breakage of large DNA was significantly inhibited by DNase inhibitors (lane 6). Results in lane 7 indicate that the *Arabidopsis* chromosomal DNAs were largely digested by *EcoRV*. Lane 8 shows that not only did the DNase inhibitors not inhibit the activity of *EcoRV*, but they seemed to support a faster digestion of the chromosomal DNA by this enzyme.

In conclusion, we have shown that the combination of 160 mM L-lysine-HCl plus 4 mM EGTA results in strong inhibition of DNase activity present in the *Arabidopsis* nuclei preparations, but these two compounds did not inhibit the activity of *I-PpoI* and the restriction enzymes *EcoRV*, *Sall*, *SacI*, *BamHI*, *PstI* and *KpnI*. However, the activities of *SmaI*, *HindIII* and *NotI* were slightly inhibited by these DNase inhibitors. We also tested DNase activity in nuclei isolated from two monocots (rice and corn) and another dicot tomato. The results were similar to those found in *Arabidopsis* nuclei in that the DNase activity was inhibited by 160 mM L-lysine-HCl plus 4 mM EGTA. Thus, we believe that combining these two compounds can play a protective role in the isolation and manipulation of large chromosomal-sized DNA molecules from a wide range of plant materials. In principle, protection of chromosomal DNAs from digestion by endogenous DNases allows one to isolate large megabase-sized DNAs for overlapping large contigs and facilitates the construction of YAC and BAC libraries for use in obtaining larger insert sizes more efficiently.

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Detection of Plasmid-Encoded *gusA* Gene in GUS-Positive *Escherichia coli*

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The β -glucuronidase gene (*gusA*) of *Escherichia coli* has been developed as an efficient and single most important reporter gene system for the analysis of gene expression in transformed plants (2). In the presence of β -glucuronide substrate, the expression of plasmid-encoded *gusA* gene can be measured as a blue color in the resultant colonies because of the lack of intrinsic activity of the *gus* gene in plants, fungi and in most bacteria. However, most of the laboratory strains of *E. coli* contain GUS activity encoded in their genome, which makes plasmid-encoded *gusA* gene detection relatively difficult. To express the plasmid-encoded *gusA* gene in *E. coli*, usually an *E. coli* host in which *gusA* locus is deleted from the chromosome is used (3,6). Unfortunately, not many such *gusA* deletion host strains are available to the scientific community. To our knowledge, only two such *E. coli* strains, SO200 (2) and

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KW1 (3), exist to date, which limits the use of *gusA* gene as a reporter when certain genetic backgrounds of laboratory strains are needed to study the *gus* gene activity. Here, we report that even the chromosomally encoded *gusA*-positive *E. coli* host strains can be used for detecting the plasmid-encoded *gusA* gene activity efficiently.

The *E. coli* Epicurian Coli® XL1-Blue, JM101 and JM109 Competent Cells from Stratagene (Heidelberg, Germany) and DH5α and HB101 Competent Cells (Institute of Himalayan Bioresource Technology collection) were used. X-gluc, 4-methylumbellifer-yl-β-D-glucuronide (MUG) and antibiotics were obtained from Sigma Chemical (St. Louis, MO, USA), and LB media was used. Restriction and modifying enzymes were obtained from New England Biolabs (Hertfordshire, England, UK) and Bangalore Genei (Bangalore, India). DNA manipulations

Table 1. Analysis of 49 Plasmid Clones (from LB Agar Selection Plate) with Respect to Their Restriction Mapping

No. of Experiments	Total No. of Colonies Analyzed ^a		
	Dark Blue	Light Blue	Uncertain
6	27(22)	19(13 + 6) ^b	3(3) ^c
^a Figures in parentheses show the number of clones that gave expected restriction pattern.			
^b Figures show that 13 clones gave restriction pattern of self-ligation of vector plasmid, while 6 clones gave restriction pattern of the <i>gusA</i> gene ligated in opposite orientation to promoter.			
^c These clones gave restriction pattern of self-ligated vector plasmid.			

and electrophoresis were performed as reported previously (5). The GUS containing chloroplast-transformation vector (pMS65LB_{gus}) was constructed in pBluescript® II plasmid (Stratagene). The *gusA* gene with its own initiation codon was cloned under tobacco chloroplast 16SrRNA gene promoter and *psbA* terminator sequences, with

the aim to express the gene and stabilize the gene product in tobacco chloroplasts (data unpublished).

Cultures of bacterial strains grown overnight were diluted in phosphate-buffered saline (PBS) to about 10⁻⁵. One hundred microliters of this diluted culture were then plated onto nutrient agar/LB agar plates containing appropri-

ate antibiotics (ampicillin and spectinomycin) and GUS substrate X-gluc (50 µg/mL each). These plates were incubated at 37°C in the dark and observed for up to 3 days. The developed dark blue colonies in the background of light blue colonies were marked as potential *gus* gene-positive plasmid clones. Then, the colonies were grown overnight in 5 mL LB, and plasmid DNA was prepared from 1.5-mL cultures (5). Ten microliters of the DNA (out of 50 µL) were digested with *Hind*III restriction enzyme and run in a 1% agarose gel electrophoresis to confirm the correct clone.

We analyzed five *E. coli* laboratory strains, XL1-Blue, DH5α, JM101, JM109 and HB101, for the development of blue color on the nutrient agar plate containing X-gluc substrate. It was observed that XL1-Blue gave the maximum dark-blue-colored colonies, while DH5α gave the least blue-colored colonies, indicating the least intrinsic GUS activity. The order of color intensity was observed as follows: XL1-Blue>JM101>JM109>HB101>DH5α. To determine the effect of media on intensity of color, we plated five strains of *E. coli* onto nutrient agar or LB agar. In general, the blue color was relatively pronounced on nutrient agar medium as compared with LB agar medium. In subsequent experiments, pMS65LBgus plasmid DNA was transfected into *E. coli* hosts, and the resulting colonies were observed for blue color development. It was observed that DH5α colonies with and without plasmid

pMS65LBgus showed a marked difference of blue color on LB agar medium (Figure 1B) as compared with nutrient agar medium, in which transformed colonies could not be differentiated from non-transformed colonies. The difference could also be observed for *E. coli* hosts HB101 and JM109, but not for XL1-Blue and JM101 (data not shown). Thus, the DH5α strain, along with LB agar medium, was found to be the best combination for the screening of plasmid-encoded *gusA* gene positive clones. The optimum period of incubation showing a marked difference of blue color in *E. coli*, with or without pMS65LBgus plasmid, was also standardized. It was observed that after a 24-h incubation at 37°C, there was almost no color in the colonies appearing on the agar plates, but the blue color began to appear in approximately 36 h and was totally developed in approximately 48 h. An important observation was that those colonies showing the early development of blue color were 100% transformed clones with pMS65LBgus plasmid in the correct orientation. Maximum difference of color was recorded after 40 h of incubation at 37°C.

To confirm whether the developing dark-blue-colored colonies are due to the expression of plasmid-encoded *gusA* gene and not the isogenic plasmids containing the *gusA* gene in the opposite orientation to promoter or vector plasmid without *gusA* gene (pMS65LB), we isolated the plasmid DNA from transformants [DH5α cells transformed with ligation mixture, which contained the *gusA* gene in the correct orientation (pMS65LBgus), the *gusA* gene in the opposite orientation and self-ligated vector plasmid (pMS65LB) without the *gus* gene]. Upon digestion with *Hind*III, we observed that approximately 80% of the dark-blue-colored colonies gave the expected restriction pattern (Table 1) (Figure 1B shows colonies marked with an arrow that were predicted *gusA*-positive plasmid clones). DNA isolated from the background light blue colonies gave either a restriction pattern of self-ligation of vector plasmid pMS65LB or the *gusA* gene inserted in the opposite orientation to promoter.

In recent years, attention has been paid towards expressing foreign

Figure 1. Media plates showing the blue color intensity as a measure of *gus* activity. (A) Nutrient agar plate with XL1-Blue cells transformed with ligation mixture. (B) LB agar plate with DH5α cells transformed with ligation mixture. The cells marked with an arrow are potential plasmid-encoded, *gusA* gene-positive clones (pMS65LBgus). Background light blue cells contain either isogenic self-ligated vector plasmid (pMS65LB) or *gusA* gene ligated in opposite orientation to promoter.

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gene(s) in chloroplasts (1,4). Chloroplast promoters have been shown to express in *E. coli* (1), and this observation signifies the need for either using *E. coli* as a host for analyzing chloroplast signal sequences, or else, for testing chloroplast transformation constructs before expressing them in plants. Unlike plants, most of the laboratory strains of *E. coli* have intrinsic GUS activity, which limits their use as a host strain for *gus* gene cloning and for the study of *gus* gene regulation. Cloning of *gusA* gene is usually achieved in a *gusA* deletion host of *E. coli* strains. Such strains are limited and often are not available. The problem is further aggravated when specific genetic backgrounds of laboratory strains are needed for analyzing the *gus* gene activity. Therefore, it was reasonable to devise a strategy to detect the *gusA* gene activity in a *gus*-positive *E. coli* host. This study shows the advantage of a multi-copy plasmid to clone the *gusA* coding sequences in a *recA*-negative laboratory strain that has a single-copy endoge-

nous *gusA* gene. We have been able to differentiate the plasmid-encoded, *gus*-positive colonies on the basis of relative intensity of blue color in a background of lighter colored colonies (Figure 1B). The higher amount of blue color in the recombinant clones (colonies marked by an arrow in Figure 1B) is probably due to the number of copies of *gus* gene-containing plasmids per cell (**Note:** pBluescript II plasmids are high-copy-number plasmids). This results in the accumulation of more gene product as compared to the single gene's product in a bacterial chromosome. The product will hydrolyse the X-gluc substrate and, consequently, intensify the blue color in proportion to the gene product. This is supported by restriction patterns of a number of selected dark blue and light blue colonies, which clearly show that dark blue colonies, indeed, contained the plasmid with the *gus* gene cloned in the correct orientation to the promoter (Table 1). The greenish, lighter blue colonies either contain plasmid with the *gus* gene in the opposite orientation or the self-ligation of the vector plasmid. When the ligation mixture was transformed in XL1-Blue and selected on nutrient agar plates, all the colonies were dark blue and could not be differentiated on the basis of blue color (Figure 1A). When the same transformants were plated on the Luria agar plates instead of the nutrient agar plates, the colonies were relatively lighter blue. Changing the host from XL1-Blue to DH5 α further widened the difference in color between transformants and non-transformants. The period of incubation is also an important consideration. We have seen that the first blue colonies to appear (in ca. 36 h) have the highest probability of being the right clone. This observation is probably due to the high copy number of plasmids and also because the plasmids replicate comparatively faster than the chromosomal genome, which results in more *gusA* gene copies per cell, more *gusA* gene product and, consequently, an increase in blue color. In plants, different cell types have been shown to have a differing metabolic activity with corresponding differences in the rates of transcription and translation and thus a nonuniform distribution of GUS activi-

ty. Peroxidases have also been shown to contribute to the variability of the *gus* spots and not the concentration of the enzymes. Unlike the plants, isogenic bacterial colonies do not give variability of color in background colonies.

In conclusion, we have shown that, even in the absence of a *gusA* deletion in an *E. coli* host, detection of plasmid-encoded *gusA* gene-positive plasmid clones (in the standard laboratory strain DH5 α and also some other strains) is possible by observing the color difference of the colonies. This technique is simple, cost-effective and timesaving and can be useful when different genetic backgrounds of a laboratory strain are needed for studying *gusA* gene activity.

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