GUS Protocols

Using the GUS Gene as a Reporter of Gene Expression

Edited by

Sean R. Gallagher

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Hoefer Scientific Instruments
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Front cover photograph: Cross section (15μ) of a loblolly pine *(Pinus taeda L.)* cotyledon showing transient gene expression from a microprojectile bombardment experiment. Photograph was taken using polarized light and interference contrast on a Leitz Diaplane microscope. Courtesy of Dr. Anne-Marie Stomp, Forestry Department, North Carolina State University, Raleigh, North Carolina.

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Foreword

"Nothing great is achieved without chimeras."
Ernest Joseph Renang
L'Avenir de la Science [1890]

Gene fusions were used in biological research well before the advent of recombinant DNA. The pioneering work of Beckwith, Signer, and their colleagues in the middle to late 1960s (Beckwith *et al.*, 1967; Miller *et al.*, 1970) in juxtaposing the *trp* and *lac* operons of *Escherichia coli* started a revolution in the analysis of biological function. The concept of using one gene with a product that is easy to detect—in this case β-galactosidase—to infer the behavior of another gene that is functionally fused to it was so powerful in its simplicity and so pervasive in its applications that there has been little recognition of the fundamental paradigm shift that it entailed. For the next two and a half decades, as recombinant DNA technologies emerged and as transformation systems were developed, thousands of scientists used and continued to develop the LAC system. The information obtained with these gene fusions in many cases could not have been obtained by any other methodology. The LAC system has tended to find its greatest use in the major laboratory model systems, such as *E. coli, Saccharomyces cerevisiae, Drosophila melanogaster,* in mammalian cells in culture, and lately in transgenic mice. The principal restriction of its use has generally been due to either a lack of suitable transformation methods for a particular system, or the presence of an endogenous β-galactosidase activity. These restrictions have been particularly troublesome in plants and other agricultural systems. Notwithstanding these limitations, the LAC system has proven the extraordinary power of gene fusions in prokaryotes, fungi and animals, and has clearly demonstrated that the development of a single tool for experimental manipulation when

broadly applied to numerous systems, can have a profound effect on the quality and type of science that emerges.

Agricultural research, especially plant molecular biology, had not been able to benefit greatly from the gene fusion paradigm until the advent of the first transformation methods for plants in 1983–1984 and of the GUS system in 1987–1988. The GUS system has been instrumental in facilitating the development of novel transformation methods for many crops, and in allowing detailed analysis of gene action in transgenic plants, bacteria, and fungi of agricultural importance. In particular, use of GUS has facilitated the routine experimental manipulation and analysis of gene action in two and three dimensions. Cell-, tissue-, and organ-specific gene expression is now routinely studied using GUS fusions in transgenic plants.

However, agricultural sciences have only just begun to exploit the important experimental paradigm of gene fusion approaches due to the intrinsic complexity of the systems studied. The gene fusion paradigm as it exists now—at least with regard to its more sophisticated applications—is still largely shaped for model systems where environmental variation and interactions are minimized. Much of plant biology, especially agricultural plant biology, cannot be readily reduced to such model systems. For agriculturalists, performance in highly complex ecosystems is the ultimate criterion for success, and extrapolation from simple models cannot be expected to deal with this enormous leap.

We are now in the midst of the development of at least two more paradigm shifts that may be specific to the agricultural research agenda and that may be required to bridge the gap between model systems and agriculture. One of these can be considered largely scientific in nature, the other apparently social. These shifts are related to what type of science can be done and who gets to do it.

The first of these paradigm shifts is necessitated by the emergence of molecular biology from the confines of laboratory walls into the real and variable conditions of farmers' fields and represents a challenge of substantial magnitude. The requirements that gene action and all its consequences be understood under uncontrolled conditions, where the vicissitudes of the environment must be accepted not as a confounding influence but as a natural and necessary component of an experimental system are daunting. And yet the imperatives of agricultural improvement and the onward rush of transformation methods for crop plants, where performance of the engineered crop in the field is critical, have hastened the application of new approaches to the field without yet achieving the change in thinking and experimental methodology that will be necessary to do it sustainably, intelligently, and equitably. We need methods and ways of approaching problem-solving that can be

used in the complex environment that dictates the biology and ultimately the performance of crops that give us new insights into the interactions between organisms and between organisms and their environment. We must ask whether these new methods can be expected to emerge as incremental improvements or whether there must be completely new approaches.

The other paradigm shift is related to who is empowered to ask questions and use these new technologies, as this ultimately determines who benefits from them. The most severe agricultural problems tend to occur where scientific research is most difficult and where solutions need to be found locally, typically in less developed countries. These problems are often socially, environmentally, and economically devastating. It is imperative, therefore, to design and distribute tools and methods that can function well in these situations and that will empower sophisticated local scientific research to address and overcome local problems.

The GUS system represents just one small step towards the necessary paradigm shifts. But it is an exciting one because it is a step that seems to be capable of very significant extension and improvement as the reconciliation between the needs of agriculture and the environment and the tools and priorities of the laboratory scientist proceeds. The possibilities for continued extension and development of the GUS system in new directions lie in the biological processes in which the enzyme is involved.

The Biology of GUS Gives Hints for New Possibilities

All vertebrates detoxify and excrete the myriad superfluous compounds that their systems encounter by one of a small number of mechanisms. These compounds will include plant secondary compounds and animal metabolites, hormones, and other endogenous compounds that are excess to requirements, and other xenobiotics, including drugs. The prevalent mechanism in most vertebrates is to conjugate these compounds with a water soluble handle, most commonly glucuronic acid, and then to excrete these conjugates through the circulatory system and ultimately through urine and bile. *Escherichia coli* has evolved as a key component of the endosymbiotic intestinal flora of most vertebrates and has developed the ability to metabolize these numerous and diverse compounds through the use of GUS and the related functions encoded by the *gus* operon (see Chapter 1), and can use the released glucuronic acid as both a carbon and energy source.

Within plants and other organisms such as fungi and insects that lack circulatory systems, conjugation with glucuronides is not generally used. Instead, these organisms typically conjugate xenobiotics with glucosides, and sequester them rather than excrete them. Additionally, β-D-glucuronides are not typically found as important structural or biochemical components of plants, and thus GUS activity in these organisms or in the organisms that are associated with them is not expected nor generally observed.

An understanding of this pathway for metabolism of glucuronides and the appreciation of its ubiquity in vertebrates and relative absence in plants raises the novel and exciting possibility of manipulating the pathway and its components to design tools that can deal with several of the key restrictions and limitations of current gene fusion technology. Now that many tens of thousands of transgenic plants representative of dozens of genera have been generated that express GUS, we can approach this task with some confidence in its innocuous effects and ubiquitous utility.

Toward *in Vivo* and *in Campo* Molecular Tools

The first and most pressing limitation is that analysis of GUS is still performed *in vitro,* in destructive assays of extracts or through histochemical analyses of dead tissues. In the development of transformation methods as well as in the analysis of gene action, the ability to analyze GUS activity nondestructively, *in vivo,* would dramatically expand our capabilities. It would also provide a major step toward developing *in campo* molecular biology—stimulating analysis of gene action and its resulting biology under field conditions.

There are two clear routes to achieving *in vivo* GUS analysis, with substantial progress being made on both fronts. The fundamental problem with *in vivo* analysis is that GUS substrates—β-D-glucuronides—are highly water soluble and therefore cannot readily traverse the lipid bilayers of living cell membranes. Additionally, enzymatic analysis, if it is to be quantitative, must be carried out in significant excess of substrate. Therefore, the resolution can be considered as either of two complementary ways of getting enzyme and substrate together; by bringing the enzyme out of the cell so it can interact with substrates in the extracellular spaces, or by transporting the substrates across the plasma membrane where they can be acted on by cytoplasmically localized GUS.

The first route derives from the observations of Iturriaga, Kavanagh, Schmitz, and their colleagues that GUS could readily and efficiently traverse many of the membrane systems within plant cells when fused to the appropriate signal or transit peptide (Kavanagh *et al.*, 1988: Schmitz *et al.*, 1990; Itturiaga *et al.*, 1989). In particular, Gabriel Iturriaga's work showed that when GUS was fused to the signal peptide from the potato tuber protein, patatin, the resulting hybrid was targeted through the endoplasmic reticulum of transgenic plants, where the protein was stable, although greatly diminished in enzyme activity. He further showed that the diminution of enzyme activity was due to an *N*-linked glycosylation of GUS, which could be prevented, with concomitant restoration of enzyme activity, by addition of tunicamycin, an antibiotic known to block glycosylation. These observations were extended by Leigh Farrell and Roger Beachy (this volume, Chapter 9), who have altered the sequence that renders GUS susceptible to this glycosylation, without losing enzymatic activity. This step, and subsequent developments that will come soon as our understanding of protein targeting in plants matures, should allow efficient secretion of GUS and its retention within the extracellular space. It is then obvious that application of highly water-soluble GUS substrates should result in cleavage and detection (or bioactivity) in and on living plant tissue. Issues remaining to be resolved will include maintenance of enzyme localization near or at the site of enzyme synthesis (probably achieved by fusion with components of cell wall proteins or simply by virtue of GUS' large tetrameric structure), transport of the substrate to the site of enzyme localization (GUS substrates are most likely highly phloem translocatable, but probably not cuticle permeant), and migration of the product of the enzyme reaction from the site of cleavage (not a serious problem with current histochemical substrates, but an important criterion for the application of fusion genetics or novel fluorochromes). A number of these steps can be dealt with by sensible design of substrates, which is very straightforward.

The second route toward *in vivo* analysis is more ambitious, but will ultimately be more general, quantitative, and powerful and involves the manipulation of the membrane transporter for β-D-glucuronides. As described by Kate Wilson and her colleagues (Chapter 1), and in unpublished work by Weijun Liang, Peter Henderson, and their colleagues at Cambridge University (W.-J. Liang, P. Henderson, T. J. Roscoe, and R. A. Jefferson, unpublished), and building on the excellent thesis work of Francois Stoeber in the late 1950s (Stoeber, 1961) the glucuronide permease from *E. coli* is now being characterized molecularly and

biochemically and seems to be a very exciting candidate for providing a general mechanism for actively transporting diverse GUS substrates into living cells. The permease is a single polypeptide proton symporter that can accumulate a very wide range of GUS substrates into *E. coli*. The manipulation of this gene to allow functional expression in transgenic eukaryotes is a likely development in the near future. When this is achieved, application of any of a number of fluorogenic, chromogenic, or bioactive substrates may then allow true, quantitative *in vivo* analysis and fusion genetics. One of the most attractive features about developing the permease for this purpose will be its ability to concentrate substrates from very low external concentrations to levels well in excess of the K_m value for GUS cleavage.

Toward Fusion Genetics

Both of these approaches to developing a viable assay system will also serve to hasten the development of effective fusion genetic methods. Fusion genetics is one of the most powerful components of the gene fusion paradigm as it was originally elaborated in bacterial genetics, but has been very poorly developed for complex eukaryotes, with some notable exceptions (e.g., Bonner *et al.*, 1984). The basic premise that underlies fusion genetics is that one can perform not only visualization or measurement of gene fusion activity, but genetic selections for variants in activity of the gene fusion, thus selecting for and obtaining both cis- and trans-acting mutations. These mutations can then be used to infer relationships between the controlling elements and components of the host genome. In bacteria, for example, this has been done in countless instances by fusing the *lac* operon to the controlling sequences of another gene (a classical operon fusion) and then selecting for constitutive or otherwise inappropriate expression of LAC. The mutations affecting the control of the fusion have then been used to define pathways of gene control.

This tool will be particularly important in complex plants where traditional genetic analysis is cumbersome relative to microbial genetics, due to long generation time, large size, genomic complexity, and developmental and spatial variations of cell types and gene action. Now that *Arabidopsis thaliana* is emerging as a powerful model system, fusion genetics, when developed in a versatile and general way, will become even more elegant and productive. Development of fusion genetic methods using the GUS system will involve the synthesis of

novel GUS substrates which will yield bioactive products upon GUS action. This synthesis is almost the exact converse of what vertebrates do; instead of rendering bioactive compounds inactive by conjugating them to glucuronic acid—which is the vertebrate detoxification rationale—we are proposing to render these conjugates active by applying them to transgenic organisms that express GUS.

It is thus obvious that design and preparation of substrates can also make use of the vertebrate conjugation system, on which there is an extensive literature (Dutton, 1966, 1981). Several thousand glucuronide conjugates have been described from the urine or feces of animals. These are mostly biologically inactive derivatives of highly bioactive substances. Chemical synthesis or biological preparation of such compounds could be tailored to show particular effects on transgenic plants only upon cleavage of the conjugate by GUS. Almost any compound with an O-glycosidic linkage in the β configuration to glucuronic acid is a substrate for GUS. One can envision plant growth regulators or toxins, for example, that could manifest their biological activity on a transgenic plant only if that plant, or some subset of the cells of the plant, was expressing GUS. This could thus provide genetic selections either to plants or cells that acquired mutations that altered the GUS expression in a particular manner. It would also provide a route to modification of the systems' biology in a conditional manner. The possibilities are limited largely by the ingenuity of the investigator.

Summary

It is clear that there are numerous and exciting possibilities for future developments of GUS to provide novel tools. It is also clear that these tools, and tools like them, need to be developed with particular constraints in mind, including the constraints of nonmodel system biology, such as agriculture, and the constraints of the resource-limited scientist. Those who have been "GUS-conscious" for some time will recognize my exhortations regarding *in vivo* analysis and fusion genetics from previous discussions, talks, or papers, and will justifiably ask why, if these methods are so near and so powerful, do they not yet exist? The answer certainly lies in the extraordinary number of questions that are already accessible by use of the first generation of the GUS system as a simple analytical tool and the limited time and energy that can be expended for tool development when substantial answers to these questions can be obtained now. Our scientific sophistication is growing as

we absorb the answers that early gene fusion experiments are providing and appreciate the limitations of both the questions and the tools. The next generation is almost upon us, and the questions will be fun indeed.

The GUS system is meant to be widely available. Those wishing clones or vectors for research should feel free to write to the author at CAMBIA to obtain these samples free of charge.

"Let's see what's out there."
Jean Luc Picard, 1990

Richard A. Jefferson

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Preface

GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression introduces researchers to the diverse applications of the GUS gene fusion system. Originally developed by R. A. Jefferson and co-workers, the importance of β-glucuronidase, or GUS, as a reporter of gene expression in plants is illustrated by recent literature citations. In the first sixth months of 1991 alone, the assay was cited over 120 times. The GUS assay also produces visually striking results, as shown by both the cover and the color plates of this book. These beautiful color photographs illustrate the blue histochemical staining of GUS activity in transgenic plants, tracing patterns of expression of tissue-, organ-, and developmentally-specific genes.

The GUS system is popular because of its simplicity, versatility, and robustness. To use the GUS gene fusion system, typically the *Escherichia coli gusA* gene is coupled to a gene of interest. Once this fusion is introduced into plant (or animal) cells, GUS expression reports activity of the other gene. Because plants normally lack endogenous GUS, detection of the GUS enzyme either in whole tissue or tissue homogenates provides a sensitive background-free measure of gene expression. GUS from *E. coli* is also a good reporter enzyme in animal cells in spite of endogenous GUS activity.

GUS Protocols is divided into five sections covering the general use of the GUS reporter gene, details of the GUS assay, histochemical detection, applications of GUS in plants and, finally, animal genetic analysis. As a reporter of gene expression, the gene isolated from *E. coli* is almost exclusively used. To gain insight into the origins and other potential uses of the *gusA* (formerly *uidA*) gene from *E. coli,* a detailed discussion of the *gus* operon is presented in the first chapter of the book. This is followed by an introduction to the various assays required to effectively analyze GUS activity, including chapters on the properties of GUS substrates and laboratory exercises for biochemistry classes.

Frequently, researchers must assay huge numbers of samples while analyzing transgenic plants. Two chapters address this, one describing automated tissue grinding and extraction and the other GUS assays with microplate fluorescence readers. Several chapters deal with histochemical localization of GUS in plants and animals, and these feature extensive discussions of the problems often encountered. The number of applications that rely on the GUS system is immense, and this kaleidoscope of research is discussed in the last part of this book with chapters covering analysis of secretory systems, molecular plant virology, transposable elements, and genetic analysis of animal cells and tissue.

Variations on the GUS gene fusion system will continue to be developed, providing new and novel applications (see Foreword). However, because this book provides a foundation made of key references, techniques, and procedures, *GUS Protocols* will also continue to stay an invaluable resource well into the future.

Without the help from both organizations and individuals the publication of *GUS Protocols* would not have been possible. I am grateful for the support and assistance provided by the following companies: Pioneer Hi-Bred, Inc. and Clontech Laboratories for generous financial contributions to offset the cost of the color plates found in this book; Hoefer Scientific Instruments for making the inception and continued work on this book a possibility; and Academic Press for their persistence and determination. Special thanks goes to Lorraine Lica of Academic Press for her skillful guidance in preparing *GUS Protocols*.

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