Genetic Transformation and Functional Promoter Analysis in Maize (*Zea mays* L.).

by
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ABBREVIATIONS

ABA	Abscisic acid
Bar	PAT gene from *Streptomyces sp.*
BSA	Bovine serum albumin
CaMV 35S	Cauliflower Mosaic Virus 35S RNA transcript
CTAB	Cetyldimethylethyl- ammonium bromide
2,4-D	2,4-Dichlorophenoxyacetic acid
DNA	Deoxyribonucleic acid
DTT	dl-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GA	Gibberellic acid
GUS	ß- glucuronidase
gusA	ß- glucuronidase gene from *E. coli*
L-PPT	L-phosphinothricin
MES	2-(N-Morpholino)ethane sulfonic acid
MOPS	3(N-Morpholino)propane sulfonic acid
MU	4- methylumbelliferone
MUG	4- methylumbelliferyl ß- D- glucuronide
NAA	Naphthaleneacetic acid
neo	Neomycin phosphotransferase II gene from Tn 5
NOS	Nopaline synthase
NPT II	Neomycin phosphotransferase II
PAT	Phosphinothricin acetyltransferase
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
T-DNA	Transfer DNA from *A.tumefaciens*
TEMED	N.N.N'.N'-Tetramethyl ethylene-diamine
Tris	Tris(hydroxymethyl) aminomethane
uidA	ß- glucuronidase gene from *E. coli*
X-gluc	5-bromo-4-chloro-3-indolyl β-D-glucuronide
Zea	Zeatin

CONTENTS

ABSTRACT

An efficient genetic transformation system based on the highly embryogenic maize cell line HE/89 has been established. The application of optimized PEG-mediated transformation protocol has resulted in a reproducible recovery of 10-20 (up to 200) independent transgenic clones from one million of treated protoplasts. Four different selection schemes utilizing kanamycin, geneticin (G418) and L-phosphinothricin as well as vital staining of transformed β-glucuronidase (GUS) gene expressing protoclones have been successfully employed to obtain over 140 independent transformed plant lines. Transgenic nature of the selected clones has been confirmed by molecular analysis (PCR, Southern hybridization) both in primary transformants and in the seed progeny.

Functional activity of different promoter sequences has been analysed in transformed maize cells and tissues. A chimeric promoter composed of viral 35S promoter derived enhancer elements in combination with the strictly tissue-specific wheat α-amylase gene promoter has conferred a distinct pattern of expression of the bacterial ß-glucuronidase gene. Cell division- dependent activity of a wheat H4 histone gene promoter has been demonstrated for the first time in transgenic maize that can be an indication of similarities in transcriptional mechanisms of these two cereal species.

The presented data indicates the existence of *cis*-interactions between promoter sequences as a general cell type independent phenomenon in control of gene expression. The obtained information confirms also the observation about the lack of correlation between the copy number of the integrated reporter gene per host genome and the level of its expression in plant cells.

1. INTRODUCTION.

Recent progress in development of technologies for genetic transformation of maize has opened the way for extensive use of gene fusions in studies on regulation of gene expression, both in cultured cells and in various tissues of regenerated plants. Maize protoplasts have been frequently utilized as experimental system for assay of transient expression of gene constructs, introduced either by electroporation or by polyethylene glycol (PEG) (Fromm *et al.*, 1986; Maas and Werr, 1989; Planckaert and Walbot, 1989; Vasil *et al.*, 1989). The application of various resistance marker genes has also made it possible to select transformed callus tissues and analyze function of different regulatory DNA sequences (Huang and Dennis, 1989; Lyznik *et al.*, 1989). Several laboratories have demonstrated the potentials in the use of microprojectile bombardment to transform embryogenic maize tissue cultures (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Walters *et al.*, 1992).The method also known as biolistics has been shown to deliver DNA, and results in the transient and stable expression of genes in cultured maize cells. Furthermore, the production of transgenic maize plants expressing agronomically significant traits was also achieved by this method (Koziel *et al.*, 1993). Regeneration of fertile transgenic maize plants was recently reported by D'Halluin *et al.* (1992) too, who electroporated plasmid molecules into wounded tissues of immature zygotic embryos or of embryogenic callus cultures. Both of these methods are proposed to be widely applicable for transformation of different breeding stocks. However, the regeneration capability was shown to be genotype-dependent in several tissue culture studies (Tomes and Smith 1985; Lupotto 1986; Petersen *et al.*, 1992). Moreover, it can be stated that the main drawback of the biolistic transformation of maize is an inability to produce multiple independent transgenic lines, which is essential when analyzing statistical events or producing transformants on a commercial scale. So far amongst the cereals the efficient system for high frequency generation of fertile transgenic plants is available only for rice. PEG-mediated direct gene transfer has resulted in transformation rates of 2-$4x10^{-4}$ which amounts to approximately 200-400 hygromycin-resistant colonies per million of treated protoplasts (Li *et al.*, 1991). While in two reports on maize transformation, however, from the several independent experiments 14 or 25 independent transgenic clones were recovered by Fromm *et al.* (1990) and Gordon-Kamm *et al.* (1990) respectively.

The continuous improvement of tissue culture techniques has contributed to the progress in plant regeneration from cultured maize protoplasts (Rhodes *et al.*, 1988; Shillito *et al.*, 1989; Prioli and Söndahl, 1990; Petersen *et al.*, 1992). A unique maize tissue culture system has been developed by Morocz *et al.*, (1990), that allows the maintenance of an efficient and reproducible plant regeneration from embryogenic maize

protoplasts over a period as long as 4 years. This synthetic genotype (HE/89) combines several advantageous traits required for rapid release of protoplasts, considerably high division frequency of protoplast-derived cells, embryogenic potential and regeneration of normal, fertile plants.

It is apparent, that the current lack of the efficient ways to regenerate a large number of transgenic plants for characterization of different regulatory elements and establishment of a collection of effective promoters with wide range of specificity in expression pattern is one of the major limitations in genetic transformation of cereals, including maize. The most commonly used promoter also in monocot transformation, is the cauliflower mosaic virus (CaMV) 5' regulatory fragment, containing 400 to 1000 base pairs of 35S upstream sequence. The 35S promoter of CaMV confers a high level of expression in a variety of cells when transferred into either dicot or monocot plants (Fromm *et al.*, 1985; Jefferson *et al.*, 1987; Lyznik *et al.*, 1989; Odell *et al.*, 1985; Rhodes *et al.*, 1988; Somers *et al.*, 1987; Terada *et al.*, 1990). Expression studies with dicot transgenic plants have revealed, that the 35S promoter has a modular organization, and synergistic interactions exist between the subdomains (Benfey *et al.*, 1989a; Benfey *et al.*, 1989b). In transgenic tobacco plants, the duplication of 250 bp of upstream sequences acted as a strong enhancer of heterologous promoters (Kay *et al.*, 1987). Transcription driven by the -208 to -46 fragment was increased by multimerization of this 162 bp element, that can function as enhancer in tobacco (Fang *et al.*, 1989).

At present, there is only limited information about functional analysis of gene fusions with monocot promoters in transgenic cereal plants. Frequently, the monocot promoters were studied in heterologous systems, after introduction of chimeric genes into dicot plants (Ellis *et al.*, 1987; Nagy *et al.*, 1987). Monocot promoters, such as the maize *Adh1*, the rice actin1 promoter or the wheat H3 histone gene promoter have been analyzed preferentially in transgenic rice plants (Vasil *et al.*, 1989; Kyozuka *et al.*, 1991; Zhang *et al.*, 1991; Terada *et al.*, 1993).

In the present study, an efficient transformation system for maize is described, that has allowed the regeneration of fertile transgenic plants from over one hundred and forty independent clones, after direct DNA uptake into embryogenic protoplasts. Selection protocols based on kanamycin, geneticin (G418) or L-phosphinothricin resistance or vital staining for GUS activity, have been successfully utilized for identification of transformants. The described transformation system has allowed the functional analysis of different promoter sequences including a chimeric promoter construct, containing the doubled enhancer region from the CaMV 35S promoter linked to the strictly tissue-specific wheat α-amylase promoter as well as wheat H4 histone gene promoter in transgenic maize plants. Based on specific enzyme activity data and histochemical localization of GUS activity, it is proposed that the viral enhancer can activate a

heterologous promoter also in this monocot plant. The study also demonstrates for the first time, differential activity of wheat histone gene promoter in maize cells. The obtained results suggest similarity in transcriptional mechanisms between the two species.

2. REVIEW OF THE LITERATURE.

2.1. Maize *(Zea mays* L.) as an object of biological research.

Maize (or 'Indian corn') is a large kerneled, highly domesticated, vigorous annual plant of tropical origin, which is well suited for biological research, including molecular studies, because of its favorable biological features, its economic importance and the resulting attention focused upon it, as well as the considerable amount of genetic and cytogenetic information currently available.

Maize is a member of the grass family, the *Gramineae*, and as in all grasses, most of the plant body is leaf tissue. Its main stem, or culm, is a slender, segmented shaft similar to the stalk of a bamboo or sugarcane. The enlarged joints along the stem, the nodes, mark the points of leaf attachment; the stem segment between nodes is called the internode. Each node bears a single leaf in a position opposite that of the neighboring leaf, giving the plant two vertical rows of leaves (so-called distichous phyllotaxy).

Maize has unisexual flowers. The male (staminate) flowers are located at the apical tip of the main stem in the tassel, a branched inflorescence. the female (pistillate) flowers are found in one to several compact ears, located on the ends of short branches near the middle of the stem. This partitioning of male and female flowers in separate structures distinguishes maize from other cereals and is one of the principal reasons that has resulted in the convenient exploration of its genetics. Thus making controlled pollinations in maize requires only placing a bag over the tassel and ear shoot, while in other cereals it is necessary to emasculate each flower used as a female parent. Another biological feature that aids enormously in performing genetic studies is the size of an ear. A normal, vigorous maize plant will usually produce an ear bearing 400 to 600 kernels with embryos consisting of many thousands of cells and often having a fresh weight of 50 mg or more. The kernels can be cross- or self-pollinated in a single act, so that very large numbers of progeny can be obtained with a minimal amount of efforts.

These distinguishing features of maize have promoted development of a detailed genetic map (Sheridan, 1982) and the Nobel Prize awarded discovery of transposable elements by Barbara McClintock (McClintock, 1984).

Molecular markers such as isozymes and RFLPs (restriction fragment length polymorphisms) have become powerful tools in the genetic analysis of various traits at DNA level and for establishing linkages with target gene(s) governing agronomical traits to facilitate the marker based selection for complex characteristics that are largely influenced by the environment and are difficult to select in conventional crop improvement programs. The RFLP map-based gene cloning could be employed to clone genes for which gene products are unknown such as disease resistance. Both, isozyme

markers and RFLPs have been successfully applied for establishing genetic linkage maps and localization of quantitative trait loci in maize (Helentjaris *et al.*, 1986; Edwards *et al.*, 1987; Stuber *et al.*, 1987; Weber and Helentjaris, 1988).

The ability to introduce foreign DNA into genome of maize plants would complement the sophisticated genetic systems currently available for this species and would facilitate research into such processes as genetic transformation and gene regulation.

2.2. Genetic transformation.

2.2.1. Overview.

The main focus of this part of the work will be on different aspects and problems of genetic transformation of cereal crops that is still one of the most difficult areas of modern plant biotechnology. The systems of transformation of dicotyledonous plants, which are well developed and rather efficient have been discussed in details in numerous recent reviews (Weising *et al.*, 1988; Davey *et al.*, 1989; Uchimiya *et al.*, 1989; Walden and Schell, 1990; Blaich, 1992; Gasser and Fraley, 1992; Van Wordragen and Dons, 1992; Feher and Dudits, in press).

The first transgenic plants have been produced less then a decade ago (Horsch *et al.*, 1984, De Block *et al.*, 1984), but at the present time genetic transformation techniques are available for most of the important plant species including cereal crops (Gordon-Kamm *et al.*, 1990; Christou *et al.*, 1992; Somers *et al.*, 1992; Vasil *et al*, 1992; Koziel *et al.*, 1993). There are already more then twenty marker genes for use in plant cells, which have been successfully employed for production of transgenic plants of over 30 crop species (Walden and Schell, 1990).

Plant cells differ in their capability to respond to triggers, a phenomenon, called competence. Cells capable of regenerating transgenic plants must be competent both for integrative transformation and regeneration. Although, theoretically, all plant cells are totipotent, the conditions for plant regeneration are not fully known for every plant tissue culture system.

Plant tissues are composed from cells competent for many responses. However, since the competence for transformation and regeneration are the most important ones, it should be taken in consideration that few plant cells are competent for both integrative transformation and regeneration. Most of the cells are competent either for transformation or for regeneration; a rather large portion of the cells are potentially competent, thus they can be triggered to shift towards reinitiation of the ontogenic program (Kahl, 1982; Dudits *et al.*, 1991) if they are exposed to a proper treatment; a

variable portion of cells is in a non-competent stage. The relative composition of particular cell population is determined by the genotype, the tissue origin of the organ, the developmental state of the organ, the physiological state of cells etc.

As shown by a large number of experimental observations wounding and hormone shock are potent triggers for shifting of potentially competent cells towards a competent state. Plants and tissues differ in their wound responses. Only plants with a pronounced wound response develop larger populations of wound-adjacent competent cells for regeneration and transformation. Monocots with noticeable wound response (e.g. *Asparagus*) are as easy to transform as dicots with wound response; and dicots without proper wound response (e.g. grain legumes?) are probably as difficult to transform as cereals (Potrykus, 1991).

For successful integrative (or stable) genetic transformation, the DNA molecules, carrying genetic information have to be transported across the cell wall and/or plasma membrane. If the DNA sequences are not degraded in the cytoplasm they have to reach the cell nucleus, where the genetic information can be expressed. The integration of the foreign DNA into host genome through illegitimate recombination resulting in a genetic modification is a final step of the stable genetic transformation.

The question of how the events leading to transformation occur is still largely unsolved, although the experiments of Okada *et al.*, (1986) and Iida *et al.*, (1991) on transformation of cells synchronized at different stages of the cell cycle, can give an insight. They found that cells synchronized at the mitosis are transformed several times more efficiently than at any other stage. Whether this is connected with the dissolution of the nuclear membrane and the liberation of chromosomes into the cytosol, thus rendering the genome exposed to incoming genetic information? Is the wound-response and its cell-cycle reactivation program influencing competence by this mechanism or other factors are also involved? These questions are still far from being answered.

As it has been outlined by Potrykus (1991) the requirements of proof of integrative transformation include: 1. Controls for treatments and analysis; 2. a tight correlation between treatment and predicted results; 3. a tight correlation between physical (e.g. Southern blot) and phenotypic (e.g. enzyme assay) data; 4. *complete* Southern analysis containing (*a*) the predicted signals in high-molecular-weight DNA, including hybrid fragments between host DNA and foreign gene, and the presence of the complete gene, and (*b*) evidence for the absence of contaminating DNA fragments or identification of such fragments; 5. data that allow discrimination between false positives and correct transformants in the evaluation of the phenotypic evidence; 6. correlation of the physical and phenotypic evidence with transmission to sexual offspring; and 7. molecular and genetic analysis of offspring populations. To these criteria that are correct and comprehensive the factor of reproducibility can be added, as there are some reports

(Hess *et al.*, 1990; Gould *et al.*, 1991; D'Halluin *et al.*, 1992) which contain all the elements delineated by Potrykus but were performed once and so far there is no information that they were repeated by the authors or other research groups.

When looking through the current publications in cereal genetic transformation it is apparent that only the experiments utilizing two methods, namely: direct DNA transfer into protoplasts (through chemical transformation, electroporation etc.) and biolistics (bombardment of cells or tissues by DNA-coated metal particles) meet the criteria outlined above. All other reports either fail to provide complete set of necessary proofs or have not been repeated so far. Of course, in the future some new approaches will emerge or some of presently unreliable protocols (or their modifications) might turn out to be more efficient and reliable, but at the present state-of-art it can be proclaimed that there are only two methods successfully exploited by scientific community for genetic transformation of cereal plants and they also have intrinsic drawbacks.

Since the early *Agrobacterium*-mediated genetic transformation experiments no gene transfer approach has met with so much enthusiasm, and in no other gene transfer approach has there been a comparable investment in experimentation and manpower as in the biolistics or particle gun bombardment approach. The method allows the transport of genes into many cells at nearly any desired position in plant without too much manual effort. The enormous investment into this technique has paid off, and fertile transgenic plants have been recovered (Klein *et al.*, 1989; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Vasil *et al.*, 1992; Somers *et al.*, 1992) that would have been difficult to produce by other methods. Generally, one to twenty independent transgenic cell lines gave rise to fertile plants in these experiments. (One wonder whether with a similar investment other methods might not have been made successful, too) (Potrykus, 1991).

Biolistics has a low transformation efficiency. If one compares (*a*) the number of transgenic cell lines and fertile transgenic maize plants recovered from biolistic treatment in large-scale experiments using embryogenic suspension cultures (Klein *et al.*, 1989; Gordon-Kamm *et al.*, 1990; Fromm *et al.*, 1990; Spencer *et al.*, 1990) with (*b*) those recovered from a comparable small-scale experiments on direct gene transfer to protoplasts isolated from such embryogenic suspensions (Rhodes *et al.*, 1988a; Lyznik *et al.*, 1989; Huang *et al.*, 1989; Armstrong *et al.*, 1990), then the low yield of independent transgenic clones from the biolistics experiments is surprising. In recalcitrant plants like cereals with limited amount of cells competent for transformation-regeneration events, the particle has to reach those rare cells by a random hit, and the DNA has to integrate into the genome of these cells, while protoplasting provides free access to all competent cells of the population under treatment. Furthermore, it has been found that 80% of the DNA-carrying metal particles bombarded into the cells end up in the vacuole (Yamashita

et al., 1991), and the cell injury from high-velocity microprojectiles (Russell *et al.*, 1992) should also be considered as a limiting factor of the efficacy of biolistic transformation.

Another potential problem and limitation of biolistics is connected with the use of multicellular structures for transformation experiments that results in recovery after selection of chimeric tissues composed of a mixture of transformed and non transformed cells. Most of the published reports in biolistics report about obtaining chimeras in the transformation experiments (Klein *et al.*, 1989; Fromm *et al.*, 1990; Spencer *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Christou *et al.*, 1991; Christou *et al.*, 1992; Spencer *et al.*, 1992; Vasil *et al.*, 1992). The recent publication of Schmulling and Schell (1993) demonstrating regeneration of periclinal chimeras from *Agrobacterium*-transformed tobacco leaf disks has shown that even constant selection pressure during callus proliferation, plant regeneration and root formation can not prevent chimerism of transformed multicellular tissues. The situation can be different if somatic embryos are produced from the transformed protoplasts.

Methods of genetic transformation through direct DNA uptake into protoplasts rely on embryogenic suspension cultures and require a well-established plant regeneration protocol from protoplasts. This, however, has now been achieved in a considerable number of plant genotypes (for review see Fehér and Dudits, in press). Protoplasts can take up DNA molecules if treated with polyethylene glycol (PEG) and/or electric pulses (Potrykus *et al.*, 1985; Saul *et al.*, 1988). Some new genetic transformation methods have been developed recently utilizing cationic liposomes like lipofectin (Antonelli and Stadler, 1990; Sporlein and Koop, 1991) or ultrasonication (Zhang *et al.*, 1991) for foreign DNA delivery into protoplasts.

The protoplast isolation procedure can promote a shift of potentially competent cells into the competent state through induction of a wound response by enzymatic digestion of the plant cell wall. The exogenous DNA introduced into protoplasts is readily integrated into genomic DNA through non-homologous recombination. When competent cells are transformed regeneration of fertile transgenic plants can be achieved (De Block *et al.*, 1984; De Block *et al.*, 1987; Rhodes *et al.*, 1988; Saul *et al.*, 1988; Terada and Shimamoto, 1990; Li *et al.*, 1991; Sporlein and Koop, 1991). The genetic transformation of embryogenic protoplasts permits obtaining of hundreds of independent transformation events in a single experiment. Moreover, the direct gene transfer to protoplasts is a convenient method for fast assessment of promoter activity by analyzing of transient gene expression.

2.2.2. Transient gene expression and stable genetic transformation.

Transient expression analysis in cultured mammalian cells through assaying the expression of non-integrated foreign genes in non-dividing cells 2-4 days after induction of DNA uptake has proved to be a valuable tool for the analysis of the promoters and the DNA binding proteins associated with them (Heard *et al.*, 1987; Evans, 1988; Ptashne, 1988). At present, this approach is being extensively exploited by plant scientists. Regulated expression from a maize alcohol dehydrogenase promoter (*Adh1*) in response to anaerobism in maize protoplasts has been described (Howard *et al.*, 1987) as well as the hormonal control of transient expression of a wheat embryo gene (Em) introduced into protoplasts derived from rice suspension culture has been shown (Marcotte *et al.*, 1988). Transient expression assay system after biolistic transformation Klein *et al.*, (1989a) has also been utilized in studies on the regulation of anthocyanin biosynthetic genes within intact aleurone and embryo tissues of maize. It has been observed that the foreign genes have been regulated in a manner similar to the endogenous genes. Transient expression analysis in oat aleurone protoplasts of chimeric genes composed of a wheat α–amylase promoter sequence positioned upstream to the coding region of the GUS reporter gene have demonstrated hormonal regulation of the gene fusions expression in a manner similar to the expression of the endogenous oat α-amylase genes (Huttly and Baulcombe, 1989). Functional transient analysis of the promoter sequences containing deletions or replacements have revealed the regions potentially containing *cis* regulatory elements (Huttly *et al.*, 1992). These results were in a good agreement with the molecular data obtained by DNase 1 footprinting method (Rushton *et al.*, 1992).

Transient expression experiments provide an opportunity to make a rapid assessment of the functional activity of different promoters, but there are certain limitations of the system. First, the protoplast is not a 'normal' plant cell. The lack of a cell wall and the physiological stress caused by the combined action of cell wall-digesting enzymes, the change of surrounding milieu, the signals released by the wounded and dying cells can affect the metabolism of the cell and produce some misleading results (Harkins *et al.*, 1990; Prof. Ives Meyer, pers. communication). The successful uptake of plasmid DNA and the subsequent expression of the introduced genes are influenced by many parameters. Some of them are of technical characteristic: purity and viability of protoplasts, quality of plasmid DNA and reagents, the physiological state of the plant source etc.

It is generally accepted that the amounts of transcripts are considerably influenced by *cis*-acting sequences, located mainly on the 5' regulatory region of the gene. However, other factors involved in control of mRNA stability may also have the

contribution to the expression level. Impact of *cis*-acting enhancers that have been found in the regulatory regions of most of the studied genes cannot account for the huge difference in the expression of the same chimeric gene in individually regenerated transgenic plants. Several factors might play a considerable role:

1. Position effect. It is well-documented that in animal systems, the expression of foreign genes is influenced by their chromosomal position (Spradling and Rubin, 1983; Levis *et al.*, 1985). There are increasing number of reports demonstrating similar events in plant systems (Elkind *et al.*, 1990; Kommari, 1990). This position effect might be related to chromatin structure at the integration site and/or the location of endogenous *cis*-acting regulatory sequences nearby.
2. Copy number. The impact of copy number on gene expression varies from one system to another. While some authors have found that the copy number of the independently integrated genes can be in positive correlation with the level of gene expression (Stockhaus *et al.*, 1987; Deak *et al.*, 1988; Newbigin *et al.*, 1990), others were not able to find such a correlation (Sanders *et al.*, 1987; Jones *et al.*, 1987; Czernilofsky *et al.*, 1987; Klein *et al* ., 1989; Steber and Willmitzer 1989; Tada *et al.*, 1990; the present study (see Results p.47 and Discussion p.64). Hobbs *et al.* (1990) have found that single copy results in higher expression when compared with multiple insertions of the gene. The last phenomenon has been linked to the methylation of genes.
3. DNA methylation. Wild-type T-DNA is shown to be subjected to methylation (Amasino *et al.*, 1984) and methylation of controlling sequences may cause inactivation of foreign genes both in animal systems and in plants (Sellem *et al*, 1985; Amasino *et al.*, 1984; Hobbs *et al.*, 1990). The methylated state might be stably inherited through meiosis, but it can be reactivated by *in vitro* culture (John and Amasino, 1989).
4. Trans-acting factors. Promoter-binding proteins and other regulatory factors involved in signal transduction can differ from species to species, and this might cause altered expression of a transgene.
5. Factors such as the physiological state of the plant, plant to plant variability and epigenetic effects in the primary transformants may result in disturbances of the gene expression.
6. Anatomical and physiological characteristics and differences in the secondary metabolism may play a considerable role and contribute to the production of controversial results when various transformed plant species are compared (to some extent reviewed by Blaich, 1992).

It has to be considered that competence for integrative gene transfer is rather different from competence for transient expression.

Ebert *et al.*, (1987) have demonstrated that transient analysis did not clearly distinguish downstream promoter elements (CCAAT-box and TATA-box) of nopaline synthase (*nos*) promoter. It appeared that crippled promoters show unusually high activity in the transient assay system. However, the upstream elements behaved similarly in both transient and stable transformation assays in that deletion of the entire upstream regulatory element resulted in no promoter activity and that duplication of the element significantly enhanced the promoter strength.

Comparison between transient expression and stable transformation experiments in two *Nicotiana* species have indicated that despite the constant and large differences in stable transformation efficiency in favor of tobacco, *N. plumbaginifolia* exhibited significantly higher levels of gene activity in transient expression assays. These results suggest that the major differences in transfection ability were not in DNA uptake, but rather in the specific fate of the transforming DNA sequences in the two species (Negrutiu *et al.*, 1990).

Another genetic transformation related phenomenon, characteristic particularly for cereal species, is an elevated gene expression level in the cells transformed by gene constructs containing introns positioned in the N-terminal non-translated regions, which has been described first in maize by Callis *et al.*, (1987). Most of the subsequent experimental data concerning the influence of intron sequences on gene expression have been established also in maize transformation system. Because of the potential significance of these findings for the genetic transformation studies in practical terms and for understanding of the molecular mechanisms of gene expression in cereal plant species a more detailed look is being given at them.

2.2.3. Introns and enhanced gene expression in cereals.

The discovery in 1977 that eukaryotic genes are interrupted by introns (intervening sequences, IVSs) catalyzed a new era of study in the control of gene expression. An initial observation in plants indicated that introns are not required in order to achieve normal expression levels. The seed storage protein phaseolin was expressed at similar levels with or without its native introns (Chee *et al.*, 1986). Later, it was demonstrated that there is a dependence on introns for adequate expression of the maize alcohol dehydrogenase gene or a reporter gene, chloramphenicol acetyltransferase (CAT) (Callis *et al.*, 1987). Subsequently these findings were confirmed by other authors in experiments utilizing various reporter genes and introns (Lee *et al.*, 1989; Oard *et al.*, 1989; Vasil *et al.*, 1989; Tanaka *et al.*, 1990; Luehrsen and Walbot, 1991; Maas *et al.*, 1991). However, it has been determined that not all introns can enhance the expression in maize protoplasts of genes lacking introns (Sinibaldi and Mettler, 1992). Enhanced

activity was not found if the intron was placed before the promoter or in the 3' untranslated region of the gene (Mascarenhas *et al.*, 1990). The experimental data have established that in monocot species introns can increase gene expression from 10-fold to as much as 1000-fold (Mascarenhas *et al.*, 1990; Maas *et al.*, 1991). The degree of intron-mediated enhancement in dicot protoplasts is not as pronounced as it is in monocots. In fact, most introns that have been surveyed in dicot protoplasts significantly reduced expression, compared to expression of the no- intron control. One intron that yields a slight enhancement (1.05-2.00-fold) is the IVS6 from the *Adh1* gene of maize.

The monocot splicing machinery is able to process faithfully introns that do not contain signals known to be absolutely essential for intron processing in other organisms like dicot plants, vertebrates and yeasts. Introns inserted in 5' untranslated leader regions of the analyzed genes have been shown to increase gene expression in cereals through increase of steady-state mRNA level. The mechanism by which the introns enhance expression probably involves the efficiency of precursor RNA splicing and/or its transport to the cytoplasm (Sinibaldi and Mettler, 1992).

However, it should be taken into consideration that most of the experimental data on this phenomenon are based on transient expression studies and the influence of introns on gene expression in stably transformed plant cells is still a question to be clarified. Klein and coworkers, (1989) could not find a difference in the number of kanamycin-resistant maize calli recovered after bombardment with intronless and intron-containing plasmid constructions. In contrast, Tanaka *et al.*, (1991) have shown that the first intron of castor bean catalase gene, *cat-1*, positioned in the N-terminal region of the coding sequence of the GUS reporter gene increased the level of the enzyme activity 10 to 40-fold and 80 to 90-fold compared with the intronless construct, in transient expression assay and transgenic calli, respectively. However, the same intron containing plasmid construct did not increase the gene expression when it was introduced into tobacco cells.

Furthermore, the information is available revealing involvement of introns not only in quantitative regulation of gene expression, but also in more complex events. As it has been reported by Yoshimatsu and Nagawa (1989), the conditional introns exhibiting cold-sensitive splicing can be inserted into a target gene to allow regulation of the gene expression to become temperature-dependent.

2.2.4. Genetic transformation of maize.

To the best of my knowledge the first publications concerning maize genetic transformation appeared in the year 1985. De Wet *et al.* (1985) reported that they succeeded in transformation of maize using pollen as a vector. The idea was based on the observation that the pollen tube, at germination, lacks a cell wall near its tip. DNA

fragments might be absorbed through this pore into the pollen tube and possibly into the sperm cell enabling incorporation in the DNA of the fertilized egg cell. But in the same year Stanford and coworkers published negative results in attempts of pollen-mediated transformation (Stanford *et al.*, 1985).

Ohta (1986) applied DNA-pollen paste onto silks of maize plants. Genetic transformation was confirmed by phenotypic changes of kernels of a recipient plant. The author concluded that in certain cases fragments of the maize genomic DNA used for transformation were transferred and expressed in seeds. He traced the fate of transformants in a second generation and found that exogenous DNA taken into embryo can be inherited to the next generation but it will be unstable and often will be lost. Lack of molecular data confirming the genetic transformation and low frequency of 'transformants' in seed progeny make doubtful the fact of transformation in this study. Booy *et al.* (1989) repeating Ohta's experiments have not been able to recover any transformant and found that nuclease activity in germinating pollen is a main reason of foreign DNA degradation and they could not find a way to solve this problem. The applied DNA degraded within 5-10 min of incubation with pollen. The authors suppose that pollen-mediated transformation could be prospective if micro- injection of DNA into the sperm cells would prove to be possible. Another alternative might be the injection of DNA into microspores or into the male inflorescence before the division into two sperm cells has taken place, whereby the nuclease activity might be partly avoided.

Graves and Goldman (1986) have found synthesis of specific opines in maize tissues inoculated by *Agrobacterium.* Later on the sensitive reporter system of agroinfection was developed by Grimsley *et al.*, (1987) in which Maize Streak Virus is transferred into maize cells at the infection site concomitantly with T-DNA. Transformed plants were then recognized by the symptoms of viral infection. More recently, obtaining of the seed progeny of two chimeric maize transformants has been reported by Gould *et al.*, (1991), after *Agrobacterium* inoculation of isolated shoot apices.

However, there is little potential in *Agrobacterium*-mediated transformation for large scale production of fertile transgenic monocot plants because the key problem in *Agrobacterium*-mediated transformation of cereals probably lies neither with *Agrobacterium* (it transfers its T-DNA to cereals) nor with the host range (cereals are probably included) but rather with the availability and accessibility of cells competent for integrative transformation and regeneration (Potrykus, 1991).

After the confirmation of transient NPTII gene expression in maize protoplasts transformed by electroporation that has been reported by Fromm *et al.* (1985), numerous publications on transient gene expression analysis as well as stable transformation experiments have followed (Rhodes *et al.*, 1988a; Huang *et al.*, 1989; Lyznik *et al.*, 1989; Maas *et al.*, 1989; Planckaert *et al.*, 1989; Vasil *et al.*, 1989; Armstrong *et al.*,

1990; Last *et al.*, 1991; Luehersen *et al.*, 1991; Lyznik *et al.*, 1991; Maas *et al.*, 1991; Sinibaldi and Mettler, 1992; Kramer *et al.*, 1993). Although, protoplast based direct gene transfer methods have provided detailed information on conditions of transformation and co-transformation of maize cells, so far there have been only two publications on obtaining fertile transgenic maize plants after PEG-mediated DNA uptake into protoplasts (Golovkin *et al.*, 1993; Omirulleh *et al.*, 1993). Previously the scientists from Hoechst AG reported obtaining of L-PPT resistant maize transformants, but full size paper has not been published by the authors up to now (Donn *et al.*, 1990).

The biolistics method proved to be rather efficient both in transient expression studies (Klein *et al.*, 1989; Oard *et al.*, 1990; Reggiardo *et al.*, 1991) and for recovery of fertile transgenic maize plants (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Spencer *et al.*, 1992; Walters *et al.*, 1992; Koziel *et al.*, 1993). However, still two problems remain, namely: low efficiency of transformation and high number of chimeric lines appearing as a result of treatment and selection of multicellular structures (Klein *et al.*, 1989; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Spencer *et al.*, 1990; Spencer *et al.*, 1992).

So far fertile transgenic maize plants have been obtained utilizing selectable marker genes conferring resistance to chlorsulfuron (Fromm *et al.*, 1990), L-phosphinothricin (Donn *et al.*, 1990; Gordon-Kamm *et al.*, 1990), hygromycin (Walters *et al.*, 1992), kanamycin (D'Halluin *et al.*, 1992) and more recently to methothrexate (Golovkin *et al.*, 1993).

There are numerous publications (Ohta, 1986; Topfer *et al.*, 1989; Antonelli and Stadler, 1990; Asano *et al.*, 1991; Sporlein and Koop, 1991; Zhang *et al.*, 1991; Christou *et al.*, 1992) informing about the development of new methods or approaches to plant genetic transformation. The scientists from the Plant Genetic Systems N.V. (D'Halluin *et al.*, 1992) have described the transformation of regenerable maize tissues by electroporation. Wounded immature embryos were electroporated with a chimeric gene encoding neomycin phosphotransferase II (NPTII). Transgenic fertile plants were regenerated from 25 independent lines after kanamycin selection. However, some of the obtained transgenic lines exhibited chimeric nature.

Recently Gupta and Pattanayak (1993) from India reported plant regeneration from mesophyll protoplasts of rice using the green inner sheath between seedlings basal part and the base of first leaf. Protoplasts isolated from eight days old plantlets were cultivated in the presence of a feeder culture . It is an important achievement that can promote development of similar protoplast regeneration systems for other important cereal crops including maize that might greatly facilitate genetic transformation of these recalcitrant species.

2.3. Gene fusions.

Much of the attention and interest in modern molecular biology is focused on the regulation of gene expression. Factors influencing or mediating such regulation are often best studied using gene fusions (or chimeric genes). Gene fusions can be defined as DNA constructions (performed *in vitro* or *in vivo*) that result in the coding sequences from one gene (reporter) being transcribed and/or translated under the influence of the controlling sequences of another gene (controller). Gene fusions can be of two general types. Transcriptional fusions are defined as fusions in which all protein coding sequences are derived only from the reporter, while in translational fusions the polypeptide produced is the result of coding information provided by both controller and reporter. Most frequently, the reporter encodes an enzyme. Use of reporter enzymes can greatly enhance the sensitivity of gene fusion detection, and can also facilitate many types of functional analysis (Jefferson, 1987). Precisely constructed gene fusions simplify the way of the monitoring of gene expression with enhanced sensitivity. They facilitate the comparison of different or altered regulatory sequences and allow study of individual members of gene families without the interfering effect of another genes. Since many regulatory components are responsible for spatial and temporal restriction of gene activity, the use of gene fusions can be a tool of functional studies. Moreover, the analysis of large number of samples that is a basic requirement for the use of statistical methods might be an overwhelming task, unless routine, high resolution techniques are available similar to the very sensitive assays developed for detection of expression of the GUS gene fusions (see below). The study of functional activity of *in vitro* generated gene fusions followed by their introduction into the plant genome has a tremendous impact on modern plant molecular biology.

2.3.1. Reporter genes.

There are several basic requirements for a suitable reporter gene:

It must have an easily detectable and quantifiable product. Most of the reporter genes encode for an enzyme with activity that is not present in host cells or safely distinguishable from endogenous activities; the product must be stable under various physiological conditions; without interference with the cellular metabolism; the gene and its product must be well characterized biochemically and genetically; highly sensitive assays for detection must be available; the enzyme should tolerate aminoterminal fusions, to allow targeting into specific compartments within the cell and for studies on the events during processing and transport of mRNAs.

The following is a more detailed description of the reporter genes that have been used in experiments in the present study.

2.3.1.1. Neomycin phosphotransferase II (*neo*, NPTII) gene.

The neomycin phosphotransferase II (*neo* or NPT II) gene is one of the most extensively used reporters (Reiss *et al.*, 1981; Salmenkallio *et al.*, 1990; D'Halluin *et al.*, 1992), due to its suitability also as a selective marker. It detoxifies aminoglycoside antibiotics of neomycin family such as kanamycin (Km) and geneticin (G418) through phosphorylation. The aminoglycosides block protein synthesis causing a rapid death of the sensitive cells. The *neo* gene was isolated originally from the prokaryotic transposon Tn 5 (Davies and Smith, 1978; Beck *et al.*, 1982) and after several cloning steps, an expression vector was constructed and utilized successfully in various vector systems for the transformation and selection of a number of species (reviewed by Reynaerts *et al.*, 1988; Van Wordragen and Dons, 1992).

The *neo* gene can tolerate large amino-terminal fusions, which makes it suitable for studying organelle transport (Van den Broeck *et al.*, 1985). Two main problems can hinder the use of the NPTII gene as a reporter: the need to separate its activity from the background activity of kinases, phosphatases that can provide competing reactions, interfering with the quantification of enzyme kinetics, and the necessity to use large amounts of radioactive ^{32}P-ATP for detection of the gene activity.

2.3.1.2. Phosphinothricin acetyltransferase (*bar*, PAT) gene.

Bialaphos is now being used in agriculture as a non-selective herbicide. It is a tripeptide that is composed of two L-alanine residues and an analog of glutamic acid known as L-phosphinothricin (L-PPT). While L-PPT is an inhibitor of glutamine synthetase in both plant and bacteria, the intact tripeptide has little or no inhibitory activity *in vitro*. In both bacteria and plants, intracellular peptidases remove the alanine residues and release active L-PPT. L-PPT is chemically synthesized (Basta, Hoechst AG) while bialaphos is produced by fermentation of *Streptomyces hygroscopicus*.

Glutamine synthetase plays a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants. It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation and photorespiration. Inhibition of glutamine synthetase by L-PPT causes quick accumulation of ammonia which leads to rapid death of the plant cell.

S. hygroscopicus is utilized for the commercial production of bialaphos. Bialaphos is synthesized from three carbon precursors in a series of at least thirteen conversions. Many of the genes which code for these enzymes as well as for a function which positively regulates their transcription have been defined by blocked mutants. Cloned DNA fragments that either restore gene function in these mutants or confer resistance to bialaphos (i.e. *bar*) were mapped to an 18-kb gene cluster. The *bar* gene was isolated from *S. hygroscopicus* genomic DNA by Murakami *et al.* (1986). Analogous *in vitro* genetically modified gene was cloned from *Streptomyces viridochromogenes* by Hoechst AG (Donn *et al.*, 1990). The *bar* gene encodes a phosphinothricin acetyltransferase (PAT) which acetylates the free NH_2 group of L-PPT making it no more inhibitory to glutamine synthase and thereby prevents autotoxicity in the active protein producing organisms (Thompson *et al.*, 1987).

Presently, the *bar* gene is actively employed in genetic transformation experiments and can be considered as the best selective marker gene currently available for cereal crop transformation projects (Deceyser *et al.*, 1989).

2.3.1.3. ß-Glucuronidase (*gusA*, *uidA*, GUS) gene.

In the year 1987, Jefferson and his coworkers proposed the use of the bacterial gene *uidA* (or *gusA*) as a reporter in plant gene expression studies and due to the properties of the encoded enzyme (ß-glucuronidase) and the versatile assays it has became the most widely used reporter gene both for quantitative measurement (level of expression) and qualitative analysis (i. e. specificity of expression in tissues and organs) in numerous dicotyledonous and monocotyledonous species (Jefferson, 1987; Battraw *et al.*, 1990; Saito *et al.*, 1991).

In the present study the ß-glucuronidase gene has been employed for quantitative and histochemical detection of activity of different promoter sequences in transgenic maize cells and tissues.

The ß- glucuronidase of *E. coli* (GUS, EC.3.2.1.31) is a hydrolase that catalyses the cleavage of a wide variety of ß- glucuronides. Fluorometric, spectrophotometric and histochemical substrates are commercially available. The enzyme is a tetramer of identical subunits with molecular weight of about 68,200 daltons each. It is stable in physiological conditions , has a pH optimum between 5.2 and 8.0 and is rather resistant to thermal inactivation (half life at 55 ^{o}C is 2 h). It is most active in the presence of thiol reducing agents like ß- mercaptoethanol and DTT. Because of the inhibition by divalent metal ions, EDTA should be included in the assay mixture.

The fluorogenic assay is the most extensively used test system for quantitative studies of gene expression. Currently the best substrate is the 4- methyl umbelliferyl ß-

D- glucuronide which, when cleaved by the enzyme, liberates a product that is fluorescent. Fluorescence methods are intrinsically 100-1000 times more sensitive then colorimetric methods, because the incident excitation light does not impinge on detection apparatus, and has a spectrum distinct and separable from that of emission. The fluorometric protocol gives a very high signal- to- noise ratio and permits detection of the GUS activity even in a single transformed plant cell (Sporlein *et al.*, 1991a).

The histochemical staining allows detection of ß-glucuronidase activity in individual cells and tissues of transgenic plants. Quality of the staining is affected by numerous variables, including all aspects of sample preparation, fixation and the reaction itself (Hu *et al.*, 1990; Hodal *et al.*, 1992). The 5- bromo- 4- chloro- 3- indolyl β-D-glucuronide (X- gluc) is the substrate of choice for the histochemical assay. The indoxyl derivative, product of the glucuronidase action on X- gluc is colorless. It undergoes an oxidative dimerisation and forms an insoluble and highly colored indigo dye. This dimerisation is stimulated by oxygen and can be enhanced by oxidation catalysts such as K^+ ferricyanide/ ferrocyanide mixture.

Various plant tissues, organs, protoplasts and entire plantlets can be assayed for GUS activity, but the procedure differs slightly for every particular case. There are refined protocols for staining of protoplasts, stem segments and seedlings (Jefferson, 1987; Jefferson *et al.*, 1987;. Battraw *et al.*, 1990). All of them can be performed with the published buffers and solutions. Sometimes the accessibility of the substrate is a problem (e.g. in cuticulated leaves). To overcome this limitation the thin sections from such organs can be prepared before staining. The localization of the activity is generally not affected by lengthy assays, but it is recommended to check the samples during the incubation and eventually to document the results.

The GUS system has been employed for a variety of studies, ranging from functional promoter analysis (Jefferson *et al.*, 1987; Benfey and Chua, 1990; Doerner *et al.*, 1990; Ohta *et al.*, 1991, Leung *et al.*, 1991; Baumlein *et al.*, 1991; Kapros *et al.*, 1993), early detection of *Agrobacterium* transformation (Castle and Morris, 1990) and detection of excision of transposons (Houba-Herin *et al.*, 1990), to use in such elaborated techniques like the inhibition of gene expression by antisense RNA (Robert *et al.*, 1990).

2.3.2. Controller sequences.

The term promoter refers to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. They are composed of discrete functional modules, each consisting of 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins. Typically, promoter modules

regulating the frequency of transcription initiation are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional modules downstream of the start site as well (Rozen *et al.*, 1986; Stenlund *et al.*, 1987; Ayer and Dynan, 1988). Enhancers were originally defined as genetic elements that increase transcription from a promoter located at a distant position. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual modules, each of which binds one or more transcriptional activator proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true for a promoter region or its component modules. On the other hand, promoters must have one or more modules that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these modules. (Dynan, 1989).

A large number of plant promoters directing transcription in either an inducible or tissue- specific manner has been studied in detail (reviewed by Sachs and Ho, 1986; Theologis, 1986; Kuhlemeier *et al.*, 1987; Weising *et al.*, 1988; Mikami and Iwabuchi, 1993; Nakayama and Iwabuchi, 1993). Plant genetic transformation has allowed the functional analysis of promoter sequences both in transient assays and after stable genetic transformation. Generally, it has been found that 5' sequences contain most, if not all, of the signals necessary for the correctly regulated expression. The studies on deleted versions of promoters revealed that a promoter might contain a combination of several modules which are responsible for qualitative and quantitative control of the expression (Benfey *et al.*, 1989; Leung *et al*, 1991; Nakayama and Iwabuchi, 1993; Terada *et al.*, 1993). These elements are likely to control RNA polymerase activity by interacting directly or indirectly with regulatory proteins. Most of the plant genes characterized to date are transcribed in a regulated rather than a constitutive manner (Kuhlemeier *et al.*, 1987; Nagata *et al.*, 1987; Terada *et al.*, 1990). Expression is frequently tissue- specific (Ohta *et al.*, 1991; Keller *et al.*, 1989), induced by environmental factors such as light (Fluhr *et al.*, 1986; Tobin and Silverthorne, 1985) and stresses (Baumann *et al.*, 1987; Pfitzner *et al.*, 1988; Keil *et al.*, 1989). Primarily cell cycle- dependent promoter activity has been demonstrated in studies of histone gene expression (Lepetit *et al.*, 1992; Kapros *et al.*, 1993; Nakayama and Iwabuchi, 1993; Terada *et al.*, 1993). The developmental regulation of plant genes is often connected with additional control mechanisms (Rocha-Sosa *et al.*, 1989; Györgyey *et al.*, 1991).

2.3.2.1. Cauliflower mosaic virus 35S RNA promoter.

Transcript mapping experiments with cauliflower mosaic virus (CaMV) have identified two viral promoters, designated 19S and 35S. During the virus life cycle, the 35S promoter is transcribed from the viral DNA minus- strand to produce an 8-kb transcript referred to as the 35S RNA (Guilley *et al.*, 1982). In the context of the virus, the 35S RNA promoter is a strong driver of transcription process, because it directs the synthesis of an RNA serving as a non- usable template for CaMV DNA synthesis. Although the normal host range of CaMV is restricted to species in the *Cruciferae* family, the promoter is active when isolated as a fragment from the viral genome and integrated into the genome of a large variety of plants both monocots and dicots (reviewed by Weising *et al.*, 1988; and Blaich, 1992; Harpster *et al.*, 1988; Battraw and Hall, 1990; Gordon-Kamm *et al.*, 1990; Terada *et al.*, 1990; Vasil *et al.*, 1992).

The CaMV 35S promoter has been considered as a constitutive regulator of transcription (Odell *et al.*, 1985; Benfey and Chua, 1990), but recent studies using highly sensitive fluorometric methods have shown a certain tissue specificity (Jefferson et al., 1987; Battraw and Hall, 1990) and cell cycle stage dependent promoter activity (Nagata *et al.*, 1987). The results of Benfey and Chua (1990) have suggested a complex structure with two domains and several subdomains, conferring, through synergistic complementation, an almost constitutive expression in tobacco.

Although the levels of activity of the promoter can vary in different tissues and it appears to be most active in leaf tissue (Jefferson et al., 1987). Expression directed by the region +8 to -90 is strongest in embryo tissue, the radical pole of the endosperm and in root, whereas the region -90 to -343 directed strongest expression in cotyledons, leaves and stem (Benfey *et al.*, 1989). This suggests that the promoter contains multiple *cis* elements able to individually confer tissue specific expression which in the intact 35S promoter work in concert in order to confer almost constitutive expression.

The monomers and multimeres of a 35S promoter fragment (-209 to -46) have been shown to act as typical enhancers to potentiate transcription from a heterologous promoter. This 162-bp promoter fragment can function in an orientation- independent manner when located either upstream or downstream of the 35S transcription unit (Fang *et al.*, 1989).

2.3.2.2. Wheat α–amylase (α-*Amy2/54*) gene promoter.

α-Amylase (EC 3.2.1.1) plays a key role in the metabolism of the plant by hydrolyzing starch in the germinating seed and in other tissues. This is accomplished primarily through the 1,4-α endoglycolytic cleavage of amylose and amylopectin, the

principal components of starch granules in plant cells (Huang *et al* 1992). Three classes of α-amylase genes *α-Amy1*, *α-Amy2* and *α-Amy3* have been identified in wheat (Lazarus *et al.* 1985; Baulcombe *et al.* 1987). The *α-Amy2* genes are a multigene family of ten to eleven individual genes spread across the constituent genomes of hexaploid wheat. The products of individual genes can be separated on the basis of isoelectric point and these isozyme proteins fall into two isoelectric point groups referred to as the high- and low- pI groups, the characteristics of which differs in a number of ways. The α-amylase genes are expressed in the aleurone cells of germinating grain under control of the plant hormone gibberellin (GA).

It has been shown by Huttly and Baulcombe (1989) that fragments of the *α-Amy2/54* low pI α-amylase gene promoter fused at the start of translation to the reporter GUS gene, when transiently expressed in oat aleurone protoplasts, were specifically controlled by GA_3 in a manner similar to the expression of the endogenous oat α-amylase genes. In addition, deletion analysis of the promoter delineated the 5' end of a region necessary for high- level GA_3- dependent expression to within 300 bp from the start of transcription. The plasmid construction *pαGT14* contained the shortest promoter fragment (deleted until position -389 upstream of ATG codon or up to -289 from the start of transcription) still retaining a considerable GA_3- regulated expression level and accommodating all of the regions of highly conserved sequences found upstream of the start of transcription of *α-Amy2* genes from wheat and barley (Huttly *et al.*, 1988).

Chimeric promoters composed of the regions of CaMV 35S and *α-Amy2/54* promoters as well as mutant *α-Amy2/54* promoters containing replacements or deletions were constructed (Huttly *et al.*, 1992) and their ability to direct expression of the reporter GUS gene in gibberellin-responsive oat aleurone protoplasts were analyzed. Based on the results obtained the authors concluded that there are at least three regions that potentially contain *cis* elements, and which together are necessary for the high- level hormonally regulated expression observed in oat aleurone protoplasts. The regions are located between -68 and -117, -164 and -175, and -241 and -289 from the start of transcription. The collected data allowed the authors to suppose that one of these elements (-68 to -117) may operate through repression of transcription in the absence of GA_3 or presence of ABA.

The outcome of these functional promoter analysis is in a good agreement with the results of Rushton *et al.*, (1992) in protein/DNA interactions *in vitro* within the *α-Amy2/54* promoter. Utilizing DNase 1 footprinting method they have localized binding of nuclear proteins from GA treated oat aleurone protoplasts to five boxes which overlap (except box 1) with the regions determined by Huttly *et al.*, (1992).

2.3.2.3. Wheat histone H4 gene (TH011) promoter.

Histones are small basic proteins represented by five major classes: four core histones, H2A, H2B, H3, H4 and one linker histone H1. These structural proteins are necessary for the assembly of newly replicated DNA into chromatin of proliferating cells. Therefore, expression of the genes encoding five major classes of histones is coupled mostly to DNA replication and is coordinately regulated at the transcriptional and post-transcriptional levels through the cell cycle. The histone mRNAs content is much lower during the G1, G2, and M phases than during the S phase of the cell cycle when these mRNA are highly accumulated. Both the 5' and 3' flanking regions of histone genes are required for regulation of histone gene expression in yeast, animal and plant cells, but some differences in their regulation mechanisms exist (Nakayama and Iwabuchi, 1993).

The primary structure of higher plant histone genes was first determined with cloned wheat H4 and H3 genes (Tabata *et al.*, 1983; Tabata *et al.*, 1984). Most of the plant histone genes were shown to be dispersed in the genome. There are 60-125 copies of H3 and H4 histone genes present in wheat and maize cells (Tabata *et al.*, 1983; Tabata *et al.*, 1984; Chaubet *et al.*, 1986). Analysis of the DNA sequences of cereal plant histone genes revealed existence of various conserved sequences (hexamer (ACGTCA), octamer (CGCGGATC) and nonamer (CATCCAACG) motifs) as possible candidates for the *cis*-acting control elements in the 5' regulatory region. Several sequence-specific DNA-binding proteins and corresponding cDNA clones have been identified: HBP-1a and HBP-1b specific for the hexamer motif (Mikami *et al.*, 1987; Tabata *et al.*, 1991); ssDBP-1 and ssDBP-2 specific for single-stranded DNA sequences containing the octamer motif (Takase *et al.*, 1991) and HBP-2 (Kawata *et al.*, 1988) specific for the nanomer motif. The information have been obtained suggesting the function of the proteins as *trans*-acting factors in regulation of histone gene transcription (Nakayama and Iwabuchi, 1993).

Recently, it has been demonstrated that wheat H3 histone gene promoter confers cell division-dependent and -independent expression of the *gusA* reporter gene in transgenic rice plants (Terada *et al.*, 1993). According the results of histochemical analysis cell division-dependent expression has been found in the apical meristem of shoots and roots and in young leaves, while cell division-independent expression has been detected in flower tissues including the anther wall and the pistil. The authors have demonstrated that the region from -909 to -1711 contains the positive *cis*-acting element(s) and that the proximal promoter region (up to -185 from the start of transcription) containing highly conserved sequence elements is sufficient to direct both cell division-dependent and -independent expression. The same research group has reported lately the direct evidence that the hexamer and octamer sequences are the *cis*-

acting elements responsible for the cell cycle-specific regulation of the wheat histone H3 gene expression (Ohtsubo *et al.*, 1993).

The wheat histone H4 gene (TH011) was isolated from the genomic library in 1983 (Tabata *et al.*, 1983) and the primary structure of the gene was determined. The quantitation of an autoradiographic image obtained after blot-hybridization of the labeled histone H4 gene fragment to wheat genomic DNA demonstrated that the copy number of the gene is in the range of 100 to 125 copies per hexaploid wheat genome. The variant wheat histone H4 gene (TH091) has been analyzed by the same researchers (Tabata and Iwabuchi, 1984). The comparison of the two genes revealed that there are 17 nucleotide replacements in the protein-coding region, causing only one amino acid substitution: a glycine in position 4 (from N terminus) in TH011 is replaced by an aspartic acid in TH091. However, the 5' and 3' regulatory sequences of the genes have been found to be less conserved. Although, the *cis*-acting hexamer and octamer elements are present in both genes, the TH091 gene does not contain the sequence motif with exact match to the nanomer element.

Functional activity of the wheat H4 histone gene (TH011) promoter which contains all the conserved sequences presumably directing S phase-specific histone gene expression has not been studied so far in homologous cereal system. Only after *Agrobacterium*-mediated introduction of wheat histone genes into sunflower cells it has been shown that the amount of H4 histone transcripts is seven times less then that of H3 histone, the values comparable to the transcription rate in wheat seedlings (Tabata *et al.*, 1987). The evidence has been obtained as well in this study suggesting that the level of transcripts of the introduced wheat H3 histone gene is parallel to that of the endogenous sunflower histone mRNA through a cell cycle. The authors concluded that these data suggest the similar regulatory manner of transcription in the two plant species. More recently, it has been demonstrated that mRNA content of wheat H4 and H3 histone genes in synchronized tobacco suspension cultured cells (BY-2) changes with the rate of DNA synthesis when the cell cycle enters the S phase (Mikami and Iwabuchi, 1993).

3. MATERIALS AND METHODS.

3.1. Protoplast culture and plant regeneration system.

Maize protoplasts have been isolated from the Ke2/2 cell suspension culture of the line HE/89, and cultured essentially as it was described by Mórocz *et al.*(1990). Briefly, the cell suspension were maintained in 50 ml N6M medium in 100-ml Erlenmeyer flasks by subculturing once in three weeks. Each new subculture was started from initial 0.2 gram fresh weight of cells. For protoplast isolation 4 g of three week old culture were transferred into 50 ml of fresh N6M medium which was substituted by the protoplast isolation solution after two additional days of cultivation.

The N6M culture medium contained the macro elements, glycine and vitamins of N6 medium (Chu *et al.*, 1975), the chelated iron and micro elements of MS medium (Murashige and Skoog, 1962), 500 mg/l Bacto Trypton, 3% sucrose and 0.5mg/l 2,4 dichlorophenoxyacetic acid (2,4D). The pH was adjusted to 5.8±0.04 by addition of 1M KOH solution; the medium was then sterilized by autoclaving. For medium solidification 0.2-0.4% of gelrite or 0.7% of agar were added.

The protoplast culture medium ppN6M/89 consisted of the components of N6M medium with the following modifications (final concentrations per liter): 370 mg $MgSO_4 \cdot 7H_2O$, 300 mg $CaCl_2 \cdot 2H_2O$, 1 g sucrose , 0.4 mg 2,4D, and additives 30 g fructose, 50 g glucose, 2.5 g maltose, 2.5 g galactose, 0.5 g galacturonic acid, 0.5 g glucuronic acid, 500 mg L-asparagine, 100 mg L-glutamine, 100 mg serine, 0.7 mg naphthalene-acetic acid and 0.7 mg zeatin-mixed isomers per liter. The filter-sterilized medium was used without pH adjustment.

The washing solution (WS) contained the N6M medium without $(NH_4)_2SO_4$, iron complex and 2,4D and with 1% sucrose (final concentration), 5.5% glucose and 5.5% fructose. The sugars were autoclaved and, after cooling, they were mixed with the tenfold concentration of other constituents. From bovine serum albumin (BSA, fraction V., Merck) a 40 mg/ml stock solution was prepared and kept deep frozen prior to use. Fifty milliliters of osmotic solution (OS) contained the following ingredients: 2.02 g KNO_3, 1.36 g KH_2PO_4, 0.47 g K_2HPO_4, 4.0 g glucose, 3.6 g fructose and 0.34 g L-proline; it was then filter-sterilized.

For the preparation of the desalted enzyme solution 4 g Cellulase RS and 0.2 g Pectolyase Y23 were dissolved in bidistilled water at 4 °C, the unsolved particles were pelleted by centrifugation, the supernatant was desalted on a 50 g swollen Sephadex G25

(with 100-300 μm bead size) containing column. The first 50-ml fraction was collected and filter sterilized.

The protoplast isolation solution (IS) was prepared just prior to use by mixing the components in the following order: 0.3 ml of the desalted enzyme solution, 2.0 ml of WS, 4.5 ml of sterile, threedistilled water, 0.5 ml of the BSA solution, 0.1 ml of 1 M $CaCl_2$, 0.1 ml of 1 M $MgSO_4$ and 2.5 ml of the OS solution, to make 10 ml of the final volume. Alternatively, the protoplast isolation solution consisted of 0.3 ml of desalted enzyme solution mixed with 9.7 ml of ppN6M/89 protoplast culture medium.

Two grams of fresh-weight material from 2-days-old suspension culture were transferred into 10 ml of the protoplast isolation medium and were incubated for several hours in dark with or without gentle shaking. After the enzyme treatment the protoplast suspension was passed sequentially through 100 μm and 50 μm mesh size stainless steel sieves, followed by centrifugation at a standard 1000 rpm. The pellet was resuspended in 10 ml of 0.6M sucrose and 1 ml of MaCa washing solution (0,2M mannitol, 0,08M $CaCl_2$, 0,1% MES pH 6.0). was overlaid for the second centrifugation. Protoplasts banding at the interphase were collected and resuspended in 10 ml of the MaCa solution and pelleted again in the third centrifugation. The number of protoplasts was counted using a Buerker chamber before the last centrifugation. The protoplasts were cultured at a density 0.5- 1 million per ml of the liquid protoplast culture medium or were embedded in low-gelling-temperature agarose (Sigma) by mixing the protoplasts gently with 2 ml of a 1:1 ratio the double strength ppN6M/89 and 1.2% melted agarose solution. For feeding protoplasts in the liquid medium, 0.5 ml of N6M medium was added after two weeks per 2 ml aliquots of the protoplast cultures. Additional 1 ml aliquots of the medium were supplemented twice in 2-3 day intervals. Then after 3-4 days the whole culture was poured onto the surface of a solid N6M culture medium. The embedded cultures were placed into 10-50 ml liquid N6M medium after 2-3 weeks of incubation.

Shoot regeneration from protoplast-derived embryogenic callus tissues was induced on a solid hormone free N6M culture medium. The *in vitro* grown plantlets were transferred into soil and the plants with emerged silks and pollen-shedding tassels were self- or sib- pollinated with different experimental breeding source material in a greenhouse.

The seeds obtained from the primary transformants were germinated *in vitro* on the hormone free N6M medium and the obtained seedlings were used for analysis of the expression of introduced foreign genes.

3.2. PEG-mediated transformation procedure.

0,3 ml aliquots of freshly isolated protoplasts (1-3x10^6) in MaCa solution or 0.4M mannitol, 0.015M $MgCl_2$ pH 5.8 (MaMg) were distributed into sterile glass tubes. Addition of 40-80 µl of plasmids dissolved in sterile water was followed immediately by mixing with 0.3 ml of 40% PEG (Mw. ca. 4000) dissolved in appropriate treatment solution. PEG solutions utilized were: 40% PEG (Mw ca. 4000) in: F-medium, 0.14M NaCl, 0.125M $CaCl_2 \cdot 2H_20$, 0.005M KCl, 0.75mM Na_2HPO_4 and 0.005M glucose (pH 7.0); 0.1M Mg, 0.1M $MgCl_2$, 0.4M mannitol; 0.1M Ca, 0.1M $Ca(NO_3)_2$, 0.4M mannitol. The PEG-protoplast mixture was incubated for 1-30 min. at room temperature and diluted then by 3 x 2ml volumes of MaCa solution. Protoplasts pelleted by centrifugation were resuspended in the protoplast culture medium ppN6M/89 at a density 1x10^6/ml. The sample aliquots for transient GUS reporter gene activity assays were taken 18-120 hours after treatment of protoplasts.

3.3. Selection procedure.

Protoplast-derived microcolonies have been embedded in agarose matrix 5-10 days after polyethylene glycol (PEG) treatment. 100 mg/l kanamycin, 100 mg/l geneticin (G418) or 100 mg/l (0.5 mM) L-phosphinothricin were used for selection of putative transformants. Agarose blocks containing embedded protocolonies were incubated in liquid protoplast culture medium with appropriate selective compound. Alternatively, three week-old microcolonies were plated onto the surface of a solid N6M selective culture medium. Proliferating individual resistant protocalli were transferred two-three weeks later onto Gelrite-solidified hormone-free N6M medium (Mórocz *et al.*, 1990) supplemented with the same amount of the same selective agent. Regenerated resistance gene expressing plantlets were further cultivated already without selection pressure.

3.4. Plasmid constructs and plasmid DNA preparation.

Construction of the pIDS 011 plasmid with the promoterless GUS gene and of the pIDS 211 plasmid with CaMV 35S promoter-GUS gene fusion has been described by Stefanov *et al.* (1991). The constructs contain the GUS reporter gene without promoter coupled with the 260 bp fragment of the polyadenilation signal from nopaline synthase

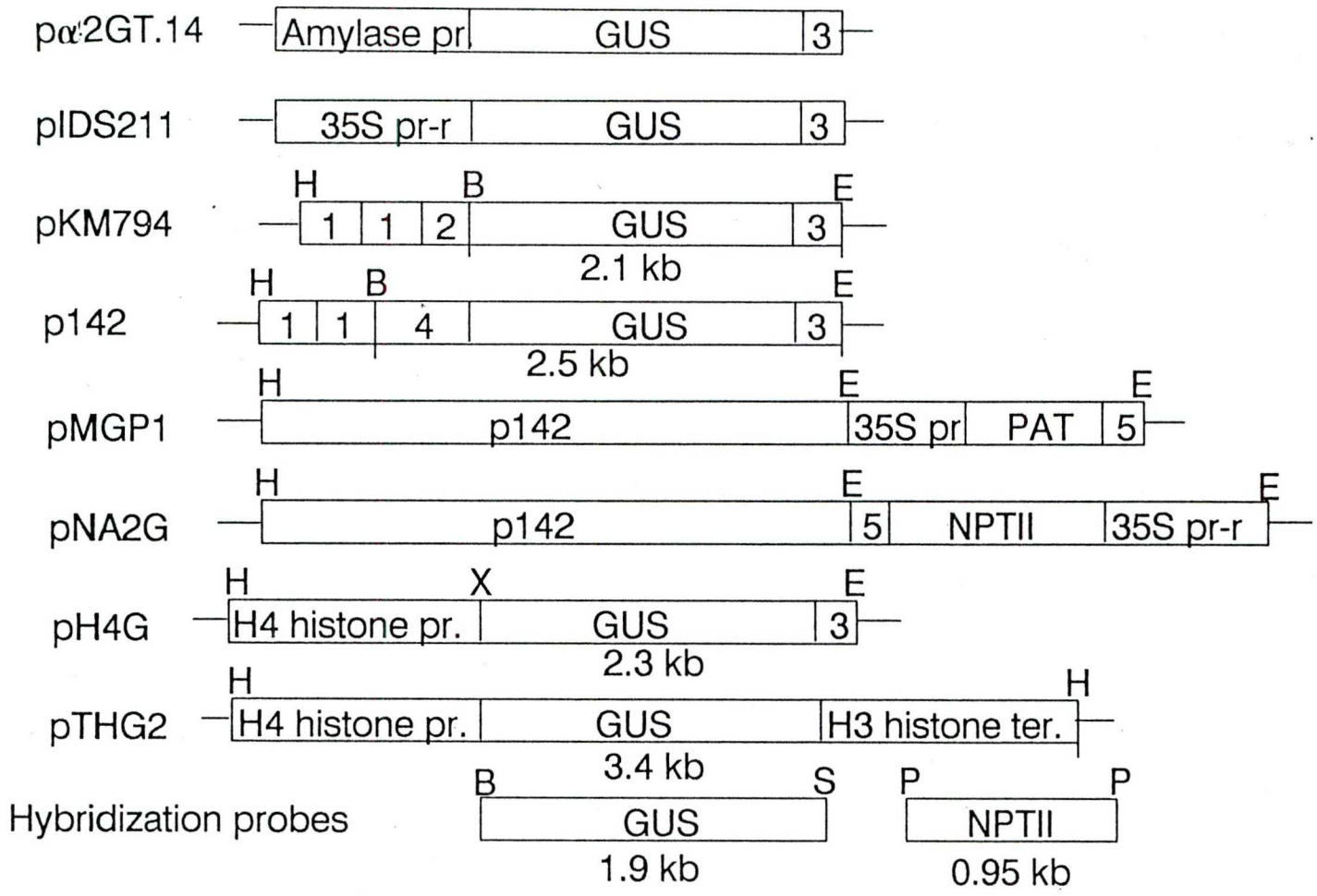

Figure 1. Schematic maps of the vector constructions used for stable transformation of maize: 1: CaMV 35S enhancer sequence (from -208 to -46 bp); 2: Domain A (-90 +8 bp) from CaMV 35S promoter; 3: Terminator sequence from the nopaline synthase gene (260 bp); 4: Truncated (up to -389 bp from ATG) promoter from wheat α-amylase gene (pα2GT.14;) 5: CaMV 35S terminator sequence; Restriction sites: B-BamHI; E-EcoRI; H-HindIII; P-PstI; S-SacI; X-XhoI.

gene (NOS) and the GUS-NOS cassette with an 800 bp fragment of 35S promoter from Cauliflower Mosaic Virus introduced upstream to the GUS gene, respectively.

The plasmid pKM794 was kindly provided by Drs E. Fejes and F. Nagy (unpublished). It contains the doubled enhancer sequences of CaMV 35S promoter (-208 to -46) inserted upstream to the domain A (-90 to +8) of the CaMV 35S promoter, linked to the GUS gene with NOS terminator.

The plasmid p142 contains doubled 35S enhancer sequences positioned upstream of truncated (up to -389 bp from ATG or up to -289 from the start of transcription) wheat α-amylase promoter of the construction pα2GT.14, which was kindly provided by Dr. D.C. Baulcombe (Huttly and Baulcombe, 1989). The pNA2G and pMGP1 constructs were developed from the plasmid p142 by insertion of *neo* (neomycin phosphotransferase, NPTII) and *bar* (phosphinothricin acetyltransferase, PAT) genes with CaMV 35S promoter and terminator sequences into the unique EcoRI site. The p35SAc gene construct was kindly provided by Hoechst AG (Frankfurt). The construct pHP23 (*neo* gene linked to CaMV 35S promoter and terminator sequences) was kindly provided by Dr. J. Paszkowszki (see Mendel *et al.*, 1989).

The wheat histone H4 and H3 gene clones in pBR322 vector plasmid (pTH012 and pTHO11) were kindly provided by Prof. M. Iwabuchi (Tabata *et al.*, 1983; Tabata *et al.*, 1984). The 720 bp long, HindIII-XhoI (filled up by Klenow polymerase) wheat H4 histone gene promoter fragment was subcloned and linked to the coding region of the GUS reporter gene in the plasmid construct pIDS011 cut by HindIII-SmaI, so that the XhoI unique restriction site was restored. Expression vectors with two types of termination signal were constructed. In pH4G vector the original terminator sequences from nopaline synthetase (NOS) gene was used , while in pTHG2 vector a 850 bp SauIII - HindIII fragment of the wheat histone H3 gene was cloned as a termination signal from the plasmid construct pTH012.

All the gene fusions constructed were subcloned into pUC vectors. Schematic maps of the vector molecules are shown by Figure 1.

Plasmid DNA concentrations were measured by fluorometric method using bis-benzimidazole (Hoechst 33258) substrate which binds specifically to A-T rich regions of DNA molecules causing the enhancement of fluorescence (Brunk *et al.*, 1979). The measurements were performed utilizing the TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, USA).

The junctions between promoters and the GUS gene were sequenced by dideoxy-mediated chain termination method using Pharmacia sequencing kit and the primer 5' GATTTCACGGGTTGGGGTTTCT3' (Jefferson *et al.*, 1987). All molecular cloning procedures were performed essentially according to the molecular cloning manual of Sambrook *et al.*(1989) with some minor modifications.

Plasmids were isolated by CsCl/EtBr gradient centrifugation as described by Maniatis *et al.*, (1982). The DH5α, JM109 or XL-1 *E. coli* strains were grown on LB or SOB culture medium at 37 °C. Competent bacterial cells utilized for transformation by plasmid DNA during the cloning work usually were prepared for a long -70 C° storage as described by Hanahan (1983) or Inoue *et al.*, (1990). Transformation of *E. coli* cells was performed using the standard protocols (Sambrook *et al.*, 1989). Selection for the transformed bacteria was carried out on LB plates supplemented with the appropriate antibiotics (100 mg/l of ampicillin or carbenicillin). Usually, two subsequent single- cell colony purifications were performed.

For miniprep plasmid DNA screening and analysing single- cell separated bacterial colonies were picked with a sterile toothpick and 2 ml of LB culture medium were inoculated by these samples and the bacteria were grown overnight at 37 ° C with vigorous shaking.

For large- scale plasmid DNA preparations 500 ml aliquots of LB culture medium in 2 l sterile flasks were inoculated with 1 ml of overnight grown bacterial cultures and incubated at 37° C with vigorous shaking until the OD 600 of the culture was equal or exceeded one unit.

3.5. Isolation of plant DNA.

Genomic DNA suitable for digestion by restriction endonucleases was routinely prepared from the callus tissues, cell suspension cultures, or leaves of young plantlets by the following method:

Plant tissue samples (1-2 g of the fresh weight) have been ground in liquid nitrogen with a mortar and pestle. The powder was mixed with 4 ml of extraction buffer (0.1 M Tris-Cl pH 9.0; 0.5% SDS). The lysate was extracted several times with equal volumes of phenol-chloroform mixture. The supernatant was separated, and DNA molecules were precipitated by adding 2.5 volume of ethanol with 0.1 volume of 3M NaAc (pH 4.8). Recovered DNA was collected with a glass rod, washed several times with 70% ethanol

and dissolved in 2 ml of TE buffer solution (10 mM Tris-Cl (pH 8.0); 1 mM EDTA). 20 ml of DNase-free pancreatic RNAase (10 mg/ml) was added, and after 30 min of incubation at room temperature samples were extracted by phenol, phenol-chloroform and chloroform solutions. Purified DNA was recovered by ethanol precipitation in the presence of 2M NH_4Ac, collected with a glass rod, washed with ethanol and dissolved in 1 ml of TE. Most of the treatments were performed at room temperature. Vacuum dried DNA pellet was dissolved to appropriate concentration in TE buffer solution (pH 8.0) or deionized water and DNA concentration was determined by fluorometric method.

3.6. Polymerase Chain Reaction (PCR) procedure.

Polymerase chain reaction (PCR) has been performed utilizing a PREM cycler machine. Primers have been chosen using the computer program OLIGO (ver.2.0, NAR). The primers were synthesized on controlled pore glass solid support on 0.2 mol scale by the standard cyanoethyl phosphoramidate methodology, using an automated DNA synthesizer (Cyclone Plus, Millipore) and purified by preparative 15 % acrylamide gel electrophoresis.

Five to ten μl (20-50 %) of obtained reaction product mixture were usually analyzed on 2% agarose gel slabs containing 2 mg/ml ethidium bromide (EtBr) solution and electrophoresis was performed at 100 V. As a fragment size marker DNA lambda phage DNA digested with PstI restriction endonuclease was applied (New England Biolabs, Inc).

Forward primer applied for the GUS gene fragment amplification is as follows: 5'-CTACACCACGCCGAACACCT-3' (+527 from ATG); reverse primer is: 5'-CAGGCACAGCACATCAAAGA-3' (+1391 from ATG). Analysed samples were subjected to Polymerase Chain Reaction in presence of the primer sequences and 864 bp long DNA fragment could be amplified in GUS reporter gene harboring maize clones. For analysis of transformants containing CaMV 35S promoter sequences the primers synthesized and kindly provided by CIBA-GEIGY (Koziel *et al.*, 1993) have been employed.

Reaction mixture included: buffer solution, 1.5mM $MgCl_2$, 200 mM dNTPs,1 mM each of the primer, 0.5 units per sample of Taq polymerase (Promega) and 0.2-0.5 μg of analyzed DNA samples.

Cycling conditions: 94 °C, 1min, 60 °C, 1 min, 72 °C, 1 min; 40 cycles.

The polymerase extension time was modified depending on the size of amplified DNA fragments.

3.7. DNA digestion with restriction endonucleases.

Restriction endonucleases have been purchased from New England Biolabs, BRL, Boehringer Mannheim and other firms and utilized according to the manufacturer's instructions. To avoid partial digestion the plant DNA samples were digested overnight with excess of enzyme (3- 6 units/μg DNA) in the appropriate buffer containing 5 mg/ml bovine serum albumin (BSA). Digested DNA was purified by phenol/chloroform extraction, precipitated and resolved in a small volume of 1x standard loading dye solution. The final reaction mixture was incubated for 5 min. at 65 °C before uncubation at 37° C.

3.8. Electrophoresis of DNA samples.

Fractionation of all kind of DNA material has been performed according to recommendations of Manniatis *et al.*, 1982 and Sambrook *et al.*, (1989). In each particular case the concentration of the gel (agarose or acrylamide) and the stringency of a current or a power applied were dependent on the aim of the experiment and the final resolution required.

Agarose gel electrophoretic experiments were done utilizing the apparatuses for the horizontal slab gels made at the Biological Research Center (BRC) (Szeged, Hungary). For DNA sequence analysis the firm vertical electrophoresis system "MACROPHOR" (LKB, Sweden) were utilized.

3.9. Southern blot analysis.

Digested DNA samples were separated in 0.7- 1% agarose gels buffered with TBE buffer (Maniatis *et al.*, 1982). For analytical electrophoresis of plasmid DNA 0.2-0.8 μg DNA per track were loaded. For separation of fragments of plant DNA the amount was increased to 10-15 μg per lane for easier detection of the single- copy introduced genes. The size of the restriction fragments was determined using as a marker lambda phage DNA digested with PstI or Hind III restriction enzymes. Gels were stained with 0.5 mg/ml ethidium bromide and the DNA fragments viewed and photographed on an UV transilluminator.

For Southern analysis electrophoretically separated plant DNA fragments were transferred and immobilized on nylon filter membranes (Amersham or Shleicher & Shuel.) with a vacuum transfer apparatus manufactured at the BRC (Szeged, Hungary) with the following treatments of the gel: exposition to UV light for 5 min., 0.25 M HCl for 5 min., denaturing for 30 min. (1.5 M NaCl, 0.5 M NaOH), neutralizing for 40 min (3 M NaCl, 0.5 M Tris-HCl pH 7.5), rinsing for 1-2 min in 2x SSC. The air dried nylon filters were exposed to subsequent UV-fixation for 2-5 min. The DNA- DNA hybridization with the radioactively labeled probe was performed according to the instructions provided by Shleicher & Shuel (Germany). Prehybridization (1-12 hours) and overnight hybridization were carried out in the buffer 5x SSPE (1xSSPE is 150 mM NaCl, 10 mM NaH_2PO_4. H_2O, 1 mM EDTA, pH 7.0), 10x Denhardt's solution (2 g/l Ficoll 400, 2 g/l Polyvinyl pyrrolydone and 2 g/l bovine serum albumin), 1% sodium dodecyl sulfate (SDS), 0.1 mg/ml denatured carrier DNA. The hybridized filters were washed two times with 2 X SSC buffer containing 0.1 %SDS at appropriate temperature and three times with 0.1 X SSC buffer with the same SDS concentration, at 65 ^{o}C. The filters were blotted dry with paper towels and were exposed to Forte Medifort RP diagnostic roentgen X-ray films (Hungary) with intensifying screens (Du Pont) for 1-10 days at - 80 ^{o}C

For the GUS gene molecules detection 10 μg of DNA samples were digested overnight with BamHI, EcoRI or HindIII restriction endonucleases, electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane filter (Hybond, Amersham). The obtained blots were hybridized with ^{32}P radioactive probe made from the agarose gel purified (see below) GUS gene fragment of pKM 794 plasmid cut by BamHI-EcoRI. The NPTII gene probe was isolated as a 920 bp PstI fragment from the pNeo plasmid (Pharmacia).

3.10. Plasmid DNA fragment isolation and labeling.

Agarose gels with preparative quantities of DNA (50-100 μg) were run overnight at low voltage. Gel slices containing the fragments of DNA to be labeled were cut out and placed in the chamber of an electroeluting device. The electroelution was carried at 100 V for 45-90 min. The DNA fragments of interest which were retained by a high- salt cushion were carefully collected and recovered. This step was followed by extraction with phenol and chloroform, washing and precipitation with ethanol (Sambrook *et al.*, 1989).

The high specific activity probes with radioactive phosphorus employed for the Southern hybridization analysis were prepared by the random nucleotide priming method

from Feinberg and Vogelstein (1983) utilizing Multiprime or Megaprime labeling kits (Pharmacia, Sweden). 40-80 ng DNA were labeled with (^{32}P) dATP or (^{32}P) dCTP to a specific activity of 0.5- 1.5 x 10^9 cpm/mg and separated from non incorporated dATP or dCTP using a chromatographic column (150 mm x 6 mm I.D.) filled up with the Sephadex G-50 (medium size) resin and equilibrated with TE buffer solution.

3.11. Vital staining procedure for detection of GUS activity.

The histochemical staining buffer consisted of 50 mM phosphate buffer pH 7.0; 10 mM Na_2 EDTA; 0.3 M glucose. X-gluc chromogenic substrate (Sigma) was dissolved in dimethylformamide (0.1 mg/ml) solution and used at final concentrations 0.1-0.5 mg/ml. The X-gluc substrate gives a blue precipitate at the site of enzyme activity after an oxidative dimerization of indoxyl derivative produced by b-glucuronidase (Jefferson, 1988).

90-95% of the culture medium was replaced by the filter sterilized staining solution and the plates were incubated at room temperature. Completely blue colonies were collected under sterile conditions by using dissecting microscope at 15x magnification and an automatic micro pipette with sterile plastic shortened tip adjusted to 2 µl. The isolated small colonies were placed into liquid medium with feeder microcolonies taken from the untreated protoplast suspension culture (HE/89) of the same age. Since the blue pellet in the stained areas can be easily recognized at later stages of colony growth, microcalli with blue spots were physically separated from the feeder cells after 2-3 weeks of culture.

The larger GUS reporter gene expressing maize protocolonies (>1 mm in diameter) could be placed directly to agar-solidified N6M medium without the use of feeder cells. They were collected using small forceps. Two weeks later each growing colony was divided into two parts. One part was restained by overnight incubation with X-gluc at 37 ^{o}C. Only second parts of colonies revealing uniform staining were used for further cultivation.

3.12. Assays of the activity of the *gusA* gene as a reporter.

The assays for GUS reporter gene activity and histochemical staining of plant tissues were performed according to Jefferson (1987) with modifications. Plant tissue samples were placed into 200 ml ice-cold extraction buffer and ground with the quartz sand in Eppendorf tubes using a glass pestles connected to a mixer head. After spinning down the cell debris utilizing an Eppendorf centrifuge at 12000 rpm for five-ten minutes,

25 µl samples were taken from the clear upper phase. Another 25 µl aliquot was used for protein determination according to Bradford (1976), the others aliquots were incubated for 0 min, 60 min, 120 min or overnight with 75 µl assay buffer solution. The reactions were stopped by addition of 25 ml of 1M Na_2CO_3 and the fluorescence of the produced 4-methylumbellyferone (MU) measured using a Perkin-Elmer fluorometer with excitation wavelength 365 nm, emission 455 nm ant the slit width in both cases set at 10 nm.

The fluorometer was calibrated with standard preparations of MU solution diluted to 100 nM and 1 µM. Specific GUS activity was calculated against the protein content of the analysed samples and the incubation time. It was expressed in nmole 4-MU/hour/mg protein.

For histochemical staining 50-100 µm thick sections from different organs were cut using a hand microtome (Griffin and Jones, UK) and a razor. After fixing for 10 min in ice-cold solution containing 0.3 % formaldehyde and 5 mM $NaPO_4$, pH 7.0 and several washes in phosphate buffer they were incubated for 12-18 h at 37 ^oC in the staining buffer. To remove the chlorophyll they were deepened in a sequentially increasing concentrations of ethanol.

3.13. Determination of the protein content of plant extracts.

Protein concentration of the prepared plant extracts have been determined by the dye-binding method of Bradford (1976) using a special kit manufactured by Bio-Rad Laboratories (California, USA). 25 µl plant extract were added to 985 µl diluted protein staining reagent and mixed well. 20- 30 min. later the adsorption of the mixture was measured against a blank reaction with a Shimadzu UV-VIS spectrophotometer at 595 nm wavelength. The protein concentrations were determined against a working calibration curve built in the range of 0-50 µg/ml using standard Bovine Serum Albumin (BSA) solution.

3.14. Reagents and chemicals.

The following reagents and chemicals have been used in the present study:

The mineral salts, vitamins, organic acids, mono- and disaccharides, phytohormones and other substances utilized in plant, tissue and protoplast culture experiments were purchased mainly from SIGMA (USA), SERVA (Germany) or REANAL (Hungary).

Other chemicals: Tris, EDTA, SDS, DTT, MOPS, PEG, Laurylsarcosyl, Spermidine, Acrylamide, Methylene bis-acrylamide, Agarose, Triton X100 - from SERVA (Germany). Ficoll, Bovine Serum Albumin, Polyvinilpyrrolidone, Chloramphenicol, Agarose (type I, V and VII) - from SIGMA (USA). Ammonium persulphate, TEMED -from REANAL (Hungary). Formamide -from MERCK (Germany). Nitrocellulose membrane filters -from Schleicher and Schull (Germany). Hybond nylon filters -from AMERSHAM (UK). Radioactive isotopes (dNTPs) - from DU PONT (USA).

Most of molecular biology enzymes were supplied by New England Biolabs (USA), BRL (USA), Boehringer Mannheim (Germany), Pharmacia (Sweden), FERMENTAS (Lithuania).

Thermostable *Taq*-polymerase for polymerase chain reaction molecular analysis was purchased from Promega (USA).

All components of bacteriological culture media - from SERVA (Germany) and Difco (USA).

Some of the reagents were additionally purified according recommendations of Maniatis *et al.*, (1982) and Sambrook *et al.*, (1989).

4. RESULTS.

4.1. Optimization of the PEG transformation protocol.

In the first set of experiments to optimize genetic transformation conditions a comparison of the influence of different polyethylene glycol (PEG) and salt solutions on the level of the GUS reporter gene transient expression in maize protoplasts has been carried out. The very sensitive fluorimetric assay has been utilized to measure intensity of a fluorescence produced by 4-methylumbellyferone (MU) which has been accumulated as a result of hydrolyses of a non fluorescent substrate 4-methylumbelliferyl ß-d-glucuronide (MUG) by the active ß-glucuronidase (GUS) enzyme produced by treated maize protoplasts. Freshly isolated protoplasts resuspended in two different salt solutions have been treated by different PEG solutions in presence of circular pKM794 plasmid DNA. The aliquots of crude protein extracts equilibrated by protein content have been incubated with MUG substrate 48 hours after protoplast treatment. As shown in Fig.2/A there have been significant differences in the GUS activity in favor of mannitol-calcium solution (MaCa) when protoplasts were resuspended in two different salt solutions, while the three PEG solutions utilized provided comparable transient expression results, although 0.1M Mg PEG solution appeared to be somewhat more efficient then the two other combinations tested. Based on these data MaCa and 0.1M Mg PEG solutions were chosen for use in subsequent transformation experiments.

When PEG-treated protoplast samples have been assayed at different time points after transformation it has been observed that the maximal level of the GUS transient expression occurred 48 hours after treatment (Fig.2/B). This can be considered as the appropriate time for conducting of the GUS reporter gene activity measurements in transient PEG-mediated transformation experiments with the maize cell line HE/89 to insure the highest sensitivity of the assay system. However, considerable level of transient GUS gene expression could be detected already after 18 hours following the treatment. The time course of transient expression level was found to be similar with both the analysed plasmid constructs, however the pKM794 provided significantly higher level of transient expression then the p142.

In the next set of experiments the effect of duration of PEG treatment on the level of transient GUS expression was examined. It was found that even a very short (one minute) treatment is sufficient to obtain detectable levels of the GUS gene expression in maize protoplasts (Fig.3/A). The optimal incubation time appeared to be 10-20 minutes, which is shorter then the treatment time applied in standard protocols (Krens *et al.*, 1982; Negrutiu *et al.*, 1987).

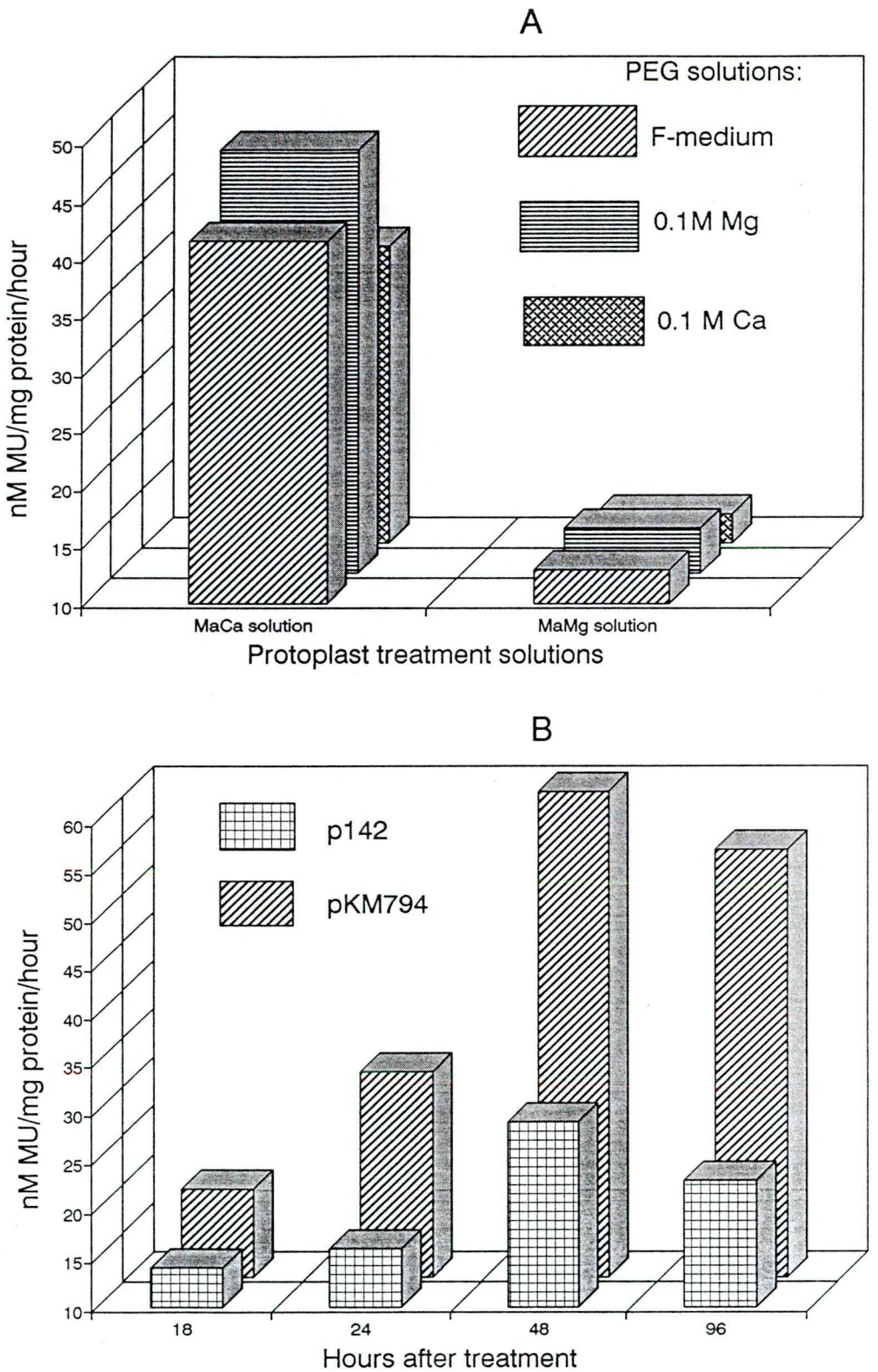

Figure 2. A, Transient GUS gene expression in protoplasts treated by different PEG solutions. B, The GUS gene transient expression at different time points after treatment.
Treatment conditions: $1x10^6$ protoplasts in 0.3 ml of treatment solution (MaCa or MaMg) + 40 mg plasmid pKM794 + 0.3 ml PEG solution; 20 min incubation; washing with 3 volumes of MaCa. MaCa, 0.2M mannitol, 0.08M $CaCl_2$, 0.1% MES, pH 6.0; MaMg, 0.4M mannitol, 0.015M $MgCl_2$ pH 5.8; PEG solutions, 40% PEG (M.w. ca 4000) in: F-medium, 0.14M NaCl, 0.125M $CaCl_2 \cdot 2H_20$, 0.005M KCl, 0.75mM Na_2HPO_4 and 0.005M glucose (pH 7.0); 0.1M Mg, 0.1M $MgCl_2$, 0.4M mannitol; 0.1M Ca, 0.1M $Ca(NO_3)_2$, 0.4M mannitol. Assayed 48 hours after treatment.

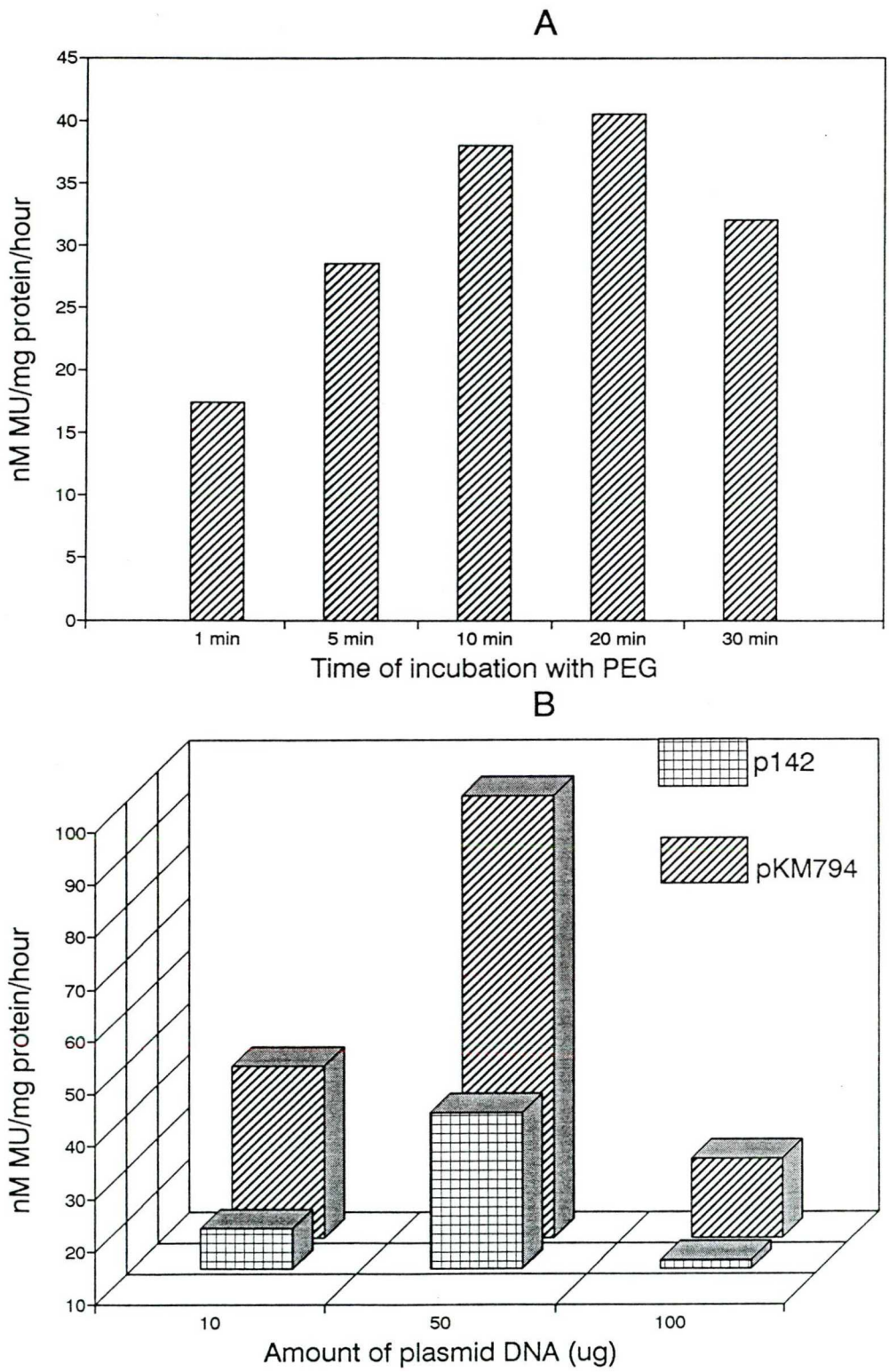

Figure 3. A, Influence of incubation time in PEG solution on the GUS gene transient expression. B, Influence of the input DNA amount on the GUS gene transient expression. Treatment conditions: as in Figure 2. (MaCa treatment solution and 0.1M Mg PEG solution).

Another important parameter influencing transformation efficiency is the amount of input DNA per treated protoplast sample. In the present transformation system 50 μg of input DNA turned out to be the optimal amount to be introduced per treated aliquot of protoplasts. The genetic transformation experiments under these conditions resulted in highest transient activity of the GUS reporter gene (Fig.3/B). The application of 100 μg of circular plasmid DNA caused clumping and subsequent bursting of treated protoplasts thus significantly decreasing survival rate and level of transient gene expression.

Based on the results obtained the established optimized PEG-mediated genetic transformation protocol is as follows: Gentle mixing of 50 μg of plasmid DNA dissolved in 50 μl of sterile water with one million of freshly isolated protoplasts resuspended in MaCa solution (0.3 ml) follows immediately by addition of equal volume of 0.1M Mg polyethylene glycol solution (0.35 ml) and gentle mixing. Dilution by three volumes (total 6 ml) of MaCa after 10 minutes of incubation at room temperature. Pelleting protoplasts by centrifugation for two minutes and then transferring them into ppN6M culture medium.

4.2. Analysis of the various vector constructs in transient GUS assay.

In search for promoter combinations with potential application in maize genetic transformation projects, different vector constructs have been tested by transient GUS assays. The schematic maps of the plasmid molecules used in the present transformation experiments are shown in Figure 1. In addition to the CaMV 35S promoter (pIDS 211) and its combination with the doubled enhancer element (pKM794) the truncated wheat α-amylase promoter (pα2GT14) and its complemented version carrying doubled CaMV 35S enhancer element as well as two constructs with wheat H4 histone promoter and NOS terminator (pH4G) or terminator sequences of the wheat H3 histone gene (pTHG2) were studied. Maize protoplasts were treated with PEG solution in the presence of the various plasmids. After 2 and 5 days of culture the GUS transient activity was determined utilizing aliquots of crude cell extracts from the treated protoplasts with equilibrated protein concentration. The results of transient GUS assays are summarized in Table 1. According to the obtained GUS activity data the analyzed α-amylase promoter region did not provide detectable GUS enzyme level. The amount of the produced 4-methylumbelliferone (MU) was similar to that of the promoterless construct (pIDS 011). If the α-amylase promoter element was supplemented with the CaMV 35S doubled enhancer sequence region a functional promoter was obtained with expression level characteristic for the CaMV 35S promoter. The doubled enhancer element was found to be active in the case of the minimal (-90 to +8) CaMV 35S promoter as well.

Table 1. **β-glucuronidase (GUS) specific activity in maize cells after transformation of protoplasts with GUS fusion constructs containing different promoter elements**

Constructs	Mean value of GUS specific activity (nM MU/mg protein/hr)	
pIDS011 (promoterless)	0.54	(3)
pIDS211 (CaMV35S)	11.00	(3)
pKM794 (2 x En 35S)	37.10	(7)
pa2GT 14 (α-amylase)	0.80	(3)
p142 (2 x En α-amylase)	12.20	(7)
pH4G	1.40	(3)
pTHG2	1.80	(3)

The number of experiments is shown in brackets. The GUS assay were carried out 2 days after transformation.

The presence of this element resulted in a considerable increase in expression level. Histone H4 gene promoter containing constructs exhibited relatively low level of GUS transient expression (1.4-1.8 units of specific activity). There were no differences in relative strength of the analyzed promoters in GUS transient assays carried out after the 2nd or 5th days of protoplast culture. According to the described transient gene expression studies the duplicated enhancer element from CaMV 35S promoter can activate both of the analyzed promoters in maize cultured cells.

4.3. Regeneration of maize plants from transformed protoplasts.

Because of the lack of the promoter function in the instance of the studied regulatory sequence element from the wheat α-amylase gene in transient GUS reporter gene activity analysis, transgenic plants were produced with the chimeric promoter, composed of the doubled enhancer element (the -208 to -46 bp upstream fragment) from CaMV 35S promoter and the -389 bp 3' end region (from ATG codon) from the wheat α-amylase promoter. The chimeric promoter-GUS gene fusion was combined with different selective marker genes, such as the neomycin phosphotransferase II (NPT-II) or the phosphinothricin acetyltransferase gene (PAT). After induction of DNA uptake into protoplasts by PEG treatment, various selection strategies were employed to identify putative transformants.

4.3.1. Selection based on NPT-II gene conferring kanamycin resistance.

Incubation of freshly isolated protoplasts ($5x10^6$) from the embryogenic culture [HE/89 (Ke2/2)] with pNA2G plasmid has resulted in 61 kanamycin resistant callus tissues. The early application of the antibiotics in the culture medium, and selection in liquid medium after embedding of cells into agarose, ensured sufficient selective pressure (Fig.4/A). From protoplast cultures, treated with plasmid molecules, numerous callus clones were obtained in the presence of 100 mg/l kanamycin. Transfer of callus tissues onto hormone-free medium initiated the formation of late embryogenic tissues, and subsequently shoots were regenerated in the presence of the selective antibiotic. A majority (82%) of the selected clones retained the embryogenic capability and regenerated plants. DNA gel blot analysis of genomic DNA from randomly selected transgenic maize lines confirmed the presence of integrated NPTII gene (data not shown).

The presence of the GUS gene in the genomic DNA from four transformants was detected by PCR amplification of 864 bp fragment, by using primers based on sequences from the coding region of the GUS gene (Fig. 4/C). Specificity of this 864 bp PCR

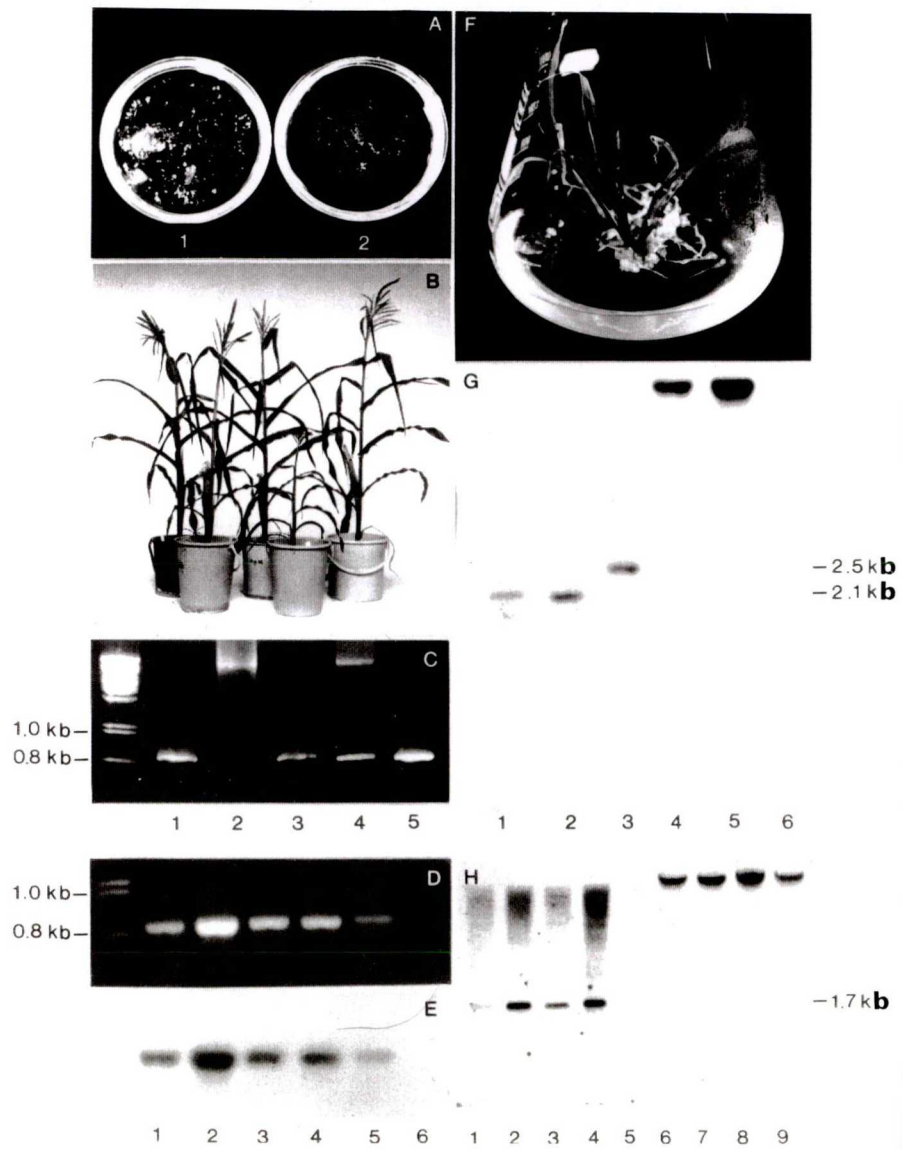

Figure 4. Various phases in production and analysis of transgenic maize: A, Selection of putative transformants in presence of 100 mg/l kanamycin. 1, clones derived from plasmid treated protoplasts; 2, control, untreated culture; three weeks after start of the selection. B, Mature transgenic maize plants exhibiting kanamycin or L-PPT resistance and GUS activity. C, PCR amplification of the GUS sequence from genomic DNA of various transformants (line 1; 3; 4; 5) and untreated control maize plant (line 2). The transformants were treated with pNA2G and selected with kanamycin. D, PCR amplification of the GUS sequence from genomic DNA of transformants (line 1-5) and the control maize plant (line 6). The transformants were selected on L-PPT after introduction of pMGP1. E, The Southern blot of PCR products shown in D hybridized with the GUS probe from the pKM794 (BamHI-SacI). F, Plantlet regenerated from a GUS-positive protoclone after vital staining with X-gluc. G, Southern blot analysis of transformants grown from GUS-positive protoclones after selection by vital X-gluc staining: 1, BamHI-EcoRI fragment from pKM794; 2, BamHI-EcoRI digested genomic DNA from transformant TG6 containing pKM794; 3, BamHI-EcoRI digested DNA from transformant TG1 obtained after treatment with p142; 4, Uncut total DNA from the TG6; 5, Uncut DNA from the TG1; 6, Total DNA from control HE/89 maize line. The hybridization probe was the same as in E. H, Southern blot hybridization of genomic DNA from GUS-expressing seedlings segregated after cross pollination of a primary transformant containing pNA2G: 1-4, DNA from GUS-positive segregants after EcoRI digestion; 5, Control DNA from HE/89 cells (EcoRI digest); 6-9, Uncut genomic DNA from the same seedlings as in 1-4. NPTII gene was used as a hybridization probe.

product was tested by Southern hybridization with the GUS probe in another experiment (Fig. 4/E).

The kanamycin-resistant protoclones were also analyzed for expression of GUS reporter gene activity in callus tissues. Both the fluorometric assay and histochemical staining showed that 89% of the selected clones also expressed the reporter gene with a defined level of expression (Fig. 5/A). Introduction of the pNA2G plasmid construct provided callus tissues with GUS specific activity at 1-5 units. The frequency of highly expressing clones was low.

Rooted shoots from the kanamycin-resistant and GUS gene expressing maize tissues were transferred into soil and the transgenic plants could be grown up to maturity (Fig. 4/B). No morphological abnormalities were found among growing transgenic maize plants and embryos were obtained after cross pollination with pollen from another breeding stock. The second generation seedlings developed from the embryos grown *in vitro*, were tested for GUS gene activity in the case of four independent transformants. Out of 36 second generation seedlings 14 showed GUS activity (above 3.0 units) in leaf tips. The presence of NPTII gene in genomic DNA from GUS gene expressing segregants was shown by Southern analysis. The DNA fragments of the proper size hybridized with the labeled NPTII gene probe after digestion of the DNA samples by appropriate restriction enzymes. Hybridization signal was also detected in the uncut high molecular weight plant DNA from offspring with GUS activity (Fig. 4/H).

4.3.2. Selection with geneticin (G418) antibiotic.

In one set of transformation experiments, geneticin (G418) antibiotic was tested in replacement of kanamycin. In a representative experiment, 6 x 10^6 maize protoplasts were incubated with pH4G and pHP23 (*neo* gene linked to CaMV 35S promoter and terminator sequences and inserted into pUC vector) plasmids in the presence of polyethylene glycol solution. The DNA treated protoplasts were cultured for 2 weeks, and proliferating microcolonies were plated onto the surface of selective medium solidified with Gelrite (0.2 %). Altogether 184 colonies with geneticin (G418) resistance were recovered from the cultures after selection on 100 mg/l of G418. Total DNA from randomly chosen 77 independent resistant protoclones was analyzed by PCR using CaMV 35S promoter specific primer sequences (kindly provided by CIBA-GEIGY; Koziel *et al.* 1993). As shown by Fig. 6/A, a DNA fragment of the proper size could be amplified by PCR from the analyzed DNA samples. The estimation of the functional co-transformation efficiency was based on the detection of GUS activity in the resistant transformants. According to these data, we observed 47% co-transformation frequency.

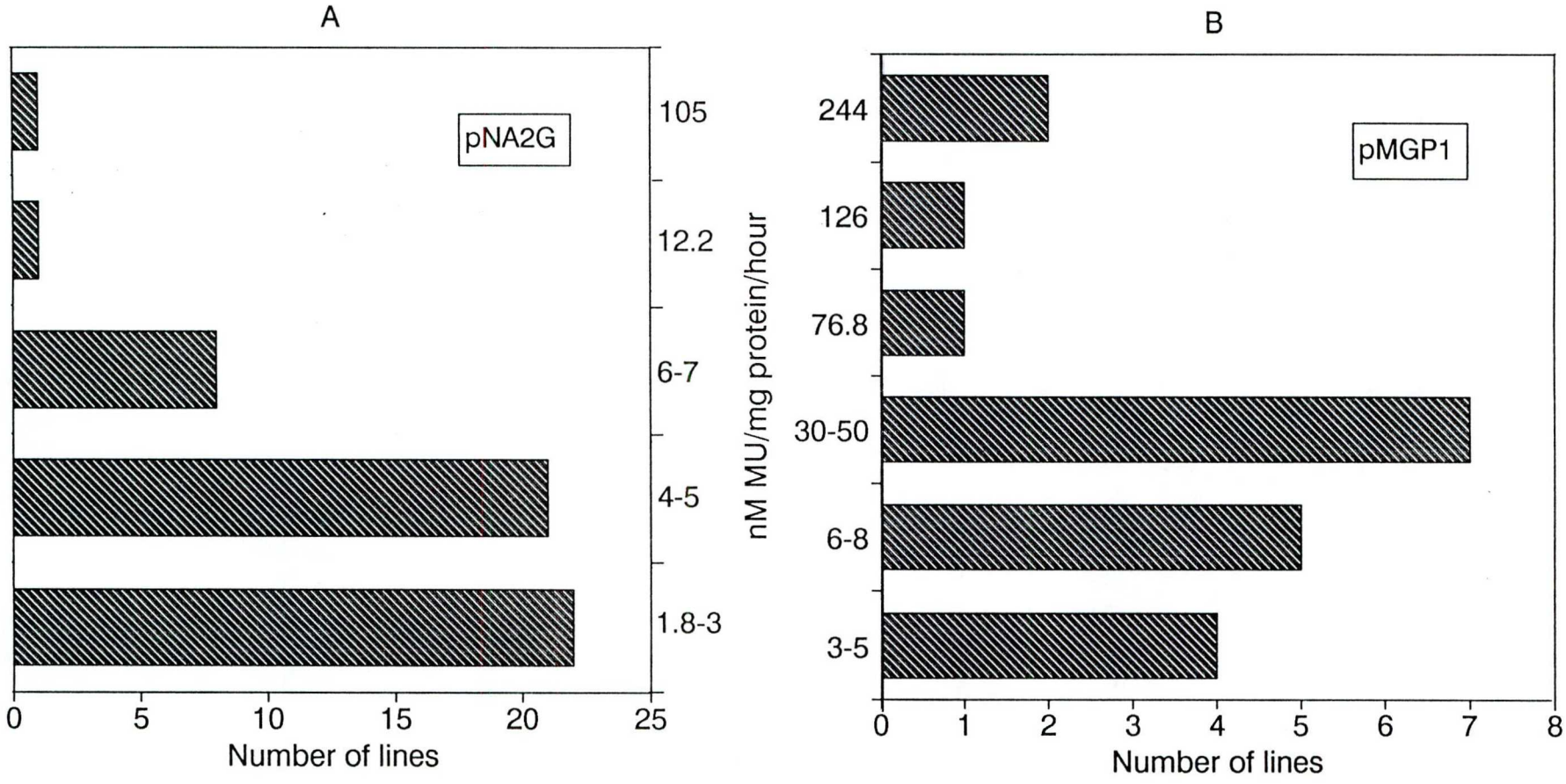

Figure 5. GUS activity measured by fluorometric assay in different lines of kanamycin-resistant (A) or L-phosphinothricin-resistant (B) protocalli.

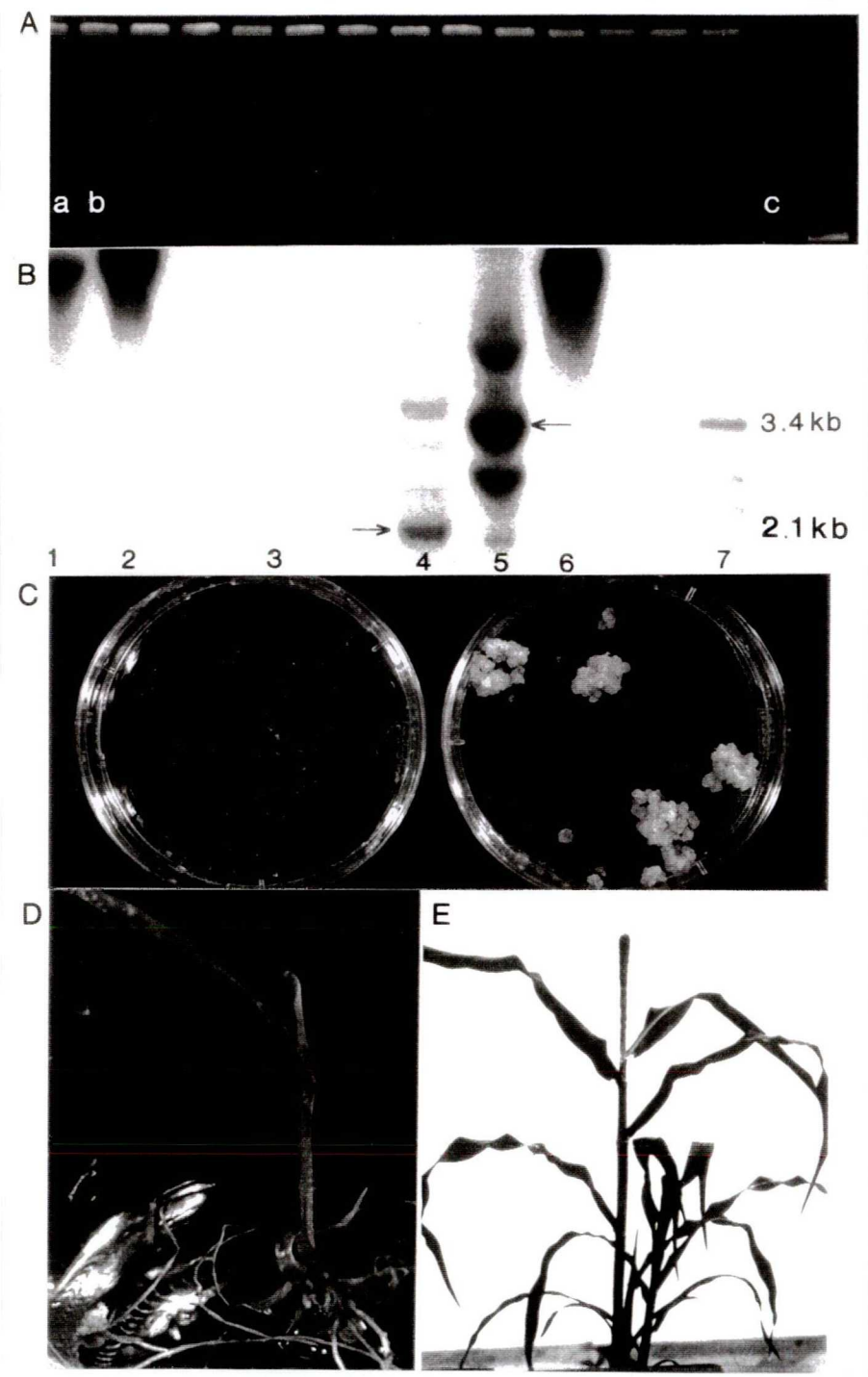

Figure 6. A, PCR amplification of 317 bp fragment of CaMV 35S promoter in DNA samples of G418 resistant protoclones: a, plasmid pHP23; genomic DNA from Southern-positive clone; untransformed control. B, Southern blot analysis of the genomic DNA from GUS-positive transformants(1.9 kb BamHI-SacI fragment of the GUS gene from pKM794 was a hybridization probe): 1, uncut total DNA from transformant HG15; 2, uncut total DNA from HG3.2; 3, DNA from untransformed HE/89 callus (EcoRI digest); 4, DNA of HG15 cut by XhoI-EcoRI; 5,6, DNA of HG3.2 cut by HindIII and EcoRI respectively; 7, 7.5 pg of plasmid pTHG2 cut by HindIII. C-E, Selection of L-PPT resistant tissues and growth of a transformant up to a flowering stage: C, Uptake of pMGP1 results in L-PPT resistant clones (right); untransformed culture (left); D, Young plantlet regenerated from L-PPT resistant callus; E, Transformant grown in a greenhouse.

Transgenic nature of two analyzed GUS gene expressing lines was also indicated by Southern blot analysis (Fig. 6/B). Integration of the GUS sequence into the high molecular weight genomic DNA can be concluded from the hybridization signal with uncut plant DNA. Digestion of plant DNA from clone HG3.2 with EcoRI which has no cutting site inside the vector, resulted in a hybridization signal also with a high molecular weight band. The DNA fragments of expected sizes hybridized with the GUS gene DNA probe after digestion of the samples by restriction enzymes releasing the GUS coding region. However, some rearranged gene copies were also observed (see Fig. 1. and Fig. 6/B 4,5). The obtained data demonstrated that transgenic protoclone HG3.2 contained approximately ten times more copies of the GUS gene per genome then the clone HG15. However, both transgenic protoclones exhibited comparable levels of the GUS gene expression when the old and fresh callus tissues were analysed by fluorometric assay (Fig. 9).

Morphologically normal healthy plants regenerated from the transgenic protocalli have been grown up to maturity in the greenhouse.

4.3.3. Selection based on PAT gene conferring phosphinothricin resistance.

Plasmid DNA construct (pMGP1) containing the chimeric promoter - GUS fusion and the PAT gene under the control of CaMV 35S promoter and terminator sequences was also introduced into maize protoplasts. 142 L-phosphinothricin (L-PPT)-resistant protocolonies were obtained out of $6x10^6$ PEG treated protoplasts. The selected callus tissues could grow in the presence of 0.5 mM L-PPT (Fig.6/C) and embryo differentiation and development of green shoots simultaneously with roots was observed on hormone-free N6M culture medium supplemented with 0.5 mM of L-PPT (Fig.6/D). The frequency of the GUS positive protoclones was 87% among the L-PPT-resistant callus tissues. The presence of the introduced chimeric genes in genomic DNA from the selected protoclones was shown by PCR amplification of the DNA fragment of the proper size by using primer sequences from the GUS protein coding sequence (Fig. 4/D,E). The L-PPT-resistant callus tissues expressed the GUS reporter gene at relatively high level (Fig. 5/B). A significant number of clones exhibited GUS specific activity above 30 units.

On hormone-free N6M culture medium, plants were regenerated from the late embryogenic callus tissues of 95 selected L-PPT-resistance protoclones (Fig.6/E). Non-transformed maize plants and primary transgenic regenerants were tested for L-PPT sensitivity. The *in vitro* grown plants were grown on N6M culture medium supplemented with 0.5 mM of L-phosphinothricin for two weeks. The control non transformed plants bleached and died within one week, while the transgenic plants grew and developed normally (Fig. 7/D).

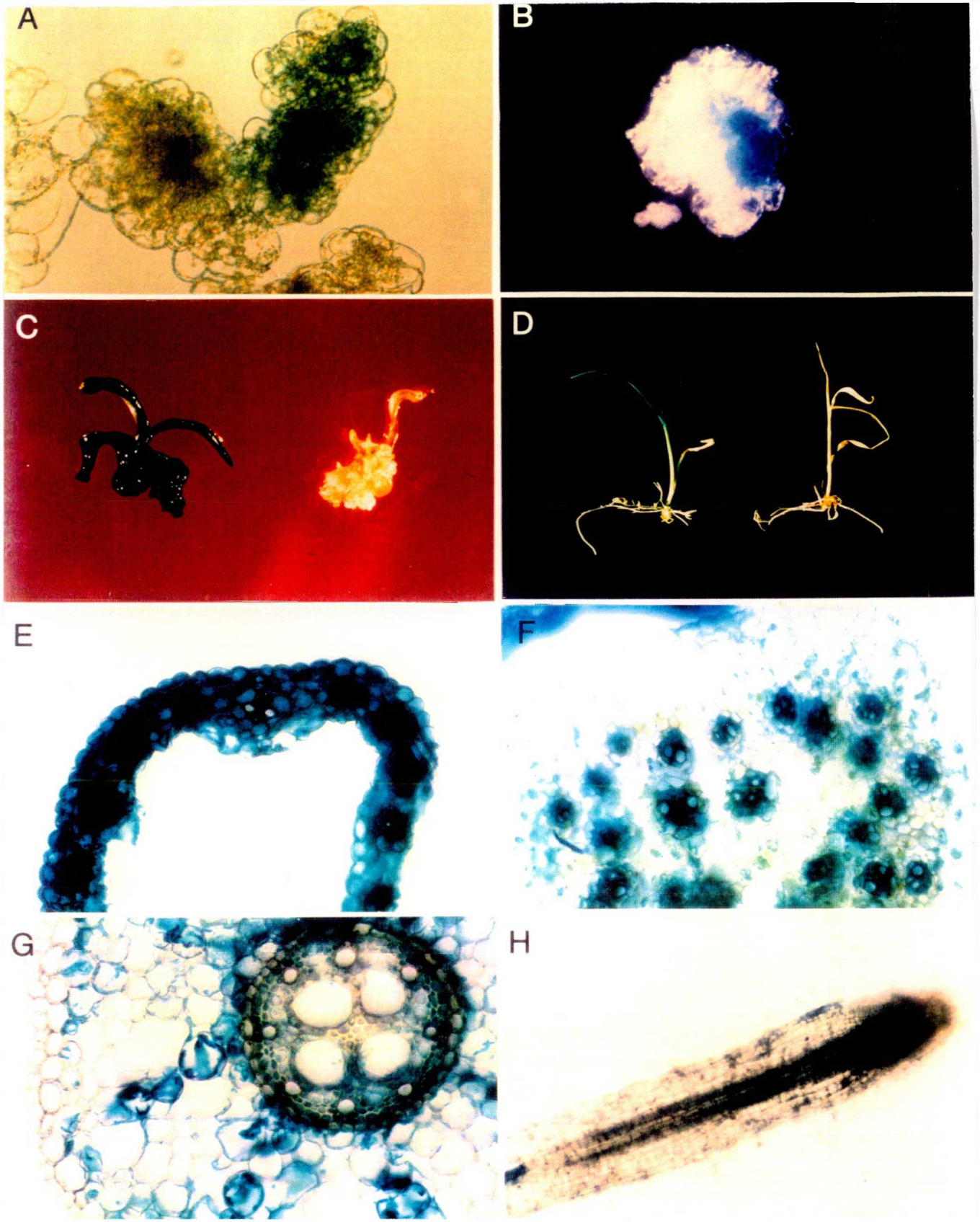

Figure 7. Histochemical localization of GUS activity in callus tissues and various organs of transgenic maize plants carrying chimeric promoter-GUS gene fusion: A, Protoplast-derived colonies after vital X-gluc staining. B, Growing callus tissues from a GUS-positive protoclone. C, Retest of GUS expression in shoot of a regenerant transformed by p142 and selected by vital staining: 1, TG1 transformant; 2, control HE/89 after overnight incubation with X-gluc. D, Analysis of resistance of regenerants to 0.5 mM L-PPT: 1, transformant (P2-47) 2, control HE/89 plantlet. E, Transverse section of young leaf from maize plant transformed with pMGP1. F, Transverse section of young stem from the same transformant as in E. G, Transverse section of root from the same transformant as in E. H, Distribution of GUS activity in root of the maize plant transformed with pNA2G.

4.3.4. Screening for GUS-positive clones by vital staining.

DNA uptake experiments were also carried out utilizing vector constructs without any selective marker genes. DNA of p142 and pKM794 plasmid constructs (see Fig.1.) were introduced into maize protoplasts. After PEG treatment, the protoplasts were cultured for 2-4 weeks until the micro-colonies reached approximately 0.5-1 mm in size. These small tissues were stained for GUS activity by the replacement of 90-95% of the culture medium with a sterile staining solution. The first blue colonies appeared within 2-4 hours of incubation (Fig. 7/A). Number of blue colonies increased with the time in the staining solution, however living colonies could be collected from cultures stained for less than 24 hours. Transformation efficiency as a number of blue colonies per 10^6 treated protoplasts was quite variable. On an average 10 (with a maximum up to 200) blue colonies were found in cultures from 10^6 protoplasts. As a negative control, the same number of protoplasts were treated with PEG in the absence of plasmid and cultured in the same way. Under similar staining conditions no blue colonies were found in these cultures.

The GUS-positive multicellular protoclones were transferred back to the same conditioned culture medium. Only 1-5% of the selected protoclones could survive the above described treatment. They developed similarly to the control untreated protoclones. The continuation of cell division and propagation of tissue material in GUS gene expressing clones was clearly manifested by the appearance of white sectors during the further culture (Fig. 7/B). When these tissues reached a proper size they were transferred onto solid, hormone-free N6M culture medium. The number of morphogenic tissues was reduced, however plantlets could be regenerated from few transformants (Fig. 4/F). The expression of GUS gene in these regenerants were indicated by GUS activity after restaining of shoot tissues (Fig. 4/C). In that particular transformant, both in root and leaf tissues, exceptionally high level of GUS expression was observed with specific enzyme activity of more than 1000 units nM MU/mg protein/hour. In the selected clones transformed with pKM 794, the GUS enzyme activity varied between 3-10 units.

Transgenic nature of two selected protoclones (TG1 and TG6) were confirmed by Southern blot analysis (Fig 4/G). Hybridization of GUS specific DNA probes to the uncut genomic DNA sequences indicated the integration of the introduced gene into the high molecular weight DNA. Detection of 2.1 and 2.5 kb hybridizing bands corresponded to the proper size of the fragments that are expected after double cutting of p142 or pKM 794 plasmid constructs by BamHI and EcoRI restriction enzymes (see Fig. 1. and Fig. 4/G).

4.4. Expression of truncated wheat α-amylase promoter under the influence of CaMV 35S enhancer sequences in maize plants.

The availability of considerable number of independent transformants has made it possible to analyze the variation in level of GUS gene expression, among the regenerated transformed plants. As shown by data in Table 2., the GUS activity ranged from a few units, up to several hundred specific activity units in leaves of individual transformants. In agreement with GUS specific activity values measured in callus tissues (Fig.5), several regenerants from transformation experiments with pMGP1 construct, exhibited a relatively high level of the gene expression. Out of 20 analyzed transformants, 6 showed GUS specific activity of more than 200 units with a maximum values in leaves of one transgenic protoclone that was found to be 656 ± 24 units. Comparison of the strength of the studied chimeric promoter clearly indicated a gradient in level of expression, with increasing order in callus < root < stem < leaf (Table 3). The behavior of this promoter also depended on the age or the position of leaves with differences between the basal, middle and tip regions (Fig. 8 and Table 3). In general, the older leaves exhibited higher GUS activity values, especially at their tips. This trend was observed in transgenic plants containing either pNA2G or pMGP1 plasmids (Table 3).

The tissue type-specific expression of the GUS reporter gene in maize transformants was examined by histochemical staining of transverse sections of leaves, stems and roots. Sectioned young leaves were stained throughout most of the leaf surface (Fig. 7/E). Intense staining was detected in the vascular bundles of the young leaf. Mesophyll and epidermis cells also expressed the GUS gene with considerable activity. GUS staining was prominent in vascular bundles of young stems of the *in vitro* grown maize plant (Fig. 7/F). Only weak expression was observed in parenchyma cells. The activity of the chimeric promoter construct in maize roots was confirmed by visualization of GUS gene function, utilizing histochemical staining protocol (Fig. 7/G,H). As shown in Fig. 7/H the chimeric promoter in this particular chromosomal location is preferentially active in root tip and vascular tissues. Incubation of transverse section of a root with X-gluc substrate, revealed a blue staining in the vascular cylinder and cortex. Pericycle layer of vascular cylinder contained cells with active promoter. The central metaxylem treachery element and the epidermal cell layer appeared to be free of any GUS activity.

Table 2. Variation in activity of chimeric promoter in leaves after transformation with various vectors

Categories in GUS specific activity values (nM MU/mg protein/hr)		1.5-10	10-100	100-200	>200
Number of lines:	pNA2G	6	10	4	-
	pMGP1	2	10	2	6
Average activity:	pNA2G	7.2+1.8	27.7+18.4	124+22	-
	pMGP1	13.9±0.2	39.9±24.1	134±44	420±207

Table 3. The level of GUS expression in various organs and tissues of transgenic maize plants (β-glucuronidase specific activity: nM MU/mg protein/hr)

A:

Tissues	Lines	
	N2-37	P2-87
callus	12	6.4
root	14	10
stem	36	23.4
leaf tip	81	65

B:

Leaf		Lines		
position	part	N2-16	N2-27	P1-05
1st	basal	3.9	28.8	72.0
	tip	47.7	52.7	98.7
2nd	basal	4.6	12.2	7.7
	tip	42.8	67.0	18.7
3rd	basal	3.4	14.6	3.6
	tip	19.1	22.0	51.5
4th	basal	4.6	4.4	6.3
	tip	33.9	23.3	19.7
5th	basal	4.1	5.8	3.2
	tip	20.9	59.0	14.5
6th	basal	9.2	4.6	-
	tip	17.0	13.1	-

The transgenic clones containing NPTII gene are marked by using "N" and those with PAT gene are indicated by "P"

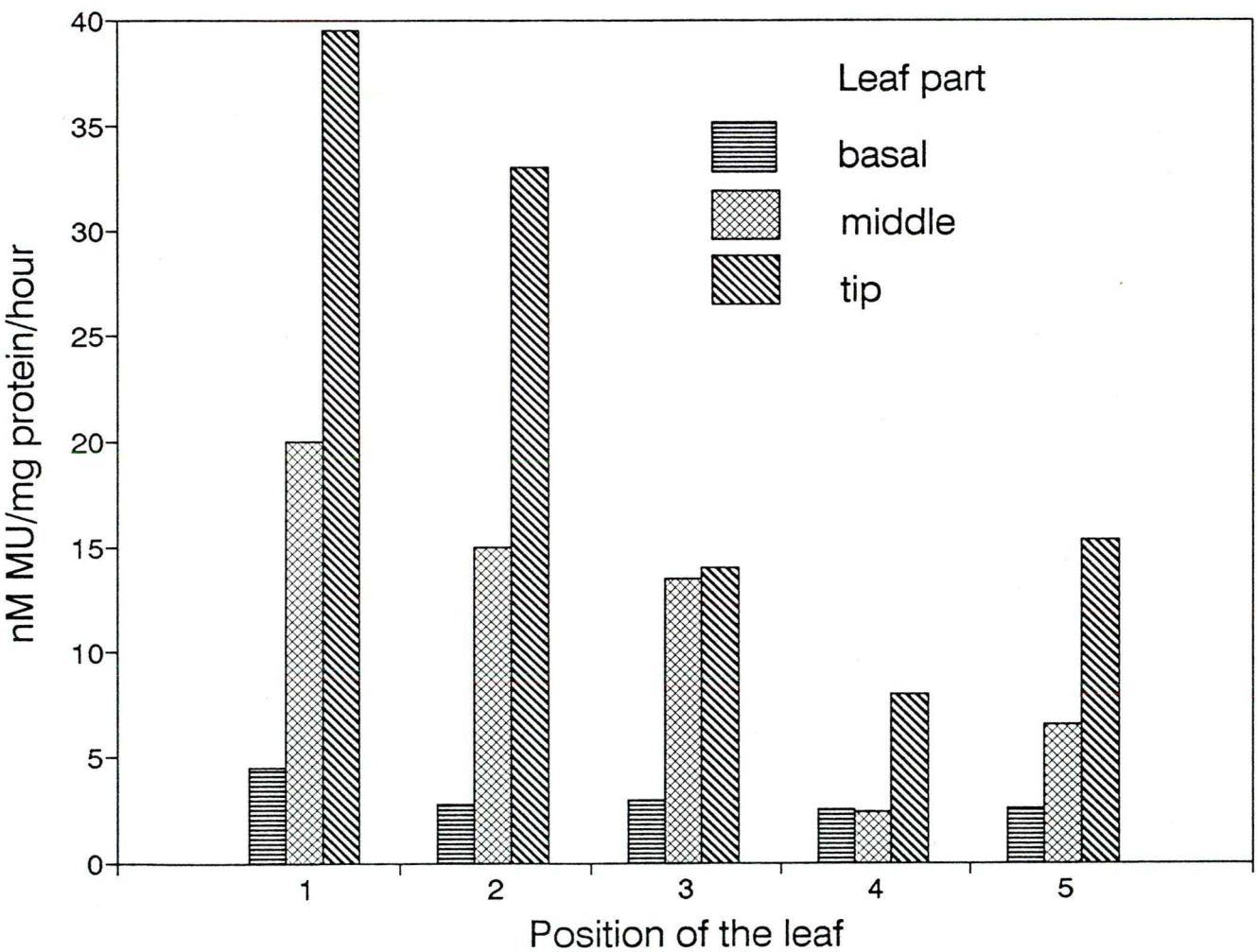

Figure 8. Pattern of expression of the GUS reporter gene in various parts of leaves of different ages under the control of the chimeric promoter after transformation with the plasmid pNA2G (line N2-01).

4.5. Differential activity of wheat H4 histone gene promoter in maize.

In experiments to characterize expression pattern of wheat histone H4 promoter - GUS gene fusions in transgenic maize, the co-transformation method was exploited by treatment of 10^6 maize protoplasts with mixture of 20 μg pHP23 or p35SAc (kindly provided by Hoechst AG, Germany) plasmid constructs with selectable genes driven by CaMV 35S promoters and of 40 μg plasmid carrying promoter - GUS fusion. In these set of experiments the reference vectors consisted of pIDS211, with CaMV 35S promoter (Stefanov *et al.*, 1992) and pKM794 with doubled enhancer sequence of CaMV 35S promoter (-208 to -46 bp) inserted upstream to the domain A (-90 to +8 bp) of CaMV 35S promoter (Fejes and Nagy, unpublished). Based on the values of activity of the GUS reporter gene, presented in Table 4., it can be suggested that in maize callus tissues, the wheat histone H4 promoter fragment with NOS terminator provides 2.5 times higher level of expression than the CaMV 35S promoters. Up to now, only one transgenic protoclone originated from transformation with pTHG2 has been characterized. In cells of this line relatively high GUS activity values ranging from 33-50 units in callus tissues up to 200 nM MU/mg protein/hour in fast growing suspension culture have been found.

The levels of activity of the GUS reporter gene, under the control of various promoter sequences, were analyzed also in freshly transferred callus tissues and in old cultures, six weeks after transfer to new culture medium. As shown in Fig.9., all transformants containing wheat histone H4 gene promoter exhibited significantly higher GUS gene expression levels in fresh callus tissues than in old ones. In contrast, the regulatory elements from CaMV 35S promoter resulted in an opposite order of GUS activity levels.

The distribution of cells expressing the GUS gene was studied by histochemical staining of different tissues from the selected transformants. As shown in Fig. 10/A, the callus tissues consist of significantly different cell types. Intense staining was detected in small cells of the meristematic regions, while the enlarged, non-dividing cells failed to accumulate the indigo dye. Staining of roots revealed a very characteristic pattern of expression (Fig. 10/B). The GUS gene expressing cells were localized to the root meristematic region. The basal regions of young leaves displayed considerable GUS activities that could not be detected in segments of mature leaves of regenerated transformants (Fig. 10/C).

Table 4. Comparison of various promoter activities in transformed maize callus tissues

Vector	Number of analyzed transgenic lines	GUS activity (nM MU/mg protein/hr)		
		minimum	average	maximum
pH4G	8	1.20	8.76	27.0
pIDS211	4	0.62	3.26	9.60
pKM794	8	0.42	3.86	10.50

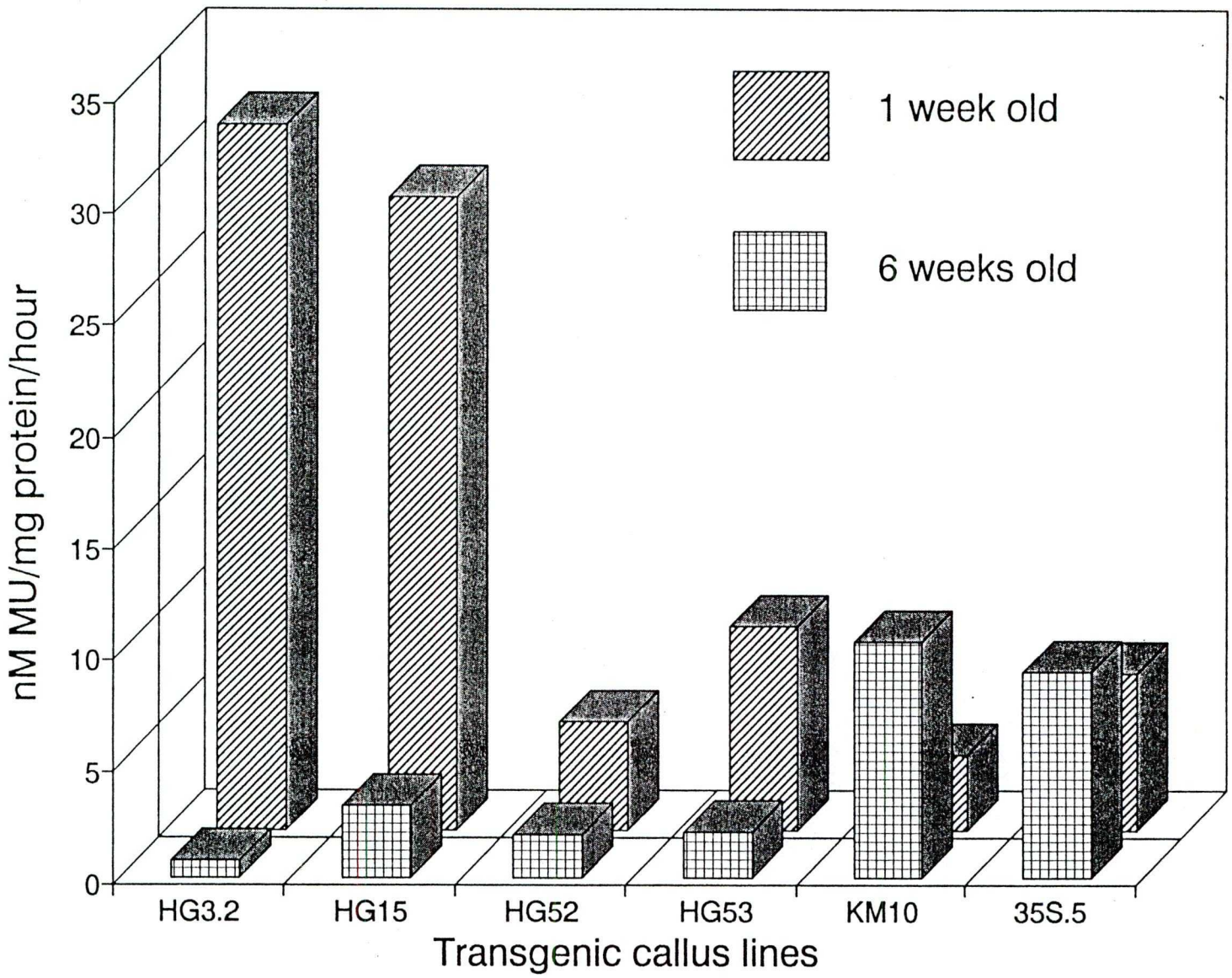

Figure 9. The level of the GUS gene expression in fresh and old callus tissues transformed by constructs with different promoters. The callus lines were transformed: HG3.2 by plasmid pTHG2; HG15, HG52 and HG53 by pH4G; KM10 by pKM794; 35S.5 by pIDS211.

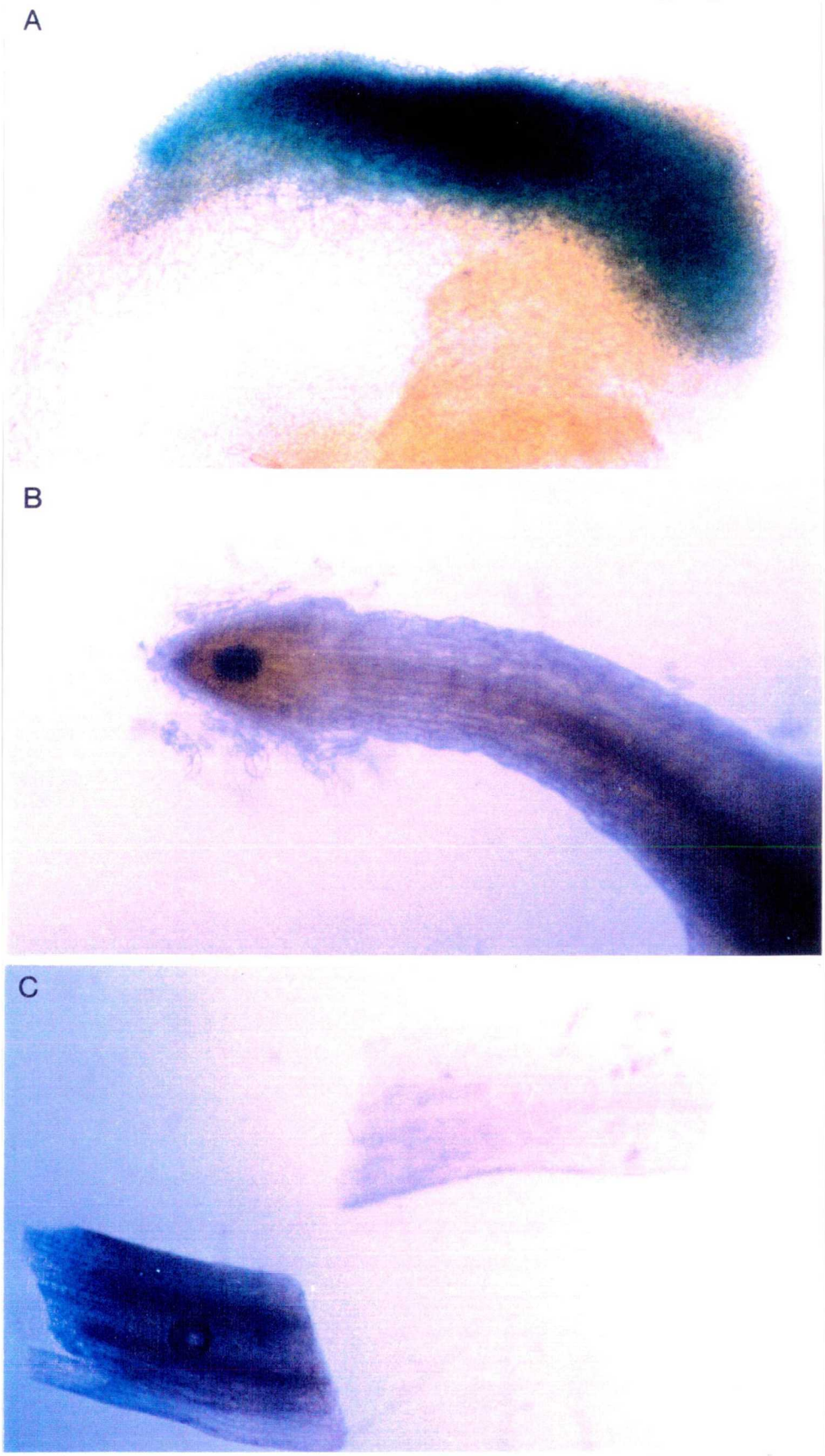

Figure 10. Staining of various tissues of the transformant with X-gluc: A, Transverse section of a maize tissue with meristematic region and callus with enlarged cells. B, Root with the intense staining of the meristematic region. C, Leaf bases of transformed and control plants.

5. DISCUSSION.

Experiments presented in this study demonstrate that direct DNA uptake into embryogenic protoplasts can be employed for efficient production of fertile transgenic maize plants and testing promoter function using gene fusions in this monocot crop plant. The described transformation system is based on a unique synthetic maize genotype (HE/89), developed by Mórocz *et al.* (1990), which has been already applied for the production of transgenic maize plants with sexual progenies (Donn *et al.*, 1990; Golovkin *et al.*, 1993). The first report by Donn *et al.*, (1990) has been a short abstract presentation informing about obtaining of the fertile transgenic maize plants of the HE/89 cell line without providing additional information about efficiency and characteristics of the genetic transformation protocol employed. In the preliminary experiments with protoplasts of the maize suspension line HE/89 by Golovkin *et al.* (1993) the protocol of Negrutiu *et al.* (1987) has been utilized. It allowed obtaining of transgenic maize protoclones with relatively low efficiency. In seven independent DNA uptake experiments only five transgenic protoclones were recovered from ca. 38 million of treated protoplasts.

Optimization of treatment conditions is an essential step to increase yield of transgenic clones in genetic transformation experiments. Currently, the GUS gene is a reporter of choice for the fast assessment of the transient expression of genes introduced into plant cells. There are two extensively used PEG transformation protocols which were developed originally for efficient genetic transformation of tobacco mesophyll protoplasts (Krens *et al.*, 1982; Negrutiu *et al.*, 1987). Briefly, according to Krens's transformation protocol protoplasts in K3 protoplast culture medium were mixed with equal volume of 40% PEG dissolved in F-medium (0.14M NaCl, 0.125M $CaCl_2x2H_20$, 0.005M KCl, 0.75mM Na_2HPO_4 and 0.005M glucose (pH 7.0)) followed by addition of DNA. After 30 minutes of incubation the mixture was diluted by ten volumes of F-medium. The optimized transformation protocol of Negrutiu included treatment of protoplast-DNA mixture resuspended in 0.4M mannitol, 0.015M $MgCl_2$ solution by equal volume of 40% PEG in 0.4M mannitol, 0.1M $Ca(NO_3)_2$ solution for 30 minutes and washing by 0.2M $CaCl_2$ or W5 solution (0.154M NaCl, 0.125M $CaCl_2x2H_20$, 0.005M KCl, 0.005M glucose).

In the present study the preliminary optimization experiments were based on the previously published results on genetic transformation of nonregenerable maize cell lines through direct gene transfer into protoplasts by PEG treatment (Maas *et al.*, 1989, Armstrong *et al.*, 1990). It had been demonstrated by the authors that the treatment of protoplasts resuspended in 0.2M mannitol, 0.08M $CaCl_2$ solution (MaCa) by polyethylene glycol dissolved in 0.1M $MgCl_2$, 0.4M mannitol resulted in considerably

higher transient gene expression levels compared to the standard PEG transformation protocols (Maas *et al.*, 1989). A similar treatment, but using PEG dissolved in F-medium (Krens *et al.*, 1982) appeared to be the most efficient combination resulting in recovery of up to 300 kanamycin resistant callus clones per million input protoplasts prepared from a nonregenerable maize variety Black Mexican Sweet cell suspension culture (Armstrong *et al.*, 1990).

Different PEG transformation protocols and treatment conditions have been compared during the establishment of an optimized transformation procedure for protoplasts of the HE/89 maize cell line (see results). Utilizing the developed genetic transformation protocol it is possible to produce routinely 10-20 (up to 200) transformants per million of treated maize protoplasts which is approximately a 100-fold higher transformation efficiency in comparison to the first experiments with the HE/89 cell line (Golovkin *et al.*, 1993). Within 3-4 months after protoplast treatment regenerated transgenic maize plants can be transferred to greenhouse to be grown up to maturity. Application of the transformation procedure described by Armstrong *et al.* (1989) gives variable results and seems to depend very much on quality and age of PEG solution.

In the present work, four different selection methods were evaluated, and all of them proved to be suitable for identification of maize transformants. Reliability of application of the NPTII and PAT genes, as selective markers in monocot genetic transformation experiments, was demonstrated by the fact that, in the present study, the majority of the selected kanamycin- or L-PPT-resistant maize tissues or plantlets turned out to be GUS-positive. This indicates the low frequency of escapees. Both of those selection protocols were already successfully applied by others in maize or rice transformation (Gordon-Kamm *et al.* 1990; Fromm *et al.* 1990; Battraw and Hall 1990). The geneticin (G418) antibiotic has been so far used for selection only in rice transformation experiments and has been shown to be superior to kanamycin in suppressing growth of non transformed protocolonies (Dekeyser *et al.* 1989). The present study demonstrates for the first time its successful application in maize transformation system.

As another new approach, an attempt has been made to establish a selection system, based on vital histochemical staining of micro-colonies for detection of the GUS gene activity and culture of individual transgenic tissues, after a gentle staining procedure. The advantage of this method would be the early and safe identification of transformants. It could be applied for a large scale screening of transformants, to search for promoter or enhancer elements, by using promoterless constructs or vectors containing a minimal promoter. It should be postulated, that recognition of transformants by this approach requires a considerable level of expression of the introduced GUS

reporter gene. This implies the possibility of employing the method in gene tagging experiments to identify strong promoters which are important in the projects where overexpression of certain genes are needed (for example, conferring a virus resistancy or other useful traits to plants through overexpression of antisense viral DNA or RNA (Mol *et al.*, 1990; Robert *et al.*, 1990).

At present, the methodology for the selection and culture of tissues after vital GUS staining, has not been fully developed. Further optimization is needed, to improve survival rate of the stained protoclones. Considering the observations by Harris *et al.* (1990) and Gordon-Kamm *et al.* (1990), about heterogeneity of X-gluc staining, it may be possible, that cells or tissue sectors only with reduced accumulation of indigo dye crystals, retained their capability for further proliferation. Therefore, shortening the time of incubation of analysed colonies in the staining solution or the separation of GUS gene expressing protoclones, as soon as the first cells appeared blue on the surface of the colonies, might increase the number of surviving transformants. Despite of the fact that regeneration of transgenic plants was achieved in several selected clones (see Fig. 2/B, Fig. 4/C) after induction of the embryogenic program in maize tissues grown from stained micro-colonies, the morphogenic potential was reduced, in comparison to callus cultures derived from other selection protocols. We have failed to obtain seeds from first regenerants selected by vital staining method and grown up in a greenhouse. Further work to optimize the staining procedure and analysis of a larger population of the protoclones, selected by the described vital GUS staining will be required to eliminate this limitation.

At present, most frequently the high-velocity microprojectile DNA delivery system has been employed to produce fertile transgenic maize plants (Gordon-Kamm *et al.* 1990; Fromm *et al.* 1990). Since this methodology relies on the bombardment of multicellular tissue material, estimation of the genetic transformation efficiency is difficult. Limited data are available about the relations between the number of bombarded cells or the amount of tissue material and the frequency of regenerated transformants. The efficiency of the PEG-mediated direct DNA transfer transformation system has been demonstrated by the present experiments, in which several hundred independent transgenic protoclones and over 140 independent transgenic plant lines with kanamycin, geneticin (G418) or L-PPT resistance and GUS activity were produced by treatment of about $17x10^6$ maize protoplasts. This amount of protoplasts can be easily released in a single experiment by enzyme treatment of 4g cells from the HE/89 embryogenic maize suspension culture. Transgenic nature of the kanamycin, geneticin (G418) or L-PPT resistant cell lines was also confirmed by detection of the GUS specific sequence by PCR amplification and Southern hybridization in the genomic DNA from selected protoclones. As shown in Fig. 4/C, the transgenic maize plants were healthy and morphological or

developmental abnormalities could be observed only rarely. Most of transgenic plants produced embryos after cross pollination. Transmission of the introduced new genes to their progenies was indicated by segregation of the GUS positive and negative seedlings in the second generation. Furthermore, Southern blot analysis demonstrated also the presence of the introduced NPTII gene sequences.

Stable co-transformation with 47% efficiency utilizing two different gene fusions inserted into separate plasmids has been achieved in these genetic transformation experiments. The observed efficiency is comparable with the data from previous publications both in dicot and monocot genetic transformation (Schocher *et al.*, 1986; Lyznik *et al.* 1989; Armstrong *et al.* 1990). The obtained results demonstrate suitability of presented co-transformation system for the application in commercial scale genetic transformation projects to introduce into maize genome new genetic traits of agronomic value where the separation of the gene of interest from the selective marker gene might be important.

One of the main focuses in the present study was the analysis of expression of the bacterial β–glucuronidase (GUS) reporter gene, under the control of different promoter sequences including an artificial chimeric promoter composed of truncated wheat α-amylase gene promoter combined with doubled enhancer sequences of CaMV 35S promoter as well as wheat histone H4 gene promoter in maize tissue cultures and regenerated transgenic plants. The utilization of the GUS gene fusions (Jefferson *et al.*, 1989) allowed quantitative measurements and histochemical localization of the gene expression. The chimeric promoter was constructed from a strictly tissue specific monocot promoter sequences, derived from wheat α-amylase gene (Huttly and Baulcombe 1989) and enhancer elements of viral 35S promoter (Kay *et al.*, 1987; Fang *et al.*, 1989). The employed -389 bp fragment of wheat α-amylase promoter in pα2GT.14 plasmid construct provided considerable expression level (48% of the full promoter) in transient expression experiments with barley aleurone protoplasts (Huttly and Baulcombe, 1989). This promoter element retained its gibberellin (GA_3) responsiveness. In transient expression studies with cultured maize protoplasts, introduction of pα2GT.14 failed to result in GUS gene expression. To generate an active promoter, a defined region represented by the duplicated -208 to -46 bp upstream fragment from the CaMV 35S RNA promoter was linked to this truncated wheat α-amylase promoter. In dicot plants, such as tobacco, this promoter element was shown to function as a transcriptional enhancer (Fang *et al.*, 1989). The present analysis of a large number of maize transformants revealed, that this regulatory DNA fragment in a duplicated form can also act as an enhancer in monocot cells and tissues. In association with another heterologous regulatory element, the constructed chimeric promoter provided considerable expression of the GUS reporter gene. The region of the CaMV

promoter between -208 and -46 bp contains three binding sites for cellular trans-acting factors, such as an activation sequence factor (ASF1), GATA motif binding factor (GATA1) and CA-rich region factor (CAF) (see the review by Benfey and Chua, 1990). Activity of this enhancer element in maize, suggests a functional role for these transcription factors in monocot plants as well.

The chimeric promoter composed of the doubled CaMV enhancer element linked to the truncated wheat α–amylase promoter and studied in the present transformation experiments provided a well defined pattern of expression of the GUS reporter gene in various organs and tissues of the regenerated transgenic maize plants. The obtained fluorometric data about the GUS activity outline a very similar expression profile, as it was described for the complete CaMV 35S promoter in other plant species (Jefferson *et al.*, 1987; Benfey and Chua 1990; Battraw and Hall 1990; Terada *et al.*, 1990; Tada *et al.*, 1991). Furthermore, the distribution of GUS-positive tissue regions and cells in sectioned organs, also followed an analogous pattern. From these observations we can conclude the dominating role of the doubled CaMV 35S enhancer element over the α-amylase regulatory sequence. This was also indicated by the failure in induction of the activity of the chimeric promoter by GA_3 treatment. Culture of transformed callus tissues in the medium, supplemented with 1 mM GA_3 did not increase the GUS activity (data not shown).

We have observed differential expression of the GUS gene fusions containing wheat histone H4 gene promoter sequences introduced into maize genome. The elevated histone promoter activity in fresh callus cells with logarithmic growth phase in comparison to old callus tissues, may be partially attributed to the increase in cell division rate. However, we have to consider as well the influence of other physiological factors on the stability of the β-glucuronidase enzyme in the analyzed transgenic maize tissues.

The histochemical analysis have revealed localization of the wheat histone promoter activity in the regions with high cell division frequency, namely, in meristematic zones and young plant tissues. In roots of transgenic plants the GUS activity was very high in the meristematic region. Very similar expression patterns were described in roots of other transgenic plants carrying histone promoter regions linked to the GUS gene, either from monocot or dicot plants (Lepetit *et al.*, 1992; Terada *et al.*, 1993; Kapros *et al.*, 1993).

The described gene expression studies suggest that the analyzed wheat histone H4 promoter region specifies cell division-dependent expression of the GUS reporter gene in transformed maize cells and tissues. This assumption is supported by the information obtained in studies of activity of the wheat histone H3 gene promoter which contains three conserved cis-acting sequence elements identical to the regulatory motifs present in the histone gene THO11. It has been demonstrated that these sequences determine the S

phase-specific expression of the wheat histone gene (Nakayama and Iwabuchi, 1993; Ohtsubo et al., 1993). However, further experiments preferably based on synchronized cultures will be required to confirm that this regulatory element is under cell cycle control. The work is in progress to study cell cycle dependency of transcriptional activity of the wheat H4 histone gene promoter combined with the GUS reporter gene utilizing synchronized suspension cultures established from transgenic callus tissues.

In agreement with findings in analysis of various transformants including maize (Klein *et al.*, 1989; Lyznik *et al.*, 1989; Terada *et al.*, 1990), wide range of GUS activity was observed among the individual transformants, both at callus and plant level. In general, the GUS specific activity did not differ considerably from the average values, reported for the full size CaMV 35S promoter - GUS gene fusion in kanamycin-resistant calli (Lyznik *et al.*, 1989). As shown in Fig. 5., several transformed callus tissues containing pMGP1 construct exhibited significantly higher GUS activity than tissues derived from transformation experiments with pNA2G. It may be important, that in this vector the chimeric promoter with the GUS coding sequence is followed by an additional CaMV 35S promoter, expressing the selective PAT gene. The orientation of the two promoters is the same. In contrast, the pNA2G construct carries the CaMV 35S promoter in a reverse orientation, and separated from the GUS block by the selective NPTII gene (see Fig. 1.). Considering the measured GUS activity values, it is evident that the pMGP1 construct provides higher promoter activity. The interaction between promoters located at the same transformation vector was already reported in dicot transformation experiments (Fang *et al.*, 1989; Beilmann *et al.*, 1992). In the present example, we can not exclude an interaction between the enhancer elements located in the chimeric and the CaMV 35S promoter. It was reported by Fang *et al.* (1989), that in transgenic tobacco, the enhancer sequences in CaMV 35S promoter, at the 3' end of the CAT gene can drive its expression, however, in reverse orientation the enhancer function was two times weaker than in the case of forward oriented one. A similar observation was made by Beilmann *et al.*, (1992) with GUS gene fusion and CaMV 35S enhancer. Here we can see an example with a monocot cell system. The observed differences in the gene expression levels can also be recognized in leaf tissues of regenerated plants (Table 2.), therefore, the *cis*-interaction between promoters can be considered as a general cell type independent feature in control of gene expression. These studies also emphasize the significance of the orientation and the distance between the interacting elements.

Another factor which can potentially influence the level of gene expression is a copy number of the introduced foreign genes per host genome. There are contradictory data concerning the character of gene expression in stably transformed independent cell clones. The results have been obtained demonstrating positive as well as negative

correlation between copy number of foreign genes and their level of expression (see Review of the literature p. 11).

However, in most of the published works on this theme (Czernilofsky *et al.*, 1987; Jones *et al.*, 1987; Sanders *et al.*, 1987; Klein *et al* ., 1989; Steber and Willmitzer 1989; Tada *et al.*, 1990) the correlation has not been found. The results of the present study also indicate the absence of interdependence between amount of *uidA* gene containing plasmid units present in genomic DNA of particular transformant and the level of the GUS expression (see p. 47, compare Fig. 6/B 4,5 and Fig. 9).

6. CONCLUSIONS.

The optimized PEG-mediated transformation procedure combined with the protoplast culture and plant regeneration protocol for the HE/89 synthetic maize cell line is an efficient system for fast and large scale production of maize cells and fertile plants with the new genetic traits and for functional analysis of promoter sequences both in transient and stable transformation experiments.

Different selection schemes can be successfully employed for selection of maize transformants utilizing *bar* and *neo* marker genes. The study demonstrates for the first time that geneticin (G418) antibiotic can be applied for safe selection of transgenic maize protoclones.

The non selected gene in a separate vector plasmid can be cotransformed in the HE/89 maize cells along with the selectable marker gene at an efficiency of ca. 50%.

The vital staining method developed here in allows recovery of the GUS gene expressing protoclones and has potentials in promoter tagging experiments.

Strictly tissue specific wheat α-amylase promoter can be activated by enhancer elements of the viral 35S promoter to drive differential expression of bacterial GUS gene in maize cells. The present analysis of a large number of maize transformants has revealed that duplicated -208 to -46 fragment of CaMV 35S promoter can act as an enhancer also in monocot cells and tissues.

Wheat H4 histone gene (TH011) promoter confers cell division- dependent expression in maize. This observation suggests similarity in transcriptional mechanisms of the two cereal species.

Interpretation of the results obtained in functional analysis of promoter sequences should take into account the existence of interactions between different promoters present in the same transformation vector. The *cis*-interaction between promoters can be considered as a general cell type independent phenomenon in control of gene expression.

The presented results demonstrate the absence of correlation between the number of molecules of the GUS reporter gene integrated into genome and its expression level in the host maize cells.

The phosphinothricin resistant maize stocks produced during the described experimental work have been distributed to the maize breeders. Since this herbicide is known as an efficient and environmentally safe compound the obtained breeding material may have potentials in further improvement of maize.

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