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Biological and Molecular Evidence for the Transgenosis of Genes from Bacteria to Plant Cells

(*Escherichia coli*/prokaryote/eukaryote/transcription/translation/metabolic function)

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ABSTRACT **Specialized transducing phages (λ and φ80) have been used as vectors in the transfer of genes (wild type and mutant) from the bacterium *Escherichia coli* to haploid cell lines of the plants *Lycopersicon esculentum* and *Arabidopsis thaliana*. The overall phenomenon of transfer, gene maintenance, transcription, translation, and function has been termed transgenosis. Transgenosis of galactose and lactose operon genes was detected by survival and growth of the plant cells on defined medium with galactose and lactose as sole sources of bulk carbon. Phages carrying a defective operon, unrelated bacterial genes, or no bacterial genes, do not affect the normal result of death on these media, nor do they prevent growth on optimal growth media. Transgenosis of the *E. coli* gene *z* (*lac* operon) was confirmed by a biochemical-immunological test specific for *E. coli* β-galactosidase. Plant cells were unable to effectively suppress an *E. coli* nonsense mutation. The *E. coli* mutant suppressor gene, *supF*$^+$, specifies insertion of tyrosine at amber (UAG) nonsense codons. Introduction of *supF*$^+$ results in a lethal transgenosis on medium normally optimal for plant cell growth. It is concluded that amber codons are vital to the life of plant cells. Differentiating cells of *A. thaliana* were not affected by *supF*$^+$.**

We have tested for the possibility that bacterial genes can be transferred to, and then expressed within, plant cells. The experiments make use of the ability of specialized transducing phage (λ, φ80) to incorporate selected bacterial genes into their genome. These vector phage are simply added to plant cells in culture on fully defined media.

For phenotypic expression, a sequence of gene transfer, maintenance, transcription, translation, and metabolic function must occur, except when the product of gene expression can function without translation (for example, a species of transfer RNA). After the initial transfer, the enzymic and structural elements for gene expression must be supplied by the plant cells. The overall phenomenon of transfer and subsequent expression has been termed transgenosis (1) to avoid a premature assumption of mechanisms inherent in the use of established terms, such as transduction, taken from bacterial genetics. Transgenosis is regarded as a particularly appropriate term when donor and recipient cells are separated widely by evolution and the mechanisms of gene transfer and maintenance (inheritance?) are obscure.

The recipient cells for transgenosis were mostly haploid cell lines of *Lycopersicon esculentum* and *Arabidopsis thaliana* recently developed in this laboratory (2, 3). Haploidy is not essential for the present experiments, but has important implications for the future development of transgenosis (1).

The biological detection of an advantageous transgenosis depended on survival and growth of plant cells on medium containing galactose and lactose as carbon sources. These environments are normally lethal (1, 4). Information for the metabolism of the sugars to usable sources of carbon was provided by the genes of the *Escherichia coli* galactose (*gal*) and lactose (*lac*) operons.

A system where transgenosis might be a disadvantage was provided by "infection" with the *E. coli* mutant *supF*$^+$ gene (also known as *SuIII*$^+$), which codes for a transfer RNA able to correct a nonsense mutation carried as a nonsense (amber) triplet in messenger RNA. Suppressor mutations lead to more mistakes in reading the genetic code and can be considered as unlikely to accumulate in higher organisms [see Watson (5)]. If nonsense codons are vital to the life of plant cells, efficient suppression resulting from transgenosis of *supF*$^+$ could be predicted to result in severe inhibition or death on normally optimal growth medium.

Preliminary reports (3, 6) and a more detailed account of evidence and a discussion of background and results (1) have been presented.

MATERIALS AND METHODS

The derivation and culture of haploid cell lines of *Lycopersicon esculentum* (tomato) ANU-H27-1 and *Arabidopsis thaliana* ANU-H872-1 have been described (2, 3). *L. esculentum* ANU-H27-1 was maintained on DBM3 medium (2),† containing, in addition, napthylene acetic acid 8 μg/ml, kinetin 0.01 μg/ml, penicillin G 1000 IU/ml (omitted in later experiments), and the appropriate carbon source. For general growth of *L. esculentum* and *A. thaliana*, glucose (2%) is used, except for *A. thaliana* on differentiation medium (2% sucrose). In transgenosis experiments, the carbon source was galactose (2%), lactose (4% or 10%), or glucose (2%), as required. Phages λ*pgal*$_8$ *c*I857 (K$^+$T$^+$E$^+$) (λ*pgal*$^+$) and λ*pgal*$_8$ *c*I857 (K$^+$T$^-$E$^+$) (λ*pgal*$^-$) were supplied by Dr. Max Gottesman (see also ref. 7) and φ80*plac*$^+$ by Dr. Y. Ohshima. We have confirmed that the φ80*lac*$^+$ strain carries the gene Z (8). The λ phages were grown on *E. coli* KB5 (*galΔ attλΔ supD*) (17) at 39° and φ80 phages were grown on *E. coli* RVSM (*lacΔ*) (supplied by Dr. J. Langridge) at 30°. At the onset of lysis, chloroform was

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†In ref. 2, the vitamins–glycine stock solution should be in 100 ml not 1000 ml.

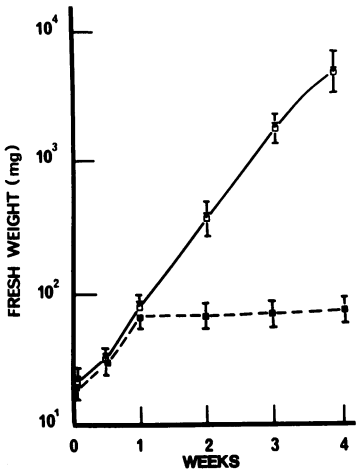

FIG. 1. Inhibition of the growth of *L. esculentum* ANU-H27-1 by the phage $\phi 80 supF^+$. Callus inoculated with $\phi 80 supF^+$ (■), $\phi 80$ (□), and no phage (▼). Note that $\phi 80$ had no observable effect. The phages were grown on *E. coli* Z32 [*trp* (amber) supplied by Dr. J. Langridge].

added to complete the lysis of phage-infected cells (9). The suspension was centrifuged at 12,000 × *g* for 10 min; the supernatant was passed through a Millipore filter (0.45 μm) and centrifuged in a Spinco 30 rotor at 27,000 rpm for 4 hr. Phage pellets were suspended overnight at 4° in 3 ml of phage adsorption buffer 0.1 M Tris·HCl (pH 7.4) containing 20 mM $MgSO_4$, or MS1 buffer (3), twice filtered through Millipores, then assayed for phage (plaque forming units, PFU) and for contaminants. This procedure gave phage titers of about 10^{12} PFU/ml. Phage stock suspensions were diluted in MS1 buffer as required for callus inoculation.

Tests for Microbial Contamination. The phage preparations and calluses were routinely sampled and tested for contaminant micro-organisms in rich liquid medium (LB medium, see ref. 10) incubated both aerobically and anaerobically at 27°. This procedure would detect most kinds of contaminants (see discussion in ref. 1). Phage preparations were stored over chloroform and twice (Millipore) filtered before use. Aseptic techniques were done in sterile laminar flow hoods.

Characterization of the T Gene Mutation. Further characterization of the mutation in the T gene of $\lambda pgal^-$ used the *E. coli* strains S1652 ($gal\Delta$ $supD^+$), its parent S165 ($gal\Delta$ his^- $supD$) (11) and KB5-1 ($gal\Delta$ $supD$) (12). Plaque and transduction assays with these bacteria were at 30° on both EMB and minimal medium plates supplemented with galactose.

Inoculation of Plant Callus with Phage. High-titer preparations of phage were applied by sterile Pasteur pipettes to small pieces of callus [20–40 mg wet weight, about 10^8 phage (PFU) per callus, or as otherwise indicated] on the appropriate medium, then inoculated at 27°.

β-Galactosidase Activity and Interaction with Antiserum. After 13 weeks, calluses sampled at random from a *lac* experiment using $\phi 80 plac^+$ plus $\lambda pgal^+$ were examined for β-galactosidase activity and for interaction with antiserum specific for *E. coli* β-galactosidase. Specific activity and molecules of β-galactosidase were calculated by the procedure and constants suggested by Rotman (13). The method of testing the ability of antisera to protect β-galactosidase activity at 60° was that of Melchers and Messer (14), except that extracts were made and heat treatment was performed in 0.1 M sodium phosphate buffer (pH 7.0)–1 mM $MgSO_4$–0.2 mM $MnSO_4$–0.1 M 2-mercaptoethanol. Specific rabbit antiserum to pure *E. coli* β-galactosidase was obtained from Dr. J. Langridge, the enzyme was prepared by Dr. A. Millerd, and the antiserum was prepared by Dr. W. Dudman. In our procedure, the amount of extract was selected to give a rate not limited by substrate. Antiserum was added in equivalent volume. The experiments were done both at relatively high and at relatively low levels of activity. Nonspecific rabbit antiserum was provided by Dr. A. Cunningham. Effects of plant cell extracts on preparations of known *E. coli* enzymes were taken into account.

RESULTS

Transgenosis of *gal*

Lycopersicon esculentum ANU-H27-1 callus usually browns off and dies within 3 weeks after inoculation on to medium containing galactose (2%) (1, 4). Very occasionally a callus has survived. Growth on 2% glucose medium is optimal, with doubling times of 4–15 days. These general results were consistent, except when calluses were inoculated with $\lambda pgal^+$. All calluses (6 replicates) grown on galactose and inoculated

TABLE 1. *β-Galactosidase from crude extracts of haploid tomato callus ANU-H27-1: specific activity and protection by specific and nonspecific antisera*

Source and nature of callus	Specific activity (units)	Activity (%) remaining after 20 min at 60° in the presence of rabbit antiserum: Specific for *E. coli* enzyme	Nonspecific
Normal callus, fast growing on glucose (2%)	158	<5	<5
Not phage infected, slow-growing control (rare) on lactose (10%)	144	<5	<5
Not phage infected, nongrowing control on lactose (10%)	264	<5	<5
Transgenosized for lac^+ with $\phi 80$-lac^+ plus λgal^+, growing on lactose (10%)	6850*	50–60	<0.01
Normal callus, fast growing on glucose (2%) plus *E. coli* extract† containing β-galactosidase	—	50–60	<5

* Corresponds to 1.78×10^{13} molecules of β-galactosidase per mg of protein or 2×10^7 molecules per cell.

† The amount of *E. coli* extract (provided by Dr. Miklos; specific activity not determined) was adjusted to give rates comparable to the activity of transgenosized cells.

with λ*pgal*$^+$ survived and grew slowly. When the experiments were abandoned such calluses had survived at least 15 weeks longer than all negative controls [2% galactose, phages (λ*pgal*$^-$ φ80, or φ80*plac*$^+$), or no phage] but were of poor appearance relative to positive controls [2% glucose, phages (λ*pgal*$^+$, λ*pgal*$^-$, φ80, or φ80*plac*$^+$), or no phage]. Doubling time was 5- to 20-times longer than the optimal positive controls, but the total increase of cell mass (and by microscopy, cell number) was between 3- and 5-fold. Subcultures to either galactose, glucose, or sucrose medium continued to grow slowly for some time, but never recovered to normal rates on glucose or sucrose.

Thus, only λ*pgal*$^+$ could contribute information necessary for survival and slow growth on galactose medium. The failure of λ*pgal*$^-$ to behave similarly to λ*pgal*$^+$ was a striking and important control, particularly since we were able to further characterize the mutational defect in the *gal*$^-$ strain (K$^+$T$^-$ E$^+$, Gottesman, personal communication and see ref. 7) as a nonsense mutation.

The nonsense mutation was detected as follows. On *E. coli* S1652 all λ*pgal*$^-$ phage plaques contained *gal*$^+$ transductants. Examples of these transductants were reisolated and shown to retain the original λ*pgal*$^-$ phage, indicating that the T$^-$ mutation had not reverted during phage replication. In contrast, phage λ*pgal*$^-$ gave no *gal*$^+$ transductants on strains KB5 and KB5-1. Strain S1652 is known to suppress amber mutations in the *gal* operon and in phages T_4 and λ (11). We concluded that the lesion in the T gene of *gal* is an amber nonsense mutation.

Thus, transgenosis for survival and growth on galactose was defeated by a minimal difference (a nucleotide pair) at the level of DNA that was known (7) to lead to a lack of UTP:D-galactose-1-phosphate uridyl transferase (EC 2.7.7.10) activity after translation. Because of the polarity of the nonsense mutation within the *gal* operon (1), the product of gene K (ATP:D-galactose-phosphotransferase, EC 2.7.1.6) most probably would not be produced. If the plant cells were able to correct nonsense efficiently, the λ*pgal*$^-$ strain would have been operationally equivalent to λ*pgal*$^+$. We concluded that the plant cells were effectively suppressor negative. This result suggested that UAG (amber) nonsense codons might be important in the regulation of translation in plant cells and that the introduction of a bacterial amber suppressor gene might lead to inhibition of growth of plant cells.

A third conclusion drawn from these experiments was that inoculation with phages not carrying relevant genes (neutral phage) did not prevent normal growth on glucose medium.

Transgenosis of gene *z* (*lac*$^+$)

Calluses of *L. esculentum* ANU-H27-1 on lactose medium (2, 4, or 10%) generally brown-off and die within 3–5 weeks (1). 5–10% of callus inocula, however, grow slowly, presumably either because of carryover of a stored general carbon source or because of epigenetic or mutational changes.

As for the *gal* experiments, these results were only perturbed by phages carrying relevant *E. coli* genes. Thus, calluses inoculated with either φ80 or λ*pgal*$^+$ behaved like uninoculated controls, but calluses inoculated with φ80*plac*$^+$ alone or φ80*plac*$^+$ plus λ*pgal*$^+$ (each about 10^8 PFU per callus) survived and grew slowly in about 80% of replicates (several experiments of 40–50 replicates for each variable). Some of these calluses eventually (about 15 weeks) died on lactose medium or deteriorated on subculture; others have survived for three or four subcultures on lactose (4 or 10%) medium (a total of 9 months of growth) and must have passed through many generations. We concluded that transgenosis of at least gene *z* had occurred.

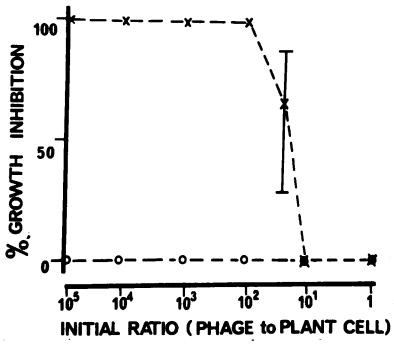

FIG. 2. Inhibition of the growth of *L. esculentum* ANU-H27-1 by the phage φ80*supF*$^+$. Effect of variation of the ratio of phage to plant cells. Similar results are obtained at any time after the first 1–2 weeks of callus growth (see Fig. 1). Callus inoculated with φ80*supF*$^+$ (×), φ80 (○).

Transgenosis of gene *z*, coding for β-galactosidase, was confirmed by the measurement of β-galactosidase activity and by the use of antiserum specific for *E. coli* β-galactosidase. This antiserum is known to protect the *E. coli* enzyme against heat denaturation (14). High-titer preparations of φ80*plac*$^+$ contained no detectable β-galactosidase activity. A callus transgenosized with *lac*$^+$ (using φ80*plac*$^+$ plus λ*pgal*$^+$) and chosen at random at 13 weeks contained a high level of β-galactosidase when compared with various control calluses (Table 1). The extent of protection of β-galactosidase at 60° was identical for the extracts of the transgenosized plant cells and for an extract of *E. coli* (Table 1). In our hands, this protection has varied from 50 to 75%, rather than about 100% as originally claimed for *E. coli* extracts (14). This difference is presumably because of changes in method (buffers, antisera, and effects of addition of plant cell extracts), but is obtained for *E. coli* and transgenosized plant cells alike. All extracts of plant cells had some β-galactosidase activity but, except for cells exposed initially to phage φ80*plac*$^+$, the activity was low and was not protected by the specific *E. coli* β-galactosidase antiserum.

Transgenosis of *supF*$^+$

These experiments were conducted on glucose medium optimal for growth of callus. The results presented in Figs. 1 and 2 are for *L. esculentum* ANU-H27-1, but similar results have been obtained with *A. thaliana* ANU-H872-1 and with a diploid culture of *L. esculentum*. Calluses inoculated with φ80-*supF*$^+$ soon stop growing and become phenotypically dead. The contrast with calluses not inoculated with phage or with calluses inoculated with φ80 is very great because of the excellent growth of these controls (for photographs, see ref. 1).

By direct count a typical *L. esculentum* ANU-H27-1 callus contains about 10^8 cells per mg of wet weight; on this basis, the callus requires an inoculum of at least 10^2 phage (PFU) per cell to ensure callus death (Fig. 2). This calculation assumed that each cell is equally exposed to phage; although the callus is loose, such may not be the case.

On rare occasions, clones of cells resistant to *supF*$^+$ have been observed. These cells can be detached and retain resistance on subsequent subculture (1).

Not all callus cultures are susceptible to transgenosis. The calluses described so far were maintained on media that do not lead to differentiation. Development and differentiation of the haploid cell lines of *L. esculentum* and *A. thaliana* can be controlled by variation of the components of the defined media (2, 3). Cultures of *A. thaliana* greening on differentiation medium (1, 3) are completely resistant to $\phi 80 supF^+$.

DISCUSSION

The results of these experiments lead us to conclude that transgenosis was accomplished for all three gene systems tested. This conclusion rests not only on the biological criterion of growth or nongrowth but, for the gene *z*, also on the direct demonstration of the synthesis of an *E. coli* gene-specific protein—the *E. coli* β-galactosidase. The biochemical–immunological test of specific protection at 60° is regarded as definitive at the molecular level. Later experiments have fully confirmed these results and, in addition, demonstrated the time course of β-galactosidase synthesis (Doy, Gresshoff, and Rolfe, in preparation). The experiments with $\phi 80 supF^+$ not only provide an example of transgenosis, but also demonstrate the natural dependence of higher plants on UAG codons.

For reasons discussed in detail elsewhere (1), we reject the possibility that transgenosis is an artifact due to contaminant micro-organisms.

Our experiments were designed as an initial probe of phage-mediated transgenosis between cells with many analogous metabolic pathways but widely separated by evolution. We recognize that phage may not be the only or the best way of achieving transgenosis. However, specialized transducing phages do provide a means for a specific selection of bacterial genes, and other of the characteristics of these phages may facilitate inheritance within the cytoplasm and organelles as an addition or alternative to nuclear inheritance (1). The long-term survival and growth, including subcultures of some cultures of *L. esculentum* ANU-H27-1 transgenosized for the *E. coli* gene *z*, leads to the tentative conclusion that a form of inheritance can sometimes occur. It should be noted that the definition of transgenosis (1) does not include a claim for inheritance.

For further development of the use of phage in transgenosis, it may be necessary to use mutant phage for which transcription of phage genes is prevented without interference with the transcription of the associated bacterial genes. This suggestion is based on the observation that calluses exposed to either neutral or effective phage frequently deteriorate on continuous subculture. Thus, although phage not carrying relevant bacterial genes were initially neutral in effect, there may be delayed or accumulated deleterious effects of transgenosis of normal phage genes. We have not detected the synthesis of new infective phage (PFU) (but see claims in refs. 15 and 16).

Transgenosis provides wide possibilities for examination of interactions between prokaryotic and eukaryotic genes and processes of control and function of fundamental—but also practical—importance (1). The mechanisms of the various stages of transgenosis require investigation, as do alternative possibilities for achieving transgenosis and gene inheritance. In addition, one wonders whether evolution has at times been accelerated by accidents of beneficial transgenosis.

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1. Doy, C. H., Gresshoff, P. M. & Rolfe, B. G. (1973) *The Biochemistry of Gene Expression in Higher Organisms* (Australia and New Zealand Book Co.) in press.
2. Gresshoff, P. M. & Doy, C. H. (1972) *Planta* **107**, 161–170.
3. Gresshoff, P. M. & Doy, C. H. (1972) *Aust. J. Biol. Sci.* **25**, 259–264.
4. Doy, C. H., Gresshoff, P. M. & Rolfe, B. G. (1972) *Proc. Aust. Biochem. Soc.* **5**, 3.
5. Watson, J. D. (1970) *Molecular Biology of the Gene* (W. A. Benjamin, New York), 2nd ed., p. 430.
6. Doy, C. H., Gresshoff, P. M. & Rolfe, B. G. (1972) *Search* **3**, 447–448.
7. Merril, C. R., Geier, M. R. & Petricciani, J. C. (1971) *Nature* **233**, 398–400.
8. Iida, Y., Kameyama, T., Ohshima, Y. & Horiuchi, T. (1970) *Mol. Gen. Genet.* **106**, 296–306.
9. Reader, R. W. & Siminovitch, L. (1971) *Virology* **43**, 623–637.
10. Bertani, G. (1951) *J. Bacteriol.* **62**, 293–300.
11. Saedler, H. & Starlinger, P. (1967) *Mol. Gen. Genet.* **100**, 178–189.
12. Rolfe, B. G. & Onodera, K. (1972) *J. Membrane Biol.* **9**, 195–207.
13. Rotman, M. B. (1970) in *The Lactose Operon*, ed. Beckwith and Zipser (Cold Spring Harbor Laboratory), p. 279.
14. Melchers, F. & Messer, W. (1970) *Biochem. Biophys. Res. Commun.* **40**, 570–575.
15. Sander, E. M. (1964) *Virology* **24**, 545–551.
16. Sander, E. M. (1967) *Virology* **33**, 121–130.
17. Onodera, K., Rolfe, B. G. & Bernstein, A. (1970) *Biochem. Biophys. Res. Commun.* **39**, 969–975.