gold particles of average 1.1 μm in diameter (Tokuriki Honten Co., Ltd., Tokyo, Japan) gave ca. 3-fold higher frequency than the particles of average 2.0 μm diameter (Alfa Chemical Co., Danvers/MA). Also, a change in the amount of gold particles from 0.05 to 0.2 mg/projectile gave ca. 2-fold higher frequency.

Interestingly, the number of shots greatly influenced the frequency of pollen showing GUS expression (Fig. 1). Multiple shots of pollen grains result in the introduction of gold particle(s) into all of the pollen cells. It is possible that all of these cells have DNA in their cellular compartment(s). Pollination using pollen grains after being subjected to multiple shots is an interesting experiment for future study.

Table 1. Effect of accelerating pressure on GUS expression frequency in mature pollen of lily after bombardment with pLAT52-7

Accelerating pressure (kg/cm^2)	GUS-expressing cells (% of total)	GUS activity (pmole 4-MU/h /μg protein)
84	0.07±0.03[a]	0.30±0.13
150	0.31±0.13	1.10±0.40
200	0.60±0.13	1.20±0.27

[a] Average of 3 experiments ± standard deviation.

Bombarded pollen was cultured for 24 h and assayed histochemically or fluorometrically. The number of pollen cells in a target sample was ca. $4x10^4$.

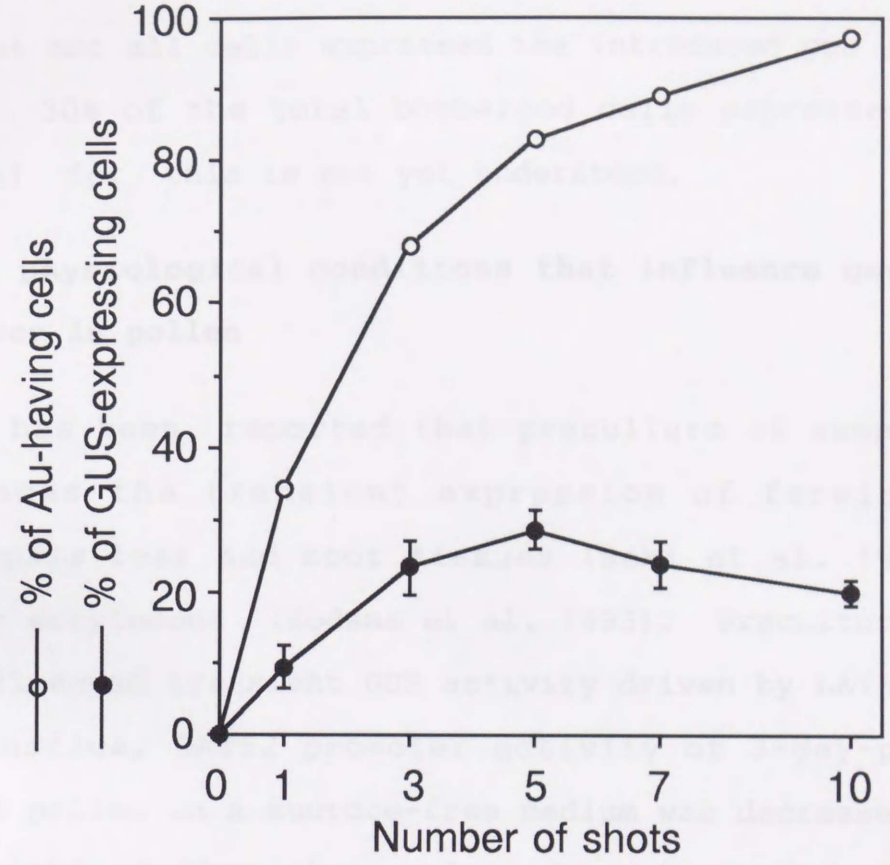

Figure 1. Effects of multiple shots on the frequencies of gene delivery and gene expression in mature pollen of lily. About $5x10^4$ cells were bombarded with the -260-GUS construct (Hamilton et al. 1992) and cultured for 24 h, after which they were assayed histochemically for GUS expression as described in chapter I, section 1. The number of Au-having and GUS-expressing pollen was determined microscopically. More than 200 and 500 cells were counted, to determine the frequencies of Au-having and GUS-expressing cells, respectively. The vertical bar indicates standard deviation of three experiments.

Note that not all cells expressed the introduced *gus* gene; at the most ca. 30% of the total bombarded cells expressed the gene. Reason(s) for this is not yet understood.

2. Cell physiological conditions that influence gene delivery efficiency in pollen

It has been reported that preculture of sample tissues influences the transient expression of foreign gene in *Arabidopsis* leaf and root tissues (Seki et al. 1991) and in cucumber cotyledons (Kodama et al. 1993). Preculture of pollen also influenced transient GUS activity driven by LAT52 promoter. In *N. rustica*, LAT52 promoter activity of 3-day-precultured immature pollen in a sucrose-free medium was decreased markedly, i.e., 1/150 of that observed in freshly isolated pollen by particle bombardment.

The stage of pollen seems to influence the expression efficiency of introduced foreign genes. In pollen of lily and *N. rustica*, no or very low GUS activities were observed when bombarded at early binucleate stage, while at the late binucleate stage the GUS activity was more than 7-fold that of the early binucleate stage in pollen of both species.

A change in the culture medium from White's modified medium (White 1963) to Murashige and Skoog's medium (1962) after bombardment did not influence the number of blue spots of GUS-expressing pollen. The effects of other components, such as, borate and Ca^{2+} that are known to stimulate pollen germination

and pollen tube growth (Vasil 1987), and osmotic stress (Armaleo et al. 1990; Vain et al. 1993) that has recently been shown to influence the efficiency of bombardment-mediated transformation are subjects of future study.

Section 3. Comparison of promoter activities of pollen-expressed genes in pollen of mono- and dicot plants

A number of pollen-expressed genes have been cloned (Mascarenhas 1990, 1992; McCormick 1991), and their 5′ upstream regulatory sequences have been isolated and studied using transient expression assay by particle bombardment and transgenic plants obtained by *Agrobacterium* method. Among them, the LAT promoters from tomato anther-expressed genes and the Zm13 promoter from maize pollen-expressed gene are most extensively studied.

Interestingly, results obtained by gene expression assay of pollen of tobacco that were bombarded with constructs containing deleted upstream sequences of various lengths connected to the reporter (*gus*) gene are in good agreement with those obtained by the analysis of pollen of transgenic tobacco that contain the same constructs in the genome. Both these results indicate that the minimal proximal sequences of regulatory region of tomato LAT52 and LAT59 promoters required for their correct temporal and spatial expression during pollen development are approximately 200 bp (-71 to +110 for LAT52 and -115 to +91 for LAT59) (McCormick et al. 1991; Twell et al. 1991a).

The upstream region of a pollen-expressed gene (Zm13) from maize (Hamilton et al. 1989; Hanson et al. 1989) has been characterized by transient expression assay using *Tradescantia paludosa* pollen by particle bombardment (Hamilton et al. 1992). These authors have shown that sequences necessary for pollen-

specific expression are present in a region from -100 to -54 and those between -260 to -100 are necessary for amplification of the pollen-specific expression of the gene. This result agrees with that obtained from the analysis of pollen of transgenic tobacco; the latter analysis shows that 375 bp sequences between -314 to +61 are necessary for correct temporal and spatial expression of the gene (Guerrero et al. 1990). These results indicate that particle bombardment is useful for rapid assay for the activity of deletion mutants to determine essential cis-elements in 5′ upstream sequences.

Tables 1 and 2 summarize GUS enzyme activities detected by histochemical and fluorometric assay, respectively, in pollen of lily, tulip, tobacco and *N. rustica* that were bombarded with three different promoter constructs (Fig. 1), in which the *gus* gene is driven by Zm13 (plasmid -260-GUS), LAT52 (pLAT52-7) and CaMV35S (pBI221) promoter. A single shot was given to each of the target pollen samples under standard bombardment conditions. The number of GUS-expressing cells per target sample of pollen highly depends on the type of promoter. In the case of lily pollen, more than 30% of the target pollen sample received gold particle(s) after a single shot (see chapter I, section 2), and more than 5% of the cells bombarded with -260-GUS expressed the *gus* gene while only 0.6% of the cells bombarded with pLAT52-7 expressed this gene (Table 1). Essentially similar results were obtained with tulip pollen. Thus, in lily and tulip pollen the Zm13 promoter from monocot maize is much more active than the LAT52 one from tomato. Bombardment with pLAT52-7 resulted in

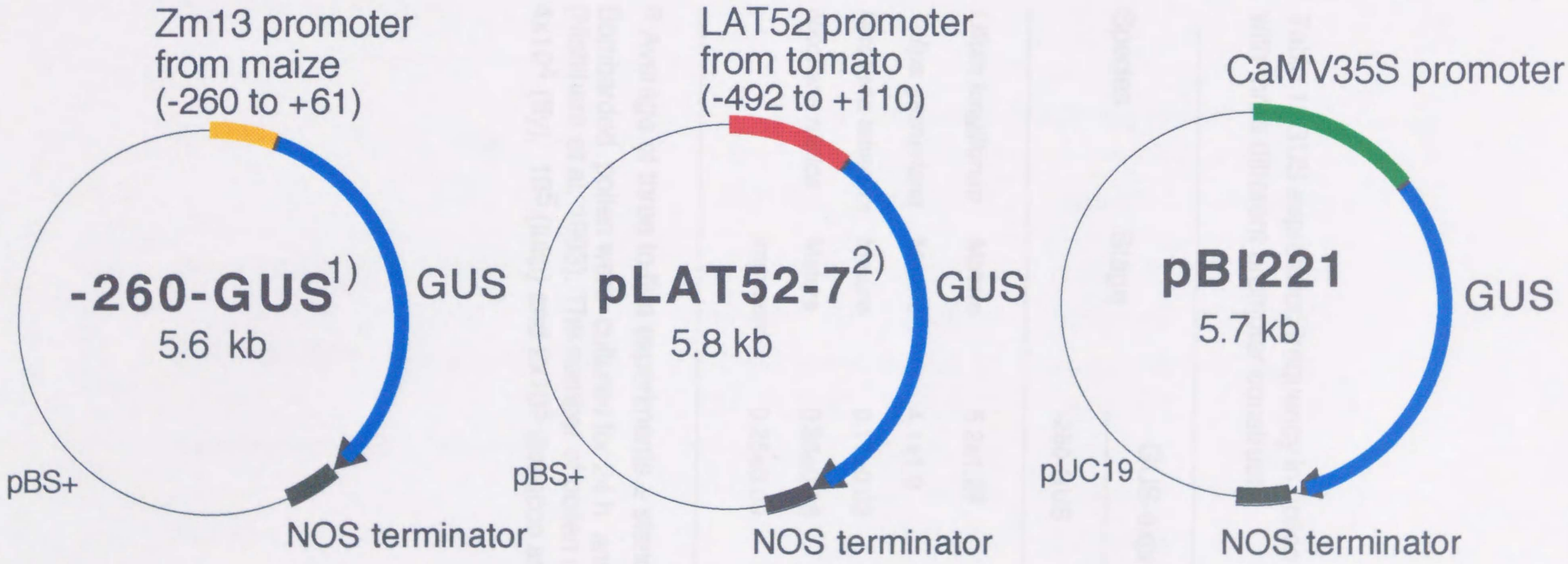

Figure 1. Plasmid constructs used for bombardment

1) Hamilton et al., (1992)
2) Twell et al., (1989b)

Table 1. GUS expression frequency in pollen of four plant species bombarded with three different promoter constructs.

Species	Stage	GUS-expressing cells (% of total)		
		-260-GUS	pLAT52-7	pBI221
Lilium longiflorum	Mature	5.2±1.2[a]	0.61±0.09[a]	0.14±0.07[a]
Tulipa gesneriana	Mature	4.1±1.9	0.10±0.03	0.34±0.08
Nicotiana tabacum	Mature	0.17±0.03	0.34±0.04	0.07±0.02
Nicotiana rustica	Mature	0.33±0.08	0.56±0.11	0.10±0.03
	Immature	0.25±0.09	0.42±0.10	0.13±0.02

[a] Average of three to five experiments ± standard deviation.

Bombarded pollen were cultured for 24 h and assayed histochemically (Nishihara et al. 1993). The number of pollen cells in a target sample was $4x10^4$ (lily), 10^5 (tulip) and $2x10^5$ (tobacco and *N. rustica*).

Table 2. GUS activity in pollen of four plant species after bombardment with three different promoter constructs.

Species	Stage	20% methanol	GUS activity (pmole 4-MU[a]/h/μg protein)			
			-260-GUS	pLAT52-7	pBI221	Control (Au)
Lilium longiflorum	Mature	+	642.0±159.7[b]	1.31±0.55[b]	0.36±0.01[b]	0.01±0.01[b]
		-	740.1±287.6	1.37±0.55	0.36±0.02	0.10±0.01
Tulipa gesneriana	Mature	+	837.6±116.7	2.24±0.87	89.6±21.6	0.24±0.24
		-	868.1±136.8	2.88±0.73	82.5±21.6	0.80±0.17
Nicotiana tabacum	Mature	+	61.6±10.4	3222±793	0.99±0.43	~0
		-	60.5±12.7	3164±849	0.99±0.43	0.10±0.09
Nicotiana rustica	Mature	+	120.8±52.7	1810±887	0.77±0.18	0.02±0.01
		-	136.6±67.4	1799±735	0.78±0.34	0.16±0.06
	Immature	+	215.5±19.1	702.4±139.2	12.2±2.2	0.05±0.03
		-	178.7±26.3	640.6±129.6	13.2±4.2	0.09±0.02

[a]4-methyl umbelliferone.

[b]Average of three experiments ± standard deviation.

Bombarded pollen were cultured for 24 h and assayed fluorometrically for GUS enzyme activity. The enzyme assay solution contained (+) or did not contain (-) 20% methanol . Control (Au) corresponds to the results of pollen cells bombarded with noncoated gold particles.

almost 2-fold higher number of GUS-expressing cells in mature pollen of tobacco and *N. rustica* than that with -260-GUS. Thus, this suggests that in tobacco and *N. rustica* pollen the LAT52 promoter is more active than Zm13 one. Accordingly, it is likely that Zm13 and LAT52 promoters have cis-acting elements in their DNA sequences that determines monocot- and dicot-pollen-specific gene expression (McCormick 1991; Mascarenhas 1992). Similar difference in cis-acting elements between mono- and dicots has been reported in rice *cab* gene promoter with somatic cells (Luan and Bogorad 1992).

Based on the histochemical analysis of bombarded pollen, the CaMV35S promoter does induce expression of the *gus* gene in pollen (see also chapter I, section 1) and its activity in pollen seems to somewhat depend upon plant species (Table 1, also see chapter I, section 1).

Fluorometric assay showed that -260-GUS gave ca. 400 to 500-fold higher GUS activity than pLAT52-7 in pollen of monocots, while pLAT52-7 gave ca. 15 to 50-fold higher activity than -260-GUS in pollen of dicots (Table 2). This is in line with the results obtained by histochemical assay of the expression of this gene shown in Table 1, but the difference in the GUS expression of each promoter between monocot and dicot pollen is much more strengthened by fluorometric assay than by histochemical assay. It is conceivable that fluorometric assay reflects the enzyme activity more directly than the histochemical assay.

Addition of 20% methanol in the X-gluc solution for GUS

enzyme activity assay greatly decreased background endogenous GUS activity (see chapter I, section 1). As shown in Table 2, addition of the same in the fluorometric enzyme activity assay solution also greatly decreased the background level of "GUS activity" in the extract from pollen grains that were bombarded with noncoated gold particles. The presence and absence of methanol in the enzyme assay solution for fluorometry did not largely affect the GUS activities detected in the extracts from pollen grains bombarded with DNA-coated gold particles, and the values obtained by assay in the presence or absence of methanol was within experimental errors in each of plasmid/pollen combinations (see Table 2).

The CaMV35S promoter seemed to have, in general, low activity than the other promoters tested in pollen grains, but interestingly tulip pollen grains are exceptional, and more than 10-fold activity was observed in mature pollen grains of tulip than those of the other plant species. The difference in GUS-activities among different constructs (driven by different promoters) were smaller in immature than in mature pollen of *N. rustica* (see Table 2), suggesting that these promoters become more specific to pollen during their development.

Section 4. Cytological observation of GUS-expressing pollen of lily

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 dense starch granules which hampered direct observation of gold particles introduced into the cells. But when bombarded pollen grains of lily, after being assayed for GUS expression, fixed with acetic ethanol, hydrolyzed with 1N HCl to remove the exine and starch granules and stained with orcein, introduced gold particles became microscopically visible as black dots in the cells (Fig. 1). In GUS-expressing pollen that had been introduced with pLAT52-7, gold particles were seen in the vegetative cytoplasm (arrow heads 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 1N HCl at 60°C for 10 min) may have caused displacement of introduced particles from the sites of their original localization. Note that no blue color was detected in pollen bombarded with noncoated gold particles (A).

The number of gold particles detected in GUS-expressing pollen did not seem to influence the intensity of blue color; its number was 11, 1 and more than 6 in B, C and D, respectively (see Fig. 2). GUS-expressing pollen that had more than 100 gold particles were also observed (data not shown) but the intensity

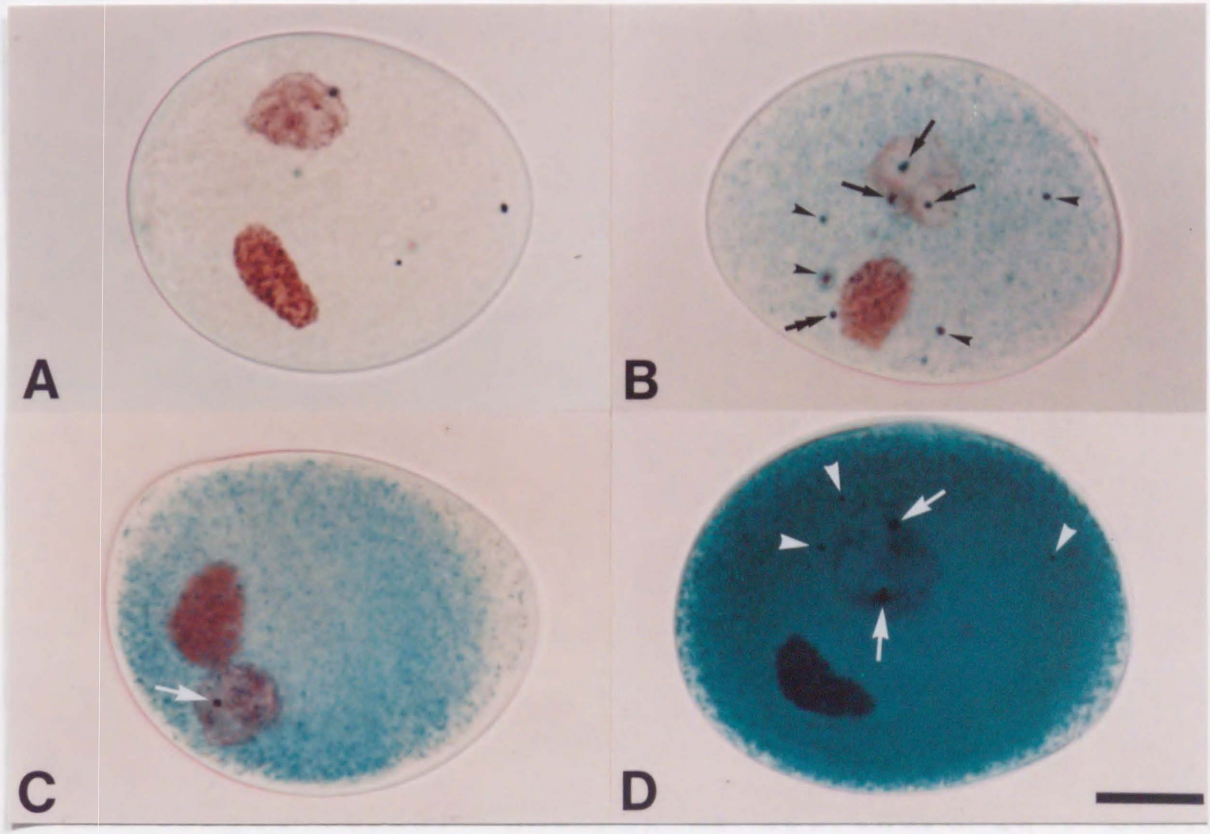

Figure 1. Typical photomicrographs for cytological observation of gold particles in GUS-expressing lily pollen. Mature pollen was bombarded with noncoated (A) and pLAT52-7- coated (B, C and D) gold particles, after which they were cultured at 26 °C for 24 h and incubated at 37 °C for 10 h in the X-Gluc solution containing 20% methanol and 0.1M sodium phosphate. They were then fixed with acetic ethanol, treated for removal of exine and starch granules, and stained with propionic orcein. Gold particles were seen as black dots in the vegetative cytoplasm (arrowheads in B and D), vegetative nucleus (arrow in B, C, and D), and generative cytoplasm (double arrow in B). Bar = 30 μm

of blue color in these cells was similar to B or D. The Reason(s) for this is not fully understood yet. The fact that lily pollen grains frequently expressed introduced foreign *gus* gene even when they had more than 100 gold particles is in contrast to the case of cultured tobacco cells, in which introduction of more than 3 gold particles per cell seemed to be lethal to the cell. Pollen cells in general are rich in cytoplasm and practically lack vacuole, while cultured tobacco cells have a large vacuole in them. Thus it is likely that the absence of vacuoles may be a cause for the high tolerance of pollen to multiple shots of gold particles.

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 of future study.

To determiner whether or not the gold particles were in fact introduced into the generative cells, bombarded lily pollen was treated enzymatically and the generative cells were isolated and stained with propionic orcein. Figure 2 shows photomicrographs of the isolated generative cell. Clearly, a black dot (gold particle) was observed in the cytoplasm of the generative cell. The number of generative cells that have a gold particle is very low. Under standard bombardment conditions, one cell per 10^3 to

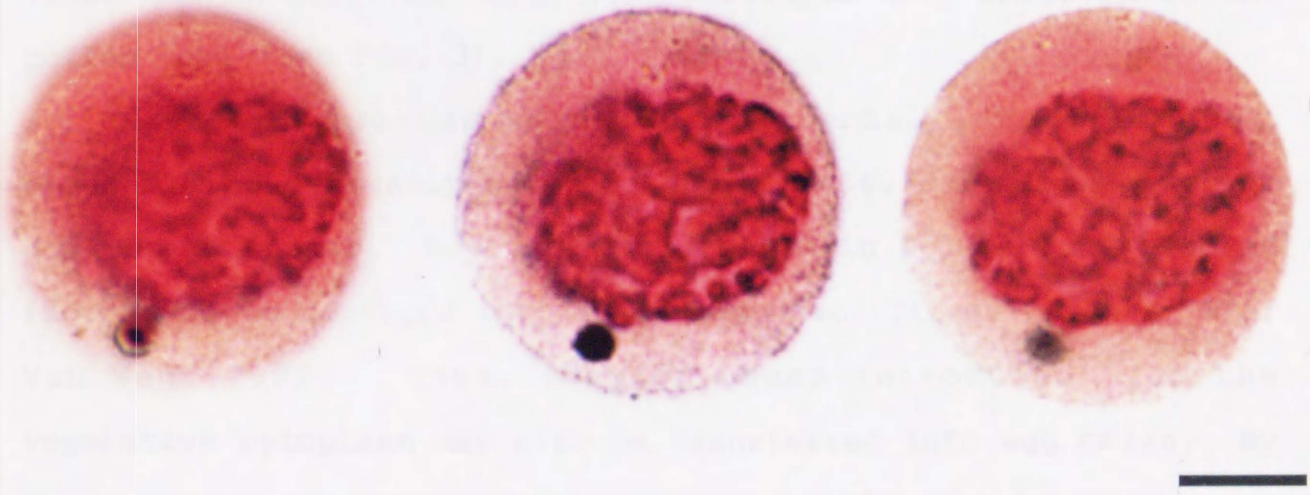

Figure 2. Generative cell isolated from bombarded lily pollen. Mature pollen was bombarded with gold particles, after which they were subjected to enzyme treatment for isolation of generative cells as described by Tanaka et al. (1989). The microphotographs were those of the same cell taken under different focuses to confirm the location of a gold particle in the cell. Bar = 30 μm

10^4 was observed to contain a gold particle.

The introduction and expression of the introduced DNA seems to be almost restricted to the vegetative nucleus. But the introduced gold particles did be seen in generative cytoplasm of pollen (Fig. 2). There have been studies to show that bombarded pollen grains retain their germination ability in tobacco (Twell et al. 1989b; Stoger et al. 1992), *Tradescantia* (Hamilton et al. 1992) and in lily, in which gold particles were observed in the pollen tube (see Fig. 3).

In plant species which show maternal inheritance of organelle DNA, usually only the sperm nuclei carry the paternal DNA to embryo sac. However, foreign DNA in pollen tube also is thought to be conveyed into the embryo sac (Tilney-Bassett 1978; Van Went 1992). Thus, foreign genes introduced into the vegetative cytoplasm may also be transferred into egg cells. My preliminary experiments showed that lily plants that were pollinated with bombarded pollen set seeds normally. In conclusion, these results strongly suggest that the possibility of the production of transgenic seeds by direct pollination of bombarded pollen.

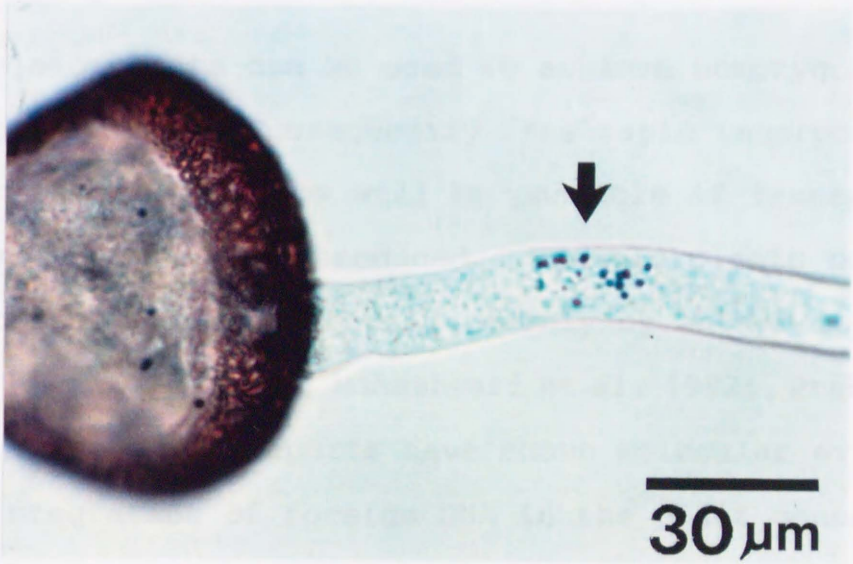

Figure 3. Germination of a mature pollen grain that was bombarded with DNA-coated gold particles. Bombarded lily pollen grains were cultured for 24 h in White's modified medium supplemented with 0.5 M sucrose at 26°C in the dark, and microscopically observed. More than ten gold particles (seen as black dots indicated by an arrow) were detected in the pollen tube.

CHAPTER II

TRANSGENIC HAPLOID PLANTS PRODUCED BY BOMBARDMENT-MEDIATED TRANSFORMATION

Section 1. Regeneration of pollen-derived transgenic haploid plants of *Nicotiana rustica*

Haploid plants can be used to achieve homozygosity within a single generation. Consequently, the rapid incorporation of new genes into higher plants will be possible if transgenic doubled haploids can be easily produced. Haploid plants of hundreds of plant species now are produced mainly by anther/isolated pollen culture (e.g. Vasil 1980; Maheshwari et al. 1982; Prakash and Giles 1987). To date, no reports have shown molecular evidence of the stable integration of foreign DNA in the plant genome that would verify the formation of transgenic haploid plants via *in vitro* androgenesis of transformed pollen. Various means of pollen transformation, including incubation of pollen in DNA solutions, cocultivation with *Agrobacterium*, microinjection and electroporation have been tried, but in no case have the plants produced been confirmed as transgenic by molecular data (reviewed by Heberle-Bors, 1991).

More Recently, production of transgenic haploid plants have been reported by electroporation of protoplasts isolated from microspore-derived cultures of maize (Sukhapinda et al. 1993). To my knowledge, however, transgenic haploid plants, in which integration of foreign genes were confirmed by molecular

evidence, produced from *in vitro* androgenesis of transformed pollen cells have nor been reported (Heberle-Bors 1991; Hahn et al. 1992).

Recently, the pollen of various higher plant species, can be transformed transiently by particle bombardment as described in chapter I. The production of stable transgenic plants by the bombardment of pollen is attractive, but to my knowledge, no successful results have been reported of stable transformation by pollination (Twell et al. 1991b) or of *in vitro* androgenesis of bombarded pollen.

High frequency regeneration of haploid plants through direct embryogenesis in *Nicotiana rustica* immature pollen has been achieved by isolated pollen culture (Imamura et al. 1982; Kyo and Harada 1985), and *N. rustica* pollen also can be transformed transiently as shown in chapter I. I here describe the first successful regeneration of transgenic haploid plants of *N. rustica* from immature pollen that had undergone particle bombardment.

1. Selection of kanamycin-resistant plants from bombarded pollen

Firstly, immature pollen of *Nicotiana rustica* was subjected to starvation treatment for 3 days as described by Kyo and Harada (1990). The treatment of *N. rustica* pollen cells at the mid-bicellular stage in 3 days in nutrient-free medium, caused starch grains to disappear and the formation of a vacuole and a characteristic cytoplasm suspended in the central area of the cell by cytoplasmic strands, but there was no division of pollen

cells as reported previously (Kyo and Harada 1990). All the bombardments therefore were made into pollen cells that had not undergone division rather than to dividing or divided pollen cells.

Following starvation treatment, the pollen was bombarded with gold particles coated with plasmid DNA pBN24 encoding neomycin phosphotransferase II (NPTII) and β-glucuronidase (GUS) genes which, respectively, are under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator in the plasmid (Fig. 1). The selection of bombarded pollen with kanamycin-containing medium produced three kanamycin-resistant pollen embryoids from a total $2x10^6$ bombarded immature pollen cells ($2x10^5$/filter paper) within 1 month after bombardment. As the embryoids were derived from three independent shots, they therefore are considered to have been originated from different transformation events. No kanamycin-resistant embryoids developed from the nonbombarded *N. rustica* pollen.

The putative transgenic pollen embryoids were transferred to solidified MS medium containing 3% sucrose and 200 mg l^{-1} kanamycin about 40 days after bombardment. Two developed into kanamycin-resistant plantlets (lines 1 and 2) about 2 months after bombardment. These plantlets were propagated vegetatively on the same medium for 4 to 10 months, then the more than 10 vegetatively propagated individuals of each line were transferred to pots.

2. Cytological observation of putative transgenic plants

Figures 2A and B show typical plants developed from the kanamycin-resistant plantlets of the two lines. All the regenerated plants of each lines were dwarf and had shortened stamen filaments, characteristics of haploid plants regenerated from immature pollen.

Figures 2C and D show typical metaphase chromosomes of root tip cells from the two lines of putative transgenic plants. The number of metaphase chromosomes counted in the root tip cells of both lines was 24, the same number as in the *N. rustica* haploid genome (Narajan and Rees 1974), evidence that both putative transgenic plant lines were haploid.

Both lines had aborted pollen grains in their anthers at the mature stage, probably because of haploidy. Greatly reduced pollen grain viability also was confirmed by the fluorochromatic reaction (FCR) test. These plants produced no seeds after self-pollination. Currently, I am using colchicine treatment to regenerate doubled haploid plants in order to obtain seeds.

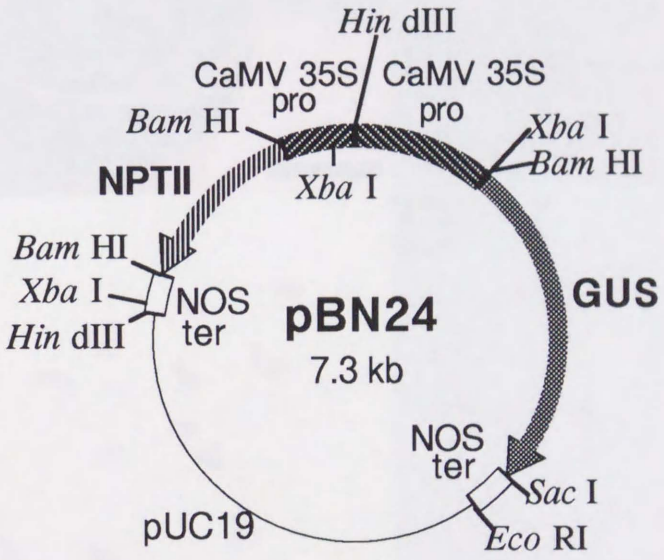

Figure 1. Map of the plasmid (pBN24) used for transformation. The restriction sites for Southern blot analysis are indicated.

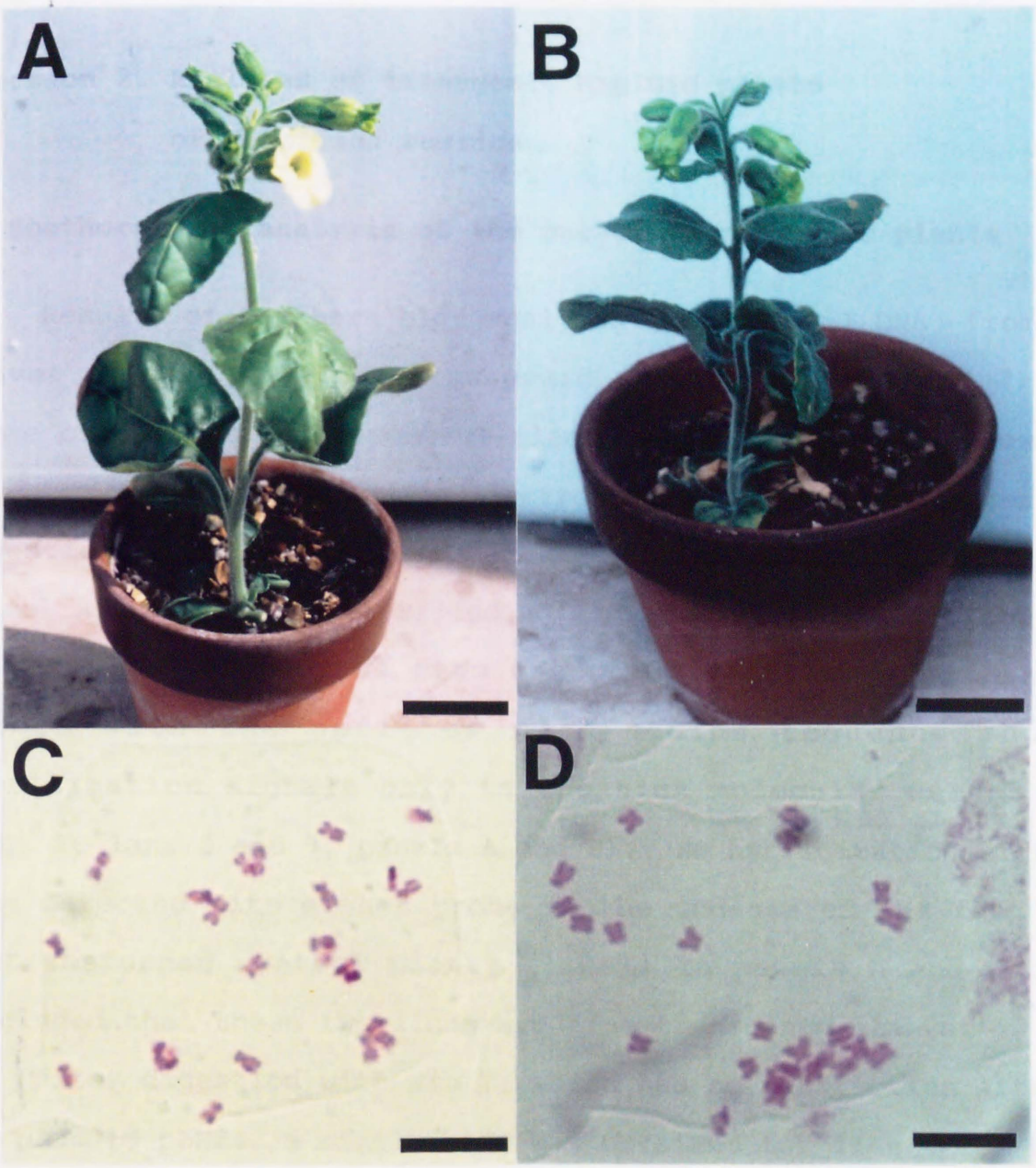

Figure 2. A typical putative transgenic plants and metaphase chromosomes taken from root tip cells. (A) About 3 months after transfer to a pot, totally 9 months after bombardment. (B) About 2 months after transfer to a pot, totally 8 months after bombardment. (C), (D) Mitotic metaphase chromosomes of a root tip cell from another plant. Preparation and staining of root tip cells were done according to Morikawa et al. (1987). Twenty-four chromosomes were counted per cell. (A) and (C) are line 1 and (B) and (D) are line 2. Bars: 3 cm for (A) and (B) and 10 μm for (C) and (D).

Section 2. Analyses of transgenic haploid plants of *Nicotiana rustica*

1. Southern blot analysis of the putative transgenic plants

Results of Southern blot analyses of the total DNA from the leaves of the putative transgenic plants (lines 1 and 2) and those of untransformed control plants of *N. rustica* are shown in Fig. 1. The total DNAs were electrophoresed before and after the digestions with the various restriction enzymes, and the *nptII* (panel A) or *gusA* (panel B) coding sequence used as the probe.

The undigested DNA from the putative transgenic plants probed with the *nptII* or *gusA* coding sequence showed hybridization signals only in the high molecular weight DNA (Fig. 1, lane 3 and 7, panels A and B). No hybridization signals were detected with either probe in the undigested DNA from the nontransformed control plants (lane 1 in panels A and B). I concluded that these two lines are true transgenic plants.

After digestion with *Stu* I, which has no restriction site in the plasmid pBN24, a single band was obtained for line 1 for both probes (lane 4, panels A and B). In contrast, the *Stu* I-digest of line 2 gave two bands for both probes (lane 8, panels A and B). These results suggest that the number of loci at which *nptII* and *gusA* gene insertions occurred was at least one for line 1 and 2 for line 2. Note that no hybridization signals were detected in the *Stu* I-digest of the control plants (lane 2, panels A and B).

In the line 1 digest restricted with *Hin*dIII, which excises

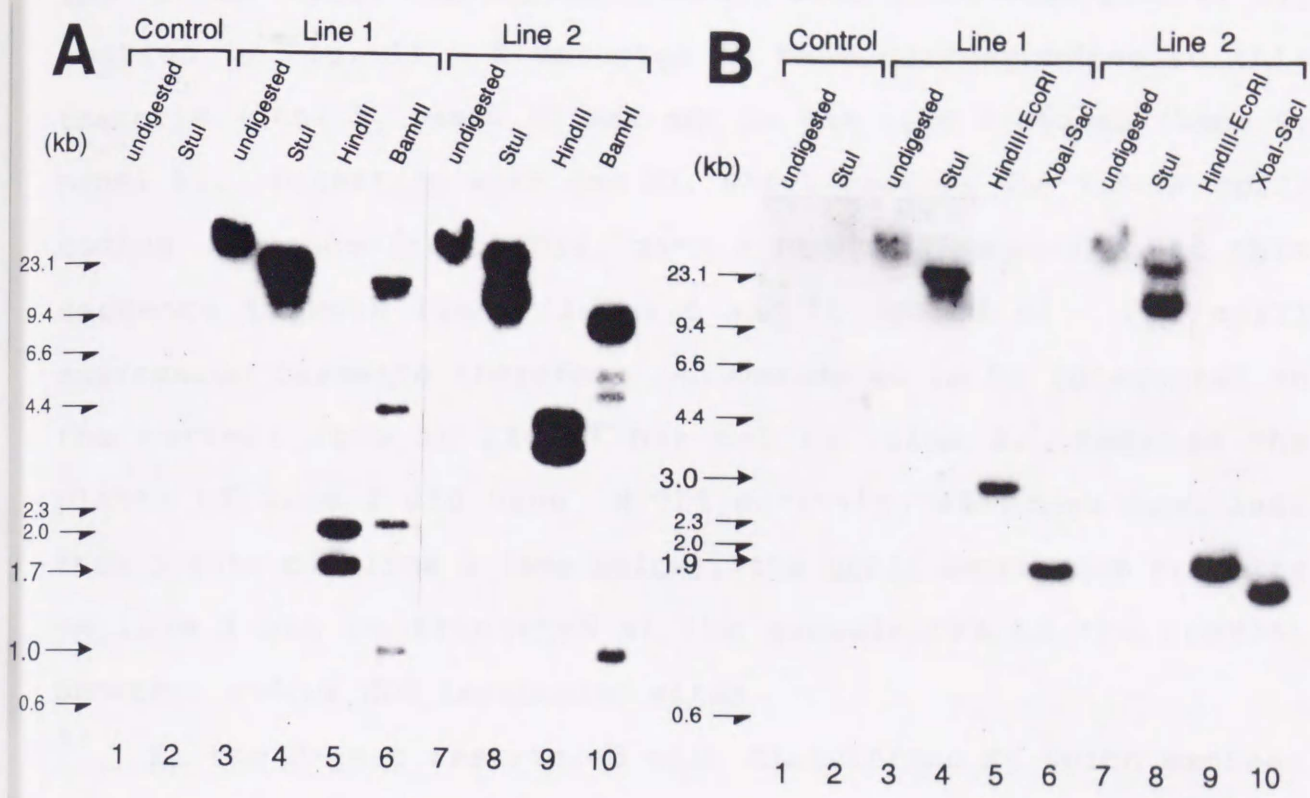

Figure 1. Southern blot analysis of the total DNA from the putative transgenic plants and an untransformed *N. rustica* plant. The total DNAs isolated from the leaves of the two transformed line plants and an untransformed control plant regenerated from nonbombarded pollen were used undigested or digested with the restriction enzyme indicated at the top of each lane then subjected to hybridization. Control: lanes with the DNA from the untransformed plant. The amount of DNA loaded per lane was 4 μg for line 1 and 8 μg for line 2 and the control. The membrane was probed with the coding sequence of the *nptII* gene excised as a 1.0 kb *Bam* HI fragment (panel A) or with the coding sequence of the *gusA* gene excised as a 1.9 kb *Xba* I/*Sac* I fragment (panel B). Sizes that correspond to the expression cassette and coding sequence of the *nptII* or *gusA* gene are indicated by arrows (⟶). The fragment sizes of a lambda DNA marker digested with *Hin*dIII are indicated by flags (⟶).

the 1.7-kb *nptII* expression cassette from pBN24 (see chapter II, section 1, Fig. 1), I detected a band corresponding to this cassette (lane 5, panel A) but not in the line 2 digest (lane 9, panel A). Digestion with *Bam* HI, which excises the 1.0-kb *nptII* coding sequence from pBN24, gave a band corresponding to this sequence in both lines (lanes 6 and 10, panel A). The *nptII* expression cassette therefore is considered to be integrated in the correct form in line 1 but not in line 2. Because the plants of line 2 did have NPTII activity, although much less than plants of line 1 (see below), the *nptII* expression cassette in line 2 may be truncated at the genomic DNA of the CaMV35S promoter and/or NOS terminator sites.

In the digest restricted with *Hin*dIII/*Eco* RI which excises the 3.0 kb *gusA* expression cassette from pBN24, I detected a band corresponding to the cassette in line 1 (lane 5, panel B) but not in line 2 (lane 9, panel B). Digests with *Xba* I/*Sac* I which excises the 1.9 kb *gusA* coding sequence from pBN24 (see Fig. 1) gave a band corresponding to this sequence in line 1 (lane 6, panel B), but not in line 2 (lane 10, panel B). These results show that the *gusA* expression cassette is integrated in the correct form in line 1 but not in line 2. Because the *Xba* I/*Sac* I digest of line 2 has a 1.7 kb fragment (lane 10, panel B) of shorter length than the intact *gusA* coding sequence (see chapter II, section 1. Fig. 1), no intact sequences of the *gusA* coding region are integrated in line 2. Consistent with this is the fact that line 2 plants had no detectable GUS activity (see below).

I estimated the number of copies of the introduced transgene from a comparison of the signal intensity of the copy-number standard and the corresponding band in the Southern blots. Line 1 respectively had four and three copies of the intact *nptII* and *gusA* gene expression cassettes per haploid genome. Line 2 was estimated to have four copies of the intact *nptII* coding sequence per haploid genome.

2. NPTII expression in transgenic plants

Table 1 shows the NPTII activity in leaf extracts of the transformant plants of lines 1 and 2. Clearly, NPTII activity in the line 1 plant is ca. 15-fold that of the control plant. The corresponding activity of the line 2 plant, however, is only ca. 1.7-fold. The reason for the low NPTII activity in the line 2 plant is not clear, but it may be related to Southern analysis results showing that this line does not contain the intact *nptII* expression cassette in its genome. It is not clear why the line 2 that shows the low NPTII activity was regenerated by kanamycin selection.

3. GUS expression in transgenic plants

Table 2 shows the GUS activities in the leaf extracts of these two lines of transgenic plants determined by fluorometric assay. The line 1 extract has GUS activity that is ca. 250-fold that of the control, whereas the line 2 extract does not show GUS activity above that of the control. This latter result is

Table 1. NPTII activities in leaves of two transgenic plant lines

Line no.	NPTII activity (cpm/μg protein)
1	1518
2	176
Control[a]	101

[a]Untransformed plants regenerated from nonbombarded pollen cells. Dot blot assay for NPTII activity was performed essentially according to An et al. (1985) as modified by Iida et al. (1990a).

Table 2. GUS activities in leaves of two transgenic plant lines

Line no.	GUS activity (pmole 4-MU[a]/min/mg protein)
1	153 ± 42[b]
2	0.8 ± 0.2
Control[c]	0.6 ± 0.2

[a]4-methyl umbelliferone

[b]Average of three independent preparations ± std.

[c]Untransformed plants regenerated from nonbombarded pollen cells. GUS activities were determined fluorometrically according to the procedure of Jefferson et al. (1987).

understandable because the line 2 plant does not have the intact *gusA* coding sequence in its genome.

The histochemical assay results for GUS expression in the transgenic plant tissues are shown in Fig. 2. Tissues excised from transformed and untransformed plants were incubated in the X-Gluc solution at 37°C overnight then fixed with 70% ethanol. Some leaves were stained uniformly (A), others showed chimeric staining (B). Vascular tissues were strongly stained both in the stem (D) and roots (E). Flowers at anthesis were well stained in parts of the sepal and faintly stained at the base of the stigma (F). A chimeric staining pattern also was frequent in stem tissues. The reasons(s) for chimeric staining of the leaves. The reason(s) for chimeric staining of the leaves and stems is not known, possibly the plants themselves have a chimeric nature. I am now using colchicine treatment to produce homozygous doubled haploid plants in order to analyze the genetic stability of the transgenes. Note that no blue staining is present in the line 2 plants (see C).

In my preliminary experiments, 3-day-cultured immature pollen cells of *N. rustica* that had been bombarded with plasmid pBN24, were cultured for 24 h then assayed histochemically for GUS expression. Blue, GUS-expressing pollen cells were present at the frequency of ca. 1.4×10^{-3}. This value is very close to a previous result showing that the frequency for freshly isolated immature pollen cells of *N. rustica* bombarded with pBI221 was 1.2×10^{-3} (Nishihara et al. 1993). This transient expression of the *gusA* gene shows that gene delivery to the 3-day-cultured

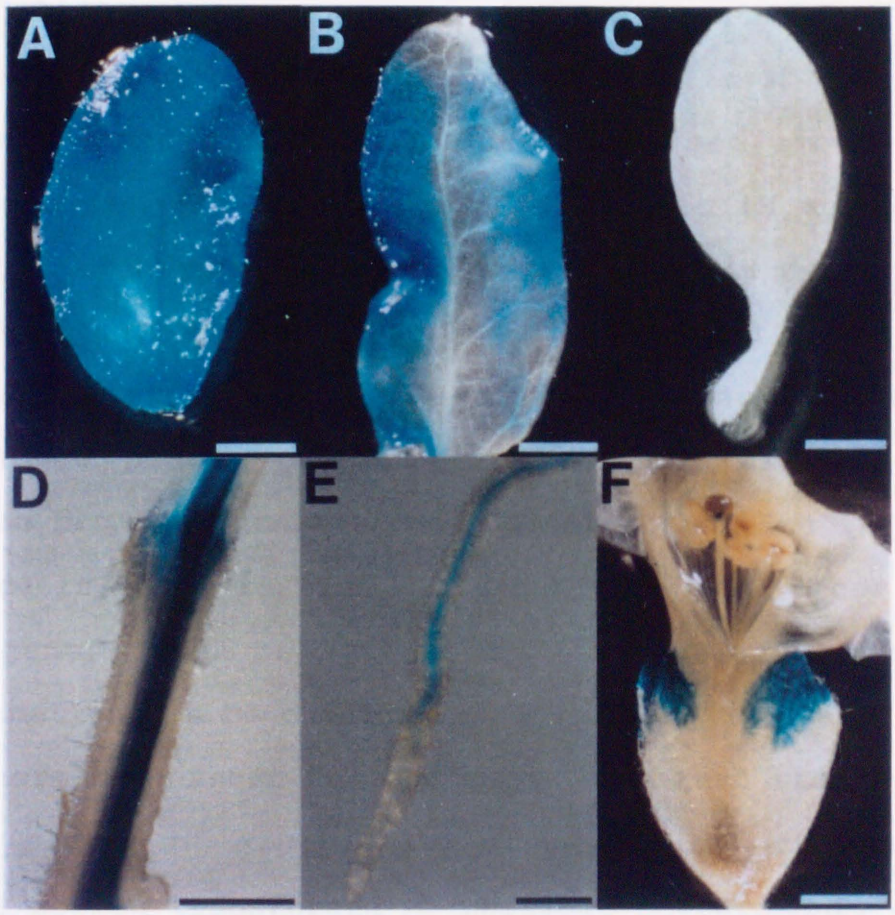

Figure 2. Typical results of the histochemical assay of GUS expression in the transgenic plants. Various transgenic plant tissues were incubated with X-Gluc overnight at 37°C then fixed with 70% ethanol. (A)-(C) Young leaves. (D) Stem (longitudinal section). (E) Root. (F) Flower bud at anthesis. All except (C) are line 1. (C) is line 2. Bars: 2 mm for (A)-(D), 1 mm for (E), and 3 mm for (F).

immature pollen cells was as effective that to freshly isolated pollen.

Two lines of transformants were regenerated from a total of $2x10^6$ bombarded cells in this study. The ratio of conversion from transient expression of the *gusA* gene (see above) to stable transformation was about 10^{-3}. This ratio is much lower than that obtained in the transformation of somatic cells by particle bombardment (Klein et al, 1988b; Finer and McMullen 1990). Because the experimental results also showed that several hundreds of plants could be regenerated without kanamycin selection from one nonbombarded filter containing $2x10^5$ immature pollen cells of *N. rustica*, I need now to test other selectable marker genes, such as *hpt* and *bar*, to see if more efficient transformation can be obtained.

I here have shown for the first time that stable transgenic haploid plants of *Nicotiana rustica* can be regenerated from immature pollen transformed by particle bombardment. Transgenic haploid plants also have been produced by electroporation of protoplasts isolated from microspore-derived cultures of maize (Sukhapinda et al. 1993). I think that transgenic haploid plants are useful materials for basic genetic studies and the breeding of higher plants because they show recessive traits and homozygosity is achieved rapidly for introduced foreign genes, thereby circumventing the problem of segregation. Such plants also should prove useful for gene-tagging and mutation studies because of the advantage of dealing with a single genome.

CONCLUSIONS

I have studied transformation of angiosperm pollen by particle bombardment. In chapter I, I have succeeded in gene transfer and expression in pollen of various plant species using a pneumatic particle gun and optimized bombardment conditions for pollen cells. Furthermore, I have compared the activities of three different promoters in pollen of mono- and dicot plants. I observed intracellular location of introduced gold particles in the GUS-expressing pollen. In chapter II, I have succeeded in production of transgenic haploid plants of *Nicotiana rustica* from bombarded immature pollen. The result demonstrates that transgenic haploid plants can be produced directly by the bombardment-mediated transformation of immature pollen. My findings reported in the preceding chapters can be summarized as follows:

CHAPTER I

Transient expression of the foreign gene (GUS) in pollen of lily (*Lilium longiflorum*), tulip (*Tulipa gesneriana*), tobacco (*Nicotiana tabacum*), *Nicotiana rustica*, and peony (*Paeonia lactiflora*) was obtained by particle bombardment. Several factors influencing detection of GUS-expression were studied using the pneumatic particle acceleration device. The presence of 20% methanol and 0.1M sodium phosphate in the X-Gluc solution for histochemical GUS assay almost reduced background GUS activity and made clear the detection of the GUS expression. Under

optimized conditions, ca. 30% of bombarded pollen expressed the *gus* gene in lily. Based on the transient expression analysis, LAT52 promoter (Zm13 promoter) has higher activity in dicot pollen than in monocot ones (in monocot pollen than in dicot ones). I have shown the evidence that some gold particle(s) can successfully be introduced into the generative cell cytoplasm. But, the frequency of the delivery of gold particles into the generative cell at present conditions is still low (ca. 10^{-3}-10^{-4} of total).

CHAPTER II

Immature pollen of *Nicotiana rustica* was bombarded with pBN24 encoding the *nptII* and *gus* genes following starvation treatment for 3 days. Two independent lines of transgenic plants were regenerated in the presence of kanamycin monosulfate. Enzyme assays showed that one has both NPTII and GUS activities and the other only weak NPTII activity. Southern blot analyses indicated that the former has a DNA fragment corresponding to the intact expression cassettes for both genes in its genome; whereas, the latter lacks intact expression cassettes for both genes and has only the intact *nptII* coding sequence in its genome. The transgenic plants of both lines have 24 chromosomes, confirming haploidy, and they are infertile.

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Chapter II

Nishihara, M., Seki, M., Kyo, M., Irifune, K., and Morikawa, H. Transgenic haploid plants of *Nicotiana rustica* produced by bombardment-mediated transformation. Transgenic Res. (in press).

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