Direct introduction of DNA into embryonic axes of Glycine max by microprojectile bombardment

Charisse Marie Buising
Iowa State University

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Direct introduction of DNA into embryonic axes of *Glycine max*
by microprojectile bombardment

Buisng, Charisse Marie, Ph.D.

Iowa State University, 1992
Direct introduction of DNA into embryonic axes of *Glycine max* by microprojectile bombardment

by

Charisse Marie Buising

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Iowa State University

Ames, Iowa

1992
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ABBREVIATIONS

2,4-D  2,4-dichlorophenoxy acetic acid
Adh1   alcohol dehydrogenase intron 1
AcCoA  acetylcoenzyme A
ANOVA  analysis of variance
BAP    6-benzylaminopurine (aka. BA, benzyladenine)
bar    phosphinothricin acetyl transferase gene
BMS    Black Mexican Sweet
bp     base pair
BSA    bovine serum albumin
CaMV   cauliflower mosaic virus
CFU    colony forming unit
DNA    deoxyribonucleic acid
DMSO   dimethyl sulfoxide
DTT    dithiotreitol
EDTA   ethylenediaminotetracetic acid
FAA    formalin acetic acid alcohol
G1-phase period of cell cycle prior to DNA replication
GUS    beta-D-glucuronidase
IAA    indole acetic acid
kb     kilo base pairs
M      Molar
ml     milliliter
mM: millimolar
mol: mole
mmol: millimole
MS: Murashige and Skoog medium
MUG: 4-methylumbelliferyl-β-D-glucuronide
NAA: α-naphthylene acetic acid
neo: neomycin phosphotransferase gene
nos: nopaline synthase gene
NPTII: neomycin phosphotransferase II
Ω': omega prime sequence
PAT: phosphinothricin acetyltransferase
PEG: polyethylene glycol
PMSF: phenylmethyl sulphonyl fluoride
ppm: parts per million
PPT: phosphinotricin (Basta®)
R₀: first plant regenerated from tissue culture
R₁: first generation progeny from R₀ regenerant
R₂: second generation progeny from R₁ plant
RNA: ribonucleic acid
S-phase: period in cell cycle of DNA replication
SD: standard deviation
SEM: standard error of the mean
SDS: sodium dodecyl sulfate
SSC: sodium citrate
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>TBS</td>
<td>transformation booster sequence</td>
</tr>
<tr>
<td>TE</td>
<td>Tris, EDTA buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>tris hydroxymethylaminomethane</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
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<td>μM</td>
<td>micromolar</td>
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<tr>
<td>μmol</td>
<td>micromole</td>
</tr>
<tr>
<td>X-GLUC</td>
<td>5-bromo-4 chloro-3 indolyl beta-D-glucuronide</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

The primary goal of this study was to examine factors that contribute to optimal transformation of soybean and other crop plants by microprojectile bombardment. In this introduction, I discuss general strategies for transformation of plants, methods of DNA transfer, methods of regenerating or recovering plants, and specific research on transformation of soybean. In section I, I characterize cells and tissues involved in de novo meristem formation in 6-benzylaminopurine (BAP)-treated embryonic axes of soybean. In section II, I examine the factors that affect the expression and persistence of plasmid molecules in bombarded embryonic axes and during regeneration to form mature plants and subsequent progeny. In section III, I characterize the effect of a specific DNA sequence on transformation frequency in a dicotyledonous and monocotyledonous plant. In the summary discussion, I discuss the major conclusions drawn from each paper and attempt to examine the relationship between the development of regenerating tissues, the application of a direct DNA transfer method (microprojectile bombardment), and the impact of selected DNA sequences on transformation frequencies: and I conclude by suggesting strategies for further experimentation.

Transformation strategies

Success in transformation of plants has varied quite dramatically depending on the species of plant, method of transformation, regeneration potential of transformed targets, vector construction, and identification and
recovery of transformed cells, tissues, or plants. Most strategies directed
toward the genetic engineering of plants involve what generally may be
considered two complementary processes: transformation and recovery.
The transformation process involves introducing exogenous DNA into the
genome of individual plant cells that can subsequently be recovered following
the treatment. This includes the development of a DNA delivery method, the
construction of vectors, and the interaction of selected DNA sequences. The
recovery process involves the regeneration or cultivation of the transformed
plant cells into organelles, cells, tissues, and/or whole, sexually competent,
plants. Transformation of some cells or tissues may require cultivation,
while others must be regenerated by tissue culture to follow a particular
developmental pattern. These de novo patterns are often categorized as being
embryogenic or organogenic. The integration of the transformation and
recovery processes is not required to be 100 percent successful, but each
aspect must have a reasonable degree of reliability and reproducibility so that
a practical number of transformed plants can be recovered. The
combination of DNA delivery, recovery of transformed tissues, and
utilization of events during DNA transfer at the nucleic and cellular level,
must be optimized for a particular plant and are presented in Table 1.
Table 1. Components in the transformation and recovery process of plants

<table>
<thead>
<tr>
<th>Components</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Agrobacterium</em></td>
<td>efficient</td>
<td>host/strain specificity</td>
<td>Zambryski, 1988; Caplan <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>DNA/RNA viruses</td>
<td>efficient</td>
<td>no control, specificity</td>
<td>Alquist and Pacha, 1990; Fütterer <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>chemical:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liposomes</td>
<td>efficient transfer</td>
<td>variable regeneration</td>
<td>Davey <em>et al.</em>, 1989; Potrykus, 1990</td>
</tr>
<tr>
<td>DEAE-dextran</td>
<td>fusion or injection</td>
<td>protoplasts/injection</td>
<td>Lucas <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>polyethylene glycol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>calcium phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>imbibition</td>
<td>simple</td>
<td>inefficient, cell wall</td>
<td>Alwen <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>electroporation</td>
<td>efficient DNA transfer</td>
<td>protoplasts</td>
<td>Lindsey and Jones, 1990</td>
</tr>
<tr>
<td>macroinjection</td>
<td>simple</td>
<td>inefficient</td>
<td>de la Peña <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>microinjection</td>
<td>independent controlled</td>
<td>tedious, difficult</td>
<td>Neuhaus and Spangenberg, 1990</td>
</tr>
<tr>
<td>microlaser</td>
<td>target specificity</td>
<td>damage</td>
<td>Weber <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>biolistic (bombardment)</td>
<td>broad range, simple</td>
<td>variable efficiency</td>
<td>Sanford, 1990</td>
</tr>
<tr>
<td>pollen tube</td>
<td>accessible</td>
<td>DNA degradation</td>
<td>Luo and Wu, 1988</td>
</tr>
</tbody>
</table>

*a* Utilization of components is not mutually exclusive.
<table>
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<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Targets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gametes (precursors)</td>
<td>natural process</td>
<td>difficult to recover</td>
<td>Heberle-Bors, 1991</td>
</tr>
<tr>
<td>immature/ mature embryos</td>
<td></td>
<td>regeneration variable</td>
<td></td>
</tr>
<tr>
<td>proplasts</td>
<td>efficient DNA uptake</td>
<td>poor regeneration</td>
<td>Potykus, 1990</td>
</tr>
<tr>
<td>somatic embryos</td>
<td>single cells, prolific</td>
<td>timing, genotype effect</td>
<td>Carman, 1990, Haccius, 1977</td>
</tr>
<tr>
<td><em>de novo</em> meristems</td>
<td>chimeras, prolific</td>
<td>induction, recovery</td>
<td>Skoog and Miller, 1957</td>
</tr>
<tr>
<td>organelles</td>
<td>possible</td>
<td>no selective markers</td>
<td>Haring and De Block, 1990</td>
</tr>
<tr>
<td><strong>Nucleic acid structure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>and Cellular function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>conformation</td>
<td>recombination</td>
<td>stability</td>
<td>Marini and Benbow, 1991</td>
</tr>
<tr>
<td>localization</td>
<td>intracellular delivery</td>
<td>multiple components</td>
<td>Howard <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>retention</td>
<td>contact likely</td>
<td>undefined</td>
<td>Krysan and Calos, 1990</td>
</tr>
<tr>
<td>amplification</td>
<td>proliferation</td>
<td>undefined</td>
<td>Wegner <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>replication</td>
<td>existing process</td>
<td>undefined</td>
<td>Benbow <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>recombination</td>
<td></td>
<td>dynamics, classifying</td>
<td>Bae, 1988.</td>
</tr>
<tr>
<td>homologous</td>
<td>target genome</td>
<td>undefined</td>
<td>Puchta and Hohn, 1991</td>
</tr>
<tr>
<td>nonhomologous</td>
<td>existing process</td>
<td>undefined</td>
<td>Pfeiffer and Vielmetter, 1988</td>
</tr>
<tr>
<td>expression</td>
<td>identification</td>
<td>process dependent</td>
<td>Negrutiu <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>cell cycle</td>
<td>existing process</td>
<td>process orchestration</td>
<td>Meyer <em>et al.</em>, 1985; Doonan 1991</td>
</tr>
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</table>
Delivery of transforming DNA

Agrobacterium tumefaciens-mediated transformation remains the simplest and most reliable means for introducing foreign DNA into the genomes of dicotyledonous plants. Hundreds of transgenic plants, typically with low copy numbers of exogenous DNA integrated into the genome, can be generated in species such as Nicotiana tabacum. In many species, however, such as crop legumes, transformation is limited by plant genotype and Agrobacterium or genotype/strain specificity (Owens and Cress, 1985). Although Agrobacterium is capable of transforming cells of monocotyledons, the transformation frequency is extremely low (Potrykus, 1990). This has spurred the development of alternative methods of DNA transfer.

Many different methods for direct DNA delivery have been utilized (reviewed by Davey et al., 1989 and Potrykus, 1990). Often these direct methods of transfer involve creating protoplasts from the recipient cells which can preclude the regeneration of whole plants. Such constraints in plant regeneration dictates use of a delivery method that directly transfers DNA into intact plant cells.

Direct introduction of DNA into plant cells by microprojectile bombardment is now recognized as an efficient system for transient expression and stable transformation of a number of plant species (Sanford, 1988; 1990). In many crop species, however, among the thousands of cells expressing transiently after particle bombardment, only 1:1,000 to 1:10,000 lead to stable integration of exogenous DNA into the genome. Perhaps only 1 in 100,000 are found in the germ line (Sanford, 1990; McCabe, 1988). A
maximal frequency of recovery and integration of exogenous DNA then
becomes increasingly important.

Recovery of transformants

The ability to recover transformed plants, tissues, and cells depends
upon which cells are targeted by a particular DNA delivery method. Transformation of a gametophyte could result in the recovery a whole plant
without the use of tissue culture; generally, however, the use of tissue
culture is usually necessary to obtain plants. The morphogenic processes of
regeneration follow two alternative pathways: embryogenesis and
organogenesis. Somatic embryogenesis is a pattern of development similar
to that of a fertilized zygote (Lupotto, 1983). The somatic embryo undergoes a
conversion process that eventually leads to germination and maturation as a
plantlet (Buchheim et al., 1989). Plant development continues beyond
embryogenesis by organogenesis, primarily in apical meristems (Stewart,
1978; Miksche, 1961). Organogenesis is the sequential organization of shoot
and root meristems of different cell origin within the callus or tissue.
Divisions in the meristem establish the new tissues and organs of the plant.

Somatic embryogenesis The culture of embryogenic cells through
plant tissue culture was reviewed by Carman (1990). There appear to be
many different factors that influence the change in developmental potential
to form somatic embryos such as: explant maturity, genotype, culture
regime, media formulation, etc. Development of embryogenic cells, which
give rise to somatic embryos, appears plastic until differentiation is largely
complete (Buising and Hoard, 1992d); however, the molecular basis for this developmental progression is unknown.

Transformation of embryogenic cells has been observed in many species (Finer and McMullen, 1991; Buising et al., 1992e). Somatic embryogenic cultures can serve as an unlimited supply of target tissue, from which transformed cells can be recovered. The technical limitations many have encountered include: induction of embryogenesis, transformation of the cell, identification of transformants, culture to mature somatic embryos, and conversion of embryos to plants.

Organogenesis The development of apical meristem and/or de novo production of multiple shoots are attractive alternatives to somatic embryogenic cultures because large numbers of shoots can be produced from a single explant. Developmental control of bud formation by kinetin was shown by Skoog and Miller (1957), who found that the relative concentrations of plant growth regulators rather than their absolute concentrations influenced the regulation of organ differentiation. Adjusting the relative concentrations of zeatin and kinetin in the culture medium induced bud formation.

In vitro culture of apical and de novo meristems can be initiated by various plant growth regulator treatments. Multiple shoots can be recovered, cultured and grown to maturity (Buising et al., 1992a). Chimeric plants that give rise to transformed progeny have been recovered from treatment of de novo meristems using microprojectile bombardment (McCabe et al., 1988, Malone-Schoneberg et al., 1992), Agrobacterium-
treatment (Hinchee et al., 1988; Chee et al., 1989; Ulian et al., 1988), or combinations of Agrobacterium and biolistics (Bidney et al., 1992).

**Prior and current research on soybean transformation**

Efforts have been made previously to transform soybean cells using Agrobacterium tumefaciens. Owens and Cress (1985) reported responsiveness of soybean cells to A. tumefaciens. However, not all Agrobacterium strains can readily infect all tissues (Byrne et al., 1987; Owens and Cress, 1985). Successful transformation of soybean using the Agrobacterium-mediated transformation system in developing meristems or de novo meristems from cotyledons has been reported (Hinchee et al. (1988), Chee et al. (1989), and Townsend and Thomas, in preparation). Transformation frequencies averaged between 1-15% and, in some cases, appeared to be limited by host/strain specificity. The transformation efficiency of Agrobacterium-mediated transfer was further optimized from 10% to 30% by wounding of tissues, application of acetosyringone, culture on selection, identification and recovery of transgenic (chimeric) shoots, followed by harvest and identification of transformed progeny (Townsend and Thomas, in preparation).

Dhir et al. (1991a; 1991b, 1991c) successfully recovered transgenic soybean after electroporation of exogenous DNA into protoplasts derived from the epidermis of immature zygotic cotyledons. These transgenic protoplasts were cultured into somatic embryos which eventually converted to plants. This technique utilizes an efficient transfer method where DNA is electroporated directly into protoplasts. The developmentally competent cells
from which these protoplasts come, are subject to the inherent problems associated with using protoplasts: poor reproducibility both in culture and recovery of transformed plants.

The direct microprojectile particle bombardment transfer system developed by Sanford (1988; 1990), has the advantages of direct transfer without the problems associated with \textit{Agrobacterium} genotype/strain specificity or the need to protoplast cells.

Christou \textit{et al.} (1988) reported stable transformation of soybean cells and plants (McCabe \textit{et al.}, 1988) using a arc-discharge particle accelerator apparatus to accelerate gold particles into apical meristems. The inheritance pattern in soybean did not appear to be Mendelian in initial experiments (McCabe \textit{et al.}, 1988); however, Christou \textit{et al.} (1989) has since reported Mendelian inheritance patterns in two \textit{R}$_1$ and \textit{R}$_2$ soybean plants. Again the transformation frequencies were extremely low, 1-2%.

Finer and McMullen (1991) reported stable transformation of the soybean genotype 'Fayette' from the bombardment of cycling somatic embryogenic cultures. Genotypic specificity has made it difficult to reproducibly apply this technique to all soybean cultivars because of the regeneration protocol. Regardless of the difficulties with the regeneration protocol, this system provides a large number of transient and, therefore, an increased number of stable transformation events. The ability to bombard, select, culture and convert thousands of somatic embryos to transgenic plants, eliminates many of the cellular and developmental limitations. The integration of the regeneration process and the DNA delivery process in agriculturally important crops has been a major obstacle in the application
of genetic engineering. Alternative strategies, for optimization of transformation, exploit cellular function and use of nucleic acid structure (see section III and summary discussion.)

Methods of increasing transformation

The effect of transferring DNA into the cell at various points of the cell cycle on transformation efficiency has been examined. Meyer et al. (1985) found that the transformation efficiency increased by approximately two orders of magnitude when protoplasts, synchronized in S- or M-phase, were transformed using PEG precipitation. Since synchronization procedures normally require creating protoplasts from cells, regeneration is limited in some species. The success in increasing the transformation frequency may take advantage of coupling cell cycle events to the localization, integration, and subsequent expression of the exogenous DNA. Increasing interactions between genomic and exogenous DNA may require optimizing access to the genome (Umek and Kowalski, 1988; 1990; Larson and Benbow, 1992), such as during S-phase when there is a more open conformation of chromatin. The transformation efficiency may be increased by extending access of foreign DNA in and to general and specific locations in genomic DNA. Sequences responsible for retention of exogenous DNA have been identified (Krysan et al., 1989; Krysan and Calos, 1991), as well as sequences that may act by direct attachment of exogenous DNA to specific locations in the genome (Puchta and Hohn, 1991).
Selected DNA sequences

Meyer et al. (1988) isolated the transformation booster sequence (TBS), a 2-kb petunia sequence that increases the efficiency of transformation about 20-fold in petunia and tobacco protoplasts by the vector containing TBS. The isolation strategy used to recover TBS was intended to enrich for autonomously replicating sequences; however, TBS was found to increase the transformation frequency. Several research groups using organisms as diverse as yeast, frogs, mice, petunia, tobacco, and human cells have independently isolated sequences that have led to increased transformation frequencies of other DNA sequences on the same supercoiled plasmid DNA vector. DNA segments resulted in promoting the integration of the supercoiled plasmid DNA vectors into high molecular weight DNA. Several different factors appear to interact and influence transformation efficiency.

The conformation of input plasmid DNA has a striking effect on whether the DNA is replicated, persists, or becomes integrated into high molecular weight DNA (Marini et al., 1988; Endean and Smithies, 1989). A linear conformation appears to facilitate formation of high molecular weight concatemers (Riggs and Bates, 1986).

Attempts to clone specific origins of DNA replication in animal cells (Marini et al., 1988; Marini and Benbow, 1991) or in plant cells (Meyer et al., 1988; Kartzke et al., 1990) have not resulted in cloned sequences that function as origins of replication for circular DNA molecules. The VirD2 protein appears to direct localization of Agrobacterium (Howard et al., 1992). Other sequences, that appear to extend residency of exogenous DNA in the nucleus, contain nuclear retention signals (Krysan and Calos, 1991), specific
DNA attachment sites (Bae et al., 1988; Benbow et al., 1992; Buising and Benbow, 1992c), or amplification signals (Wegner et al., 1989; 1990).

The effect of repeated DNA sequences on direct gene transfer in animals (Folger et al., 1982) and plants also has been examined (Paszkowski et al., 1988; Marchesi et al., 1989). Such sequences may promote homologous recombination (Baur et al., 1990; Puchta and Hohn, 1991; Lyznik et al., 1991) by the preferential integration of the transforming DNA into sites adjacent to genomic repeated sequences. This proposed mechanism of homologous recombination would allow directing exogenous DNA to specific location in the genome. The random integration observed in many direct transformation systems suggests that nonhomologous recombination of DNA also can occur (Pfeiffer and Vielmetter, 1988).
EXPLANATION OF DISSERTATION FORMAT

This dissertation is written in an alternative format. The sections of this dissertation are manuscripts that will be revised to accommodate editors suggestions and be submitted for publication in the following journals: Developmental Biology, In Vitro Cellular and Developmental Biology, and Molecular and General Genetics. Each section contains and is arranged according to the following: abstract, introduction, materials and methods, results, discussion, acknowledgements, references, tables, figure legends, and figures. Following these three sections is a general discussion of the entire dissertation.
SECTION I: EARLY EVENTS OF MULTIPLE SHOOT DEVELOPMENT IN SOYBEAN EMBRYONIC AXES TREATED WITH THE CYTOKININ, 6-BENZYLAMINOPURINE
EARLY EVENTS OF MULTIPLE SHOOT DEVELOPMENT IN SOYBEAN EMBRYONIC AXES TREATED WITH THE CYTOKININ, 6-BENZYLAMINOPURINE

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Randy C. Shoemaker 1, 2
Robert M. Benbow 1, 3

To be resubmitted to Developmental Biology

1 Department of Zoology and Genetics
2 USDA-ARS-FCR, Department of Agronomy,
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Iowa State University
Ames, Iowa 50011-3223
ABSTRACT

Early events of multiple shoot development in germinating soybean embryonic axes treated for 24 hr with the cytokinin, 6-benzylaminopurine (BAP) were compared to the development of untreated control axes using four different techniques: photomicrography, scanning electron microscopy, histology, and autoradiography. Apical meristem and axillary bud development in BAP-treated embryonic axes by 66 hr post-imbibition seemed normal morphologically, but was delayed by about 9 to 15 hr relative to control axes. By 114 hr post-imbibition, numerous (25-100), rapidly proliferating meristematic regions (*de novo* vegetative buds) were observed in BAP-treated axes around the perimeter of the apical dome at and above the level of the axillary buds. These visible morphological changes were preceded by transient inhibition of DNA synthesis in the primary apical meristem and axillary buds as early as 21 to 36 hr post-imbibition. Subsequent changes in cell division patterns in these regions were observed from 42 to 90 hr post-imbibition. Some of the new meristematic regions subsequently developed into multiple shoots. These shoots were excised, subcultured, and used to generate mature plants. In the absence of BAP, excision of the primary apical meristem or axillary buds did not result in multiple shoot formation. These results suggest that transient exposure to BAP interrupted chromosomal DNA replication and reprogrammed the developmental fate of a large number of cells in the shoot apex. We postulate that interruption of DNA synthesis, either directly, by interfering with DNA
replication, or indirectly, by preventing entry into S-phase, caused this reprogramming.
INTRODUCTION

Although early events in normal shoot apex development of soybean have been extensively characterized, events associated with multiple shoot development after treatment with the cytokinin, 6-benzylaminopurine (BAP) have not. A mature soybean seed [Glycine max (L.) Merrill] contains a dormant embryo consisting of two large cotyledons and an embryonic axis (Sun, 1957; Miksche, 1961). The embryonic axis consists of a hypocotyl-radicle, two cotyledonary axillary buds, a plumule with two primary unifoliolate leaves, and a primary shoot apex. The primary apical meristem measures 80 to 120 μm in diameter, is 30 to 50 μm in height, and consists of a two-cell layered tunica and large corpus (Miksche, 1961). A central initiation zone containing large cytoplasmically-dense isodiametrically-shaped cells, a surrounding peripheral zone of deeply staining cells, and a rib meristem underlying the central initiation zone comprise the corpus (Sun, 1957; Miksche, 1961). The central initiation zone and the first trifoliolate leaf primordium both contain numerous mitotic figures (i.e., were active in cell division prior to dormancy). The two smaller axillary bud primordia each consist of a single layered tunica, which divides anticlinally and generates the epidermis, and an underlying corpus, which divides periclinally and contributes to the remainder of the shoot.

Germination is initiated by imbibition of water. Development proceeds by proliferation of vascular tissue in a peripheral zone followed by downward growth of the root apex and cell divisions within the shoot apices (reviewed by Lersten and Carlson, 1987). Within 36 hr after imbibition, cell divisions
are observed in the corpus of the apical meristem, the mesophyll of the primary unifoliolate leaves, and the corpus of the axillary bud primordia (Miksche, 1961; also see Fig. 4 and Fig. 5 in Results below). Cell divisions in the primary apical meristem and the axillary buds begin within 40 to 48 hr after imbibition.

Treatment with 6-benzylaminopurine (BAP) induces multiple shoot formation in many plant species (reviewed by Flick et al., 1983), including soybean. Axillary buds and tissues of the cotyledonary node of Glycine max have been diverted from normal development (described above) to form multiple shoots on medium containing BAP (Cheng et al., 1980; Saka et al., 1980). Stimulation of multiple shoot formation was initiated at high (50-100 μM) BAP concentrations, but required subsequent reduction of BAP concentration. Callus formation also was reported, but emergence of new meristems was not observed (Saka et al., 1980). Kartha et al., (1981) subsequently observed regeneration of plantlets from shoot apical meristems of Glycine max cultured with 1 to 10 μM BAP. Widholm and Rick (1983) reported shoot regeneration from calli of cotyledons and hypocotyls of Glycine canescens cultured on 25 μM BAP. Dramatic cultivar-dependent differences in the number of shoots formed per axis were noted by Barwale et al. (1986a; 1986b).

Wright et al., (1986a, 1986b) characterized de novo multiple shoot production from excised cotyledonary nodes during the period of 6 to 28 days after continuous culture on 5 μM BAP followed on occasion by transfer to 1 μM BAP: proregenerative tissues were produced by 6 days. In addition, Wright et al., (1986a; 1986b) and Barwale et al., (1986a; 1986b) reported that
BAP interfered with subsequent development and elongation of multiple shoots. Wright et al. (1986a) concluded, however, that continuous exposure to BAP was required for additional bud production. They also showed histologically that basal regions of cotyledons and epicotyls developed meristematic regions (histologically defined as densely staining) adjacent to the axillary bud. A ridge of vegetative buds subsequently developed around the circumference of the primary apical meristem by 14 days after germination at the node between the attachment points of the two cotyledons.

Early events in the development of BAP-induced multiple shoots have not been characterized in Glycine species. Moreover, previous studies were carried out with BAP present continuously. Since BAP interferes with shoot development, but not shoot initiation, it has been previously been difficult to distinguish the initial effects of BAP from its subsequent perturbations of normal development. In this study we show that a single 24 hr exposure to even low concentrations of BAP reprograms development throughout the shoot apex. Multiple de novo vegetative buds are induced, each of which is capable of giving rise to a mature plant. These morphological changes are preceded by earlier changes in DNA replication and cell patterns. BAP transiently inhibits DNA synthesis for 9 to 15 hr throughout the shoot apex. A similar effect of transient inhibition of DNA synthesis on gene amplification in mammalian cells has been reported (i.e. dissociation of the normal progression through the cell cycle followed by overreplication, Johnston et al., 1986; Schimke, 1988): By analogy with these studies in mammalian cells, we postulate that BAP acts by reprogramming DNA replication patterns throughout the soybean embryonic axis.
MATERIALS AND METHODS

Plant material

Cultivars and unadapted lines used in this study were *Glycine max* (L.) Merr. cvs. Corsoy, Dunfield, Hack, Harosoy, Illini, Mandarin, Medium Green, Peking, Pioneer 9341, Richland, Seneca, Virginia; *Glycine gracilis* Skorv. PI 79593, PI 153292, PI 326580, PI 65388; and *Glycine soja* Zucc. E. Zieb. PI 65549, and PI 424078. Taxonomy classification according to Hymowitz, T. and Singh, R. J., 1987. Mature seed can be obtained from the U.S. Department of Agriculture Soybean Germplasm collection at Urbana, IL, USA. Pioneer 9341 was the generous gift of Robert Freestone (Pioneer Hi-Bred International, Inc.).

Culture of embryonic axes

Mature soybean seed were surface sterilized for 15 min in 0.5% sodium hypochlorite (10% solution of Chlorox® in distilled water) containing two drops of Triton X-100 (Sigma, St. Louis, MO), rinsed five times in sterile distilled water, and allowed to imbibe for 18 hr. The seed coat, cotyledons and unifoliate leaves were then excised from the embryonic axis. Embryonic axes were placed abaxial surface down on Murashige and Skoog (MS) medium under aseptic conditions (Murashige and Skoog, 1962) and were incubated in the dark at 27°C. The basal medium, with no growth regulators added, (referred to as MS or control media), was supplemented with either gibberellic acid or BAP (6-benzylaminopurine, also referred to as benzyl-adenine (BA) or N⁶-benzyl adenine) which were obtained from
Sigma. After 24 hr, axes exposed to BAP or gibberellic acid were moved to half strength MS medium and incubated for the times noted. Axes were incubated at 28°C under approximately 100 μE m⁻² s⁻¹ of light and a 16-hr day length.

Leaf nomenclature in different plant species remains inconsistent; therefore, we follow Lersten and Carlson, 1987. In soybean, the first leaves are unifoliate in morphology and termed the unifoliolate leaves. The second, third, fourth, etc. leaves to emerge exhibit a compound trifoliolate morphology, and are termed the first trifoliolate, second trifoliolate, etc. leaves.

**Plantlet regeneration**

Shoots greater than 1 cm were excised, transferred to MS medium in Magenta boxes (Magenta Corporation, Chicago, IL) and allowed to elongate. Shoots were dipped in rooting hormone of 0.1% indole-3 butyric acid (Dip'n Root®, Kansas City, KS), planted in a 3:1 mixture of peat and perlite, watered until well soaked, enclosed in a plastic bag and incubated under light for 2 days. Plants were gradually acclimated to lower humidity by opening the plastic bag until leaves began to wilt. Exposure to the light and atmospheric conditions for increasing lengths of time was repeated for 5 days before plants were transferred to the greenhouse where they were grown to maturity under a 16-hr daylight cycle.
Photomicrography

Embryonic axes were harvested at the indicated times after imbibition and photographed with a Nikon FX-35 camera and exposures adjusted with a Nikon AFX-11 using Kodak Ektachrome 160 tungsten ET 135 slide film.

Scanning electron microscopy

Embryonic axes were harvested at various times after imbibition and fixed in FAA [45 ml of 50% ethyl alcohol, 2.5 ml glacial acetic acid, 2.5 ml (37%) formaldehyde]. Axes were washed in 0.1 M sodium cacodylate buffer (pH 7.2), post-fixed in 1% osmium tetraoxide, washed in water, and dehydrated in a graded ethanol series. Specimens were critical point dried by CO₂, mounted and sputter coated with gold/palladium (ratio of Au/Pd, 60/40) on a Polaron E5100 Sputter Coater. Microscopy was carried out using a JEOL JSM-35 Scanning Electron Microscope operated at an accelerating voltage of 10 KeV, 40 μamp current. Axes were viewed at the magnifications described and photographed with Polaroid 665-P/N film.

Histology

Embryonic axes were harvested at various times after imbibition, fixed in FAA, and dehydrated in a graded ethanol series to xylene. Axes were embedded in paraffin, cut into 10 μm ribbons floated on distilled water, and placed on slides without adhesive. Slides were dewaxed in xylene and rehydrated in a graded ethanol series. The triple staining procedure of McDaniel et al., (1982) was employed to highlight meristematic regions. Meristematic cytoplasm appears as intense blue staining regions (deep gray
tones in black and white prints). Triple stain containing 1% aqueous safranin, 0.5% aniline blue and 2% orange G (Sigma) in 0.1 M sodium citrate buffer, pH 3.5. After staining, slides were again dehydrated in a graded ethanol series, cleared in xylene and mounted under a cover slip with resin.

**Autoradiography**

Control and BAP-treated embryonic axes were labeled with 10 μCi/ml of [methyl-3H] thymidine (6.7 Ci/mmol, NEN-DuPont) in liquid MS media for 1 hr at the times indicated in Fig. 9, washed three times with liquid MS media, and cultured for 5 additional hr to allow incorporation into high molecular weight chromosomal DNA. Labeled axes were fixed in FAA, and treated as described in Histology (above) without staining. 10 μm sections were overlaid with emulsion from Kodak AR-10 fine-grain autoradiographic stripping plates (Eastman Kodak Co.) and processed as suggested by the manufacturer.

**Statistical analysis**

Statistical analysis was carried out according to Daniel (1974) using Statview II software (Abacus Concepts, Inc., Berkeley, CA, 1987). The effect of growth regulator treatment on the appearance of shoots from soybean embryonic axes was examined by ANOVA (Fisher, 1966).
RESULTS

Morphology of multiple shoot development in BAP-treated Glycine max embryonic axes: Photomicrographs

The visible morphology of events in apical meristem development in control embryonic axes was compared with events in multiple shoot development in BAP-treated axes during the same time period. Excised embryonic axes (Fig. 1a) were cultured on 22 μM BAP for 24 hr, transferred to MS media, and cultured for the indicated times. Embryonic axes exposed to BAP developed a characteristic morphology (Fig. 1b, left pair); by 168 hr they were significantly shorter and showed a 2-fold increase in hypocotyl diameter relative to control (no growth regulator) axes (Fig. 1b, central pair). BAP-treated axes also exhibited prominent axillary buds (Fig. 1b) and retarded root growth when compared to either control embryonic axes or to axes treated with the growth regulator, gibberellic acid (Fig. 1b, right pair).

It had been suggested previously (Evans, 1984) that gibberellic acid, like BAP, appears to induce cell division. Since multiple shoot formation was never observed (unpublished observations) after treatment with gibberellic acid, however, we focused on early events following treatment with BAP. BAP-treated axes showed the emergence of multiple buds (at least 25 are visible in Fig. 1d) 5 to 7 days after imbibition and suppression of primary apical meristem growth (compare Fig. 1c with Fig. 1d). At 10 days after imbibition (Fig. 1e), multiple buds proliferated at the level of the axillary buds and at the junction of prior cotyledon attachment. Buds also were observed surrounding the preexisting apical meristem. By 14 days after
imbibition, up to six buds had elongated to form shoots, which were defined 
nascent vegetative buds that had elongated at least 0.3 cm; at least two are 
visible in addition to the axillary shoots (Fig. 1f). The remaining vegetative 
buds persisted, but only a limited number elongated. At 21 days after 
imbibition, an average of five to six shoots (see Fig. 7, Fig. 8, and Table 1 
below) had elongated (Fig. 1g), some as much as 1 cm. Unless the longest 
shoots (> 1 cm) were excised at this time, they exhibited an apical 
dominance-like effect; the remaining shoots stopped growing and additional 
vegetative buds did not elongate. At 28 days after imbibition in BAP-treated 
embryonic axes from which the two or three longest shoots had been excised, 
some of the remaining smaller shoots elongated, leaflets emerged, and 
additional vegetative buds appeared (Fig. 1h). The morphology of emerging 
leaves in nascent vegetative buds was similar to the trifoliolate leaves of 
preexisting axillary buds. It is important to note that de novo vegetative buds 
were never observed in axes cultured on basal MS media without the 
addition of BAP.

**Morphology of multiple shoot development in BAP-treated *Glycine max* 
embryonic axes: Scanning electron micrographs**

At 42 hr post-imbibition (ie. 18 hr imbibition plus 24 hr exposure to 
BAP) there were no visible morphological differences between control and 
BAP-treated embryonic axes (compare Fig. 2a with Fig. 2b), even though we 
will show there were dramatic differences in patterns of DNA synthesis at 
this time (see Fig. 9 below). At 66 hr post-imbibition there were still no 
visible morphological differences in development of the apical meristem and
axillary buds, but inhibition of trichome outgrowth was detectable (compare Fig. 2c with Fig. 2d). In control axes at 90 hr post-imbibition, there was extensive trichome outgrowth and prominent development of the first trifoliolate leaf. Leaflet development at the axillary bud also was well defined (Fig. 2e). In BAP-treated axes at 90 hr post-imbibition, trichome outgrowth was delayed. The axillary buds had enlarged, but there were still no visible morphological changes in the region adjacent to the axillary buds that would give rise to multiple shoots (Fig. 2f). At 114 hr post-imbibition control embryonic axes displayed normal apical meristem development and axillary bud proliferation (Fig. 3a; largely obscured by the abundant trichome outgrowth in this micrograph). By contrast, in BAP-treated embryonic axes at 114 hr post-imbibition, apical meristem development and trichome outgrowth was delayed and the proliferation of multiple nascent vegetative buds was apparent (Fig. 3b). These were the first visible morphological changes in the shoot apex during multiple shoot development.

The development of a prominent nascent vegetative bud immediately to the right of the existing axillary bud is shown at a higher magnification in Fig. 3c. The proliferation of another vegetative bud to the left of the existing axillary bud is shown in Fig. 3d. BAP induced vegetative buds developed in a nested array extending outward from the existing axillary bud around the circumference of the cotyledonary node. The first induced buds developed adjacent to the axillary buds. These were the buds most likely to elongate.

Normal apical meristem development in a control embryonic axis at 162 hr post-imbibition is shown in Fig. 3e. Trifoliolate leaf development
flanking the apical meristem was apparent. The axillary buds had not visibly elongated (concealed in Fig. 3e by extensive trichome outgrowth). A higher magnification view of axillary bud leaf development at 114 hr post-imbibition (before it became obscured at 162 hr by extensive trichome outgrowth) in a control embryonic axis showed no nascent vegetative bud formation (Fig. 3f). The elongation and proliferation of nascent vegetative buds in BAP-treated embryonic axes at 234 hr are shown in Fig. 3g. The embryonic axis in Fig. 3g was the same used in the photomicrograph in Fig. 1e (note that the prominent axillary buds seen in Fig. 1e were out of the field of view in Fig. 3g). A higher magnification view of nascent vegetative buds (Fig. 3h) adjacent to the axillary shoot showed proliferation of at least four buds (compare Fig. 3h and Fig. 1e). A single 24 hr exposure to BAP has, therefore, resulted in a developmental reprogramming of the shoot apex such that axillary bud elongation was stimulated, apical meristem development was delayed, and rapid proliferation of multiple vegetative buds was induced. A subset of the induced vegetative buds subsequently gave rise to elongated multiple shoots.

**Histological characterization of multiple shoot development**

An opposite pair of unifoliolate leaves (which have been excised in Fig. 4), axillary bud meristems, and the first trifoliolate leaf primordium on the primary apical meristem were already evident in imbibed *Glycine max* seed. As expected (see Introduction), mitotic figures (cells that had recently completed telophase) were observed at higher magnification in longitudinal
sections of the primary apical meristem taken at 18 hr post-imbibition (see Miksche, 1961).

Effects of exposure to BAP from 18-42 hr post-imbibition were already evident by 42 hr in histological sections (Fig. 5a, Fig. 5b), although these effects were not visible in whole mounts (Fig. 1 above). Few periclinal divisions in the first trifoliolate leaf buttress were observed in BAP-treated axes (Fig. 5b), whereas they were numerous in control axes (Fig. 5a; see Miksche, 1961). By 66 hr post imbibition, the first trifoliolate leaf buttress had developed in BAP-treated axes to about the same extent as in 42 hr control axes (compare Fig. 5d with Fig. 5a). By contrast, in control axes at 66 hr post-imbibition, the second trifoliolate leaf buttress was visible and the first trifoliolate leaf had elongated considerably (Fig. 5c). At 90 hr post-imbibition in BAP-treated axes, periclinal divisions of the second trifoliolate leaf were visible (at higher magnification) and the first trifoliolate leaf had elongated: this was comparable to the control axis 24 hr earlier (compare Fig. 5e, Fig. 5f with Fig. 5c). Leaf formation in axillary buds also was noted on both the control and BAP-treated axes at this time. At 114 hr post imbibition in BAP-treated axes, emerging lateral leaflets were just visible (Fig. 5h) and the second trifoliolate leaf had begun elongation. The primary differences between BAP-treated and control axes were the dramatic increase in hypocotyl diameter (compare Fig. 5h with Fig. 5g) and the appearance of de novo vegetative buds in some sections (see Fig. 6 below). At 114 hr, de novo vegetative buds exhibited cell division patterns similar to those of preexisting axillary buds (Fig. 6a). Leaf development in nascent vegetative buds was initiated by periclinal divisions of several cells in the
outer layer of the corpus, and later involved anticlinal divisions of the tunica (Fig. 6b).

Dependence of multiple shoot development on BAP

The number of multiple shoots per embryonic axis for cv. Harosoy observed at 28 days after imbibition appeared to be a direct function of the initial BAP concentration up to 22 μM (Fig. 7; Table 1). Concentrations of BAP greater than 25 μM resulted in the same number of vegetative buds, but subsequent outgrowth of multiple shoots was inhibited. Concentrations of BAP greater than 100 μM were toxic. The mean number of shoots observed per embryonic axis incubated on 22 μM BAP was 5.59 ± 0.09 (n=350) for Glycine max (Harosoy) at 4 weeks post-imbibition. No significant differences in the mean number of multiple shoots was observed for 17 additional cultivars and unadapted lines (Table 1) when analyzed by ANOVA, including those characterized by Barwale et al., (1986a, 1986b) either as low or high shoot responders (i.e. a low or a high number of multiple shoots per embryonic axis). These results suggest that the high and low shoot responses reported by Barwale et al., (1986a; 1986b) may have been the result of subsequent culture dependent perturbations of development by the continued presence of BAP.

Developmental reprogramming of shoot apices by BAP

Exposure to BAP resulted in the induction of numerous (25-100) vegetative buds on each embryonic axis (Fig. 1 d; Fig. 1e). Serial harvesting of elongated shoots (> 1 cm) from BAP-treated axes at 4 week intervals for at
least 4 months resulted in elongation of additional vegetative buds. Usually only about five of these buds elongated at any one time to form shoots (Fig. 8); thus, the number of multiple shoots per axis was consistently maintained. It appears that transient exposure to BAP altered numerous cells throughout the shoot apex such that most, and probably all, of the numerous vegetative buds observed at 7 and 10 days post-imbibition were competent to form elongated shoots. Presumably, outgrowth of these vegetative buds was inhibited by the longer shoots. A major factor that appeared to limit the number of multiple shoots harvested from a single axis was the accumulation of damage during repeated excisions of shoots.

The number of multiple shoots depended on BAP treatment and was not influenced by excision of either the primary apical meristem or of axillary meristems as shown by the following control experiments. If the apical meristem was excised at 90 to 114 hr post-imbibition, the mean number of multiple shoots formed in serially harvested BAP-treated axes was (mean ± SEM) 5.2 ± 0.46, 4.9 ± 0.45, 4.5 ± 0.46, and 4.1 ± 0.44 at 4, 8, 12, and 16 weeks respectively. By contrast, the number of shoots formed in the absence of BAP on control axes from which the apical meristem had been excised was 1.2 ± 0.12, 0.1 ± 0.06, 0, and 0 at 4, 8, 12, and 16 weeks respectively. When axillary buds were removed at 90 to 114 hr post-imbibition, the number of multiple shoots formed in BAP-treated serially harvested axes was 5.2 ± 0.54, 4.8 ± 0.37, 4.4 ± 0.40, and 4.6 ± 0.43 at 4, 8, 12, and 16 weeks respectively. By contrast, when axillary buds were removed from serially harvested control axes, the number of shoots formed was 0.9 ± 0.1, 0, 0, and 0 at 4, 8, 12, and 16 weeks respectively. Similar results were
obtained when both the axillary buds and the apical meristem were excised (data not shown). *De novo* multiple shoot formation in the *Glycine* species examined in this study, therefore, required exposure to external BAP.

**BAP inhibits chromosomal DNA synthesis in embryonic axes**

There was no incorporation of [³H]-thymidine in either control or BAP-treated embryonic axes pulse labeled for 1 hr at 18 hr post-imbibition. Incorporation of [³H]-thymidine into cells of the first trifoliolate leaf buttress and axillary buds was first observed at 27 hr in control embryonic axes pulse labeled for 1 hr at 21 hr post-imbibition (Fig. 9a). By contrast, no [³H]-thymidine incorporation was observed in these regions at 27 or 30 hr in BAP-treated embryonic axes labeled at 21 hr (not shown) or 24 hr (Fig. 9b). At least 15 independent axes were examined for each time point. Extensive incorporation of [³H]-thymidine into the first trifoliolate leaf buttress, axillary buds, leaf stipules, and vascular tissues was observed in control embryonic axes pulse labeled for 1 hr at 30 hr (Fig. 9c) and 36 hr (Fig. 9e). Only a trace level of incorporation was observed in the first trifoliolate leaf buttress of BAP-treated axes labeled at 30 hr (Fig. 9d). [³H]-thymidine incorporation into BAP-treated embryos labeled at 36 hr post-imbibition (Fig. 9f) closely resembled the pattern of incorporation into control embryos labeled 15 hr earlier at 21 hr post-imbibition (compare Fig. 9a with Fig. 9f). labeling at later times was difficult to interpret because of asynchronous replication throughout the shoot apex. These data suggest that BAP treatment induces a delay of between 9 and 15 hr (depending upon the sections examined) in DNA synthesis in the first cells which synthesize
DNA in the primary apical meristem. BAP treatment did not appear to alter the developmental pattern (order) in which cells in the shoot apex entered into S-phase, but more cells were observed to synthesize DNA at later times in BAP-treated axes. Since inhibition of DNA synthesis was observed within 3 hr of the first exposure to BAP (when DNA synthesis occurred in control axes), and persisted for 9 to 15 hr, it was likely that BAP either interfered with chromosomal DNA replication or with entry into S-phase.

Concomitantly, cells in other regions of the shoot apex were being reprogrammed to form nascent vegetative buds. Exposure to BAP, therefore, not only broke apical dominance, but also rapidly and transiently inhibited DNA synthesis throughout the shoot apex, and triggered the chain of events resulting in reprogramming of multiple cells in the shoot apex.
DISCUSSION

Early events in BAP induction of multiple shoot formation

Since Ali and Fletcher (1970; 1971) have suggested that BAP stimulates cell division in axillary buds, we elected to examine early events (DNA synthesis prior to the first cell divisions) in soybean embryonic axes exposed transiently to BAP. The first effect we observed was a delay in DNA synthesis in the primary apical meristem at 24-30 hr post-imbibition. DNA synthesis was first observed in untreated control axes 9 to 15 hr before any synthesis was detected in the apical meristem of BAP-treated embryos (compare Fig. 9a with Fig. 9d). Similar delays were seen in the initiation of DNA synthesis in axillary buds. Therefore, the first effect of exposure to BAP we observed was rapid inhibition of chromosomal DNA synthesis. The next effect we observed was a contingent delay of cell division in the apical meristem and axillary buds (Fig. 5a; Fig. 5b). Thus, the initial effect of the cytokinin BAP in rapidly dividing meristematic cells was, paradoxically, inhibition rather than stimulation of cell division (see also Van't Hof, 1968). This inhibition of DNA synthesis and the resulting delay in cell division presumably accounts for the initial breakage of apical dominance.

BAP is required for de novo bud formation in Glycine species

Excision of the apical meristem and/or axillary buds without exposure to BAP did not give rise to de novo vegetative buds and multiple shoots [although splitting of existing apices can give rise to two or more shoots (J. Ranch, personal communication)]. The cells that give rise to de novo
vegetative buds appear, therefore, to be developmentally determined not to do so, rather than to be inhibited from doing so by the apical and axillary meristems. Since it has been shown by autoradiography that BAP is selectively accumulated by cells that respond to cytokinins (Brandes and Kende, 1968), these results suggest that transient exposure to BAP is the causative agent (direct or indirect) in reprogramming the developmental fate of these cells.

In BAP-treated embryonic axes, the number of shoots > 0.3 cm was consistently maintained at about five per axis suggesting that a regulatory feedback mechanism exists within the shoot apex. If no shoots were harvested, the mean number of total shoots was 1.4 for control axes and 5.6 for BAP-treated axes. If shoots were harvested at any time, a corresponding number of vegetative buds elongated so as to maintain the number of shoots per axis at about five. Axes exposed to concentrations of BAP lower than 22 μM (suboptimal for multiple shoot formation) developed numerous vegetative buds, but fewer multiple shoots elongated (Fig. 7). Excision of the apical and axillary meristems from axes cultured on low BAP concentrations, however, resulted in increased outgrowth of multiple shoots until an average of five per axis was obtained. Thus, exposure to even low concentration of BAP reprograms the entire shoot apex. The lower concentrations do not break the apical dominance imposed by larger existing meristems, however, which presumably accounts for the elongation of fewer multiple shoots at low BAP concentrations in non-excised axes.

Elongated multiple shoots could be harvested and regenerated into plantlets with greater than 90% success rate. A major factor leading to the
success of shoot survival was the size of the explant at harvest (Hu and Wang, 1983). Shoots > 0.3 cm could survive, root, and give rise to plants; however, larger shoots (1 cm) had a greatly increased rate of survival.

**How does BAP inhibit DNA synthesis?**

McGaw (1987) has suggested that cytokinins act at the level of tRNA; however, most of the biologically active cytokinins do not exist as constituents of tRNA and incorporation of externally applied cytokinins such as BAP into tRNA is extremely low and non-specific. It is also clear that cytokinins stimulate protein synthesis and cause an increased rate of RNA synthesis (Kulaeva, 1981).

Although it is possible for these mechanisms to cause the inhibition of DNA synthesis shown in Fig. 9, we observed inhibition of DNA synthesis within 3 hr after treatment with BAP. Our data raise the possibility, therefore, of a more direct and immediate effect by BAP.

DNA synthesis was rapidly inhibited by BAP, but synthesis resumed within 9 to 15 hr, even in the continued presence of BAP. This would appear to contradict earlier studies using cultured cells (Simard, 1971; Fosket and Short, 1973) that suggested that the effects of cytokinin were not mediated by modifications of DNA synthesis. The effect of cytokinin on DNA synthesis, however, may depend on the state of differentiation of the cells (Evans, 1984; MacLeod, 1968). Van't Hof (1968) has shown cytokinin-induced retardation of cell division in rapidly dividing meristematic cells. Possible mechanisms by which BAP could transiently inhibit DNA synthesis include: (1)
interference with synthesis of DNA precursors; (2) direct interference with DNA replication machinery; or (3) blockage of entry into S-phase.

Inhibition of DNA synthesis by BAP may occur by interference with reactions involved in pathways for the biosynthesis of nucleic acid precursors. Other purine compounds, such as psicofuranin (6-amino-9-D-psicofuranosylpurine), inhibit synthesis of guanylic acid by interfering with the ammonia-mediated amination of xanthyclic acid (Mahler and Cordes, 1971). Subsequent induction of nucleic acid biosynthetic salvage pathways to increase precursor levels or subsequent increases in the levels of the affected enzymes (Van't Hof, 1974) may explain the transient nature of the inhibition of DNA synthesis. Although the majority of compounds described as cytokinins are adenyl derivatives, the presence of a purine ring is not essential for cytokinin activity. For example, 8-azakinetin is active as a cytokinin, even though it is not a N6-monosubstituted adenyl derivative (Steward and Krikorian, 1971) and 1,3-diphenylurea lacks a purine ring but, nonetheless, possesses cytokinin activity. Other cytokinins could, therefore, also interfere with precursor synthesis (similar to hydroxyurea) and result in a transient inhibition of DNA synthesis.

Alternatively, BAP is a nucleotide analog that may interfere directly or indirectly (after conversion to other forms) with the enzymes involved in DNA replication. In soybean tissue, labeled 6-benzylaminopurine exists only for a short time as a free base and rapidly becomes phosphorylated and glycosylated (Fox, 1970). The labeled ribonucleotide disappears within a few hours and a new metabolite, possessing cytokinin activity, appears. Direct interference with replication machinery by nucleotide analogs can block
DNA polymerases, DNA helicases, DNA topoisomerases, and DNA ligases (Kornberg and Baker, 1991; Fry and Loeb, 1986). For example, 8-azaadenine, 2,6-diaminopurine, and 8-azaguanine are incorporated in place of adenine and guanine and inhibit DNA replication (Lewin, 1980). No direct effect of BAP, however, was observed (Jiyong Zhao, personal communication) in the cell-free *Xenopus laevis* DNA replication system of Hutchison et al. (1987; 1988).

Another alternative is that BAP may block entry into S-phase. The G1-S transition is the boundary prior to the replication of DNA and is a regulatory point in the cell cycle (see review, Lewin, 1990). In almost all organisms, cyclins have been identified that may be involved in regulation of the cell cycle. Thus, BAP could function by interfering with cyclins or other molecules involved in the entry into S-phase. In the latter two alternative mechanisms, the transient nature of the inhibition of DNA synthesis may be explained by the metabolism of BAP by (1) inactivation by inducible enzymes; (2) irreversible conjugation with sugars or amino acids; or (3) reversible conjugation leading to less active or inactive compounds (Horgan, 1987). For example, an inducible cytokinin oxidase catalyzes the oxidative cleavage of the N-6 side chains of some naturally occurring cytokinins.

**BAP also reprograms cell fates**

BAP treatment of a soybean embryonic axis causes cells that would normally remain relatively quiescent (at this time) to instead divide repeatedly and give rise to *de novo* vegetative buds. These cells have, therefore, been reprogrammed and are now determined to form new
meristematic foci. Similar reprogramming of human erythroleukemia cells by aphidicolin, which specifically blocks DNA synthesis, has been reported by Murate et al. (1990). Regardless of the reprogramming mechanism, in each case the first observed effect of BAP was a rapid but, transient inhibition of DNA synthesis. In addition, Johnston et al. (1986) have shown that appropriate concentrations of either hydroxyurea (an inhibitor of DNA precursor synthesis) or aphidicolin (an inhibitor of DNA polymerase α) cause transient inhibition of [H³] thymidine incorporation in cultured mammalian cells (Johnston et al., 1986) followed by subsequent reprogramming. In yeast there is a commitment point in G1 termed START, after which a cell is irreversibly committed to DNA synthesis. By analogy, the BAP induced embryonic axis cells have passed a START commitment point. The initiation of additional rounds of DNA synthesis induced by BAP in soybean embryonic axes (as well during the transient inhibition described above) are strikingly similar to events observed in mammalian cells during drug induced gene amplification (Schimke et al., 1986; Johnston et al., 1986; Schimke, 1988). Methotrexate, hydroxyurea, and aphidicolin all transiently inhibit DNA synthesis, then lead to subsequent overreplication of specific regions in the genomic DNA. Schimke et al.,1986; 1988 has suggested that disruptions in DNA synthesis, with the subsequent accumulation of cell cycle-regulated enzymes involved in DNA synthesis, results in DNA overreplication. We speculate that similar mechanisms operate in soybean embryonic axes and result in reprogramming of shoot apex cells, although the molecular basis for these and for the newly determined state of the reprogrammed cells remains obscure.
ACKNOWLEDGEMENTS

The technical assistance of Deborah Bleile and Ann Bassart, is gratefully acknowledged. We also thank Bruce L. Wagner of the Bessey Microscopy Facility for scanning electron microscopy and JiYong Zhao for DNA replication studies in Xenopus extracts. We appreciate the critical reviews and suggestions of Robert Graybosch, Drena Larson, Reid Palmer, Kayla Polzin, and Jerry Ranch. This work was supported in part by the Iowa Lottery Economic Development fund for Biotechnology, the Iowa Soybean Promotion Board, and Iowa State University.

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REFERENCES


Table 1. Multiple shoot formation at 4 weeks post-imbibition in BAP-treated embryonic axes of 18 soybean cultivars and unadapted lines

<table>
<thead>
<tr>
<th>Designation</th>
<th>diversity</th>
<th>MS (control)</th>
<th>BAP (22 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corsoy</td>
<td>G. max</td>
<td>N</td>
<td>1.33 ± 0.08 (n=43) c</td>
</tr>
<tr>
<td>Dunfield</td>
<td>G. max</td>
<td>N</td>
<td>1.41 ± 0.08 (n=41)</td>
</tr>
<tr>
<td>Harosoy</td>
<td>G. max</td>
<td>N</td>
<td>1.43 ± 0.03 (n=428)</td>
</tr>
<tr>
<td>Hack</td>
<td>G. max</td>
<td>N</td>
<td>1.25 ± 0.03 (n=217)</td>
</tr>
<tr>
<td>Illini</td>
<td>G. max</td>
<td>C</td>
<td>1.29 ± 0.08 (n=48)</td>
</tr>
<tr>
<td>Mandarin</td>
<td>G. max</td>
<td>C</td>
<td>1.35 ± 0.08 (n=49)</td>
</tr>
<tr>
<td>Medium Green</td>
<td>G. max</td>
<td>C</td>
<td>1.31 ± 0.09 (n=45)</td>
</tr>
<tr>
<td>Peking</td>
<td>G. max</td>
<td>C</td>
<td>1.22 ± 0.05 (n=118)</td>
</tr>
<tr>
<td>Pioneer 9341</td>
<td>G. max</td>
<td>nt d</td>
<td>1.38 ± 0.06 (n=124)</td>
</tr>
<tr>
<td>Richland</td>
<td>G. max</td>
<td>N</td>
<td>1.28 ± 0.07 (n=47)</td>
</tr>
<tr>
<td>Seneca</td>
<td>G. max</td>
<td>N</td>
<td>1.28 ± 0.09 (n=46)</td>
</tr>
<tr>
<td>Virginia</td>
<td>G. max</td>
<td>C</td>
<td>1.21 ± 0.08 (n=38)</td>
</tr>
<tr>
<td>PI 79593</td>
<td>G. gracilis</td>
<td>C</td>
<td>1.18 ± 0.12 (n=11)</td>
</tr>
<tr>
<td>PI 153292</td>
<td>G. gracilis</td>
<td>C</td>
<td>0.90 ± 0.18 (n=10)</td>
</tr>
<tr>
<td>PI 326580</td>
<td>G. gracilis</td>
<td>C</td>
<td>1.22 ± 0.15 (n=9)</td>
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<td>PI 65388</td>
<td>G. gracilis</td>
<td>C</td>
<td>1.20 ± 0.2 (n=10)</td>
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<tr>
<td>PI 424078</td>
<td>G. soja</td>
<td>C</td>
<td>1.10 ± 0.18 (n=10)</td>
</tr>
<tr>
<td>PI 65549</td>
<td>G. soja</td>
<td>C</td>
<td>1.41 ± 0.23 (n=12)</td>
</tr>
</tbody>
</table>

a Barwale et al., (1986a) categorized shoot responses as high (Richland), intermediate (Dunfield, Illini, Medium Green, and PI 153292; and low (Corsoy, Mandarin, and Seneca).

b Classified according to degree of nuclear (N; Keim et al., 1989) or cytoplasmic (C; Close et al., 1989) genetic diversity.

c Data are given as mean ± SEM, n = the number of embryonic axes observed. Germinated embryonic axes that did not exhibit a green color and hypocotyl elongation by 66 hours were not included.

d nt, Not tested.
Fig. 1. Photomicrographs of control *Glycine max* embryonic axes and axes cultured on BAP. (a) Excised germinating embryonic axes 18 hr after imbibition. The cotyledons have been removed from all three axes. The unifoliolate leaves have been removed from the right- and left-most axes. Stipules are visible on the right axis flanking the apical meristem. (b) Excised embryonic axes cultured from 18 to 42 hr on MS + 22 μM BAP (left pair), MS (control, central pair), or MS + 2.5 μM gibberellic acid (right pair), followed by culture on modified MS until 168 hr post-imbibition. (c) control embryo axis 138 hr after imbibition. (d) BAP-treated embryonic axis 138 hr after imbibition. (e) BAP-treated embryonic axis 238 hr after imbibition; the arrows indicate vegetative buds adjacent to the axillary buds (ax). (f) BAP-treated embryonic axis 14 days post-imbibition. (g) BAP-treated embryonic axis 21 days post-imbibition. (h) BAP-treated embryonic axis 28 days post-imbibition, after excision of the 3 longest shoots at 21 days post-imbibition. Magnification, a, c, d, e, f, g, h: bar = 1 mm; b: bar = 5 mm.
Fig. 2. Scanning electron micrographs of control and BAP-treated Glycine max embryonic axes. (a) Control embryonic axis at 42 hr; 100x. (b) BAP-treated embryonic axis at 42 hr; 100x. (c) Control embryonic axis at 66 hr; 75x. (d) BAP-treated embryonic axis at 66 hr; 80x. (e) Control embryonic axis at 90 hr, 65x. (f) BAP-treated embryonic axis at 90 hr, 75x. Magnification, bar = 100 μm.
Fig. 3. Scanning electron micrographs of control and BAP-treated *Glycine max* embryonic axes. (a) Control embryonic axis at 114 hr post-imbibition; 47x. (b) BAP-treated embryonic axis at 114 hr post-imbibition; 33x. (c) BAP-treated embryonic axis at 114 hr post-imbibition; 100x. The arrow points to the existing axillary bud. (d) BAP-treated embryonic axis at 114 hr post-imbibition; 100x, different angle than Fig. 3c. The arrow points to the existing axillary bud. (e) Control embryonic axis at 162 hr post-imbibition, 30x. (f) Axillary bud of a control embryonic axis at 114 hr post-imbibition, 130x. (g) BAP-treated embryonic axis at 234 hr post-imbibition, 30x. The arrow indicates multiple shoots. (h) BAP-treated embryonic axis at 234 hr post-imbibition, 130x; ax = axillary bud; arrows indicate nascent buds. Magnification, bar = 100 μm.
Fig. 4. Histology of the primary apical meristem in *Glycine max* embryonic axes 18 hours after imbibition and excision from cotyledons. Magnification, bar = 50 μm.
Fig. 5. Histology of the primary apical meristem in control and BAP treated *Glycine max* embryonic axes. (a) Axis cultured on MS medium, 42 hr post-imbibition. (b) Axis cultured from 18-42 hr post-imbibition on 22 μM BAP, 42 hr post-imbibition. (c) Axis cultured on MS medium, 66 hr post-imbibition. (d) Axis cultured from 18-42 hr on 22 μM BAP, 66 hours post-imbibition. (e) Axis cultured on MS medium, 90 hr post-imbibition. (f) Axis cultured from 18-42 hr on 22 μM BAP, 90 hr post-imbibition. (g) Axis cultured on MS medium, 114 hr post-imbibition. (h) Axis cultured from 18-42 hr on 22 μM BAP, 114 hr post-imbibition. Magnification, a, b: bar = 25 μm; c, d, e, f, g, h; bar = 50 μm.
Fig. 6. Histology of *de novo* buds in *Glycine max*. (a) 18 hr post-imbibition, control pre-existing axillary bud. (b) 114 hr post-imbibition, *de novo* meristem induced on BAP-treated embryonic axis. This nascent vegetative bud was located ~150 μm from a developing preexisting axillary bud (compare nascent and preexisting bud in Fig. 5f and Fig. 5h). Magnification, bar = 25 μm.
Fig. 7. Effect of BAP concentration on multiple shoot formation in *Glycine max* cv. Harosoy. The mean number (± SEM) of shoots per embryonic axis at 4 weeks after imbibition was determined after a single 24 hour exposure to BAP at the indicated concentration. The number of axes examined was 428 at 0 μM BAP, 110 at 2.2 μM BAP, 85 at 12.5 μM BAP, and 350 at 22.2 μM BAP. All axes were subsequently cultured on a modified MS medium as described in Materials and Methods.
Fig. 8. Serial harvesting of elongated multiple shoots: effect on vegetative bud outgrowth in *Glycine max* cv Hack. The mean number (± SEM) of shoots per embryonic axis at 4 weeks and at 4 week intervals after harvesting shoots greater than 1 cm was determined. Untreated embryonic axes cultured on MS (•); embryonic axes exposed for 24 hours to 22 μM BAP (△). The cumulative (total) number of shoots per BAP treated embryonic axis ([]), this value is the sum of the multiple shoots remaining on the embryonic axis and all of the harvested shoots from that axis).
Fig. 9. Incorporation of $[^3\text{H}]-\text{thymidine}$ in *Glycine max* embryonic axes cultured from 18 to 42 hr post-imbibition on 22 $\mu$M BAP and in control axes. (a) 27 hr post-imbibition control embryonic axis labeled at 21 hr. (b) 30 hr post-imbibition, BAP-treated embryonic axis labeled at 24 hr. (c) 36 hr post-imbibition, control embryonic axis labeled at 30 hr. (d) 36 hr post-imbibition, BAP-treated embryonic axis labeled at 30 hr. (e) 42 hr post-imbibition, control embryonic axis labeled at 36 hr. (f) 42 hr post-imbibition, BAP-treated embryonic axis labeled at 36 hr. Magnification, bar = 50 $\mu$m.
SECTION II: EXPRESSION AND PERSISTENCE OF EXOGENOUS GENES
INTRODUCED BY MICROPARTICLE BOMBARDMENT
INTO SOYBEAN EMBRYONIC AXES TREATED WITH THE
CYTOKININ, 6-BENZYLAMINOPURINE
EXPRESSION AND PERSISTENCE OF EXOGENOUS GENES
INTRODUCED BY MICROPROJECTILE BOMBARDMENT INTO
SOYBEAN EMBRYONIC AXES TREATED WITH THE CYTOKININ,
6-BENZYLAMINOPURINE

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ABSTRACT

Embryonic axes of *Glycine max* (L.) Merr. (soybean) were bombarded with microprojectiles to which supercoiled plasmid DNA containing an *E. coli* *uidA* (β-glucuronidase) and/or *neo* (neomycin phosphotransferase) gene(s) had been adsorbed. Gene expression was observed in 93% of bombarded axes within 24 hr and persisted in about 1% of the plants until maturity. The plasmid(s) that showed the highest levels of expression contained a partial duplication of the cauliflower mosaic virus (CaMV) 35S promoter, an omega prime (Ω') sequence, and a nopaline synthase polyadenylation (nos) sequence. Maximal expression was observed in embryonic axes cultured from 18 to 42 hr post-imbibition on 6-benzylaminopurine (BAP) and bombarded at 66 hr. Control embryonic axes not exposed to BAP had 2-3 fold lower levels of expression and the optimal time of bombardment was 42 hr. Expression of β-glucuronidase and neomycin phosphotransferase was observed in one or more nodes from 12 out of 1,034 plants regenerated from excised multiple shoots. Every node in which β-glucuronidase expressed was also shown to contain plasmid DNA sequences by polymerase chain reaction (PCR) analysis. Nine of the 12 plants showed expression and persistence of plasmid DNA at multiple nodes. Most plants exhibited distinctive patterns of β-glucuronidase expression that were consistent in all positive nodes throughout the plant. Germ line transmission of the exogenous genes was not detected in R1 plants by PCR or Southern analysis.
INTRODUCTION

Microprojectile bombardment (Klein et al., 1987) has been widely used to introduce new genes into plants (Potrykus, 1990). McCabe et al. (1988) and Christou et al. (1988; 1989) obtained rare transgenic soybean plants by bombarding immature meristems with plasmid constructs. Multiple shoot formation was induced in the bombarded meristems using the cytokinin, 6-benzylaminopurine (Christou et al., 1989). Potentially transformed plantlets were regenerated from excised shoots. In principle, these plantlets should be enriched for lineages derived from cells receiving exogenous DNA.

Early events in multiple shoot development in soybean embryonic axes treated with BAP have been characterized previously Buising et al. (1992a). BAP transiently inhibited DNA synthesis and resulted in a 9-15 hr delay in the cell cycle. In addition, BAP developmentally reprogrammed cells throughout the shoot apex such that hundreds of new meristematic foci were formed, each of which could be excised and regenerated to give rise to a mature plant.

In this study we have systematically examined parameters that affect levels of expression and persistence of exogenous genes introduced into BAP-treated soybean embryonic axes by microprojectile bombardment. Gene expression was analyzed relative to early events in multiple shoot formation. Cell lineages were determined by histological analysis of exogenous gene expression in every node of regenerated plantlets.
MATERIALS AND METHODS

Plant material

Cultivars, unadapted soybean lines, and histological techniques used in this study were described previously (Buising et al., 1992a). Nicotiana tabaccum cv. Xanthi leaves were used in control experiments from 15 day old plants.

Plasmids

Plasmids and probes are illustrated in Figure 1. Transformation of E. coli (DH5) was carried out as described by Maniatis et al. (1982). Plasmids were prepared essentially as described by Hines and Benbow (1982). All plasmids designated as pPHI were gifts from Dr. Larry Beach of Pioneer Hi-Bred, International, Inc.

pPHI419, a 4.783-kb plasmid carrying the neo gene (Beck et al., 1982), was created using the plasmid pCaMVNeo (provided by Michael Fromm; Fromm et al., 1986). pCaMVNeo was digested with BglII and inserted into pUC18. To prepare the partial duplication of the CaMV 35S promoter described by Kay et al. (1987), part of the CaMV 35S promoter (-90 to -421; Gardner et al., 1981) was isolated by digestion with EcoRV, ligated to an Xbal linker, then ligated into the Xbal site upstream of the CaMV 35S promoter. The TMS RNA virus 5'-untranslated leader sequence, also called the omega prime (Ω') sequence (provided by T. Michael A. Wilson; Gallie et al., 1987a; 1987b) was isolated as a 67-bp HindIII-SalI fragment. The Ω' sequence was digested with mung bean nuclease and fused to an intact CaMV 35S
promoter by insertion of a PstI linker. The synthetic polylinker (5'-TCGACCCGGGATCCACCATGGTTAACGTGCA-3') was inserted and the fragment was introduced 3' of the Ω' leader creating a complementary SalI overhang. The other end of the polylinker had an overlap complementary to a PstI site, but in a context that did not reconstitute a PstI site. This made the Pst I site between the Ω' leader and the CaMV 35S promoter unique.

pPHI413, a 5.65-kb plasmid carrying the uidA gene (Novel and Novel, 1973), was pPHI419 from which the neo gene had been removed and uidA inserted. The neo gene was removed with BamHI. A SalI-EcoRI fragment containing the uidA gene was excised from pRAJ275 (Jefferson et al., 1987a), the EcoRI site was filled in, and a PstI octameric linker was added. This SalI-PstI fragment was inserted between the Ω' and nos polyadenylation site (Fraley et al., 1983).

pPHI 456, a 7.79-kb plasmid, was prepared by excising the 2.13-kb neo cassette of pPHI419 with BglII. A NotI site was created 3' to nos of pPHI413 and the BglII fragment of pPHI419 was inserted 3' to the uidA cassette.

pCB209, a 8.8-kb plasmid, was created by excising the 1.1-kb transformation booster (TBS) sequence with EcoRI from pTBS-1 (Meyer et al., 1988) and inserting it into the unique EcoRI site of the pPHI456 polylinker region.

pBI221 was purchased from Clontech (Jefferson et al., 1986). The plasmid consists of a 3.0 kb Hind III-EcoRI fragment that contains an 0.8-kb HindIII-BamHI fragment of the CaMV 35S promoter (Odell et al., 1985), a 1.87-kb fragment of the uidA gene and a 250-bp fragment of the nos polyadenylation site cloned into pUC19.
Microprojectile bombardment

Microprojectiles [1.2 μm; either tungsten (General Electric Co., Cleveland, OH) or gold (Morton Thiokol, Inc., Danvers, MA)] were prepared as described by Klein et al. (1988a) using 10 μg of DNA (except as noted). Control microprojectiles were prepared without DNA. There appeared to be an upper limit to the amount of DNA adsorbed to the microprojectiles (Ben Bowen, personal communication). Higher DNA concentration adsorbed to the microprojectiles inhibit transient expression (perhaps because of aggregation of microprojectiles).

Embryonic axes were bombarded three times (except as noted) essentially according to Tomes et al., 1990. Bombarded embryonic axes were transferred to one-half or full strength MS media (Murashige and Skoog, 1962) supplemented with the antibiotic kanamycin sulfate at 100-, 200-, or 300 mg/l (Sigma, St. Louis, MO) where noted.

Protein quantitation

Approximately 5-7 mg of fresh leaf tissue or shoot apices (see Fig. 2b) were homogenized in 50-200 μl of buffer A (Jefferson, 1987b) using a disposable polypropylene pestle in a Kontes tube (Kontes Glass Co., Vineland, NJ) and centrifuged at 16,000 x g for 15 min at 4°C. Buffer A consists of 50 mM sodium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 10 mM disodium EDTA, 0.1% (w/v) sodium lauryl sarcosine, and 0.1% (v/v) Triton X-100 (Sigma). One μl aliquots of the supernatant were used to determine protein concentration (Bradford, 1976) with a Bio-RAD™ protein
assay kit (Bio-RAD, Richmond, CA) using bovine serum albumin as a standard.

**β-Glucuronidase assay**

Aliquots corresponding to 2 and 4 μg of protein (see above) were assayed in duplicate using 4-methylumbelliferyl-β-D-Glucuronide (MUG, Sigma) as substrate. β-glucuronidase was quantitated on a Titertek Fluoroskan II microplate fluorometer (Flow Laboratories, McLean, VA) using opaque microtiter plates from Perkin-Elmer (Norwalk, CT) following the method of Rao and Flynn (1990). The lower linear range of the assay was 0.7 pg β-glucuronidase/μg total protein. β-glucuronidase type VII from _E. coli_ (Sigma) and extracts from a previously identified transformed tobacco plant (Tomes _et al._, 1990) were used as positive controls.

Histochemical analyses were performed using 5-bromo-4 chloro-3 indolyl beta-D-glucuronide (X-GLUC, Research Organics, Cleveland, OH). The substrate was prepared as described by Jefferson (1987b). Embryonic axes were immersed in X-GLUC at 37°C for 24 hours then sectioned.

**Neomycin Phosphotransferase assay**

Aliquots corresponding to 1 or 2 μg of protein were monitored for phosphorylation of neomycin as described by Staebell _et al._ (1990). Blots were dried and analyzed on an AMBIS 2-D beta scanner (Nye _et al._, 1988). Positive controls included transformed calli and plants (Tomes _et al._, 1990) and purified NPT enzyme (5 Prime -> 3 Prime, Inc., West Chester, PA). Negative
controls included reaction mixtures lacking homogenate, reaction mixtures lacking neomycin, and extracts of non-transformed plants.

**Polymerase chain reaction amplification**

The polymerase chain reaction (PCR; Saiki et al., 1988) was utilized to identify the *neo* and *uidA* genes in genomic DNA isolated from R₀ and R₁ plants. Oligonucleotide primers (Fig. 1) were synthesized at the Iowa State University Nucleic Acid Research Facility (Ames, IA). Reaction mixtures consisted of 2.5 μl of buffer B [670 mM Tris-HCl, pH 8, 166 mM NH₄SO₄, 6.7 mM MgCl₂, 100 mM dithiothreitol (Sigma), 0.1% Triton-X 100 (Sigma), 0.1% Tween 20 (Sigma)], 0.6 μl of a mixture containing 10 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia LKB Biotechnology, Milwaukee, WI), 20 μM of each oligonucleotide, 1 unit TAQ polymerase (Perkin Elmer Cetus, Norwalk, CT), and 200 ng of genomic DNA or 1 ng of plasmid DNA.

Reaction mixtures were diluted to 25 μl total volume and overlaid with sterile mineral oil. An Ericomp Thermal Cycler (San Diego, CA) was programmed as follows: 93°C for 2.5 min, followed by 30 cycles of 30 sec at 93°C, 1 min at 65°C, and 72°C for 2 min. Amplified DNA was electrophoresed in a 1.4% agarose gel (Maniatis et al., 1982).

**Southern analysis**

DNA was isolated from plant tissues essentially as described by Keim et al. (1988) and subjected to Southern analysis (Southern, 1975). Probes, labelled with ³²P-dCTP (ICN, Irvine, CA), were prepared by random priming according to the method of Feinberg and Vogelstein (1984). Filters
were probed with a single copy gene (Keim, et al., 1989) to determine whether transfer of DNA was successful.

**Statistical analysis**

Statistical analysis was carried out according to Daniel (1974) using Statview II software (Abacus Concepts, Inc., Berkeley, CA, 1987). Gene expression in soybean embryonic axes was examined by ANOVA (Fisher, 1966).
RESULTS

Expression of exogenous genes in embryonic axes

Embryonic axes of *Glycine max* were bombarded with plasmid pBI221, which contains the *E. coli* β-glucuronidase open reading frame (uidA; Fig. 1). β-glucuronidase expression was observed in the primary apical dome and around the circumference of the shoot apex at the level of the axillary buds (Fig 2b). These regions contribute to nascent multiple shoots (Buising *et al.*, 1992a). Expression was also observed in organized primary unifoliolate leaf tissue (Fig. 2a) that does not contribute cells to nascent multiple shoots.

Embryonic axes bombarded with control (without DNA) microprojectiles did not stain blue (Fig. 2c), although a faint blue-green color was noted. Embryonic axes bombarded with plasmid pPHI413, which contains the *E. coli* β-glucuronidase open reading frame, stained a deep blue color (Fig. 2d). At 2 wk after bombardment (17 days post-imbibition), β-glucuronidase expression was seen throughout R₀ plantlets regenerated from excised multiple shoots. Sectors were formed in stem sections (Fig. 2e) and in leaves (Fig 2f). In about 1% of the regenerated plantlets (see below), β-glucuronidase expression persisted until maturity in the absence of any selection pressure.

Continued survival and growth of soybean embryonic axes in the presence of kanamycin requires persistence and expression of an exogenous *E. coli* neo gene that encodes the enzyme neomycin phosphotransferase II. Multiple shoot and leaf formation was observed in embryonic axes cultured
on 100-300 mg/l kanamycin sulfate that had been bombarded with pPHI419, pPHI456, or pCB209 (Fig. 1). Multiple shoot formation was significantly inhibited if axes were cultured on kanamycin immediately after bombardment. Few shoots were recovered and most died, even if subsequently removed from selection. The few axes bombarded with pPHI419 alone that were observed to produce multiple shoots under selection were stunted in comparison to those not under selection. As shoots elongated and leaves began to expand under selection, green sectors were observed in predominantly unpigmented leaves (Fig 2g). Plantlets arising from harvested multiple shoots appeared chimeric (Fig. 2h), regardless of whether or not axes were cultured on kanamycin.

**Optimization of β-glucuronidase expression in embryonic axes**

To quantitate β-glucuronidase expression, the region demarcated on the embryonic axis in Fig 2b was removed and assayed using the fluorometric assay of Rao and Flynn (1990). Three bombardments of the same axes consistently resulted in the highest levels of β-glucuronidase expression, while one, two, and four or more bombardments resulted in lower levels. DNA concentrations lower than 5 µg and greater than 15 µg resulted in lower β-glucuronidase expression (Table 2; B. Bowen personal communication). A distance of 10.0 cm from the stopping plate to the axes was found to result in the highest levels of β-glucuronidase expression. Much lower levels of β-glucuronidase expression were obtained when the distance between axes and stopping plate was less than 7.5 cm or greater than 12.5 cm. Axes placed between 1- and 2 cm from the center of the petri
dish exhibited highest expression levels. Axes placed at the epicenter (0-1 cm) of the bombardment or greater than 2 cm from the center of the petri dish showed lower levels of expression. Almost all surviving embryonic axes bombarded at 66 hr with plasmid pPHI413 displayed high levels of β-glucuronidase expression (see Fig. 2f) throughout the meristematic regions. Using optimal conditions, 93% of all surviving meristems displayed β-glucuronidase expression in one or more regions of the axis by 90 hr post-imbibition.

As shown in Fig. 3a and Table 1, β-glucuronidase expression from constructs such as pPHI413 was typically about 10-fold higher than from pBI221, irrespective of time of bombardment. Optimal expression (Table 1 and unpublished observations) was obtained with constructs containing a partial duplication of the cauliflower mosaic virus 35S promoter, the omega prime (Ω') translational enhancer sequence, and the nos polyadenylation sequence. Embryonic axes bombarded with various constructs at 42, 66, and 90 hr post-imbibition were compared for β-glucuronidase expression (Table 1). It should be emphasized that the level of expression was highly variable among different axes (note standard deviations in Table 1).

Temporal analysis of gene expression

Since we planned to regenerate plantlets containing the bombarded constructs from multiple shoots, the effect of particle bombardment on the number of multiple shoots formed was examined. A similar mean number of shoots was observed in control embryonic axes whether or not they had undergone particle bombardment. By contrast, BAP-treated embryonic axes
formed significantly more multiple shoots (P = 0.0001, ANOVA) after undergoing three particle bombardments (Table 1).

The effect of time of bombardment after imbibition on \( \beta \)-glucuronidase expression was compared in control and BAP-treated embryonic axes (Fig. 3b). Bombarded control axes exhibited the highest levels of expression at 42 hr post-imbibition (Fig. 3b). \( \beta \)-glucuronidase expression levels were two to three-fold higher and expression persisted longer in BAP-treated embryonic axes than in control axes. Axes cultured on 22 \( \mu \)M BAP exhibited the highest levels of expression after bombardment at 66 hr post-imbibition (Table 3). The time of bombardment had a dramatic effect on the level of expression, particularly with the construct that exhibited the highest levels of expression. All constructs showed an increased level of expression in BAP-treated embryonic axes bombarded at 66 hr post-imbibition (Fig. 3a and Table 1).

**Histological localization of \( \beta \)-glucuronidase gene expression**

Cells expressing \( \beta \)-glucuronidase were localized to determine whether cells capable of giving rise to multiple shoots (Buising et al., 1992a) had received DNA. Embryonic axes bombarded with control tungsten particles did not exhibit \( \beta \)-glucuronidase expression in sections (Fig. 4a). Thus, the faint blue-green color seen in whole embryonic axes (Fig 2c) was not \( \beta \)-glucuronidase expression. By contrast, embryonic axes bombarded with pH\( \text{PHI}413 \) showed several foci of \( \beta \)-glucuronidase activity either localized at the surface (Fig 4b, right) or extending several cell layers deep (Fig 4b, left). As shown in Fig. 4c and Fig. 4d, cells expressing \( \beta \)-glucuronidase are found
at least 5 layers deep (and perhaps as far as 10 layers). This included all cell layers involved in multiple shoot formation.

**Clonal analysis of β-glucuronidase and neomycin phosphotransferase expression in R0 plants**

Approximately 24,000 embryonic axes were bombarded. Each axis gave rise to an average of five multiple shoots (Buising et al., 1992a). R0 plants were regenerated from a subset of the ~120,000 possible multiple shoots. Shoots and plantlets used for regeneration were selected from bombardments that had resulted in either: (i) high levels of expression in multiple embryonic axes on the same plate; (ii) formation of multiple shoots after culture on kanamycin; or (iii) persistent expression at 5, 14, and 28 days post-bombardment. Thus, the regenerants were not randomly chosen, but represented those most likely to have received, replicated and expressed the exogenous DNA.

Leaf pieces from at least four nodes above the cotyledonary node per plant were assayed for β-glucuronidase and neomycin phosphotransferase expression. Thirty nodes were identified that exhibited β-glucuronidase and/or neomycin phosphotransferase expression (referred to below as "positive") in 12 R0 plants (Fig. 5). Therefore, approximately 1% of the regenerants (12 out of 1,034) of selected multiple shoots received, replicated for many cell divisions, and expressed exogenous DNA.

Leaf samples exhibited β-glucuronidase expression primarily at cut edges (Fig. 4e). Expression was observed in anthers (Fig. 4f, right flower) and could be distinguished from the faint blue-green background in anthers
of control plants (Fig. 4f, left flower). Transections of petioles and stems from the 30 nodes displayed varying patterns of β-glucuronidase expression (compare the negative control, left most petiole in Fig. 4g, with various patterns in Fig. 4g and Fig. 4h).

Many of the nodes exhibited β-glucuronidase expression in the phloem and xylem (Table 4). Staining was also observed in tissues (classified according to Lersten and Carlson, 1987) of the endodermis, cortical parenchyma, sclerenchyma, interfascicular parenchyma, and pith (summarized in Table 4 and legend of Fig. 4). Since the tissue staining patterns were usually invariant in all positive nodes of a single plant, and were usually distinct from patterns in any other plant, we assume that the patterns usually derive from clonal descendants of a single "hit" (see Discussion). Only one plant expressed β-glucuronidase activity throughout the transection. In one petiole transection (plant 836, node 6), β-glucuronidase activity was observed in the short basal and surrounding epithelial cells of the trichome, but not the long terminal cell (Fig. 4g, center). The majority of nodes expressed β-glucuronidase in the epidermis and endodermis (Table 4).

Clonal analysis of persistence of plasmid DNA

Leaf samples from each node of the 12 positive plants were retested by PCR to confirm the presence of exogenous DNA sequences (Fig. 6). The neo gene was amplified (876-bp) in 19 of the 28 nodes expressing the uidA gene (Table 4). In addition, neo constructs were detected in nodes that were negative in neomycin phosphotransferase assays (Table 4). The uidA gene
was detected in all R0 plant nodes (Fig. 6, panels B and C; Table 4) that expressed β-glucuronidase. The $uidA$ or $neo$ genes were not detected in any of the 141 nodes that did not express β-glucuronidase and/or neomycin phosphotransferase.

**Analysis of R1 plants**

The 12 R0 plants were allowed to self pollinate and R1 seed was harvested after recording the node of origin. Seeds (n= 539) representing all of the nodes in Fig. 5 and Table 4 were planted. No β-glucuronidase or neomycin phosphotransferase expression was detected in any R1 plant. Some R1 plants were germinated in 100 µg/l kanamycin sulfate, but were subsequently found to be negative by PCR and enzymatic analysis. They eventually died. These negative results were confirmed by Southern analysis probing for the $uidA$ and $neo$ genes.
DISCUSSION

Expression of the *uidA* and *neo* genes introduced into embryonic axes by microprojectile bombardment was monitored within the first 2-5 days post-bombardment, at 14-28 days post-bombardment, and at maturity (about 150 days post-bombardment under greenhouse conditions). Factors necessary for efficient (>90%) expression in bombarded axes included gun design, microprojectile workup, and bombardment geometry (Klein, *et al*., 1988b; reviewed by Sanford, 1990), as well as plasmid construct, number and timing of bombardments, and treatment with BAP.

The effect of plasmid construct on levels of expression

Since 93% of optimally bombarded axes exhibited one or more sectors of β-glucuronidase expression, direct transfer of *uidA* DNA by bombardment was efficient. The levels of expression depended on the constructs. Bombardment with pPHI413 resulted in dramatically higher levels of β-glucuronidase expression relative to pBI221 (Table 2, Fig. 3a). In addition to the *uidA* gene, plasmid pPHI413 has two cauliflower mosaic virus 35 S promoters and an omega prime (Ω') sequence. Kay *et al*., (1987) have suggested that two CaMV 35 S promoters could act as a transcriptional enhancer. Translational enhancement with the omega prime sequence due to increased 70S and 80S ribosome binding has been reported (Gallie *et al*., 1987a; 1987b).
The effect of number and timing of bombardments on levels of expression

Microprojectile dispersion patterns, aggregation of microprojectiles, and cartridge discharge were highly variable. Multiple bombardments probably minimize variability, thus resulting in increased levels of expression. Three bombardments per sample was optimal.

Control axes bombarded at 42 hrs post-imbibition exhibited higher levels of expression than those bombarded at earlier or later times (Fig. 3a and 3b). Buising et al. (1992a) showed that cells throughout the control shoot apices enter S phase from 21 to 24 hrs post-imbibition. Mitotic divisions in control axes occur between 36-42 hrs post-imbibition (Sun, 1957; Miksche, 1961). A large fraction of cells would, therefore, be early in G1 at 42 hrs post-imbibition. Since RNA and protein synthesis double during each cell cycle, it seems logical that exogenous gene expression would be higher at 42 hrs than at earlier times.

The lower levels of expression in control axes bombarded at later times probably results from a combination of factors. The optimal time of bombardment depends critically upon the cell cycle. As cells lose synchrony a smaller fraction will cycle together and enter G1 synchronously. Moreover, a small fraction of the axis is represented by the cells receiving DNA (see below) resulting in lower expression per μg protein. No significant differences were observed in levels of expression at similar times of bombardment for all genotypes and unadapted lines tested.
The effect of BAP treatment on levels of expression

BAP treatment resulted in a 24 hr delay in the optimal time of bombardment (Fig. 3). We have shown previously that BAP causes a 9-15 hr delay in entry into S phase of cells in the apical meristem and axillary buds (Buising et al., 1992a). It seems likely that the delay in the optimal time of bombardment results simply from this delay in the cell cycle.

In addition, BAP treatment resulted in an increased level of expression at 66 hr post-imbibition relative to control axes at 42 hrs. Cytokinins have been shown to increased the rate of RNA (Kulaeva, 1981) and protein synthesis which could result in increased expression. Moreover, since BAP reprograms cells to divide that ordinarily would not, it also increases the number of cells (and fraction of the plant) entering into G1. The increased number of de novo buds would also explain why expression in BAP-treated embyonic axes does not decay as rapidly as in control embryos.

Subsequent decreases occur in levels of expression in axes bombarded at later times because of loss of synchrony of cells entering into G1 and because a smaller fraction of the axis is represented by these cells. DNA synthesis was observed to occur synchronously at replicons after BAP treatment in meristems of Sinapis alba during the vegetative to floral transition (George Bernier, personal communication). Each 24 hr delay in bombardment resulted in the approximate halving of the level of expression. At times later than the optimal bombardment time the surface area of the apical meristem does not increase dramatically (Buising et al., 1992a), but there is nearly a two to threefold increase in cell number and a concomitant
increase in protein content during the next plastochron. The number of cells receiving microprojectiles, therefore, would not increase significantly. Expression per μg of protein would decrease. When gene expression was quantitated per axis, rather than relative to protein, there was little change in overall expression.

**Patterns of expression in R0 plants**

Nine of 12 plants showed expression in multiple nodes. Histochemical staining usually showed identical patterns at every positive node (for example, plant 62 had the pattern of cortical parenchyma, endodermis, interfascicular parenchyma, pith, phloem, xylem at node 4, 9, and 10; whereas, plant 190 had phloem, sclerenchyma, xylem at node 1A, 1B, 5, 17, 26 and phloem, sclerenchyma, xylem, plus epidermis at node 25). Moreover, every plant (except 377 and 476) had a unique staining pattern. This strongly suggests that expression in each multiple node was derived from a single "hit". Most expressing nodes, therefore, are likely to represent a single clonal population.

The exceptions to identical patterns of β-glucuronidase expression shown in Fig. 4 and Table 4 may be due to shifts in cell fate. Two plants exhibit nonidentical patterns in different nodes: plant 190, where expression in node 25 differed from other expressing nodes; and plant 93 where expression was lost in the endodermis and epidermis. The cells involved in sectors of *liguleless-1* in maize (Becraft and Freeling, 1991) show movement between adjacent cell layers and changes in cell fate. It has also been demonstrated that cell lineage does not limit cell fate (Poethig and Sussex,
1985a; 1985b; McDaniel and Poethig, 1988). Invasion of cells from one lineage into adjacent lineages, such as when epidermal (LI) cells invade LII and become mesophyll have been demonstrated previously (Stewart and Dermen, 1970; Stewart, 1978).

Persistence of exogenous DNA in R0 plants

In every node that exhibited β-glucuronidase expression, persistence of $uidA$ gene was confirmed by PGR. Moreover, every node that contained the $uidA$ gene by PCR analysis expressed β-glucuronidase. Conversely, every node that did not contain the $uidA$ gene by PCR also showed no expression (141 of 141 negative nodes). Therefore, the inability to obtain $uidA$ expression in regenerants was due to lack of persistence of the DNA.

Bombardment of embryonic axes with two separate plasmids always resulted in expression of both constructs in the same axes (but not necessarily in all nodes, see Table 4). We have not determined whether the constructs become concatenated (Marini et al., 1988; Marini and Benbow, 1991; Kaiserman et al., 1990), integrated (Meyer et al., 1988) or persist as extrachromosomal elements.

Interestingly, neomycin phosphotransferase persistence differed in different nodes. Whenever $neo$ expression was observed, persistence of the $neo$ gene was also observed. Most plants showing $neo$ gene persistence, however, did not show NPTII expression above background levels. Presumably NPTII expression was below the level of detection of the assay or problems may have existed at the level of transcription or translation. In 5 of the 12 plants persistence of the $neo$ gene was seen only in some nodes. Since
all 141 negative nodes were tested by multiple assays at several locations on the leaves, one would expect sampling errors to be negligible. This suggests, therefore, that the neo gene persisted extrachromosomally and was lost at a relatively high frequency during mitosis.

Most plants that expressed in one node showed expression in multiple nodes (75%; Fig. 5). Moreover, as expected, all R0 plants that expressed exogenous genes were chimeric (since direct transfer of DNA occurs in a small fraction of the cells giving rise to each plantlet and leaves develop from about 100 cells in tobacco [Poethig and Sussex, 1985a; 1985b]). Embryonic axes bombarded with pPHI419 (containing the neo gene) and grown on kanamycin containing medium were observed to produce multiple shoots that were stunted in comparison to those not under selection. As these shoots elongated the developing leaves were chimeric (white and green sectors in Fig. 2g). This is consistent with only one (or a few) cells receiving DNA. Although chimeras can be vegetatively propagated, we elected not to do this because we wished to determine the probability of obtaining R1 progeny containing the exogenous genes.

Persistence and expression of exogenous DNA in R1 plants

The progeny of a chimera are determined by the tissues that produce the egg cells and pollen grains. The germ line in soybean can be traced to an LII subepidermal origin (Groose et al., 1988). Seven of 10 β-glucuronidase expressing plants showed histochemical staining in cells derived from the LII cell layer (exceptions were 836, 377, 476, and 190). Seeds from any of these plants (Table 4) could potentially give rise to a transformed R1 plant.
151 seeds representing the LII (n=60), LI (n=6) and LIII (n=85) were tested. No exogenous gene constructs were detected by PCR and Southern analysis in plants grown from any of these seed. Based on the transformation frequencies (2%) observed by Christou et al., 1988, this result was not unexpected.

Gene expression and persistence of plasmid in R₀ plants without the transmission to progeny could be explained by the persistence of extrachromosomal plasmid molecules. Persistence of extrachromosomal DNA resulting in transformation has been described in C. elegans (Stinchcomb et al., 1985), and has been reported with the Mu transposable element in maize (Sundaresan and Freeling, 1987). Heritability of linear extrachromosomal elements, however, has not been observed (Peleman et al., 1991). The formation of double minutes provided extrachromosomal expression and persistence of the dihydrofolate reductase gene in CHO cells after transient inhibition of DNA synthesis (Schimke, 1988).

**Efficient transformation of R₁ soybean plants: an hypothesis**

Microprojectile bombardment is an efficient means of directly introducing DNA into embryonic axes: 93% of bombarded axes expressed the exogenous DNA. Only 1% of the plantlets regenerated from multiple shoots, however, showed expression in mature R₀ plants. Other strategies for increasing frequency of transformation through the use of Agrobacterium (Hinchee et al. 1988; Chee et al., 1989), protoplasts and electroporation (Dhir et al., 1991a; 1991b; 1991c), and a embryogenic culture system (Finer and McMullen, 1991) have been used.
Based on the data in this study, we postulate that frequency of transformation of R1 plants can be increased dramatically by factors that simply increase the persistence of the DNA. Three DNA sequence elements have been described that have strong probabilities of increasing persistence: nuclear retention sequences (Krysan et al., 1989; Krysan and Calos, 1991), amplification promoting sequences (Wegner et al., 1989; Wegner et al., 1990), and/or homologous recombination sequences (Meyer et al., 1988; Kartzke et al., 1990; Marchesi et al., 1989). We postulate that the addition of these sequence elements to the pPHI413/419 vectors would dramatically increase germline transmission.

Several laboratories have selected for genomic DNA fragments with positive effects on transformation frequencies (Meyer et al., 1988; Paszkowski et al., 1988; Marchesi et al., 1989, Buising and Benbow, 1992b). Meyer et al. (1988) isolated a transformation booster sequence (TBS), from petunia. A 1.1 kb internal fragment of TBS was found to increase the efficiency of transformation of protoplasts about 20-fold by expression vectors containing TBS. Previously Meyer et al. (1985) have shown that the transformation efficiency was increased by approximately two orders of magnitude when tobacco protoplasts, synchronized in S- or M-phase, were transformed via polyethylene glycol precipitation. Stimulation of transformation by TBS was intimately related to the cell cycle.

The effect of TBS on transformation of non-synchronized and synchronized M- or S-phase protoplasts was analyzed by Kartzke et al. (1990). Transformation of non-synchronized protoplasts with expression vectors containing TBS resulted in a 20-fold increase in transformation frequencies.
By contrast, in S-phase TBS did not lead to increased transformation frequencies, but did lead to increased copy numbers. Kartzke suggested that TBS may carry an S-phase specific signal that leads to increased copy numbers. Rearrangements of vector sequences integrated in the DNA were observed in S-phase transformants, but not with M-phase or non-synchronized transformants.

When the origin of replication was removed from Epstein-Barr virus vectors that normally replicate autonomously (Krysan et al., 1989), plasmid molecules were retained in the nuclei of human cells for an extended period of time. Krysan and Calos (1991) showed that these vectors can persist as long as they contain nuclear retention sequences. The vectors used in this study do not contain similar nuclear retention sequences.

Cis-acting sequences from mouse rDNA have been identified which promote plasmid DNA amplification in mouse cells (Wegner et al., 1989; 1990). These DNA sequences confer the ability for linked exogenous DNA to persist in high copy number. The vectors in this study do not contain similar amplification promoting sequences.

Homologous recombination has been used to promote the integration of linked exogenous DNA sequences into the genome (Marchesi et al., 1988; Folger et al., 1982; reviewed by Capecchi, 1989). The vectors used in this study do not appear to contain sequences that stimulate homologous recombination.
This research was supported in part by Pioneer Hi-Bred International, Inc. and by a grant from the Iowa Soybean Promotion Board.

We acknowledge and appreciate the excellent technical support of Pam Flynn and Kellie Winter of Pioneer Hi-Bred Int'l, Inc, with the numerous expression assays. The expertise of Mike Daywalt of Pioneer Hi-Bred Int'l, Inc, in PCR analysis is also greatly appreciated.
REFERENCES


Buising, C. M., Shoemaker, R. C., and Benbow, R. M. 1992a. Early events of multiple shoot development in soybean embryonic axes treated with the cytokinin, 6-benzylaminopurine. To be resubmitted.


Table 1. β-glucuronidase expression in bombarded *Glycine max* cv. Pioneer 9341 embryonic axes: Effect of construct and time of bombardment

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>DNA (μg)</th>
<th>42</th>
<th>66</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPHI413</td>
<td>10</td>
<td>2.1 ± 1.5</td>
<td>10.3 ± 10.1</td>
<td>5.6 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>n = 48</td>
<td>n = 111</td>
<td>n = 48</td>
<td></td>
</tr>
<tr>
<td>pPHI456</td>
<td>10</td>
<td>1.2 ± 0.6</td>
<td>3.8 ± 1.3</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>n = 30</td>
<td>n = 86</td>
<td>n = 31</td>
<td></td>
</tr>
<tr>
<td>pCB209</td>
<td>10</td>
<td>1.1 ± 0.6</td>
<td>4.4 ± 3.8</td>
<td>1.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>n = 30</td>
<td>n = 30</td>
<td>n = 30</td>
<td></td>
</tr>
<tr>
<td>pBI221</td>
<td>10</td>
<td>0.75 ± 0.74</td>
<td>0.94 ± 0.1</td>
<td>0.77 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>n = 18</td>
<td>n = 18</td>
<td>n = 18</td>
<td></td>
</tr>
<tr>
<td>pPHI419</td>
<td>10</td>
<td>≤ 0.70 ± 0</td>
<td>≤ 0.70 ± 0</td>
<td>≤ 0.70 ± 0</td>
</tr>
<tr>
<td></td>
<td>n = 29</td>
<td>n = 33</td>
<td>n = 25</td>
<td></td>
</tr>
<tr>
<td>pPHI413/pPHI419</td>
<td>5/5</td>
<td>3.1 ± 3.3</td>
<td>9.4 ± 5.1</td>
<td>4.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>n = 26</td>
<td>n = 42</td>
<td>n = 31</td>
<td></td>
</tr>
<tr>
<td>pPHI413/pPHI419</td>
<td>7.5/7.5</td>
<td>1.4 ± 0.6</td>
<td>4.5 ± 2.5</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>n = 26</td>
<td>n = 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPHI413/pPHI419</td>
<td>10/5</td>
<td>1.1 ± 0.9</td>
<td>2.2 ± 1.5</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>n = 53</td>
<td>n = 26</td>
<td>n = 34</td>
<td></td>
</tr>
</tbody>
</table>

Note: A construct consisted of 5 mg of pPHI413 and 5 mg of pPHI419.

a Embryonic axes of Pioneer 9341, bombarded with control tungsten particles (minus DNA) at 18, 42, 66, 60, or 114 hr had baseline levels of ≤ 0.70 ± 0 pg/μg total protein. Expression is presented as the mean ± standard deviation, n = the number of axes observed. The three time points for the plasmids in Fig. 3 are included to facilitate comparison of data.
Table 2. Multiple shoot formation in soybean embryonic axes: Effect of bombardment

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Diversity</th>
<th>MS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BAP&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not Bombarded</td>
<td>Bombarded</td>
<td>Not Bombarded</td>
</tr>
<tr>
<td></td>
<td>n=42</td>
<td>n=34</td>
<td>n=53</td>
</tr>
<tr>
<td>Corsoy</td>
<td>N</td>
<td>1.33 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.41 ± 0.09</td>
</tr>
<tr>
<td>Dunfield</td>
<td>N</td>
<td>1.41 ± 0.08</td>
<td>1.37 ± 0.08</td>
</tr>
<tr>
<td>Hack</td>
<td>N</td>
<td>1.25 ± 0.03</td>
<td>1.47 ± 0.35</td>
</tr>
<tr>
<td>Harosoy</td>
<td>N</td>
<td>1.43 ± 0.03</td>
<td>1.39 ± 0.06</td>
</tr>
<tr>
<td>Illini</td>
<td>C</td>
<td>1.29 ± 0.08</td>
<td>1.32 ± 0.09</td>
</tr>
<tr>
<td>Mandarin</td>
<td>C</td>
<td>1.35 ± 0.08</td>
<td>1.49 ± 0.09</td>
</tr>
<tr>
<td>Medium Green</td>
<td>C</td>
<td>1.31 ± 0.09</td>
<td>1.49 ± 0.08</td>
</tr>
<tr>
<td>Peking</td>
<td>C</td>
<td>1.22 ± 0.05</td>
<td>1.39 ± 0.70</td>
</tr>
<tr>
<td>Pioneer 9341</td>
<td>nt&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.38 ± 0.06</td>
<td>1.30 ± 0.04</td>
</tr>
<tr>
<td>Richland</td>
<td>N</td>
<td>1.28 ± 0.07</td>
<td>1.41 ± 0.08</td>
</tr>
<tr>
<td>Seneca</td>
<td>N</td>
<td>1.28 ± 0.09</td>
<td>1.35 ± 0.07</td>
</tr>
<tr>
<td>Virginia</td>
<td>C</td>
<td>1.21 ± 0.08</td>
<td>1.36 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>n=47</td>
<td>n=51</td>
<td>n=52</td>
</tr>
<tr>
<td></td>
<td>n=46</td>
<td>n=49</td>
<td>n=59</td>
</tr>
<tr>
<td></td>
<td>n=38</td>
<td>n=44</td>
<td>n=68</td>
</tr>
</tbody>
</table>

<sup>a</sup> Classified according to degree of nuclear (N; Keim et al., 1989) or cytoplasmic (C; Close et al., 1989) genetic diversity.

<sup>b</sup> Axes were bombarded 66 hr post-imbibition with control particles. The number of multiple shoots/axis was scored at 4 wk post-imbibition.

<sup>c</sup> Data are given as mean ± SEM, n = the number of axes observed. Imbibed axes that did not exhibit hypocotyl elongation by 66 hours were not included.

<sup>d</sup> nt, Not tested.
Table 3. β-glucuronidase expression in embryonic axes: Effect of soybean cultivar or unadapted line and time of bombardment

<table>
<thead>
<tr>
<th>Designation</th>
<th>n</th>
<th>Mean ± SD</th>
<th>n</th>
<th>Mean ± SD</th>
<th>n</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>42 hr</td>
<td>66 hr</td>
<td>90 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corsoy</td>
<td>45</td>
<td>1.3 ± 1.0</td>
<td>41</td>
<td>9.7 ± 6.7</td>
<td>32</td>
<td>5.8 ± 4.7</td>
</tr>
<tr>
<td>Dunfield</td>
<td>30</td>
<td>1.0 ± 0.8</td>
<td>37</td>
<td>8.2 ± 5.6</td>
<td>33</td>
<td>6.2 ± 3.9</td>
</tr>
<tr>
<td>Hack</td>
<td>34</td>
<td>1.4 ± 1.1</td>
<td>47</td>
<td>9.4 ± 7.6</td>
<td>41</td>
<td>4.8 ± 3.2</td>
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<tr>
<td>Harosoy</td>
<td>39</td>
<td>1.1 ± 1.0</td>
<td>59</td>
<td>9.6 ± 8.4</td>
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<td>5.2 ± 3.9</td>
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<tr>
<td>Illini</td>
<td>41</td>
<td>0.9 ± 0.7</td>
<td>39</td>
<td>11 ± 9.7</td>
<td>42</td>
<td>6.2 ± 4.9</td>
</tr>
<tr>
<td>Mandarin</td>
<td>38</td>
<td>1.3 ± 0.9</td>
<td>48</td>
<td>13 ± 10.3</td>
<td>47</td>
<td>6.1 ± 5.1</td>
</tr>
<tr>
<td>Medium Green</td>
<td>36</td>
<td>0.8 ± 0.7</td>
<td>48</td>
<td>9.2 ± 6.8</td>
<td>47</td>
<td>5.6 ± 4.3</td>
</tr>
<tr>
<td>Peking</td>
<td>49</td>
<td>1.1 ± 0.9</td>
<td>48</td>
<td>10 ± 8.4</td>
<td>47</td>
<td>5.7 ± 4.1</td>
</tr>
<tr>
<td>Pioneer 9341</td>
<td>48</td>
<td>2.1 ± 1.5</td>
<td>111</td>
<td>10.3 ± 10.1</td>
<td>48</td>
<td>5.6 ± 4.2</td>
</tr>
<tr>
<td>PI 153.292</td>
<td>31</td>
<td>0.9 ± 0.7</td>
<td>37</td>
<td>7.8 ± 6.1</td>
<td>34</td>
<td>4.9 ± 3.8</td>
</tr>
<tr>
<td>PI 326.580</td>
<td>42</td>
<td>0.7 ± 0.7</td>
<td>33</td>
<td>8.1 ± 7.3</td>
<td>38</td>
<td>5.3 ± 4.7</td>
</tr>
<tr>
<td>PI 424.078</td>
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<td>1.1 ± 0.8</td>
<td>38</td>
<td>8.7 ± 6.5</td>
<td>42</td>
<td>6.1 ± 5.2</td>
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<tr>
<td>PI 65.388</td>
<td>32</td>
<td>0.91 ± 0.4</td>
<td>39</td>
<td>8.5 ± 6.7</td>
<td>41</td>
<td>5.7 ± 4.5</td>
</tr>
<tr>
<td>PI 65.549</td>
<td>41</td>
<td>1.1 ± 0.52</td>
<td>46</td>
<td>8.6 ± 5.1</td>
<td>45</td>
<td>4.3 ± 2.4</td>
</tr>
<tr>
<td>PI 79.593</td>
<td>32</td>
<td>0.9 ± 0.7</td>
<td>42</td>
<td>8.3 ± 6.5</td>
<td>37</td>
<td>4.1 ± 2.6</td>
</tr>
<tr>
<td>Richland</td>
<td>45</td>
<td>1.2 ± 0.7</td>
<td>49</td>
<td>11 ± 9.2</td>
<td>46</td>
<td>4.9 ± 3.6</td>
</tr>
<tr>
<td>Seneca</td>
<td>48</td>
<td>1.05 ± 0.4</td>
<td>45</td>
<td>9.7 ± 4.6</td>
<td>34</td>
<td>4.7 ± 2.7</td>
</tr>
<tr>
<td>Virginia</td>
<td>46</td>
<td>0.9 ± 0.3</td>
<td>51</td>
<td>9.4 ± 6.6</td>
<td>42</td>
<td>5.8 ± 3.1</td>
</tr>
</tbody>
</table>

a The source of diversity were described in Table 1.

b All embryonic axes were treated with BAP as described in Buising et al. (1992a) and bombarded three times with pPHI413 (10 μg).

c Values are expressed as mean ± standard deviation, n = the number of axes observed.
Table 4. Enzymatic and PCR analysis of nodes from R₀ plants

<table>
<thead>
<tr>
<th>Plant</th>
<th>node</th>
<th>β-glucuronidase</th>
<th>NPTII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Histochemical (X-GLUC)</td>
<td>Fluorometric (pg/μg total protein)</td>
</tr>
<tr>
<td>62</td>
<td>4</td>
<td>cp,end,ip,p,ph,x</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>cp,end,ip,p,ph,x</td>
<td>4</td>
<td>+ (B, 2)</td>
</tr>
<tr>
<td>10</td>
<td>cp,end,ip,p,ph,x</td>
<td>5</td>
<td>+ (B, 3)</td>
</tr>
<tr>
<td>93</td>
<td>4</td>
<td>end,ep,ph,sc,x</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>end,ep,ph,sc,x</td>
<td>1</td>
<td>+ (B, 5)</td>
</tr>
<tr>
<td>14</td>
<td>ep,ph,sc,x</td>
<td>1</td>
<td>+ (B, 6)</td>
</tr>
<tr>
<td>15</td>
<td>ep,ph,sc,x</td>
<td>1</td>
<td>+ (B, 8)</td>
</tr>
<tr>
<td>17</td>
<td>ph,sc,x</td>
<td>1</td>
<td>+ (B, 9)</td>
</tr>
<tr>
<td>130</td>
<td>5</td>
<td>ip,p,ph,sc,x</td>
<td>14</td>
</tr>
<tr>
<td>190</td>
<td>1A</td>
<td>ph,sc,x</td>
<td>1</td>
</tr>
<tr>
<td>1B</td>
<td>ph,sc,x</td>
<td>1</td>
<td>+ (B, 12)</td>
</tr>
<tr>
<td>5</td>
<td>ph,sc,x</td>
<td>1</td>
<td>+ (B, 14)</td>
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<td>17</td>
<td>ph,sc,x</td>
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<tr>
<td>25</td>
<td>ep,ph,sc,x</td>
<td>1</td>
<td>+ (B, 16)</td>
</tr>
<tr>
<td>26</td>
<td>ph,sc,x</td>
<td>1</td>
<td>+ (B, 17)</td>
</tr>
</tbody>
</table>

a Tissues in which β-glucuronidase staining was observed.
Abbreviations: cp-cortical parenchyma, end-endodermis, ep-epidermis, ip-interfascicular parenchyma, ph-phloem, p-pith, sc-sclerenchyma, t-trichomes, w-whole, and x-xylem (Lersten and Carlson, 1988).

b Negative sign denotes that the expression was not detected.
nc Letter and number in parenthesis corresponds to the panel and lane number of Fig. 6.
d Negative sign denotes that the gene was not detected, absence of a lane number for a positive or negative analysis indicates the analysis was done on another gel (not shown).
Table 4 (continued)

<table>
<thead>
<tr>
<th>Plant node</th>
<th>Histochemical (X-GLUC)</th>
<th>β-glucuronidase Fluorometric (pg/μg total protein)</th>
<th>PCR c</th>
<th>Enzyme b PCR c, d NPTII</th>
<th>PCR c</th>
</tr>
</thead>
<tbody>
<tr>
<td>366</td>
<td>6 cp,ep,ip,p,sc,x</td>
<td>12 + (C, 1)</td>
<td>-</td>
<td>+ (A, 9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 cp,ep,ip,p,sc,x</td>
<td>10 + (C, 2)</td>
<td>-</td>
<td>+ (A, 10)</td>
<td></td>
</tr>
<tr>
<td>368</td>
<td>7A w, except ep</td>
<td>13 + (C, 3)</td>
<td>-</td>
<td>+ (A, 11)</td>
<td></td>
</tr>
<tr>
<td>377</td>
<td>15 ph, x</td>
<td>2 + (C, 4)</td>
<td>-</td>
<td>+ (A, 12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 ph, x</td>
<td>1 + (C, 5)</td>
<td>-</td>
<td>+ (A, 24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19 ph, x</td>
<td>1 + (C, 6)</td>
<td>-</td>
<td>+ (A, 14)</td>
<td></td>
</tr>
<tr>
<td>476</td>
<td>2 ph, x</td>
<td>6 + (C, 7)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 ph, x</td>
<td>1 + (C, 8)</td>
<td>-</td>
<td>+ (A, 15)</td>
<td></td>
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<tr>
<td>720</td>
<td>1 -</td>
<td>&lt;0.7 - (C, 14)</td>
<td>-</td>
<td>+ (A, 16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 -</td>
<td>&lt;0.7 - (C, 15)</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>832</td>
<td>2 w</td>
<td>3 + (C, 9)</td>
<td>2</td>
<td>+ (A, 17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 w</td>
<td>5 + (C, 10)</td>
<td>3</td>
<td>+ (A, 18)</td>
<td></td>
</tr>
<tr>
<td>836</td>
<td>6 ep, t</td>
<td>4 + (C, 11)</td>
<td>-</td>
<td>+ (A, 19)</td>
<td></td>
</tr>
<tr>
<td>849</td>
<td>5 cp,end,ep</td>
<td>4 + (C, 12)</td>
<td>-</td>
<td>+ (A, 20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 cp,end,ep</td>
<td>6 + (C, 13)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 cp,end,ep</td>
<td>2 + (C, 16)</td>
<td>-</td>
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</tr>
</tbody>
</table>
Fig. 1. Schematic representation of plasmid constructs

a. pPHI419; b. pPHI413; c. pPHI456; d. pCB209; e. pBI221. The location and sequence of oligonucleotide primers used for PCR amplification are indicated.
Fig. 2. Visualization of exogenous gene expression in BAP-treated embryonic axes. a. Unifoliolate leaf of Glycine max (L.) Merr. cv. Harosoy from an embryonic axis bombarded with pBI221 at 42 hr post-imbibition; stained at 66 hr. b. Embryonic axis bombarded with pBI221 42 hr post-imbibition; stained at 66 hr. The unifoliolate leaves have been removed. c. Embryonic axis bombarded with control tungsten particles at 66 hr post-imbibition; stained at 90 hr. d. Embryonic axes bombarded with pPHI413 at 66 hr post-imbibition, stained at 90 hr. e. Two week old shoot of an embryonic axis bombarded with pPHI413 at 66 hr; stained at 2 weeks. f. Center leaflet from a trifoliolate leaf on an embryonic axis four weeks after bombardment with pPHI413 at 42 hr; stained at 4 weeks. g. Green and unpigmented sectors in leaves of embryonic axes bombarded with pPHI419 at 42 hr post-imbibition. Axes were cultured on kanamycin (200 mg/l) from 90 hr post imbibition and were photographed at 2 weeks after bombardment. h. Kanamycin sensitive leaf (unpigmented) and kanamycin resistant leaf (green) growing from the same shoot. Magnification, a, b, c, d, e, f, g, h: bar = 1 mm.
Fig. 3. Expression of β-glucuronidase in bombarded embryonic axes of *Glycine max* cv. Pioneer 9341. Axes were assayed for expression 48 hr post-bombardment using the fluorescence assay of Rao and Flynn (1990). Panel A. Effect of plasmid construct and time of bombardment on expression of β-glucuronidase. Embryonic axes were bombarded with pBI221(Δ), pPHI456 (○), and pPHI413 (♦) at the indicated time post-imbibition.

Panel B. Effect of BAP treatment on expression of β-glucuronidase.

Control (○, MS) and BAP-treated (♦) embryonic axes from 18 to 42 hr post-imbibition were bombarded with pPHI413 at the indicated times post-imbibition.
Fig. 4. Persistence and expression of exogenous genes in BAP-treated embryonic axes. β-glucuronidase expression was visualized by X-GLUC staining. a. Axis of soybean cultivar Hack bombarded 66 hr post-imbibition with control tungsten particles (minus DNA). b. Axis bombarded at 66 hr post-imbibition with pPHI413, stained at 90 hr, and fixed at 114 hr in FAA. c. Axis bombarded at 66 hr post-imbibition with pPHI413, stained at 114 hr, and fixed at 138 hr in FAA. d. High magnification of an embryonic axis bombarded at 66 hr post-imbibition with pPHI413, but not counter stained. e. Cut section of leaf from R0 plant 832, node 10, stained in X-GLUC. f. Expression of β-glucuronidase in anthers of R0 plant 368, node 7A (right) and control (left). Petals have been removed to view anthers. g; h Transections of petioles and stems from R0 plants stained with X-GLUC. Staining patterns observed were: negative control (g, left), epidermal staining especially at the base of trichomes (g, center; R0 plant 836, node 6), and phloem and xylem (g, right; R0 plant 476, node 2). Sectors of β-glucuronidase expression were observed in epidermis, sclerenchyma, phloem and xylem (h, left; R0 plant 93, node 12). Expression was also detected in epidermis, sclerenchyma, cortical parenchyma, interfascicular parenchyma, xylem, and pith (h, center; R0 plant 366, node 7). The entire transection stains a deep blue, with the exception of the epidermis that appears as a light halo of unstained epidermal cells and trichomes (h, right; R0 plant 130, node 5).
Fig. 5. Schematic of representation of plants with β-glucuronidase and/or neomycin phosphotransferase expression (darker branches). Nodes of R₀ plants expressing β-glucuronidase were identified by staining with X-GLUC and by the fluorometric method of Rao and Flynn (1990). Neomycin phosphotransferase expression was identified by enzymatic analysis.
Fig. 6. PCR analysis of genomic DNA from nodes of R₀ plants.

Oligonucleotide primers used to detect the *uidA* and *neo* genes are diagrammed in Fig. 1. The identification of specific plants and nodes are summarized in Table 4 and Fig. 5. Panel A. Persistence of the *neo* gene (predicted fragment size 876-bp). Controls included were: DNA from a transgenic tobacco plant (lanes 26 and 27; predicted fragment size 768 bp), R₀ plant 439 (lanes 28 and 29; bombarded with control (minus DNA) tungsten microprojectiles, control PCR reaction lacking genomic DNA (lanes 30 and 31), and plasmid pPHI419 (lane 32). Panel B. Persistence of the *uidA* gene (predicted fragment size 498-bp). Controls included were: DNA from a transgenic tobacco plant (lanes 20 and 22), R₀ plant 439 (lanes 7, 18 and 19; bombarded with control (minus DNA) tungsten microprojectiles, control PCR reaction lacking genomic DNA (lanes 23 and 24), and plasmid pPHI419 (lane 25). Panel C. Persistence of the *uidA* gene (predicted fragment size 498-bp). Controls included were: DNA from a transgenic tobacco plant (lanes 21), R₀ plant 439 (lanes 19 and 20) bombarded with control (minus DNA) tungsten microprojectiles, control PCR reaction lacking genomic DNA (lanes 22 and 23), and R₀ plant 720 bombarded with pPHI419 (lanes 14 and 15).
Fig. 6

Panel A

Panel B

Panel C
SECTION III: MOLECULAR ANALYSIS OF TRANSGENIC PLANTS AND CELLS GENERATED BY MICROPARTICLE BOMBARDMENT: EFFECT OF PETUNIA TRANSFORMATION BOOSTER SEQUENCE
MOLECULAR ANALYSIS OF TRANSGENIC PLANTS AND CELLS
GENERATED BY MICROPROJECTILE BOMBARDMENT:
EFFECT OF PETUNIA TRANSFORMATION BOOSTER SEQUENCE

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ABSTRACT

The effects of petunia transformation booster sequence (TBS) on the expression and persistence of supercoiled plasmid constructs in dicotyledonous (tobacco) and monocotyledonous (maize) plant cells were compared. Expression vectors containing bacterial genes coding for β-glucuronidase and neomycin phosphotransferase, or phosphinothricin acetyltransferase, were introduced by microprojectile bombardment into leaves and cotyledons of *Nicotiana tabacum* cv. Xanthi, or into suspension cultures of *Zea mays* cv. Black Mexican Sweet (BMS). Petunia TBS increased the transformation frequency 7.8- to 16-fold (Wilcoxon matched-pairs signed-ranks test) in tobacco, and 1.7- to 2.4-fold in maize. Although TBS contains a well defined transcription enhancer element, no significant differences were observed on the enzyme levels of β-glucuronidase and neomycin phosphotransferase in transgenic tobacco, or of β-glucuronidase and phosphinothricin acetyltransferase in transgenic BMS cells. TBS did not appear to alter the integration patterns of exogenous DNA in either tobacco or maize transformants and, in particular, no effect was observed in the complexity of the integration patterns. In tobacco, transformants usually contained at least one copy of the introduced gene in a non-rearranged form, and often contained multiple copies of the gene in rearranged forms as well. In maize, however, most transformants contained only rearranged copies. In tobacco, TBS had no significant effect on the segregation of the exogenous genes in *R*1 progeny, or on the linkage of two genes introduced in the same construct.
INTRODUCTION

Microprojectile bombardment is increasingly being used to directly transfer foreign DNA into plant cells. In *Zea mays* cv. Black Mexican Sweet (BMS) and in embryogenic maize cells, Klein *et al.* (1988a) observed transient expression of a bacterial chloramphenicol acetyltransferase gene after microprojectile bombardment. Stable transgenic tobacco were generated after microprojectile bombardment of leaf cuttings and suspension cultures with various expression vectors (Klein *et al.*, 1988b). Stable transformation of soybean cells Christou *et al.* (1988) and plants (McCabe *et al.*, 1988) by microprojectile bombardment has also been reported. In initial reports, segregation of exogenous genes in soybean did not appear to be Mendelian (McCabe *et al.*, 1988), but Christou *et al.* (1989) have subsequently shown Mendelian inheritance in transformed R1 and R2 plants. Both Mendelian and non-Mendelian segregation ratios have been observed in transgenic tobacco generated by microprojectile bombardment (Tomes *et al.*, 1990).

Genomic DNA fragments that increase transformation frequencies of linked genes have been isolated by several laboratories (Meyer *et al.*, 1988; Paszkowski *et al.*, 1988; Marchesi *et al.*, 1989). A 2-kb genomic petunia fragment called transformation booster sequence (TBS), isolated by Meyer *et al.* (1988), and an internal 1.1-kb fragment of TBS, were both found to increase the efficiency of polyethylene glycol-mediated (PEG) transformation about 20-fold in petunia and tobacco protoplasts. Recently, the effect of TBS on transformation of non-synchronized and synchronized M- or S-phase protoplasts was analyzed by Kartzke *et al.* (1990). Rearrangements of vector
sequences integrated in the DNA were observed in S-phase transformants, but not in M-phase or non-synchronized transformants. Integration patterns were similar for plasmids containing or lacking TBS. Since TBS did not lead to increased transformation frequencies in S-phase transformants, but instead led to an increased number of integrated copies, Kartzke et al., (1990) suggested that TBS may carry an S-phase specific signal.

In this study we have characterized the effect of TBS on transformation frequency in monocotyledonous cells (maize) and dicotyledonous plants (tobacco). We have examined the effect of TBS on gene expression, integration patterns in transformants, and segregation ratios in R1 progeny of transformants. Sequence pattern analysis of the TBS sequence suggests that it contains common modular elements found in eukaryotic chromosomal DNA replication origin regions (Benbow et al., 1992). The possible roles of these elements in the observed stimulation of transformation is discussed.
MATERIALS AND METHODS

Plasmids

The plasmids and probes used are diagrammed in Figure 1. Plasmid were prepared essentially as described by Hines and Benbow (1982). pPHI419, pPHI413, pPHI456 and pCB209 were described previously (Buising et al., 1992).

pCB106, a 5.8-kb plasmid carrying the neo gene, was created by excising the 1.1-kb TBS with EcoRI from pTBS-1 (Meyer et al., 1988), and inserting it into the unique EcoRI site of the pPHI419 polylinker region.

pPHI485, a 7.93-kb plasmid containing the bar (Murakami et al., 1986; Thompson et al., 1987) and uidA (Novel and Novel, 1973; Jefferson et al., 1987) genes, was created from plasmids pPHI408 and pPHI463. pPHI408 contains the cauliflower mosaic virus 35S promoter (CaMV 35S; Kay et al., 1987), omega prime sequence (Ω'; provided by T. Michael A. Wilson; Gallie et al., 1987a; 1987b), maize alcohol dehydrogenase intron 1 (Adh1; Klein et al., 1988a), uidA gene, and is terminated by the nopaline synthase polyadenylation sequence (nos; Fraley et al., 1983). pPHI463 was created by excising the uidA gene with BamHI-PvuII from pPHI408 and instead inserting a 0.57 kb BamHI-BglII fragment of the bar gene (DeBlock et al., 1987). pPHI485 was created by excising the 2.5-kb fragment, containing the CaMV 35S promoter, Ω' sequence, Adh1 intron, bar gene, and nos polyadenylation site, with BglIII from pPHI463 and inserting it into the polylinker region of pPHI408 (see Fig. 1).

pPHI502, a 9.05-kb plasmid, was was created by excising the 1.1-kb
transformation booster sequence with XbaI from pTBS-1 (Meyer et al., 1988), and inserting it into the unique XbaI site 5' to pPHI485 (see Fig. 1).

**Generation of transformants**

Microprojectiles [1.2 μm; either tungsten (General Electric Co., Cleveland, OH) or gold (Morton Thiokol, Inc., Danvers, MA)] were prepared as described by Klein et al. (1988a) using a 10 μg/ml preparation of plasmid DNA (except as noted).

**Tobacco**  *Nicotiana tabacum* cv. Xanthi were aseptically grown from surface sterilized seed on MS medium (Murashige and Skoog, 1962) for approximately 14 days. Tobacco leaves and cotyledons were bombarded as described by Tomes et al. (1990).

After bombardment, leaves and cotyledons were separated at the petiole and incubated on fresh MS medium for 2 days. Tissue was cultured on modified MS medium containing: MS salts (Murashige and Skoog, 1962), 4% sucrose (w/v), B5 vitamins (Gamborg et al., 1968), 0.5 mg/l benzylaminopurine (BAP; Sigma, St. Louis, MO), 2 mg/l 1-naphthylacetic acid (NAA; Sigma) and 100 mg/l kanamycin sulfate (Sigma). 3-4 weeks post bombardment, leaves with kanamycin resistant colonies were transferred to modified MS medium lacking NAA. Colony forming units (CFU) were identified as opaque, round, inseparable masses. Leaves were turned over each week and scored for CFUs.

Individual CFUs were used to regenerate plants in the presence of kanamycin. Regenerated plants were rooted on a medium consisting of MS salts, MS vitamins (Murashige and Skoog, 1962), and 50 mg/l kanamycin.
Plants were transferred to the green house, and screened for both neomycin phosphotransferase and β-glucuronidase activity.

MaizeSuspension cultures of *Zea mays* cv. Black Mexican Sweet (BMS) non-embryogenic maize (supplied by Shiela Maddock, Pioneer Hi-Bred Intl., Inc.) were subcultured to MS liquid medium 2 days prior to bombardment. One day prior to bombardment, BMS cells were sieved through a Buchner funnel containing a Whatman 617 filter; approximately 1,000 mg of cells were resuspended in MS suspension medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose and 0.25M mannitol. On the day of bombardment, 100 mg (wet weight) BMS suspension cells were layered upon two-stacked Whatman 617 filters soaked with MS medium containing 0.25M mannitol.

Two days after bombardment, cells were washed from the filters, separated into four-25 mg aliquots, and cultured on medium containing: MS salts, MS vitamins, 0.1 g/l myo-inositol, 1 mg/l 2,4-D, 3% sucrose, and 5 mg/l phosphinothricin (PPT, Basta®, Hoechst AG). Colonies were visible approximately two weeks after bombardment. Individual colonies were maintained on phosphinothricin selection for 6 weeks, removed from selection for two weeks, then returned to selection.

Expression analysis

Protein quantitation Approximately 5-7 mg of fresh tobacco leaf tissue or BMS callus were used to determine protein concentration (Bradford, 1976) with a Bio-RAD™ protein assay kit (Bio-RAD, Richmond, CA) as described by Buising *et al.*, 1992.
β-Glucuronidase assays Aliquots corresponding to 2 and 4 μg of protein (see above) were assayed in duplicate using 4-methylumbelliferyl-β-D-Glucuronide (MUG; Sigma) as substrate following the method of Rao and Flynn (1990). Histochemical analyses were performed using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide (X-GLUC, Research Organics, Cleveland, OH). The substrate was prepared as described by Jefferson (1987b). Tobacco leaves or maize calli were immersed in X-GLUC at 37°C for 24 hours.

Neomycin Phosphotransferase assays Aliquots corresponding to 1 or 2 μg of protein were monitored for phosphorylation of neomycin as described by Staebell et al. (1990) and Buising et al. (1992).

Phosphinothricin Acetyltransferase assays Phosphinothricin acetyltransferase assays were done using thin layer chromatography essentially according to De Block et al. (1987; 1989). Maize calli (50 mg) were homogenized in 100 to 200 μl of buffer C (25mM Tris-HCl [pH 7.5], 1 mM Na2-EDTA). Homogenates containing 5 and 10 μg protein, were diluted to 21 μl with buffer C, and the following were added to the reaction mixture: 3 μl of 1 mM acetyl Coenzyme A (AcCoA; Sigma) in 0.01 M sodium acetate, pH 5, 4 μl [14C] acetyl Coenzyme A (58.1 mCi/mmol; New England Nuclear), and 2 μl of phosphinothricin (Basta®). The reaction mixture was incubated for 30 minutes at 37°C in a water bath, spotted on a silicagel thin layer chromatography plate (Whatman #4410-222), and dried. Ascending chromatography was carried out in a 3:2 mixture of 1-propanol and ammonium hydroxide. After drying, the plate was analyzed on an AMBIS 2-D beta scanner (Nye et al., 1988).
DNA analysis

Southern analysis DNA was isolated from plant tissues essentially as described by Keim et al. (1988; 1989) and subjected to Southern blot analysis (Southern, 1975; Maniatis et al., 1982). Probes, labeled with $^{32}$P-dCTP (ICN, Irvine, CA), were prepared by random priming according to the method of Feinberg and Vogelstein (1984).

Segregation analysis Following the initiation of flowering, individual tobacco plants were selfed to determine transmission of the foreign genes. Seed were surface sterilized in 0.5% sodium hypochlorite (1/10 dilution of Chlorox®) for 15 minutes, rinsed four times in sterile deionized water, and germinated on MS medium containing 2% sucrose and 200 mg/l kanamycin. Seed was also germinated on MS medium without kanamycin to check germination frequency and segregation of β-glucuronidase. Plants were grown at 28°C under approximately 100 μE m$^{-2}$ s$^{-1}$ of light with a 16-hour day length, and were scored after 21 days for green or white leaf or cotyledon color and appearance of secondary leaves. Segregation ratios were expressed relative to the total number of germinated seeds.

Statistical analysis

Parametric methods The effect of TBS was characterized by analysis of variance (ANOVA; Fisher, 1966). Statistics were calculated according to Daniel (1974) using Statview software (Abacus Concepts, Inc., Berkeley, CA). Data was analyzed by the chi-square test (Pearson, 1900) and
differences were considered significant when the calculated probability, against expected Mendelian segregation ratios, was $p < 0.05$.

**Non-parametric methods** The Wilcoxon matched-pairs signed-ranks test was used (Wilcoxon, 1945) to analyze the effect of TBS, since much of the data (see results) did not meet standard criteria for parametric analysis. The programs from SPSS-X version 3.0 or Statview software (Abacus Concepts, Inc., Berkeley, CA) were used to calculate the Wilcoxon matched-pairs signed-ranks test.
RESULTS

Effect of petunia TBS: kanamycin resistant colony formation in tobacco

Petri dishes containing tobacco leaves and/or cotyledons (10 per dish) were bombarded with tungsten microprojectiles to which plasmid constructs (Fig. 1) had been adsorbed. An average of 1.22 ± 0.08 (SEM) CFU per dish (n=100) were recovered from explants bombarded with pPHI456 and cultured for 4 to 8 weeks on 100 mg/l of kanamycin. Most of the individual colonies were on separate explants. The mean number of kanamycin resistant colonies per dish from explants that had not been bombarded or had been bombarded with microprojectiles minus DNA was 0.00 ± 0.0 (n= 10). These control explants enlarged for about a week, than began to atrophy. Similar explants cultured in the absence of kanamycin showed rapid callus proliferation and formed more than 100 colonies per plate (n = 20).

The mean number of colonies recovered per dish from explants bombarded with pCB209 (pPHI456 containing, in addition, the petunia transformation booster sequence) was 4.85 ± 0.2 (SEM; n=96). Many explants had more than one discrete colony. Analysis of variance (ANOVA) indicated that TBS resulted in a highly significant increase in the frequency of kanamycin resistant colonies (p = 0.0001). Since the variance of the population bombarded with pPHI456 was different from that of the population bombarded with pCB209, however, the distributions were not normal. ANOVA understates the real effect of TBS on transformation efficiency. The results of a Wilcoxon matched-pairs signed-ranks test (Wilcoxon, 1945), comparing pPHI456 with pCB209 is summarized in Table
1B. We conclude that a 7.8-(including ties) to a 16-fold (excluding ties) increase in transformation frequency was observed with the TBS containing plasmid, pCB209, relative to the same construct lacking TBS.

**Effect of petunia TBS: phosphinothricin resistant colony formation in maize**

Maize suspension cultures (100 mg) were bombarded with pPHI485 and cultured on 5 mg/l phosphinothricin. A total of 327 phosphinothricin resistant colonies were recovered from 76 dishes, for a mean of 4.3 ± 0.3 colonies/dish (Table 2). Non-bombarded control cells cultured on 5 mg/l phosphinothricin did not proliferate and turned brown. Bombardment of BMS cells with pPHI502 (pPHI485 containing, in addition, petunia TBS), resulted in the recovery of 426 phosphinothricin resistant colonies from 76 dishes for a mean of 5.6 ± 0.33 colonies/dish (Table 2). Analysis of variance (ANOVA) showed a small, but significant, increase (p = 0.0079) in the number of colonies recovered after bombardment with TBS present in plasmid constructs. The Wilcoxon matched-pairs signed-ranks test indicated that TBS resulted in an increase in transformation frequency from 1.7-fold (including ties) to 2.4-fold (excluding ties). This experiment was repeated using 200 additional plates with similar results.

**Effect of petunia TBS: gene expression in tobacco and maize:**

Fifty-eight kanamycin resistant tobacco colonies recovered after bombardment with pPHI456, and 145 after bombardment with pCB209 (all from Table 1) were analyzed for neomycin phosphotransferase and β-glucuronidase expression. As expected, 100% (180/180) of the colonies
expressed neomycin phosphotransferase. β-glucuronidase expression was
detected in 79.3% of colonies after bombardment with pPHI456 compared to
80.1% in colonies after bombardment with pCB209 (Table 3). There were no
significant differences in the overall levels of β-glucuronidase expression:
4.13 ± 0.32 pg/µg total protein (pPHI456) versus 4.41 ± 0.22 pg/µg total protein
(pCB209), suggesting that petunia TBS had no effect on exogenous gene
expression in tobacco in spite of a substantial increase in kanamycin
resistant colony formation.

Forty-one phosphinothricin resistant maize colonies recovered after
bombardment with pPHI485 and 55 after bombardment with pPHI502 were
analyzed for phosphinothricin acetyltransferase (PAT) and β-glucuronidase
expression levels. Expression of PAT was detected in 80.5% (pPHI485)
versus 65.1% (pPHI502) of the colonies (Table 4). Presumably, the level of
expression in the remaining colonies was below the level of the detection
threshold of the assay, since they persisted on phosphinothricin selection. β-
glucuronidase expression was detected in 65.9% and 74.5% of the colonies
bombarded with pPHI485 and pPHI502, respectively. There were no
significant differences in the overall levels of β-glucuronidase expression: 19
± 6.8 pg/µg total protein (pPHI485) versus 12 ± 3.5 pg/µg total protein
(pPHI502). We conclude that petunia TBS had no effect on exogenous gene
expression in either maize or tobacco.

Effect of TBS in $R_0$ transformed tobacco plants and $R_1$ progeny

A total of 99 $R_0$ plants were regenerated from explants bombarded
with the following plasmid constructs: 33 from pCB209, 47 from pPHI456, 3
from pPHI419, and 3 from pCB106. There were 13 negative control plants. All 86 tobacco plants regenerated after bombardment on kanamycin selection expressed neomycin phosphotransferase activity. β-glucuronidase expression was observed in 75% of plants derived from bombardments with pPHI456 and 83% of from bombardments with pCB209; however, these differences were not significant. Table 5 summarizes foreign gene expression in 36 of 86 putative transformants and 3 of 13 negative control plants.

Kanamycin resistance of R₁ progeny from the 36 transformed R₀ tobacco plants was best characterized by a three-to-one segregation ratio (Table 5). Cytochemical staining with X-GLUC also indicated segregation ratios of three-to-one for seed germinated on MS medium without kanamycin. Expression levels of β-glucuronidase or neomycin phosphotransferase in other R₁ plants were not significantly different from levels observed in parent plants. No segregation analysis was carried out for the BMS suspension cultures because they were not embryogenic.

**Effect of TBS on integration and segregation of exogenous genes in tobacco**

Regenerated R₀ tobacco plants were examined for neomycin phosphotransferase and β-glucuronidase (Table 5) expression. Plasmid constructs, pPHI456 and pCB209, contain both the neo and uidA genes, while pPHI419 and pCB106 contain only the neo gene (Fig. 1). Neomycin phosphotransferase activity was observed in all 36 plants resulting from bombardments with neo gene constructs. β-glucuronidase expression was observed in 18 of 22 plants derived from bombardments with pCB209
(containing TBS) and 9 of 12 plants from bombardments with pPHI456 (lacking TBS). All R0 plants (Table 5) were examined by Southern analysis to determine integration patterns of the neo and uidA genes (Figs. 2, 3, 4). R0 plants were selfed and progeny were examined for neomycin phosphotransferase and β-glucuronidase expression (Table 5). The segregation of both genes in the R1 progeny was best explained by a 3:1 segregation ratio.

All R1 progeny of R0 plants that did not express β-glucuronidase also did not express β-glucuronidase (Table 5). Only 3 out of the 679 R1 plants from R0 plants expressing both neomycin phosphotransferase and β-glucuronidase failed to express β-glucuronidase. This suggested that the uidA and neo genes were tightly linked. Therefore, R0 plants expressing foreign genes were observed to pass these genes to their R1 progeny in an expected 3:1 segregation ratio (Table5) with little loss of the ability to express the gene. TBS appears to have little, if any effect on either segregation or expression.

Southern analysis of selected R0 and R1 plants was carried out to analyze transmission of integration patterns (Fig. 2, 3, 4). Genomic DNAs from 10 R0 plants expressing β-glucuronidase were digested with BamHI and probed with the uidA gene. All showed an expected 3.0 kb (Fig. 2, Panel A) band, as well as other bands of higher molecular weight. There was considerable variation in the apparent copy number regardless of the presence or absence of TBS. In contrast to the results of Kartzke et al. (1990) and Meyer et al. (1988), there was no apparent influence of TBS on integration patterns in transformants (see Discussion). Note that Plant 140,
which did not express β-glucuronidase, contained at least one copy of the
uidA gene. The same filter was reprobed with the neo gene: all plants
showed the expected 1-kb fragment (Fig. 2, Panel B), and frequently
contained rearranged copies as well.

Genomic DNA digested with the restriction enzyme *NheI*, which does
not cut either pCB209 or pPHI456, was probed with the *uidA* gene (Fig. 3).
R₀ plant 122, which expressed β-glucuronidase, was compared to one of its
R₁ progeny, 122.3, which did not express β-glucuronidase activity. R₀ plant
122 showed two integration sites, whereas 122.3 had lost at least one of these
at ~15 kb. The integration pattern of the *uidA* gene in 173.7, which did not
express β-glucuronidase, was strikingly different from the three fragments
observed in the R₀ plant 173 (Fig. 3). R₀ plants 139 and 153, as well as a
subset of progeny (all of which retained the ability to express β-
glucuronidase) were compared. The progeny 139.2 and 139.9 showed
apparent changes in copy number but retained the fragments observed in the
R₀ parent. Similarly all R₁ progeny 153.3, 153.5, and 153.7 exhibited the
same integration patterns of the R₀ parent.

Genomic DNA from R₁ plants that had not been selected on
kanamycin was digested with *NheI* and probed with the *uidA* gene (Fig. 4).
The R₀ plant 171, which expressed β-glucuronidase, was compared with R₁
progeny 171.5 that did not. Plant 171.5 showed differences in the integration
pattern compared with the parent 171: a band at ~6 kb was lost in the
progeny. Plant 101, which had not been selected on kanamycin and
expressed β-glucuronidase was compared with 7 progeny that expressed β-
glucuronidase and 3 that did not. R₁ plants 101.2 and 101.6 had lost both
fragments observed in the R₀ parent and lost both neomycin phosphotransferase and β-glucuronidase expression. Plant 101.4 lost one fragment and β-glucuronidase expression, but retained neomycin phosphotransferase activity. In contrast to most results, in this case results suggest that expression of the *neo* gene was derived from a different locus than the *uidA* gene in the R₀ parent.

**Effect of TBS on the integration and segregation of exogenous genes in BMS cells**

BMS calli from suspension cultures bombarded with pPHI502 or pPHI485 and cultured on 5 mg/l phosphinothricin were examined by Southern analysis (Fig. 5). Genomic DNA, digested with *BglII* and *BamHI*, was probed with the *uidA* (Fig. 1) gene. In contrast to the results with tobacco, the expected 2.0 kb fragment was seen in only one of ten calli examined. Since the calli in lanes 1, 2, 4, 5, 6, 8, 9, and 10 expressed both β-glucuronidase and phosphinothricin acetyltransferase, it seems likely that the genes were integrated in such a fashion as to modify the construct (except perhaps in lane 1). The integration patterns appear, on average, to be much more complex in maize than in tobacco. Lanes 3 and 7 contain genomic DNA from calli that did not express β-glucuronidase yet, nonetheless, contain multiple copies of the *uidA* gene. There appeared to be no influence of TBS on integration patterns in maize.
DISCUSSION

Effect of TBS on transformation frequencies and expression of exogenous DNA

Transgenic plants are generated at low frequencies following direct transfer of foreign DNA into intact cells by electroporation or microprojectile bombardment (review by Potrukus, 1990). Several laboratories have previously isolated genomic DNA fragments that increase transformation frequencies of linked genes following direct transfer (Meyer et al., 1988; Paszkowski et al., 1988; Marchesi et al., 1989). In the present study we show that petunia transformation booster sequence (TBS; Meyer et al. 1988) increased the frequency of transformation 7-to 16-fold in a dicotyledonous plant (Table 1) and 2-fold in a monocotyledonous plant (Table 2) after direct transfer by microprojectile bombardment. These results confirm the TBS mediated increase in transformation frequencies in tobacco protoplasts transformed using PEG (Meyer et al., 1988), and suggest that TBS may also mediate lesser increases in transformation frequencies in maize. TBS did not appear to have an effect on levels of gene expression.

Effect of TBS on integration

In this study, TBS apparently had no influence on integration patterns in either tobacco (Table 5, Figs. 2, 3, 4) or maize (Fig. 5). Meyer et al. (1985) found that transformation efficiency increased by approximately two orders of magnitude when tobacco protoplasts were synchronized in S- or M-phase, then transformed using PEG precipitation. Meyer et al. (1988) have reported
a preference for single copy integration in non-synchronized dicotyledonous cells for plasmids containing TBS. Kartzke et al. (1990) reported differences in integration patterns between synchronized and non-synchronized protoplasts transformed with plasmids containing or lacking the TBS segment, and suggested that TBS may direct S-phase specific integration.

Relatively complex integration patterns were observed in this study (Figs. 2, 3, 4, and 5). Segregation analysis confirmed integration at a single dominant locus, however, and no significant TBS-dependent differences were observed between the integration patterns or copy numbers of foreign DNAs in transformed cells or plants. The tissues and cells (tobacco and maize) are more likely to be non-synchronized, so no firm conclusions can be drawn regarding single copy integration.

The strategy used by Meyer et al. (1988) to isolate TBS was originally intended to enrich for autonomously replicating sequences; however, TBS was found to increase transformation frequencies, at least in part, by some other mechanism. Similar attempts to clone specific origins of DNA replication in animal cells (Marini et al., 1988) or in plant cells (Meyer et al., 1988; Kartzke et al., 1990) have not resulted in cloned sequences that function as origins of replication for circular DNA molecules. In all cases, the goal of the original projects had been to isolate autonomously replicating segments (ARS), analogous to those discovered a decade ago in yeast.

In most organisms, DNA segments were isolated that promoted the integration of the plasmid DNA vectors into high molecular weight DNA. Moreover, in animal and plant cells, the conformation of input plasmid DNA has a striking effect on whether the DNA is replicated, persists, or becomes
integrated into high molecular weight DNA (Marini et al., 1988; 1989; Endean and Smithies, 1989; Lyznik et al., 1991). It seems likely that input DNA must either be introduced in a linear conformation or must contain specific sequences that facilitate formation of high molecular weight concatemers. In most cases, the high molecular weight plasmid DNA was found as concatemers (Riggs and Bates, 1986).

TBS may act by several different interacting mechanisms. TBS may increase transformation frequencies by providing replication origin activity, by stimulating homologous or non-homologous recombination, by promoting nuclear retention of foreign sequences or by amplification of plasmid molecules.

TBS shares common modular elements with replication origin regions

The 1.1-kb TBS fragment used in this study and the full length 2-kb sequence pTBS-2 from Meyer et al. (1988), were analyzed for modular elements that were likely to be associated with chromosomal DNA replication origin regions in eukaryotes (reviewed by Benbow et al., 1992; Gale et al., 1992). Analyses were carried out using using the Wisconsin Genetic Computer Group (GCG) Sequence Analysis Software Package and the National Biosciences Oligo program (Benbow et al., 1992; Larson and Benbow, 1992). A schematic diagram of the modular elements found in pTBS2 is shown in Figure 6.

DNA unwinding elements Umek and Kowalski (1988) have shown that ARS (autonomous replication sequence) activity in yeast is in large part determined by the ease of DNA unwinding within A+T-rich 3' flanking
regions adjacent to the 11-bp ARS consensus sequence. DNA unwinding elements (DUEs) are extended duplex DNA regions with reduced Tm that exhibit hypersensitivity to single-strand specific nucleases (Umek and Kowalski, 1990). The helical instability of a DUE is not strictly correlated with A+T content, but can be predicted using calculated nearest neighbor free energy values (Breslauer et al., 1986; National Bioscience Oligo program). Petunia TBS contained several DUEs (Fig. 6).

**Scaffold-associated regions** TBS showed homology to consensus sequences found in scaffold attachment regions (Fig. 6; SARs or MARs: Mirkovitch et al., 1984; Gasser and Laemmli, 1986; Cockerill and Garrard, 1986; Mirkovitch et al., 1988; Dijkwel and Hamlin, 1988). Scaffold attachment regions have been postulated to specify the attachment sites of chromatin loops and contain DNA topoisomerase II cleavage consensus sites (Adachi et al., 1989). SARs are important for the organization of transcriptionally active domains (Gasser and Laemmli, 1986) and replication units (Marini and Benbow, 1991) in genomic DNA. Moreover, DNA topoisomerase II transiently produces double-stranded breaks and could potentially mediate integration of foreign DNA at cleavage consensus sites.

**Transcription Enhancers** Cis-acting transcriptional control elements and/or recognition sites for transcription factors were also identified in TBS (GTGGTATG; Weiher et al., 1983; Gillies et al., 1983; Meyer et al., 1988). The transcriptional enhancer was suggested to contribute to the effect of TBS by Meyer et al. (1988) because a 10-fold increase in transformation frequency was observed when TBS was used in conjunction with the Cauliflower Mosaic Virus 35S promoter, which also contains a
transcriptional enhancer. Transcriptional enhancer sequences have been found in association with replication origin regions (DePamphilis, 1988; Mohr et al., 1990; Rivier and Rine, 1992). Transcription enhancers may direct replication enzymes to DNA, interact with replication machinery, or may cause changes in DNA conformation that increases DNA unwinding.

**Other modular elements**

TBS contains pyrimidine tracts, which have been shown to be preferential DNA polymerase-primase start sites (Yamaguchi et al., 1985a; 1985b) and have been observed as elements associated with origin regions (Benbow et al., 1992). ARS-like consensus sequences, which were also observed in the TBS sequence, may serve as initiator protein binding sites (Campbell and Newlon, 1991).

**TBS may provide nuclear retention or amplification**

The combination of modular elements found in TBS may also provide the ability to be retained within the nucleus as an extrachromosomal plasmid (Krysan and Calos, 1991). Modular elements may promote amplification of the plasmid construct (Wegner et al., 1989; Wegner et al., 1990).

**TBS may effect recombination**

Recombination of foreign and genomic DNA may be enhanced by elements involved in DNA replication. Repeated DNA sequences affect direct gene transfer in animals (Folger et al., 1982) and plants (Paszkowski et al., 1988; Marchesi et al., 1989). There may be preferential integration of transforming DNA by homologous recombination into sites within the repeated genomic sequences by double strand break repair or by single
strand annealing (Puchta and Hohn, 1991). Puchta and Hohn (1991) supported the suggestion of Baur et al. (1990), that single-stranded intermediates occur during the process of extrachromosomal recombination in plant cells. This proposed mechanism differs from the guiding role that was described by Meyer et al. (1988) for TBS. Double stranded breaks in DNA, supported by DNA-binding proteins such as topoisomerase II, may allow stabilization of the breaks and allow opportunities for illegitimate recombination of plasmid DNA into the genome (Pfeiffer and Vielmetter, 1988; Bae et al., 1988).
ACKNOWLEDGEMENTS

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REFERENCES


Pearson, K. 1900. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. The London, Edinburgh and Dublin Philosophical Magazine and Journal of Science, Fifth series, 50: 157-175.


Table 1. Statistical analysis of the effect of TBS on kanamycin resistant colony formation in tobacco

A. Frequency distribution

<table>
<thead>
<tr>
<th>Number of Colonies per plate</th>
<th>pPHI456 (- TBS)</th>
<th>pCB209 (+ TBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>10</td>
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<td>-</td>
</tr>
<tr>
<td>Number of plates</td>
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B. Wilcoxon matched-pairs signed-ranks test *

<table>
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<tr>
<th>Number of cases</th>
<th>pairs</th>
<th>mean rank</th>
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<tbody>
<tr>
<td>5</td>
<td>pPHI456 &gt; pCB209</td>
<td>8.80</td>
</tr>
<tr>
<td>6</td>
<td>pPHI456 = pCB209</td>
<td>-</td>
</tr>
<tr>
<td>80</td>
<td>pPHI456 &lt; pCB209</td>
<td>45.84</td>
</tr>
</tbody>
</table>

* Z = -7.8149, p = 0.00001
Table 2. Statistical analysis of the effect of TBS on phosphinothricin resistant colony formation in BMS maize cells

A. Frequency distribution

<table>
<thead>
<tr>
<th>Number of Colonies per plate</th>
<th>pPHI485 (-TBS)</th>
<th>pPHI502 (+TBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
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<tr>
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<td>Number of plates</td>
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</tr>
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</table>

B. Wilcoxon matched-pairs signed-ranks test *

<table>
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<th>Number of cases</th>
<th>pairs</th>
<th>mean rank</th>
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</thead>
<tbody>
<tr>
<td>20</td>
<td>pPHI485 &gt; pPHI502</td>
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<tr>
<td>8</td>
<td>pPHI485 = pPHI502</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>pPHI485 &lt; pPHI502</td>
<td>34.7</td>
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</table>

* Z = -3.01, p = 0.0026
Table 3. Effect of TBS on β-glucuronidase expression in kanamycin resistant tobacco colonies

<table>
<thead>
<tr>
<th>Expression of β-glucuronidase (+/-) a</th>
<th>Number of Expressing Colonies</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pPHI456</td>
<td>pCB209</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(- TBS)</td>
<td>(+TBS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>+</td>
<td>46</td>
<td>79.3</td>
<td>117</td>
</tr>
<tr>
<td>-</td>
<td>12</td>
<td>20.7</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>100</td>
<td>146</td>
</tr>
</tbody>
</table>

a Colonies were recovered on 100 mg/l of kanamycin; all expressed neomycin phosphotransferase when assayed by the method of Staebell et al. (1990). No colonies were recovered from non-bombarded or bombarded with tungsten particle minus DNA when cultured on kanamycin.
Table 4. Effect of TBS on β-glucuronidase expression in phosphinothricin resistant BMS colonies

<table>
<thead>
<tr>
<th>Expression of β-glucuronidase (+/−)</th>
<th>Number of Expressing Colonies a, b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pPHI456 (−TBS)</td>
</tr>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>+</td>
<td>27</td>
</tr>
<tr>
<td>−</td>
<td>14</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>41</strong></td>
</tr>
</tbody>
</table>

a Bombarded BMS cells were cultured on 5 mg/l phosphinothricin. No drug resistant calli were recovered from non-bombarded negative control plates or cells bombarded with tungsten minus DNA and cultured on 5 mg/l phosphinothricin.

b In 8 of pPHI485 and 17 of pPHI502 phosphinothricin resistant colonies, phosphinothricin acetyltransferase activity was below the detection level of the assay.
Table 5. Summary of exogenous gene expression in 39 R\textsubscript{0} tobacco plants and their R\textsubscript{1} seed \textsuperscript{a}

<table>
<thead>
<tr>
<th>Plant</th>
<th>Neomycin Phosphotransferase Seedling number</th>
<th>Beta-glucuronidase Seedling number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NPTII Green White</td>
<td>(\chi^2)</td>
</tr>
<tr>
<td>not bombarded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB006</td>
<td>&lt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>plasmid, pCB209</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB100</td>
<td>894</td>
<td>93</td>
</tr>
<tr>
<td>CB101</td>
<td>4906</td>
<td>109</td>
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<td>CB102</td>
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<td>27</td>
</tr>
<tr>
<td>CB116</td>
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<td>101</td>
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\textsuperscript{a} Neomycin phosphotransferase (NPTII) and \(\beta\)-glucuronidase (GUS) activity is expressed as pg/\(\mu\)g total protein. R\textsubscript{1} seed was germinated on 200 \(\mu\)g/ml kanamycin or germinated on MS medium and stained with X-GLUC.

\textsuperscript{b} Not calculated.

\(^*P = \chi^2\) probability with 1DF.
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Figure 1. Schematic representation of plasmids and probes: pPHI419 (4.78-kb); pPHI413 (5.65-kb); pPHI456 (7.79-kb); pCB209 (8.89-kb); pCB106 (5.89-kb); pPHI485 (7.93-kb); and pPHI502 (9.05-kb).
Figure 2. Southern blot analysis of transgenic tobacco plants transformed with pPHI456 or pCB209. Panel A. Genomic DNA was digested with BamH1, and probed with the *uidA* gene (probe b, Fig. 1). Panel B. Southern blot analysis of the same gel probed with the *neo* gene (probe a, Fig. 1). Lanes contain (left to right): lane 1, negative control plant R0-CB006; lanes 2-6, R0 plants CB111, CB145, CB146, CB151, and CB183; lanes 7-12, R0 plants CB114, CB100, CB118, CB138, CB140, and CB142.
Figure 3. Southern blot analysis of transgenic tobacco plants and selected progeny transformed by pPHI456 or pCB209. Genomic DNA was digested with NheI and probed with the *uidA* gene. Lanes contain the following (left to right, 1-13): control plant R0-CB006, 2.5-kb pPHI456 fragment, R0-CB122, R1-CB122.3, R0-CB173, R1-CB173.7, R0-CB139, R1-CB139.2, R1-CB139.9, R0-CB153, R1-CB153.3, R1-CB153.5, and R1-1CB53.7.
Figure 4. Southern blot analysis of transgenic tobacco plants and selected progeny transformed by pPHI456 or pCB209. Genomic DNA was digested with *NheI* and probed with the *uidA* gene. Lanes contain the following (left to right, 1-15): Undigested genomic DNA of Ro-120, control Ro-CB186, Ro-CB171, R1-CB171.5, R0-CB101, R1 plants of CB101 (lanes 6-15); 01, 02, 03, 04, 05, 06, 07, 08, 09, and 10.
Figure 5. Southern blot analysis of transgenic maize calli transformed by pPHI485 or pPHI502. Genomic DNA was digested with *BglII* and *BamHI* and probed with the *uidA* gene.
Figure 6. Common modular elements in TBS. Modular elements are diagramed schematically, with sequence elements depicted by symbols. Major DNA unwinding elements were predicted as 100 bp regions at 50mM salt, 250pM DNA, in which the Tm 10-20°C is lower than average for the sequence. Minor DNA unwinding elements, are those in which the overall Tm of 7-10°C is lower than average for the sequence. ARS sequences matching 9, 10, or 11 bases of the 11 bp ARS consensus sequences of WTTTATRTTTTW and WRTTTATTTAW are noted. Sequences matching ≥14/16 and ≥15/17 bp of the pyrimidine tracts, YYYYYYYYYCTTTYYYY and YYYYYYYRCCCYYYYY are indicated. Sequences matching 9 or 10 bases out of 10 bp consensus sequences for scaffold-associated regions: AATAAAAYAAA, TTWTWTTWTT, WADAWAYAWW, and TWWTDTTWW. Sequences matching 13 out of 15 bp for the consensus topoisomerase II binding site element, GTNWAYATTNATNNG are indicated.
Major DNA Unwinding Element

Minor DNA Unwinding Element

ARS Consensus (≥9/11 match)

Pyrimidine Tracts (≥15/17, ≥14/16 match)

Scaffold-Associated Regions (≥9/10)

Topoisomerase II binding site (≥13/15)

TBS EcoRV fragment (1.1 kb)

Transcription Enhancer element GTGGTATG
SUMMARY DISCUSSION

The development of efficient means for transferring foreign genes into plant germ lines will be an important tool for understanding the structure, function and developmental regulation of plant genomes. This study shows that the soybean apical and de novo meristem is an appropriate target for direct DNA transfer using microprojectile bombardment. Delivery of DNA to meristem cells results in the persistence and expression of exogenous DNA. These DNA sequences can be recovered from their developmental descendants, which includes cells capable of giving rise to germ line transmission. Transformation frequencies can be increased in both monocotyledonous and dicotyledonous plants by utilizing linked DNA fragments such as TBS.

Early events in the development of multiple shoots

The earliest event observed in the formation of de novo shoots from soybean embryonic axes was an interruption in chromosomal DNA synthesis within 3 hr of a transient exposure to the cytokinin, BAP. Exposure to BAP was required for formation of new shoots; however, it was unnecessary for continued shoot growth and development to maturity. We postulate that interruption of DNA synthesis, either directly, by interfering with DNA replication, or indirectly, by preventing entry into S-phase, causes this reprogramming of cells.

The change of development induced by BAP could result from a transient inhibition of DNA synthesis. BAP could transiently inhibit DNA
synthesis by interference with DNA precursor synthesis (Johnston et al., 1986; Schimke, 1988), by incorporation into chromosomal DNA that interferes with DNA replication (Kornberg and Baker, 1991; Fry and Loeb, 1986), or by a block at G1-S phase of the cell cycle (reviewed by Pardee, 1991; Laskey et al., 1991; Murray and Kirschner, 1991).

In vivo, very few plant cells are triggered to express alternative developmental patterns. Results from the first section suggest that, at the time of germination, the developmental fate of cells or groups of cells in the apical meristem, is not firmly established. The development of these cells can be significantly affected by intra- and extracellular signals. Cytokinins appear to be primarily responsible for formation of the initial meristematic zones and not for later shoot organogenesis.

Persistence and expression of exogenous DNA in developing and maturing multiple shoots

Gene expression was observed in 93% of bombarded soybean axes within 24 hr, but persisted in only about 1% of the plants until maturity. Since there is little or no control over which tissues receive exogenous DNA when bombarded, the need for identification, multiplication and harvest of selected tissues is evident. This also includes the recovery of progeny from specific cells giving rise to germ line transmission (LII, Miksche, 1960). Parameters identified to affect the frequency of expressing cells over extended periods of development were DNA concentration, time and number of bombardments, vector construction, and identification of transformed tissues.
Clonal analysis in nodes of the R0 plants revealed that exogenous DNA persisted and expressed in too few nodes for efficient recovery in the germ line. This method does present itself to cell lineage analysis and molecular analysis; however, the frequency of recovering transformation events must be increased. The use of sequences to increase nuclear localization, retention, amplification, and/or replication should increase the recovery of transformants in germ lines.

Increasing the frequency of transformation

Petunia TBS increased the transformation frequency 7.8- to 16-fold in tobacco, and 1.7- to 2.4-fold in maize. Although TBS contains a well defined transcription enhancer element, no significant differences were observed on the gene expression levels. TBS did not appear to alter the complexity of integration patterns of exogenous DNA in either tobacco or maize. In tobacco, transformants usually contained at least one copy of the introduced gene in a non-rearranged form, and often contained multiple copies of the gene in rearranged forms as well. In maize, however, most transformants contained only rearranged copies. In tobacco, TBS had no significant effect on the segregation of the exogenous genes in R1 progeny, or on the linkage of two genes introduced in the same construct.

The increase in the transformation frequency may be the effect from (1) the various modular origin elements found in the TBS sequence (Buising and Benbow, 1992c; Benbow et al., 1992); (2) nuclear localization sequences (Howard et al., 1992), nuclear retention sequences (Krysan and Calos, 1990), amplification elements (Wegner et al., 1989), and/or an increased potential
for homologous (Puchta and Hohn, 1991) or illegitimate recombination activity (Pfeiffer and Vielmetter, 1988).

Microprojectile bombardment should offer an alternative route for direct DNA delivery in intact plant cells and subsequent recovery of transgenic plants and progeny. The the use of TBS to increase the frequency of recovering chimeric soybean multiple shoots (1%) was also examined. The potential 7-fold increase of transformed, but chimeric multiple shoots required too large an effort to recover transformed progeny (20,000 control plants would be required, for example) for a single student, so I did not attempt to quantitate the increase in transformation frequency in soybean.

**Future Experiments**

The optimization of transformation and recovery will provide a valuable tool that will enable us to better understand molecular, cellular and developmental cell events. Many different parameters involved in the transformation process must be adjusted to maximize the frequency of stable transformation in the plant genome. Future experiments with *de novo* multiple shoots may include studying the effect on growth and differentiation by the alteration of endogenous cytokinin levels (Medford *et al.*, 1989); inhibition of DNA replication (Schimke, 1988), and/or examination of genes that interact with the cell cycle (Laskey *et al.*, 1991; Murray and Kirschner, 1991). The study of cell differentiation in *de novo* meristems could be accelerated if one could develop a precise targeting system to specific cells, and maintain the ability to target those cells *in vivo*. The use of specific DNA sequences that provide nuclear retention (Krysan and Calos, 1991),
amplification (Wegner et al., 1990), or localization (Howard et al., 1992) of exogenous DNA would be more effectively studied in a system with a higher frequency of recovery, such as microprojectile bombardment of cycling somatic embryo cultures (Buising et al., 1992e) or an embryogenic cell suspension of soybean.
GENERAL REFERENCES


Buising, C. M., Shoemaker, R. C., and Benbow, R. M. 1992a. Early events of multiple shoot development in soybean embryonic axes treated with the cytokinin, 6-benzylaminopurine. To be resubmitted.


for homologous recombination between injected plasmid DNA molecules.


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A special thank you goes to my husband, Bob, for his patience.

This work is dedicated to the memory of my father, Robert J. Myers.