TISSUE AND ORGAN SPECIFIC EXPRESSION OF DEVELOPMENTALLY REGULATED CHIMERIC GENES IN TRANSGENIC PLANTS

Ph. D. Thesis

Written by Ivan Stefanov
Under the supervision of Professor Dr. Dénes Dudits

Institute of Plant Physiology,
Biological Research Center,
Hungarian Academy of Sciences, Szeged

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University "Attila József" Szeged.

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LIST OF ABBREVIATIONS

BA	N^6- benzyladenine.
CaMV 35S	Cauliflower Mosaic Virus 35S RNA transcript.
CTAB	Cetyldimethylethyl- ammonium bromide.
DNA	Deoxyribonucleic acid.
GA	Gibberelic acid.
g4	Gene # 4 from *Agrobacterium* T- DNA.
GUS	ß- glucuronidase.
IBA	Indole- 3- butyric acid
mas	Mannopine synthase
MU	4- methylumbelliferone
MUG	4- methylumbelliferyl ß- D- glucuronide.
NAA	Naphthaleneacetic acid.
neo	Neomycin phosphotransferase gene from Tn 5 of *Escherichia coli*.
NOS	Nopaline synthase
NPT II	Neomycin phosphotransferase II.
P	Promoter.
t	Transcription terminating signal.
T- DNA	Transfer DNA from the tumor- inducing plasmid of *Agrobacterium*.
uid A	ß- glucuronidase gene from *E. coli*.
x-gluc	5-bromo-4-chloro-3-indolyl b-D-glucuronide

1. INTRODUCTION

A plant, like any multicellular organism consists of an intricated array of tissues. Each of these tissues differ from the others, and each stand in a precise physiological and morphological relation to the others. Similarly, the control of the developmental process that affects the organization of complex tissues is often exceedingly specific. In animals, these control elements are largely internalized and normally are not influenced considerably by outside environmental changes and environmental cues are not used during the development. The mode of such control steps in animals, involves both the movement of cells and the irreversible commitment of groups of cells to a particular developmental path. Animal development proceeds according to a faithfully followed blueprint. At each stages particular and highly specific cell movements linked to changes in gene expression occur in a way that both timing and order are crucial from the point of the overall result- the adult animal.

In contrast, plants are always capable to change development program. They develop, like all others organisms, according to a genetic blueprint, but the way in which their particular blueprint is expressed is very flexible and greatly influenced by external factors. Related to this flexibility it is a fact, that almost any differentiated cell stage can be completely reversible. In a fully differentiated plant cell, division and gene expression can be reactivated that can lead to an alternative modes of differentiation, even to the development of a complete plant. The processes of plant differentiation have been studied all along the 20th century. Most of the impetus for this study has come from a single hypothesis: in 1902 the German botanist Gottlieb Haberlandt, born in Mosonmagyaróvár, proposed that all living plant cells are *totipotent*, each of them possess the full genetic capacity for the control of development entire organism. Haberlandt explained the differentiation of tissues by proposing a hypothesis that only part of this potential is normally realized in any given differentiated tissue. He went on to suggest, one ought to be able to develop a whole mature plant from isolated plant cells if the correct medium to support growth is provided. His hypothesis remained unconfirmed for more than 50 years. A solution of the problem was devised by Steward and his coworkers in 1958. They were able to induce somatic embryos ("embryoids") in carrot cell suspension cultures and to regenerate whole plants. Since that time, the potential for asexual embryogenesis and organogenesis has been shown to be characteristic of a wide range of tissue culture systems from both dicot and monocot plants.

Therefore morphogenesis, and especially somatic embryogenesis provides a bridge between the cellular and plant levels and opens the use of cultured cells in genetic manipulation experiments (Dudits et al., 1991).

The development of these techniques has considerably promoted the recent advances in plant molecular biology as well. While it has been hailed as a huge new step in the "Green revolution", its main impact for biology and genetics is due to its tremendous power not only to change, but also to analyze gene structure, function and regulation. Extensive studies led to a considerable accumulation of information on the structure and activity of genetic material, so our knowledge about phenomena occurring in plant cells in the intact plant and during *in vitro* morphogenesis has multiplied by an order of magnitude within a couple of years. But, at the same time, the complexity of the problems has increased. The use of more sensitive methods of molecular biology, including molecular cloning of genes, the determination of coding sequences, *cis* and *trans* acting factors regulating gene expression and the use of chimeric genes revealed a much more complex regulation. During the continuous progression some of the proposed postulates have been already questioned by new facts.

The aim of this work was to use the potentials in studies on the expression of a bacterial marker gene linked to promoters with different patterns of transcriptional activity as a model system for monitoring the transcriptional changes occurring during plant development and *in vitro* embryogenesis and organogenesis. At the same time we wanted to asses the possibilities in use of the GUS fusion system as a tool for studies on plant morphogenesis. For this purpose we applied both quantitative measurements and histochemical staining of plantlets, organs, tissues and individual cells.

Three plant tissue culture and regeneration systems were used: the *Nicotiana tabacum* Petit Havana SR 1 genotype, able to form shoots directly from the explant, three cultivars of *Brassica napus* which needed the establishment of a complex regeneration protocol and the genotype A2 of *Medicago varia* regenerating through somatic embryogenesis. Transgenic plants were produced that carry chimeric promoter-gene fusion. This methodology has opened substantially new ways to revealed essential changes in gene expression during plant development.

2. LITERATURE REVIEW

2.1. REPORTER GENES

The mechanism by which an organism controls the production of proteins through differential expression of its genes has challenged many researchers since the link between protein production and gene expression was first discovered. It has been shown that in most cases the control of protein level is mostly achieved during the transcription, although stability of mRNAs, proteins and posttranslational modifications and restructuring are also important. The classical approach to study gene expression is based on protein and RNA separation techniques, combined with radioactive and immunodetection methods (Galston and Davies, 1969; Nagao et al., 1986; Györgyey et al., 1991). Although these approaches have been successfully used in the analysis of genes encoding for abundant proteins during specific development stages as seed storage proteins (Goldberg et al., 1981; Higgins, 1984), some hormone- induced genes (Theologis, 1986) and defence genes (Ecker and Davis, 1987), the detection and quantitative determination of polypeptides of interest are in many instances rather complex and tedious procedures. This is especially the case if a gene product can not be easily detected through enzymatic or functional activities.

The use of gene fusions between the regulatory or targeting sequences from a gene of interest (*controller*) and the coding sequence of a gene whose product is easily detectable (*reporter*) can broaden the possibilities for deep studies on regulation of gene expression. These later genes are called *reporters*, because they supply information on the regulation or action of the analyzed gene. 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 individual members of gene families without the interfering effect of an other members (see as an example Kapros et al., in press). 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. The use of *in vitro* generated gene fusions followed by their introduction into the plant genome gave a tremendous pouch to modern plant science.

Studies on gene function in intact systems are intrinsically descriptive, because experimental changes of the environment without seriously injuring the regulatory network of the cell are not possible.

On the other hand, the use of cell free systems can not represent the complex interaction characteristic for a living organism, even the simplest unicellular one. The use of transgenic plants avoids some of these drawbacks allowing the study of the behaviour of manipulated genes in different environment, without the crude mechanical intervention employed by the classical methods.
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.

Several reporter genes have been used in studies of gene expression in higher plants. These include the ß-galactosidase (*lac Z*), the neomycin phosphotransferase II (NPT II, APH3' II), the chloramphenicol acetyltransferase (CAT) and the ß-glucuronidase from *E. coli*, the nopaline synthase (NOS) and octopine synthase (OCS) from *A. tumefaciens*, the luciferases (LUX) from *Vibrio harveii* and *V. fisheri* and the firefly luciferase (LUS). For some special purposes, like the monitoring of transposon excision, the streptomycin resistance gene (SPT) was used (Jones and al., 1989). New, readily assailable and versatile gene systems are also on the way. All currently used reporter gene systems have its positive and negative properties.

In spite the remarkable success in use of *lac* fusions in bacterial and animal cell systems (Itakura et al., 1977; Rose et al., 1981; Lis et al., 1983), β-galactosidase gene fusions have limited potentials in plants (Helmer et al., 1984) since it is difficult to assay them due to high level of endogenous β-galactosidase- like activities in virtually all plant tissues.

The use of the *Agrobacterium* Ti- plasmid encoded genes such as nopaline synthase (NOS, Bevan et al., 1983) and octopine synthase (OCS, De Greve et al., 1982) eliminates the problems related to endogenous enzyme activities. Theoretically, they should be very good reporter genes, since there is no equivalent of neither their gene product, nor products of the enzyme activity normally in plant cells.

These genes are not widely used in expression studies mainly because of the difficulties in quantification procedures requiring tritiated substrates (Otten and Schilperoort, 1978; Herrera- Estrella et al., 1988). Another serious drawback is that the OCS can not tolerate amino- terminal fusions (Jones et al., 1985). Nevertheless they find their place as scorable markers proving information about the integration of modified T- DNAs (Rogers et al., 1987).

Historically, the chloramphenicol acetyltransferase (CAT) was the first bacterial gene successfully expressed in plant cells and thus demonstrated the feasibility of the reporter gene concept (Herrera- Estrella et al., 1983). The gene product specifically acetylates chloramphenicol thereby it inactivates the antibiotic. CAT gene fusions were used to characterize the regulatory sequences of *Bacileus subtilis* (Goldfarb et al., 1981), yeasts (Cohen, 1980) and animal cell systems (Gorman, 1985). The assay is very sensitive and quantitative. The main disadvantages are the high cost of the reagents, the tediousness of the procedure and the intrinsic non-specific acetylating activities that are present in some plants (Gorman et al., 1982).

The neomycin phosphotransferase II (NPT II) gene is one of the most widely used reporters (Reiss et al., 1981; Van den Broeck et al., 1985; Salmenkallio et al., 1990), due to its suitability also as a selective marker. It can tolerate large amino-terminal fusions, making it usable for studying organelle transport (Van den Broeck et al., 1985). Two main problems can hinder the use of the NPT II 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 by enzyme kinetics, and the need to use large amounts of radioactive ^{32}P-γATP for detection.

Genes involved in bioluminescent reactions have been isolated, cloned and expressed in different systems. The best characterized *lux A* and *lux B* genes are from the marine microorganisms *Vibrio harveyi* and *V. fisheri* (Baldwin et al., 1984; Engebrecht and Silverman, 1984) and the luciferase (*luc*) from the firefly *Photinus pyralis* (De Luca and Mc Elroy, 1978; De Wet et al., 1985). Both enzymes were successfully used in transgenic plants (Ow et al., 1986; Koncz et al., 1987; Schneider et al., 1990; Langridge et al., 1989). New luciferase genes were cloned from click beetles that encoded enzymes producing light with different colours (Wood et al., 1989). These genes are expected to broaden the possibilities of the system by the simultaneous detection and analysis of the activity of several reporters. The most serious drawback for the extensive use of the luciferases is the need of very sophisticated and expensive equipment in order to measure accurately the photoemission. The *in vivo* or *in situ* detection requires video imaging techniques.

In the 1987 year, Jefferson and his coworkers have proposed the use of the bacterial gene *uid A* as 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 several dicotyledonous and monocotyledonous species (Jefferson, 1987; Saito et al., 1991; Battraw et al., 1990; Omirulleh et al., in press).

In the present work we used the ß-glucuronidase for quantitative and histochemical detection of promoter activity in transgenic tobacco, rapeseed and alfalfa plants.

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 (Fig. 1./A). Fluorometric, spectrophotometric and histochemical substrates are commercially available (Fig. 1./B). The enzyme is a tetramer of identical subunits with mol. weight of about 68,200 daltons each. It is stable under physiological conditions, has a pH optimum between 5.2 and 8.0 and 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 is included in the assay mixture.

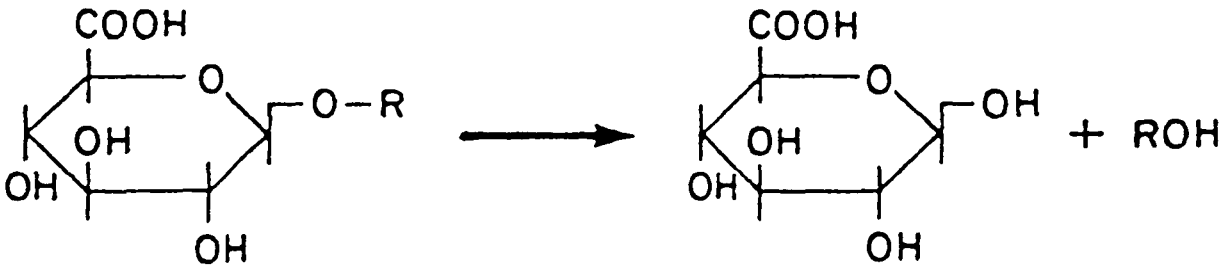
For quantitative studies on gene expression, most frequently a fluorogenic assay is used. The substrate, when cleaved by the enzyme, liberates a product that is fluorescent. Currently the best substrate is the 4- methyl umbelliferyl ß- D-glucuronide (M.U.G. Jefferson, 1987).

The histochemical staining allows us to detect ß-glucuronidase activity in individual cells and tissues in plants. Its quality is affected by numerous variables, including all aspects of sample preparation, fixation and the reaction itself. The 5-bromo- 4- chloro- 3- indolyl glucuronide (X- Gluc) is the substrate of choice for that 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 coloured indigo dye. This dimerisation is stimulated by oxygen and can be enhanced by oxidation catalysts such as K^+ ferricyanide/ ferrocyanide mixture (Jefferson, 1987; Castle and Moris, 1990).

The GUS system was used for a variety of studies, ranging from 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., in press), 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).

A

β-glucuronidase reaction



B

ASSAYS

histochemical	R = napthol napthol ASBI
fluorogenic	R = 4-methylumbelliferone R = fluorescein 3-O-methylfluorescein R = resorufin
colorimetric	R = p-nitrophenol R = phenolphthalein

Fig. 1. ß- glucuronidase activity and substrates.
A. Cleavage of ß- glucuronides catalyzed by the enzyme.
B. Commonly used and commercially available substrates.

2.2. SPECIFIC PROMOTERS

From a broader perspective, the growth, development and the entire life of an organism entails for a long series of biochemical reactions, each of them delicately tuned to achieve a precise effect. Specific enzyme activities are responsible for a particular metabolic or structural change. Once the change has occurred and the cell does not need them, those defined enzyme activities cease. A cell must know not only how to synthesize a particular protein, but also when to start the synthesis. As it was mentioned earlier, organisms control the expression of their genes largely by specifying the time when the transcription of individual genes has to begin. Most genes possess special nucleotide sequences called regulatory sites or promoters, which act as points of control. It must be pointed out that the term **promoter** is used in two different manners: some authors (mostly molecular biologists) define it as the RNA- polymerase recognition site together with the transcriptional start. According to the second usage, the term includes all upstream regulatory elements of a gene at 5' sequence region. In the present work we will use the later terminology.

A large number of plant promoters directing transcription in either an inducible or tissue- specific manner have been studied in details (reviewed by Sachs and Ho, 1986; Theologis, 1986; Kuhlemeier et al., 1987; Weising et al., 1988). Plant transformation has allowed the functional analysis of promoter sequences both in transient assays and after stable transformation. Generally, it has been found, that 5' sequences contain most of, 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 motifs which are responsible for qualitative and quantitative control of the expression (Benfey et al., 1989; Leung at al, 1991). These elements are likely to control RNA polymerase activity by interacting directly or indirectly with regulatory proteins. Fig. 2. shows a simplified picture of the 5' regulatory structures of a plant gene.

Most of the plant genes characterized to date are transcribed in a regulated rather than a constitutive manner (Kuhlemeier et al., 1987). 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). The developmental regulation of plant genes is often connected with additional control mechanisms (Rocha- Sosa et al., 1989; Györgyey et al., 1991). A brief overview of the promoters used in this work would help to understand the basis of our choice.

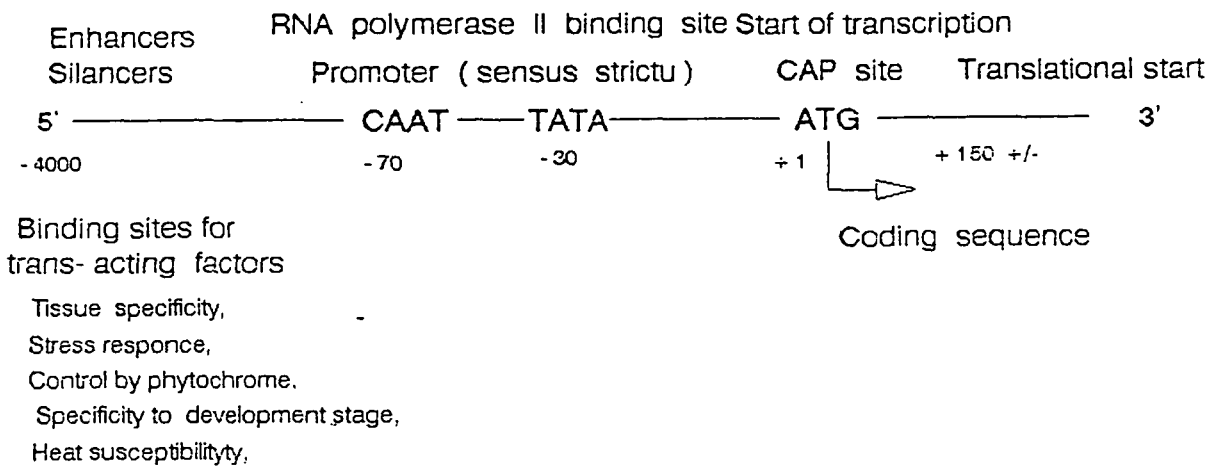


Fig. 2. Simplified scheme of the 5' regulatory elements of a plant gene. (From Blaich, 1992, modified)

Ribulose-1,5-bis phosphate carboxylase (*rbcS*) is the most abundant protein in chloroplasts, where it accounts for up to 50% of the total organelle protein (Ellis, 1979). The carboxylase holoenzyme is made of eight copies each of a large and a small subunits. The large subunit (*rbcL*) is encoded in the chloroplasts, while the small (*rbcS*) is encoded in the nucleus by a multigene family. Expression of *rbcS* genes is induced by light and the regulation is exercised primarily at the transcriptional level (Tobin and Silverthorne, 1985). The *rbcS* transcripts are distributed on a tissue-specific manner, being most abundant in photosynthetic organs, such as leaves and perycarps (Coruzzy et al., 1984). The light-inducibility and organ specificity of the *rbcS* gene family made it attractive as system for studying gene regulation, especially *cis*-elements.

It has be shown that the *rbcS* gene is expressed in a light regulated and leaf-specific manner in transgenic tobacco and petunia plants (Nagy et al., 1985; Jefferson et al., 1987)

Promoters from non- plant sources can work poorly, if at all, in plants (Weising et al., 1988). Among the few exceptions we can mention the promoter of the *Drosophila* heat- shock genes (Spena and Schell, 1987) and plant- specific promoters derived from pathogens, i. e. the T- DNA genes from *Agrobacterium* and genes from plant viruses, which have been often used to direct foreign gene expression in plants.

The promoter of the gene IV of the cauliflower mosaic virus (CaMV) encodes for a 35S RNA transcript. In the context of the virus, the 35S RNA promoter is a strong one, because it drives 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).

The CaMV 35S promoter was considered as constitutive regulator (Odell et al., 1985; Benfey and Chua, 1990), but recent studies using highly sensitive 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) suggested a complex structure with two domains and several subdomains, conferring, through synergistic complementation an almost constitutive expression int tobacco.

The dual bidirectional promoter called TR 1'-2' or mass was derived from the genes for mannopine synthase on the TR-DNA of an octopine-type Ti plasmid (Velten et al., 1984).

This promoter is often used for expression of chimeric genes in transgenic plants and in transient expression studies (Velten and Schell, 1985; Deblaere et al., 1987; Koncz et al., 1987; Sarito et al., 1991; Stefanov et al., 1991). It has been assumed that the expression of this promoter is constitutive (Deblaere et al., 1987; Rogers and Klee, 1987). Recent results, however, reported tissue-specific, organ dependent and auxin enhanced expression of this promoter (Langridge et al, 1989; Leung et al, 1991). The two units of this promoters are leading transcription in opposite directions and confer different level of expression in transgenic plants. Modular structure with multiple controlling elements was also suggested (Leung et al, 1991)

As the developmental control or the tissue specificity of a promoter are concerned, the results of the experiments based on the use of different reporter genes and expression systems can lead sometimes to rather different conclusions and they should be carefully interpreted (Benfey and Chua, 1990; Schneider et al., 1990).

It is generally accepted that the amounts of transcripts are regulated by *cis*- acting sequences, mainly located on the 5' end of the gene. However, factors involved in the quantitative control of the expression level are still a twilight zone. *Cis*- acting enhancers have been found in the vicinity of some genes, they cannot account for the huge difference in expression of the same chimeric gene in individual regenerated transgenic plants. Several factors might play a considerable role:

1. Position effect. In animal systems, the expression of foreign genes is influenced by their chromosomal position (Spradling and Rubin, 1983; Levis et al., 1985). This position effect might be related to chromatin structure at the integration site and/or the location of endogenous *cis*- acting regulatory sequences near by. It was demonstrated that 'stuffer' fragments which presumably should buffer these effects are of little use (G. Donn, personal communication), and the level of expression of two very closely linked genes can vary independently (Nagy et al., 1985; Eckes et al., 1986; Stefanov, unpublished).
2. Copy number. The impact of copy number on gene expression varies from one system to an another. While some authors 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), others were not able to find correlation (Sanders et al., 1987; Jones et al., 1987; Czernilofsky et al., 1987). Hobbs et al. (1990) have found that single copy results in higher expression. The last phenomenon has been linked to the methylation of genes.

3. DNA methylation. Wild- type T- DNA is subject 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 at 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 characters and differences in the secondary metabolism may play a considerable role and contribute to the production of controversial results when various systems are used (to some extend reviewed by Blaich, 1992)

To summarize, limited information is available about the factors influencing transcription of a foreign gene introduced into a transgenic plant. The purpose of this work is to approach the problem of gene regulation, by studies on the activity of well characterized promoters in various plants with different pathways of development both *in vitro* and under normal growth conditions.

2.3. TRANSGENIC PLANTS

Since 1984, when the first transgenic plants expressing engineered alien genes were reported, rapid progress has been echieved in plant molecular biology. Several techniques have been established for the precise and controlled addition of genes to the genome of plant cells. The regeneration of such transformed cells leads to transgenic plants with new traits. A large number of strategies have been developed to attempt to broaden the possibilities of the gene transfer, but up to now, only few of them are used successfully (critically reviewed by Potrykus, 1990) (Fig. 3.).

Uncovering the biology of gene transfer might be helpful to understand why some methods work and others never do.

First of all, a transgenic plant can only be resulted from integrative (stable) transformation of the novel gene in a totipotent cell. Several barriers lay between our willingness to introduce a gene and the integration event.

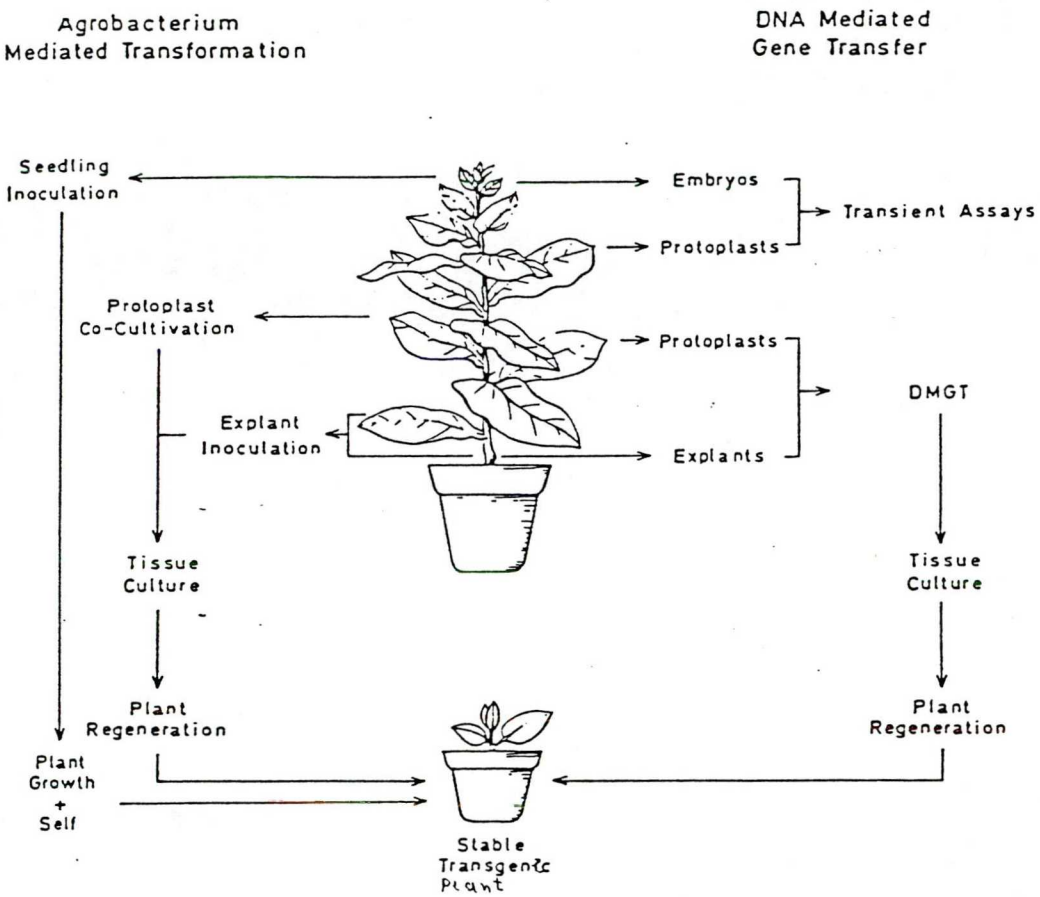


Fig. 3. **Production of transgenic plants. The scheme shows the most popular methods that are currently available for introduction of foreign genes into the plant genome and the essential steps for transformation of plants. (From Walden and Schell, 1990)**

1. However, according to the theory, all plant cells are totipotent, the conditions for plant regeneration are not fully known for every plant tissue culture systems.
2. Plant cells differ in their capability to respond to triggers, a phenomenon, called competence. Cells supposed to regenerate transgenic plants must be competent both for integrative transformation and regeneration.
3. Plant tissues are composed from cells competent for many response ever since the competence for transformation and regeneration are the most important ones, several situations can be imagined: - few cells are competent for both integrative transformation and regeneration. Most of the cells are competent for transformation or 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 cell population is determined by the genotype, the tissue origin of organ, the developmental state of the organ.

As shown by large number of experimental observation wounding and hormone shock are potent triggers for shifting of potentially competent cells towards competent state.

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

In any case, the DNA molecules, carrying genetic information have to be transported across the cell wall and/or plasmamembrane. If the DNA is not degradated in the cytoplasm it has to reach the cell nucleus, where the genetic information can be expressed. The question how that transport across the nuclear membrane occurs is still largely unsolved, although the experiments of Okada and coworkers (1986) 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 dissolving 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.

Plant cell walls are efficient barriers and traps for DNA molecules, so, either they have to be removed (protoplasts) or a vector must brake through to reach the cytoplasm and nucleus, as the conjugation-line process involving *Agrobacterium,* or the microinjection of DNA solution directly into the target cell (Neuhaus et al., 1987). The use of high velocity particles coated with DNS is also a route to transport genes across the cell wall (Kelin et al., 1988)

Protoplasts can take up DNA if treated with polyethylenglycol (PEG) and/or electroporation (Potrykus et al., 1985; Saul et al., 1988). The protoplast isolation procedure can promote the shift of potentially competent cells into the competent state. The exogenous DNA is easily integrated through non-homologous recombination. When regeneration-competent cells are transformed transgenic plants can be recovered (Saulet et al., 1989; Shimamoto et al., 1989; Omirulleh et al., in press). In addition, the direct gene transfer to protoplasts, is suitable for fast assessment of promoter activity by transient expression. A limitation in direct DNA-transformation method is the need for a well established plant regeneration protocol from protoplasts, however it has been achieved up to now with considerable number of plant genotypes (for review see Fehér and Dudits, 1993).

The *Agrobacterium* mediated transformation is an efficient way of production of transgenic plants from numerous dicotyledonous species (recently reviewed by van Wordragen and Dons, 1992). Only plants with a pronounced wound response develop a large population of wound- adjacent competent cells for efficient transformation.

The virulent strains of *Agrobacterium* contain a large, 170- 200 kb tumor inducing (Ti) plasmid (Zaenen et al., 1974; Chilton et al., 1977) (Fig.4.). When these strains infect a wounded plant, a discrete portion of the Ti- plasmid (called T- DNA) is transferred to and integrated within the nuclear genome of the infected cells. The T-DNA encodes for genes possessing eukaryotic regulatory signals, therefore they are transcribed and translated in the plant tissue to produce new enzymes. A set of such enzymes directs the synthesis of plant hormones through an unusual pathway. In transformed plants mechanisms for control of phytohormones synthesis are by-passed, what leads to cell proliferation and the formation of a callus or crown gall (Weiler and Spanier, 1981; Amasino and Miller, 1982) (Fig.5.). Other genes encode for enzymes responsible for the synthesis of amino- acid derivatives, called opines, which can be used as the sole carbon and nitrogen source for growth of bacteria containing the appropriate Ti- plasmid. Ti- plasmids are classified according to the opine type they specify, viz. octopine-, nopaline-, agropine- type, just to name the most important ones.

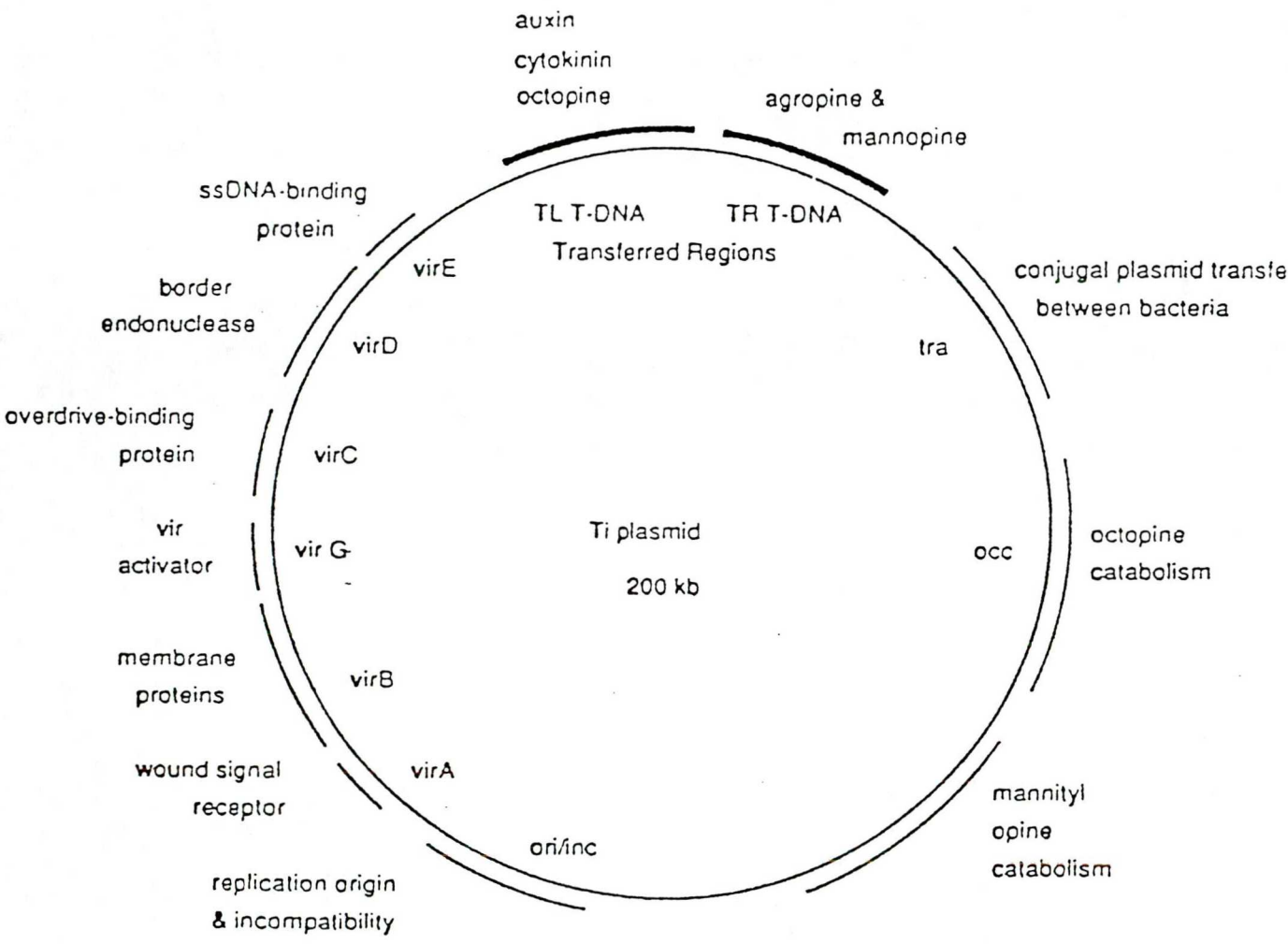


Fig. 4. **Genetic map of an octopine- type plasmid (From Ream, 1989).**

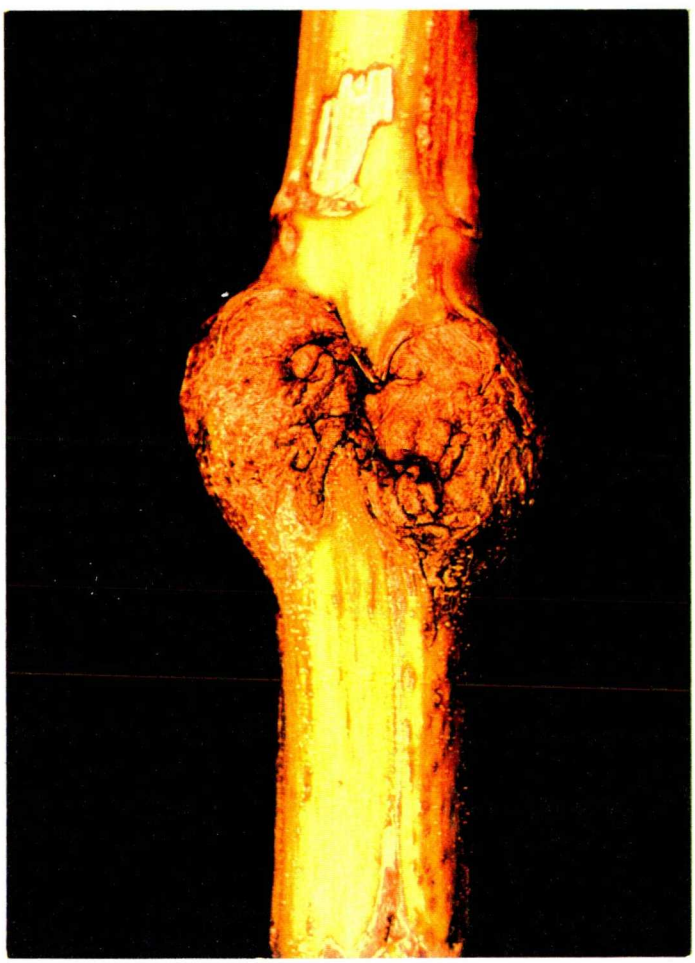


Fig. 5. Crown gall tumor on the stem of a sunflower.

Genetic analyses revealed multiple components required for the T- DNA transfer to plant. First, two chromosomal genes ,*chv A* and *chv B* express products required for the binding of the bacteria to the plant cell (Douglas et al., 1985). Second, a large region of the Ti- plasmid (40 kb) called the virulence *(vir)* region encodes for *trans*- acting functions, promoting T- DNA transfer (Rogowski et al., 1987; Stachel and Nester, 1986). *Vir* DNA itself never integrates into the plant genome. Third, the T- DNA is flanked by an essential 25 bp *cis* elements as perfect direct repeats, called respectively the left and right borders (LB and RB). The deletion of the RB leads to the loss of T- DNA transfer.

Experiments (Wang et al., 1984) have demonstrated, that the RB is sufficient to promote T- DNA transfer, but it is functional only in one direction, that is the same direct as found normally in the Ti-plasmid. Beside these essential components, which vary in structure in the different types of Ti- plasmids, the transformation is influenced by numerous genes potentially affecting every step of the process. Extensive studies were carried out over the past years and considerable progress has been made in understanding the key molecular events (Zambryski, 1988; Binns and Tomashow, 1988). Numerous experiments (Stachel et al., 1986; Albright et al., 1987; Jayaswal et al., 1987) showed that the mechanism of transfer is similar to the bacterial conjugation and involves the production of single- stranded copies of the T- DNA, called T- strands, which form polar DNA- protein complexes.

The exact mechanism of the T- DNA integration into the plant genome is still rather unknown. Single integrated copies are most often recorded, but an average of three T- DNA copies per genome were detected in several dicotyledonous plants (Thomasow et al., 1980; Slightom et al., 1985; Chyi et al., 1986; Wurtele and Bulka, 1989; Fang and Grumet, 1990; Sangwan et al., 1991). Occasionally even 20- 50 copies have been observed (Barton et al., 1983). The occurrence of multiple integrations is either in groups of tandem repeats or in sparse single copies, scattered throughout the genome. The type of distribution is dependent on the Ti-plasmid, generally, the octopine ones generating unlinked copies, while the nopaline type - tandem repeats (J. Jones, personal communication). Nucleotide sequence of cloned T- DNA/ plant junctions demonstrated, that the target sequences are unrelated, with the higher AT content being the only common feature (Holsters et al., 1983; Simpson et al., 1982; Zambryski et al., 1982).

In summary, there are at last seven steps leading to the transformation of a plant cells during *Agrobacterium* infection: 1. recognition of a susceptible plant cells, 2. induction of the *vir* genes, 3. production of the T- strand, 4. formation of the T- DNA/protein complex and its transport to the bacterial membrane, 5. transfer across the bacterial and plant membranes (conjugation ?),

6. transport of the complex through the plant cell cytoplasm and targeting to the nucleus, and 7. integration of the T- DNA as a linear fragment into the plant genome (Fig.6.).

The very early realization that no *trans*- acting functions are encoded by the T-DNA itself, that the border repeats (even only the right one) are the only requirement for successful transfer and integration of any DNA sequence placed between them, led to the use of the Ti- plasmid as a vector for plant genetic engineering. But several obstacles were on the way of its 'domestication':

1. The genes located on the T- DNA region interfere with the normal plant development.
2. Due to their size, the Ti- plasmids are not very convenient for directed and precise genetic manipulation.
3. Since the transformation events are rather rare, a selectable marker should be present within the transferred DNA, in order to identify by positive selection the transformed cells or plants.

In general, two approaches were used to eliminate the unwanted oncogenic genes naturally occurring in the T- DNA: the simplest is to 'disarm' the Ti plasmid. Using homologous recombination, the hormone genes or even the entire T- DNA can be removed, leaving behind a 'disarmed' *Agrobacterium* (Ooms et al., 1982; Zambryski et al., 1983; Koncz and Schell, 1986). Such a Ti plasmid can be used as a helper, providing *in trans* the transfer functions with the so called binary vectors (Hoekema et al., 198) (Fig.7./D). Small plasmids that are able to replicate both in *E. coli* and *Agrobacterium* and contain a selectable marker, a multiple cloning site, and sometimes additional elements within the borders can be used. They are easily manipulated in *E. coli* and then introduced into *Agrobacterium* to be used for transformation. Several such systems, based on different replicons and Ti- plasmid elements were developed (Bevan, 1984; Koncz and Schell, 1986; An, 1987; Deblaere et al., 1987; Rogers et al., 1987; Zyprian and Kado, 1990). Another approach is to manipulate a plasmid that is not capable to replicate in *Agrobacterium*, but contains regions of homology with sequences located within the borders of partially disarmed Ti-plasmids with deleted hormone genes. Upon transfer to the bacterium, the vector is integrated into the T- plasmid by homologous recombination and functions as a part of the T- DNA (Fig.7./B). Two systems are widely used for plant transformation (Zambryski et al., 1983; Fraley et al., 1985, Deblaere et al., 1987) The cointegrated system is more difficult to use than the binary vectors, but is more stable and there are reports on higher transformation efficiency, e. g. with tomato (Mc Cormick et al., 1986).

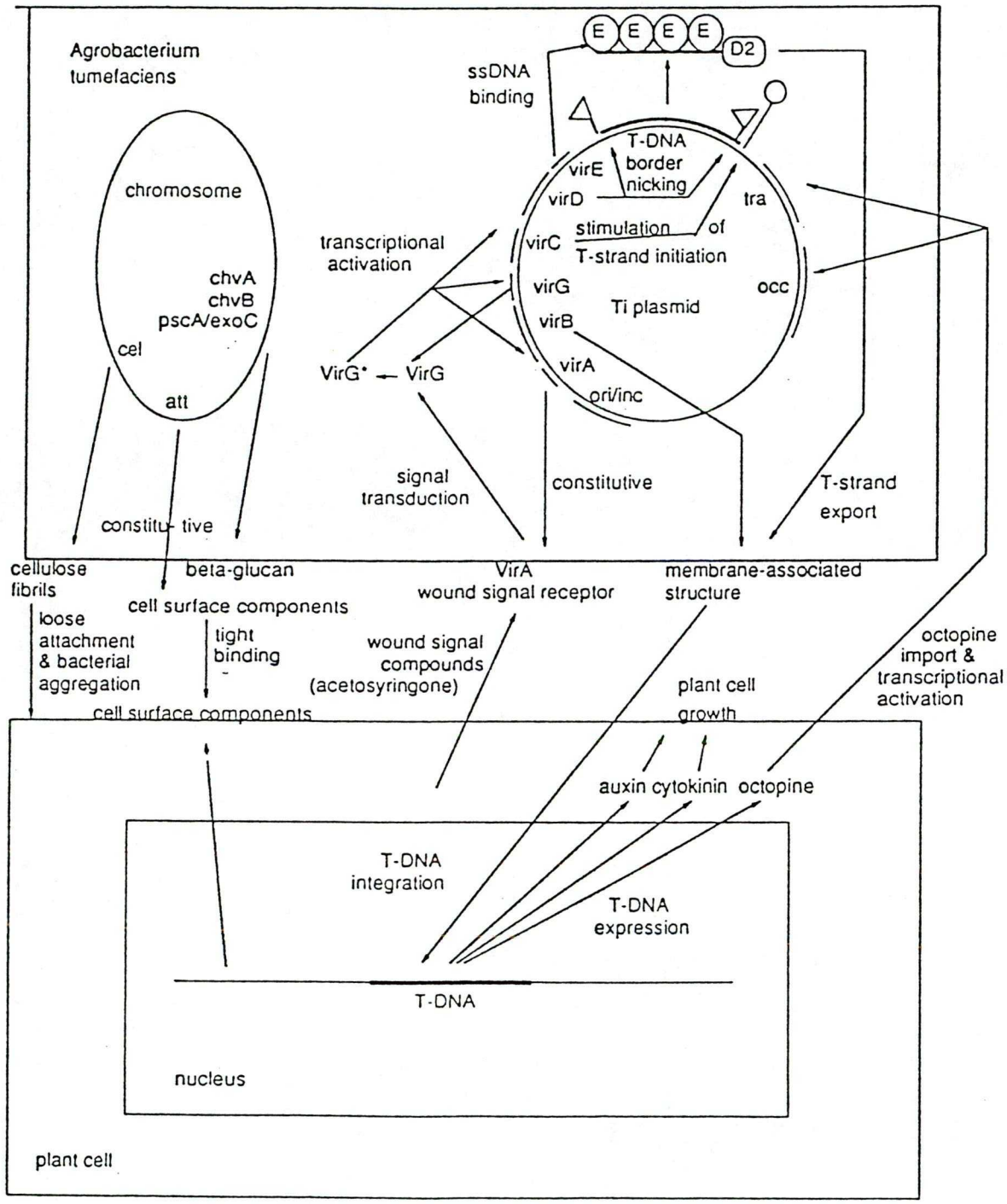


Fig. 6. General scheme of the interaction between *Agrobacterium* and plant cell. The temporal sequence of events is from left to right. (From Ream, 1989)

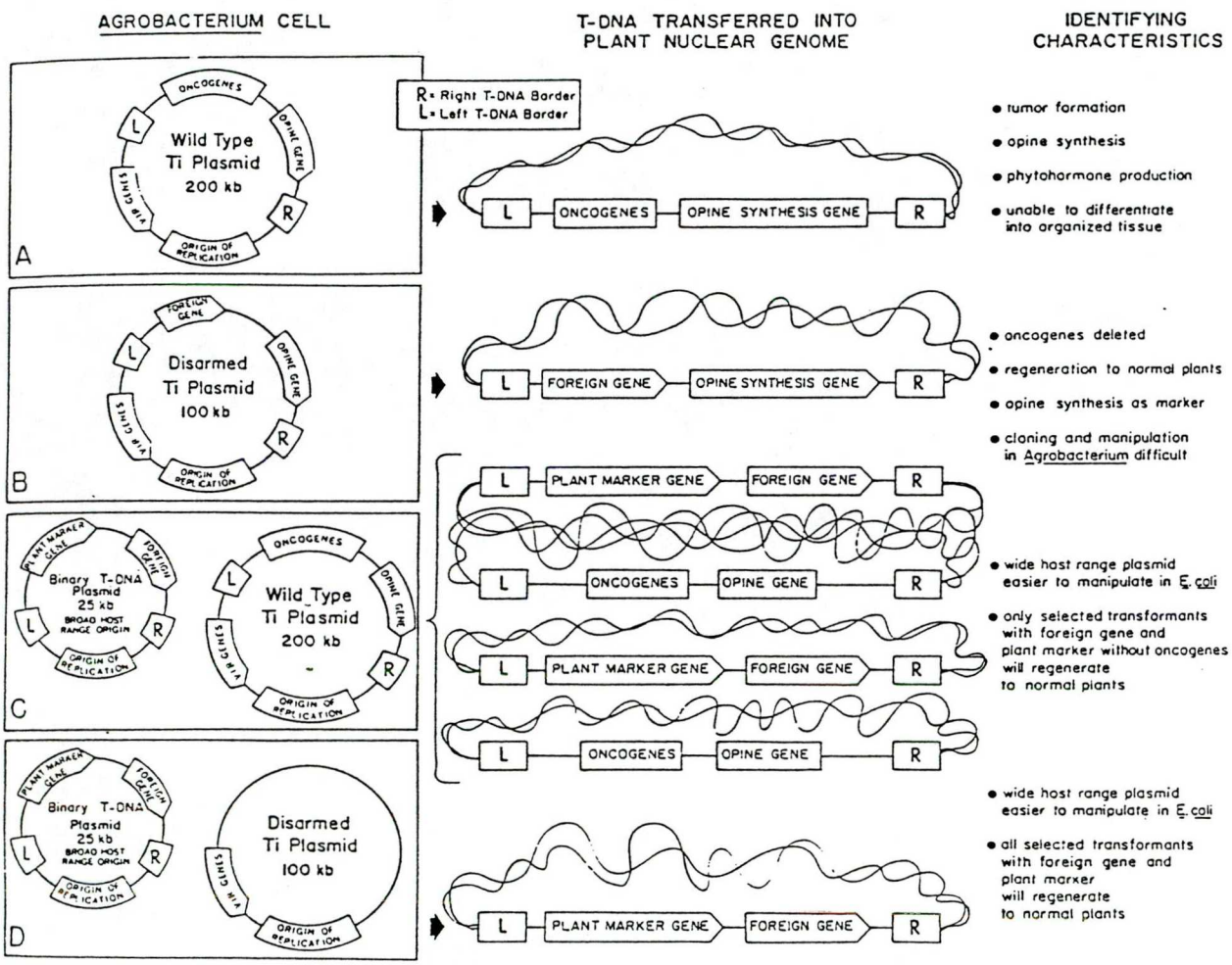


Fig. 7. Ti- plasmid mediated gene transfer:

A. Natural, wild- type *Agrobacterium* Ti- plasmid.
B. Disarmed Ti-plasmid. Oncogenes have been removed and foreign gene inserted instead.
C. Binary vector working in combination with a wild- type Ti- plasmid as a helper. The *vir* functions will either transfer both T- DNA or each of them separately.
D. Binary vector combined with a disarmed Ti- plasmid. (From Perani et al., 1986)

Most of the *Agrobacterium* strains used in gene transfer experiments are the wild type or disarmed variants of 'broad host range' isolates. Although they are capable to infect a wide variety of plant species, very often the plant genotype plays an important role for the successful transformation. In part, this might be due to different stress responses, thus differentially acts on the *vir* genes of the bacteria, that react also selectively. This can be an explanation for the observation that not every combination between plant and bacteria is able to produce transgenic plants or in some instances differences between various cultivars and bacterial strains in efficiency have been detected (Byrne et al., 1987; Charest et al., 1988; Chabaud et al., 1988). The matching of the *vir* functions that is present on the helper plasmid and the sequences adjacent to the T- borders, which are not essential for the transfer but can have an impact on it is also plays a role in the process, especially in wild type strains, where a multitude of border sequences are competing (Zambryski, 1988). Even the efficiency of transport of the T- strand across the bacterial membrane, was correlated with some *vir* genes represented in supervirulent strains as A 281 that is important for the transformation of recalcitrant species (Jin et al., 1987). Briefly, all the seven steps of the transformation can be a limiting event in the efficiency of the process.

An important aspects in development of an effective transformation protocol is the choice of the proper explant, with a pronounced wound- response and with high regeneration capability (Potrykus, 1990). As a general rule, meristematic tissues, with actively dividing cells are the most susceptible starting material. Often, seedling explants as cotyledons and hypocotyls, or young leaves and shoot tips were successfully used for generation of transgenic plants with both oncogenic and disarmed strains (Van Wordragen and Dons, 1992).

Reduction of the stress is also crucial for the establishment of an efficient transformation- regeneration methodology. The combined effect of wounding, hormone shock, habitat change and *Agrobacterium* assault can easily overwhelm the fragile homoeostatic balance in the cell. One way to reduce the initial stress is the careful selection of the infection protocol, in order to allow optimum T- DNA transfer, while keeping the damage caused by the bacteria to be low. Three general methods are used to bring the *Agrobacterium* and the target plants into contact:

1. Even under the best conditions, only few of the available cells are transformed. This is the reason why the development of an effective transformation protocol requires the availability of a selection method to be able to recognize these rare transformation events. A selection system must fulfil several requirements. Most crucial one is the inhibitory effect of the selective agent on the plant cells lacking the resistance gene.

However not all toxic compounds are good selectable markers. Cells can be killed in such a way that they become toxic to the adjacent, transformed cells. In such cases the dying cells leak toxic substances. The best selective agents arrest the non-transformed cells and their killing effect act slowly, without stress. At least 13 markers have been tested for dominant positive selection of transformed plant cells with more or less success. Currently five of them are used more often, although each combinations between plant and selection system should be carefully evaluated.

2. By far the most widely used selectable marker is the neomycin phosphotransferase, type II (NPT II) enzyme. It detoxifies aminoglycosides, such as kanamycin (Km) and geneticin (G 418) by phosphorylation. Its gene was isolated originally from the prokaryotic transposon Tn 5 (Beck et al., 1982) and after several cloning steps expression vector was constructed and used successfully in several vector systems for the transformation and selection of a number of species, reviewed most recently by Van Wordragen and Dons (1992). In spite its versatility, a single resistance gene is not enough to fulfil all the needs of molecular biologists, plant physiologists and breeders. First and most important reason is that this marker is not working equally well in all plant species. Sometimes it is the consequence of lack of toxicity of the kanamycin, due to special features of the secondary metabolism of the plant. Or, for similar reasons, the enzyme is not conferring resistance to the transformed cells. Another reason for developing alternative systems for transformation /selection is the need to introduce sequentially more genes into the target plant (Matzke et al., 1989) and the use of transgenic plants as partners in protoplast fusions and the subsequent selection for double resistance (Deak et al., 1988; Komari et al., 1989; Thomas et al., 1990). Extensive studies in the field leaded to the production of several suitable selection agent/ resistant gene combinations.
3. Hygromycin B (hm B) is an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus*. This compound inhibits protein synthesis by interfering with ribosomal translocation and aminoacyl tRNA recognition. The phosphotransferase encoded by the *hph* gene of *E. coli* can inactivate the drug, therefore, it can be used in plant transformation experiments (Van den Elzen et al., 1985; Rogers et al., 1987; Dekeyser et al., 1989).

Bleomycin is a potent mutagene, creating single- and double- stranded breaks in eukaryotic DNA. The introduction of a chimeric resistance gene, whose product binds the drug, conferred resistance to high levels of the antibiotic (Hille et al., 1986).

Methotrexate is blocking the activity of the enzyme dihydrofolate reductase (DHFR) that causes cell death through induction of thymidilate starvation. A mutant DHFR isolated from mouse has about 500 times lower affinity towards methotrexate. When a construct containing this mutant enzyme is transferred to plant cells it confers resistance towards high concentration of the agent present in the medium (Rogers et al., 1987; Hauptman et al., 1988; Dekeyser et al., 1989).

Phosphinotricin (PPT, Basta, Bialaphos) through inhibition of glutamine synthase, induces the accumulation of ammonia and subsequently cell death. A gene from *Streptomyces hygroscopicus*, coding for the enzyme phosphinotricin acetyltransferase (*bar* gene) is used to counter the effect of PPT in transformed plants (De Block et al., 1987; Dekeyser et al., 1988; Deak et al., 1988; De Block et al., 1989; Golovkin et al., submitted).

It is likely others, more advanced selection systems will appear in the near future, and the above mentioned ones will be further improved. A prerequisite for the production of transformed plants is the availability of a method to regenerate a complete plant from the altered cell. This holds for almost all systems of genetic manipulation. In many dicots phenotypically completely normal and fertile plants were regenerated from various tissues, including protoplasts, either with or without callus phase.

Various aspects of tissue culture of tobacco were studied in details, (Murashige and Skoog, 1962; Thorpe and Murashige, 1968; Ross et al., 1973). Callus and shoot formation is highly predictable on a chemically defined medium (Tran Than Van, 1973). These features made it a widely used model for transformation and gene expression experiment (Rogers et al., 1987; An, 1987; Saul et al., 1988; Nagy et al., 1985; Benfey and Chua, 1990).

Various studies, however indicated different patterns of expression of transferred genes when heterologous system are used (Eckes et al., 1986; Benfey and Chua, 1989).

These observations lead us to use two more plants with different habitues, growth pathways and responses *in vitro*.

Beside its economic significance, rapeseed is a highly amenable laboratory plant. They are examples of successful plant regeneration from leaves (Stringam, 1979; Dunwell, 1981), stem (Kartha, 1974, Stringam, 1977), roots (Lazzeri and Dunwell 1984), thin cell layer explants (Klimaszewska and Keller, 1985) and from mesophyll and hypocotil protoplasts (Glimelius, 1984). Transformation with genetically engineered *Agrobacterium* strains led to the production of normal transgenic plants expressing foreign genes (Fry et al., 1987; Pua et al., 1987; De Block et al., 1989; Moloney et al., 1989; Swenson and Erickson 1990; Boulter et al., 1990; Misra, 1990).

However, with very few exceptions the spring cultivar Westar was used in these experiments. Here, we describe a transformation and regeneration scheme for a broader range of genotypes, including some recalcitrant winter cultivars.

Alfalfa is a highly valued forage legume extensively cultivated in warm, temperate and cool subtropical regions. It has the highest feeding value of commonly grown hay crops and accounts for more than 60% of the total hay production in USA.

Because of its impact on world economics, alfalfa became a target for improvement by unconventional methods. Reports have outlined somatic embryogenesis on alfalfa calli, derived from a variety of tissues including mature embryos (Saunders and Bingham, 1975; Walker et al., 1978) petioles (Novak and Conecna 1982; Walker et al., 1978), stems (Novak and Conecna 1982; Denn, 1986), cotyledons (Novak and Conecna 1982), hypocotyls (Novak and Conecna, 1982; Retsch and Bingham, 1980) and leaves(Dos Santos et al., 1980; Atanassov and Bromm, 1981). Process of induction of somatic embryogenesis has been recently reviewed by Dudits et al. (1991).

Transformation experiments led to production of transgenic calli and plants from different species (Webb 1986; Deák et al., 1986; Chaband et al., 1988; Thomas et al., 1990; Damian and Acron, 1991). In virtually all cases, the regeneration on alfalfa was achieved throughout somatic embryogenesis.

3. MATERIALS AND METHODS

Since most of the techniques used by us are well known, their description will be mentioned only briefly, with providing the appropriate references. Some essential protocols are described in more details in APPENDIX 2.

3.1. MICROBIOLOGICAL TECHNIQUES.

3.1.1. Bacterial strains and plasmids

The *E. coli* strain HB 101 (Maniatis et al., 1982) was used as a host during cloning work and as a donor strain for triparental mating with *Agrobacterium* strains.

Due to the recalcitrance of some of the plant species used, several *Agrobacterium* strains were tested for the efficiency of transformation. LBA 4404 is a disarmed octopine strain carrying the Ti- plasmid pAL 4404, from that the entire T-DNA was deleted (Ooms et al., 1982). It contains a selectable marker through the presence of the plasmid (Sm resistance) and have been used extensively for transformation experiments (Shahin et al., 1986; Jefferson et al., 1987; Ledger et al., 1991; Sangvan et al., 1991). The strain GV 31019 (pMP 90 RK) was engineered as a specific host for the replication of the pPCV- type binary vectors- an advanced system for plant transformation and cloning (Koncz and Schell, 1986).

Table 1. shows the plasmids used in the present work. The plant transformation vectors pBI 101.1, pBI 121.1 and pBI 131.1 are a kind gift from Dr. R. A. Jefferson (Institute of Plant Science Research, Cambridge, U. K.) and described by Jefferson et al. (1987). The plasmid pPCV 701 is a gift from Dr. Cs. Koncz (present address: Max- Planck- Institut for Zuchtungsforschung, Koln, F. R. Germany) The plasmid pPCV701 GUS is a kind gift from Dr A. Zilberstain (Tel-Aviv University, Tel-Aviv, Israel). It contains the GUS gene coding sequence controlled by the *CaMV 35S* promoter and the *nos* termination signal. pGA 482 and pUC 18 are commercially available and were purchased from Pharmacia PLC International, Uppsala, Sweden.

3.1.2. Handling of bacteria and culture conditions

The *E. coli* strains were grown on LB medium (Maniatis et al., 1982) at 37 ^{o}C.

Transformation of *E. coli* was performed using the $CaCl_2$ method (Maniatis et al., 1982). Selection for the transformed bacteria was carried out on LB plates supplemented with the appropriate antibiotics. Two subsequent single- cell colony purifications were done.

Table 1. Basic features of the plasmids used in this work.

PLASMID	LENGHT	FEATURES			REF.
	k.b.p.	BACT.SEL.	PLANT.SEL. CASS.	PLANT EXPR. CASS.	
pUC 18	2.7	Ap	=	=	111
pGA 482	13.2	Tc / Km / Cb	NOS - NPT II - nos	=	5
pPCV 701	9.5	Cb	NOS - NPT II - nos	not active / -Cod. Seq.	100
pPCV 701GUS	11.0	Cb	NOS - NPT II - nos	35S - GUS - nos	-
pBI 101.1	12.0	Km	NOS - NPT II - nos	not active / -Prom.	87
pBI 121.1	12.8	Km	NOS - NPT II - nos	35S - GUS - nos	87
pBI 131.1	13.0	Km	NOS - NPT II - nos	rbc S - GUS - nos	87
pIDS 401	3.5	Ap	=	not active / -Cod. Seq.	164
pIDS 011	4.6	Ap	=	not active / -Prom.	164
pIDS 211	5.4	Ap	=	35S - GUS - nos	164
pIDS 311	5.6	Ap	=	rbc S - GUS - nos	164
pIDS 411	5.1	Ap	=	MAS - GUS - nos	164
pIS 012	12.8	Tc / Km	NOS - NPT II - nos	not active / -Prom.	T.W.
pIS 212	13.6	Tc / Km	NOS - NPT II - nos	35S - GUS - nos	T.W.
pIS 312	13.8	Tc / Km	NOS - NPT II - nos	rbc S - GUS - nos	T.W.
pIS 412	13.3	Tc / Km	NOS - NPT II - nos	MAS - GUS - nos	T.W.
pRK 2013		Km	==	==	44

Abbreviations:

Bact. Sel.- Bacterial selection; Plant Sel. Cass.- Expression cassettes for positive selection of plant transformants. they contain promoter- resistance gene coding sequence- polyadenilation signal. Plant Expr. Cass.- Expression casette for monitoring the transcriptional activity of the studied promoter.

For miniprep plasmid screening single- cell colonies were picked with a toothpick and 2 ml LB medium were inoculated and the bacteria were grown overnight with vigorous shaking.

For large- scale plasmid preparation 500 ml LB medium in 2 l flasks was inoculated with 1 ml overnight culture and incubated with vigorous shaking until the OD600 was equal to 1.

The *A. tumefaciens* strains were cultivated on ABM medium with the required antibiotics at 25- 28 ^{o}C.

The purity of the *Agrobacterium* strains was verified by culturing on lactose medium. When distinct colonies were seen, the LM plates were flooded with Benedict's solution and incubated for 1- 2 hours. Around the *Agrobacterium* colonies, a yellow- gold cooper oxide ring appeared, indicating the presence of 3- ketolactoses, a test reaction for the genus (Bernaerts and De Ley, 1963; Hooykaas, 1988)

The introduction of binary vectors in *Agrobacterium* was done by a slightly modified triparental mating protocol, initially described by Ditta et al., (1980). In brief, the *E. coli* donor strain carrying the binary vector, a helper strain- RK 2013, and the *Agrobacterium* recipient strain were cultivated separately at the optimum growth conditions with the required selective antibiotics. 300 l from each cultures were mixed in a sterile Eppendorf tube, spin down for 20 sec in an Eppendorf centrifuge and washed with LB medium to remove the antibiotics. After on more pelleting, they were resuspended in 200 ml LB medium and plated on a freshly prepared MGL plate without selecting agents. The plates were incubated at 28 ^{o}C overnight. Using an inoculation loop, a small quantity was transferred in 2 ml ABM medium, resuspended and different dilutions plated on selective ABM medium.

In 2- 3 days putative transconjugant colonies appeared and after two purifications on ABM medium, were checked by Quick Screen minipreps (An, 1988). Single colonies, containing unaltered plasmid were used for plant transformation.

All bacterial strains were stored at -70 ^{o}C as glycerol stocks.

To prepare the *Agrobacterium* strains for plant transformation, 25 ml MGL medium with antibiotics were inoculated with 500 l of ABM- grown overnight bacterial culture and grown for one more day until late log- phase was reached. These suspensions were used for the different transformation protocols.

3.2. *IN VITRO* PLANT TISSUE CULTURE: PLANT MATERIAL, MEDIA AND PROCEDURES.

3.2.1. Tobacco

N. tabacum cv Petit Havana, genotype SR 1 (Maliga et al., 1973) plants were propagated as axenic culture on solid MS medium. Young, but expanded leaves were used for transformation and regeneration. The MS (Murashige and Skoog, 1962) medium was used with the required antibiotics and growth regulators added for the different steps:

MS.1 for inoculation: liquid, basic medium hormone- and antibiotics free.

MS.2 for incubation: 0.8 % agar; 1.0 mg/l benzyladenine (BA); 0.1 mg/l naphthaleneacetic acid (NAA).

MS.3 for washing: liquid, like MS.2 with 500 mg/l carbenicillin (Cb)

MS.4 for selection: as MS.2 but with Cb 500 and Km 100 added.

MS.5 for rooting: basal medium solidified with 0.6 % agar with antibiotics (Cb 500, Km 100).

For transformation and regeneration of the SR 1 genotype, the protocol of Deblaere et al. (1987), was used.

Young leaves were cut in square pieces of about 1 cm^2 in a petri dish with MS.1 medium. After 15- 20 min they were washed with fresh medium and 1/10 volume bacterial suspension was added. After 10- 15 min coincubation the leaf pieces were blotted on a stack of sterile filter paper to remove the excess bacteria and plated in petri dishes containing MS.2. 3- 4 days later, depending of the state of the explants, they were washed two times with MS.1 and left for overnight in flasks with MS.3 medium with moderate shaking (150 rpm). Next day they were plated on MS.4 for selection of the putative transformants. The hormone balance in the medium supported the direct regeneration through organogenesis. Organized structures appeared one week after transformation and developed shootlets during the next 15- 20 days. They were transferred to rooting medium. At that stage GUS assays were carried out to confirm the presence of the gene in an active form

3.2.2. Rapeseed.

Seeds from the cultivars Hanna (spring type) Arabella, Santana, Ujfertody and Varma (all winter type) were kindly provided by Dr. L. Nagy, VEV Experimental Station, Szentes, Hungary. Pilot experiments demonstrated, that the K3 medium (Nagy and Maliga, 1976) superior to the MS (Murashige and Skoog, 1962) or B5 (Gamborg et al., 1968) mediums for plant regeneration in the case of studied cultivars.

Thus it was used throughout the experiments. If not stated otherwise it was always supplemented with 1% glucose and 2% sucrose and solidified with 0.7% agar. For the different steps hormones and antibiotics were added as follows:

Coincubation medium (K3.1): 1 $mg.l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D); 0.5 $mg.l^{-1}$ N6-benzyladenine (BA) and 0.1 $mg.l^{-1}$ a-naphthaleneacetic acid (NAA).

Selection medium (K3.2): as above with 500 $mg.l^{-1}$ carbenicilin (Cb); 300 $mg.l^{-1}$ claforan (Cf) and 100 $mg.l^{-1}$ kanamycin (Km).

Conditioning medium (K3.3): 1 $mg.l^{-1}$ BA; 0.1 $mg.l^{-1}$ 2,4-D; 500 $mg.l^{-1}$ Cb; 300 $mg.l^{-1}$ Cf and 100 $mg.l^{-1}$ Km.

Regeneration medium (K3.4): 1 $mg.l^{-1}$ BA; 0.1 $mg.l^{-1}$ NAA; 5 $mg.l^{-1}$ $AgNO_3$; 300 $mg.l^{-1}$ Cb; 200 $mg.l^{-1}$ Cf and 100 $mg.l^{-1}$ Km.

Shoot strengthening medium (K3.5): K3 salts with sucrose lowered to 1% and 100 $mg.l^{-1}$ Km.

Rooting medium (K3.6): 1/2 strength salts of K3 with 1% sucrose and 1 $mg.l^{-1}$ indole-3-butyric acid (IBA). An original protocol combining callus induction before regeneration of plant and enhancement of the morphogenesis with $AgNO_3$ was developed (Stefanov et al. manuscript in preparation).

Hypocotils from 3- 4 days- old plantlets grown in the dark were used as starting material. Two schemes were used to transform rapeseed. In both cases 5-6 cm long hypocotils were cut in 4-5 mm pieces and washed with K3.1 medium. For the octopine strain LBA 4404(pIS 412) we used the protocol of Deák et al., (1987) with slight modifications. Explants were placed in 100 ml Ehrlenmeyer flasks with 40 ml liquid K3.1 medium and 200 l of fresh overnight grown bacterial culture was added. The cocultivation was carried out for 3 days with shaking (200 RPM) with daily change of the medium to prevent overgrowth. For inoculation with the strain GV3101 (pMP90RK/ 702 GUS) the explants were immersed in overnight culture diluted 10 times with K3.1 medium to allow efficient attachment of the bacteria to the cell surface.

The hypocotil pieces were blotted dry and transferred to agar-solidified K3.1 medium for 2-3 days. At that time, the bacterial growth was visible. The segments were washed thoroughly and transferred to K3.2 medium for selection and elimination of bacteria. 2-3 weeks later small green calli appeared on the cut surface. We removed them and cultivated further on K3.3 medium where they grew vigorously for another 2 weeks. A this stage samples were taken for detection of GUS activity in individual calli. Only calli with activity more than 0.1 units were cultivated further. After the green calli reached 3-4 mm in diameter we transferred them on K3.4 medium to induced shoot formation. Small plantlets formed within 2-6 weeks. Upon transfer to K3.5 medium they grew fast and in two weeks they were

rooted in K3.6 medium. We transferred the plantlets into soil after 5-6 weeks. The winter type rapeseed genotypes needed a prolonged vernalization at low temperature.

3.2.3. Alfalfa.

M. varia genotype A2 exhibiting high regeneration capability *in vitro* was kindly provided by Dr. Atanas Atanassov, Institute for Genetic Engineering, Kostinbrod, Bulgaria. Plants were propagated as axenic shoot culture on hormone free UM medium. For transformation experiments young shoots were used. The UM (Uchimiya and Murashige, 1974) and B5 (Gamborg et al., 1968) basal media with 3 % sucrose were used sequentially for plant regeneration, with the following modifications:

Coincubation medium: liquid UM medium supplemented with 0.5 mg/l 2,4- D; 0.2 mg/l BA and 250 mg/l bacto tryptone.

Callus induction and selection medium: As the coincubation medium with Km 100 and Cb 400 added. Solidified with 0.6 % Agar Noble (Difco).

Embryo induction medium (B5N): liquid b5 medium supplemented with 500 mg/l meso- inositol; 10 mg/l glutathione; 1 mg/l adenine; 1 mg/l 2,4- D; 0.2 mg/l BA; Km 100 and Cb 400.

Embryo development medium (B5.3MPEG): liquid B5 medium supplemented with maltose, instead of sucrose, 0.2 mg/l BA and 1 % PEG 6000.

Embryo maturation medium: agar-solidified UM medium with 30 mM ABA.

Conversion medium: agar-solidified UM medium with 27 mM GA_3

The transformation and regeneration of the transformants was done according combination of the protocols of Deak et al., 1986, as a modification of the cocultivation method for transformation and the protocol of Denchev et al., 1991 for the regeneration.

Vigorously growing axenic shoots were cut in 4- 5 mm- long pieces and washed two times with coincubation medium. The coincubation was carried out for 3 days in 100 ml flasks with 40 ml medium with 200 l overnight bacterial culture. The explants were washed and incubated overnight with coincubation medium supplemented with 500 mg.l-1 carbenicilin. After *Agrobacterium* cocultivation kanamycin-resistant calli were induced and selected on callus induction medium. Three- four weeks later small yellow- green calli appeared at the wounded surfaces of the explants. They were plated separately and after several passages, during which time they grew vigorously in the presence of inhibitory concentration of kanamycin, were placed in flasks with embryo induction medium.

The subculture was done twice a week. After several weeks large quantities of globular embryos appeared. The medium was changed to embryo development. The use of 1% PEG 600 greatly improved the formation of healthy embryos One or two weeks later (depending on the cell line) the embryos reached the stage of elongated globular- torpedo and were plated on medium for embryo maturation. Two weeks later they were plated on conversion medium. On this medium the embryos regenerated into plants.

3.2.4. SR 1 tobacco protoplast isolation and transformation.

Leaf protoplasts were isolated from axenically grown plants according to Shillito et al., (1983). Direct gene transfer by naked plasmid DNA uptake using PEG as a mediator was performed according to Saul et al., (1988) with modifications. The isolated and purified leaf protoplasts were resuspended in transformation buffer (MaMg buffer, 0.5 M mannit; 15 mM $MgCl_2$; 0.1 % MES, pH 5.6) to a concentration of 2 x 10^6 protoplast per ml. and 0.5 ml. samples were separated in vasserman tubes. 30-40 ml of the appropriated plasmid DNA were added and mixed carefully. 10 min. later 0.5 ml PEG solution (40 % PEG 4000 or 6000; 0.1 M $Ca(NO_3)_2$; 0.5 M mannit; pH 8.0) were added with extreme care to every test tube and mixed very well. The mixture was incubated for 20 min. at room temperature and diluted carefully with 3- 4 volumes W5 solution (154 mM NaCl; 125 mM $CaCl_2$; 5 mM KCl; 5 mM glucose; pH 6.0 adjusted with KOH). After a short centrifugation at 700 rpm for 5 min. the supernatant was removed and the protoplasts resuspended in K3 medium (Nagy and Maliga, 1976) and plated at a density of 2 x 10^5 protoplasts/ml. Cultivation was carried out in dark at 27 ^oC.

3.3. MOLECULAR BIOLOGY AND BIOCHEMICAL TECHNIQUES.

3.3.1. *E. coli* plasmid DNA isolation.

For analytical purposes ("miniprep") the procedure of Ish- Horowitz and Burke (1981) was used. Large quantities ("maxiprep"), were isolated using the protocol published by Maniatis et al., (1982) with a CsCl gradient centrifugation.

3.3.2. A. *tumefaciens* plasmid DNA isolation.

The isolation of plasmid DNA for controlling the integrity of the introduced vector (Quick Screen) was done according An (1988).

3.3.3. Isolation and purification of plant DNA.

Plant DNA was isolated using the CTAB method (Rogers and Bendich, 1988) with subsequent purification by isopicnic CsCl gradient (Maniatis et al., 1982).

3.3.4. Digestion with restriction endonucleases:

Restriction enzymes were purchased from New England Biolabs, BRL, Boeringer and other firms and used according to the manifacturer's instructions. To avoid partial digestion the plant DNA samples were digested with excess enzyme (3- 6 units/mg DNA).

3.3.5. Electrophoresis of DNA fragments and DNA- DNA molecular hybridization ("Southern analysis"):

Digested DNA samples were separated in 0.7- 1 % agarose gels buffered with TBE buffer (Maniatis et al., 1982). For analytical electrophoresis of plasmids 0.2-0.8 mg DNA per track were loaded. For separation of fragments of plant DNA the amount was increased to 10-15 mg per lane for easier detection of the single- copy alien genes. The size of the restriction fragments was determined using lambda phage DNA digested with Pst I or Hind III restrictases as marker. 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 nitrocellulose membranes (Amersham or Shleicher & Shuel.) with a vacuum transfer apparatus manufactured in the B. R. C. with subsequent heat- fixation for 2 hours at 80 ^{o}C under vacuum (Maniatis et al., 1982). The DNA- DNA hybridization with the radioactively labelled probe was performed according the instructions provided by Shleicher & Shuel (Germany). The filters were washed two times with 2 X SSC buffer containing 0.1 %SDS at room 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 Forte Medifort RP diagnostic röntgen films were exposed for 3-10 days at - 80 ^{o}C

3.3.6. Plasmid DNA fragment isolation and labelling:

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

Labelling of DNA fragments with radioactive was done by using the random nucleotide priming method from Feinberg and Vogelstein (1983). 40-80 ng DNA were labelled with ($-^{32}P$) dATP to a specific activity 0.5- 1.5 X 109 cpm/mg and separated from nonincorporated dATP using a chromatographic column (150 mm X 6 mm I.D.) filled with Saphadex G 25 resin and equilibrated with TE buffer.

3.3.7. Neomycin phosphotransferase tests.

The activity of the enzyme conferring resistance towards kanamycin can be assayed by several methods (Reiss et al., 1984; Mc Donnell et al., 1987; Platt and Yang, 1987). Although the last two ones require small quantities of plant material, are fast and a great number of samples can be assayed at once, the presence of internal phosphatases, kinases and other unspecific activities can provide a high background that often makes the evaluation of the result to be difficult. We used the method described by Reiss et al., (1984) based on electrophoretic separation of the NPT II enzyme activity from the unspecific intrinsic background of the plant cell. Crude plant extracts containing 100 mg total protein were loaded in the pockets of a vertical polyacrilamide gel (10 % PAGE, 10 cm long and 2 mm thick) and ran overnight at 4 ^{o}C with constant current (10 mA). The gels were washed in assay buffer and overlaid with molten agarose- containing buffer supplemented with ^{32}P-labelled ATP and kanamycin. After 45 min incubation, a sheet of phosphocellulose paper slightly larger than the gel was placed on it and a capillary blot of paper towels and a weight of 0.5 kg was formed. After 2 hours of transfer the paper was washed twice in water at 80 ^{o}C, blotted dry and the results scored using a X- ray film.

3.3.8. Analysis of the activity of the promoters using the *uid A* gene as a reporter.

The assays for GUS activity and histochemical staining of plant tissues were performed according Jefferson (1987) with modifications. Plant tissues were ground into 200 µl ice-cold extraction buffer with quartz sand in an Eppendorf tube using a glass pestile connected to a mixer head. After spinning down the cell debris with an Eppendorf centrifuge for five minutes, 25 ml samples were taken from the clear phase. 25 µl was used for protein determination according to Bradford (1972), the others were incubated for 0 min, 60 min and 120 min with 75 µl assay buffer. The reactions were stopped with 25 mls of 1M Na_2CO_3 and the fluorescence of the produced methylumbellyferone (MU) measured using a Perkin-Elmer fluorimeter with excitation wavelength 365 nm, emission 455 nm ant slight width in both cases set at 10 nm.

The fluorimeter was calibrated with standards of MU diluted to 100 nM and 1 M. Specific activity was calculated against the protein content of the samples and the incubation time. It is expressed in nmole 4-MU/min/mg protein.

For histochemical staining 50-100 mm tick 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 % formol and 5 mM $NaPO_4$, pH 7 and several washes in phosphate buffer they were incubated for 12-18 h at 37 ^{o}C in staining buffer. To remove the chlorophyll they were deepened in a sequentially increasing concentrations of ethanol.

3.3.9. Determination of the protein content of plant extracts.

Protein concentration of plant extracts was determined by the dye- binding method of Bradford (1976) with a kit manufactured by Bio- Rad Laboratories, California, USA. 25 ml plant extract were added to 985 ml diluted 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 concentration was determined with a calibration curve built in the range 0- 50 mg Bovine Serum Albumin (BSA).

4. RESULTS

4.1. CONSTRUCTION OF VECTORS FOR TRANSIENT EXPRESSION AND STABLE INTEGRATION OF THE MARKER GENES INTO THE PLANT GENOME.

The plant expression cassettes from the binary vectors for plant transformation pBI 101.1 (promoterless vector for testing of promoters with the ß- glucuronidase gene as reporter), pBI 121.1 (containing the *uid A* gene under the control of the promoter of 35S RNA transcript of CaMV, Odell et al., 1985) and pBI 131.1 (with the transcription of the *uid A* gene directed by a tobacco *rbcS* promoter, Mazur and Chui, 1985) were recloned into the Hind III- Eco RI sites of the bacterial cloning vector pUC 18 to produce the transient expression vectors pIDS 011, pIDS 211 and pIDS 311 respectively.

The strategy used to construct the transient expression vector pIDS 411 is summarized in Fig.8. The gene assay cassette containing the poly A signals from gene 7 of *Agrobacterium* Ti- plasmid, the dual promoter of mannopine synthase (Velten and Schell, 1985) and the octopine synthase termination signal with appropriate unique cloning sites between them was excised from the plant transformation vector pPCV 701 with Eco RI and Hind III and ligated in the corresponding sites of pUC 18 to create the intermediate vector pIDS 401. The mannopine synthase promoter was excised from this vector by digestion with Sal I and Bam HI and ligated to the appropriate sites of pIDS 011 to form the transient expression vector pIDS 411. In this vector the promoter is placed in order to have the TR 1' as controller to the *uid A* gene. Some essential parameters of the constructed vectors are listed in Table 1.

The plant expression cassettes from the vectors for transient expression pIDS 011, pIDS 211, pIDS 311 and pIDS 411 were excised with Eco RI and Hind III, purified from the cloning vector by electrophoretic separation in agarose gel and ligated to the appropriate sites of the plant transformation vector pGA 482 (An, 1987), thus replacing a 2.7 kb fragment. The resulting plant transformation vectors were named respectively pIS 012, pIS 212, pIS 312 and pIS 412 (Fig.9.) and were used for stable transformation of *N. tabacum* SR 1, *M. varia* A 2 and *B. napus* Arabella and Santana.

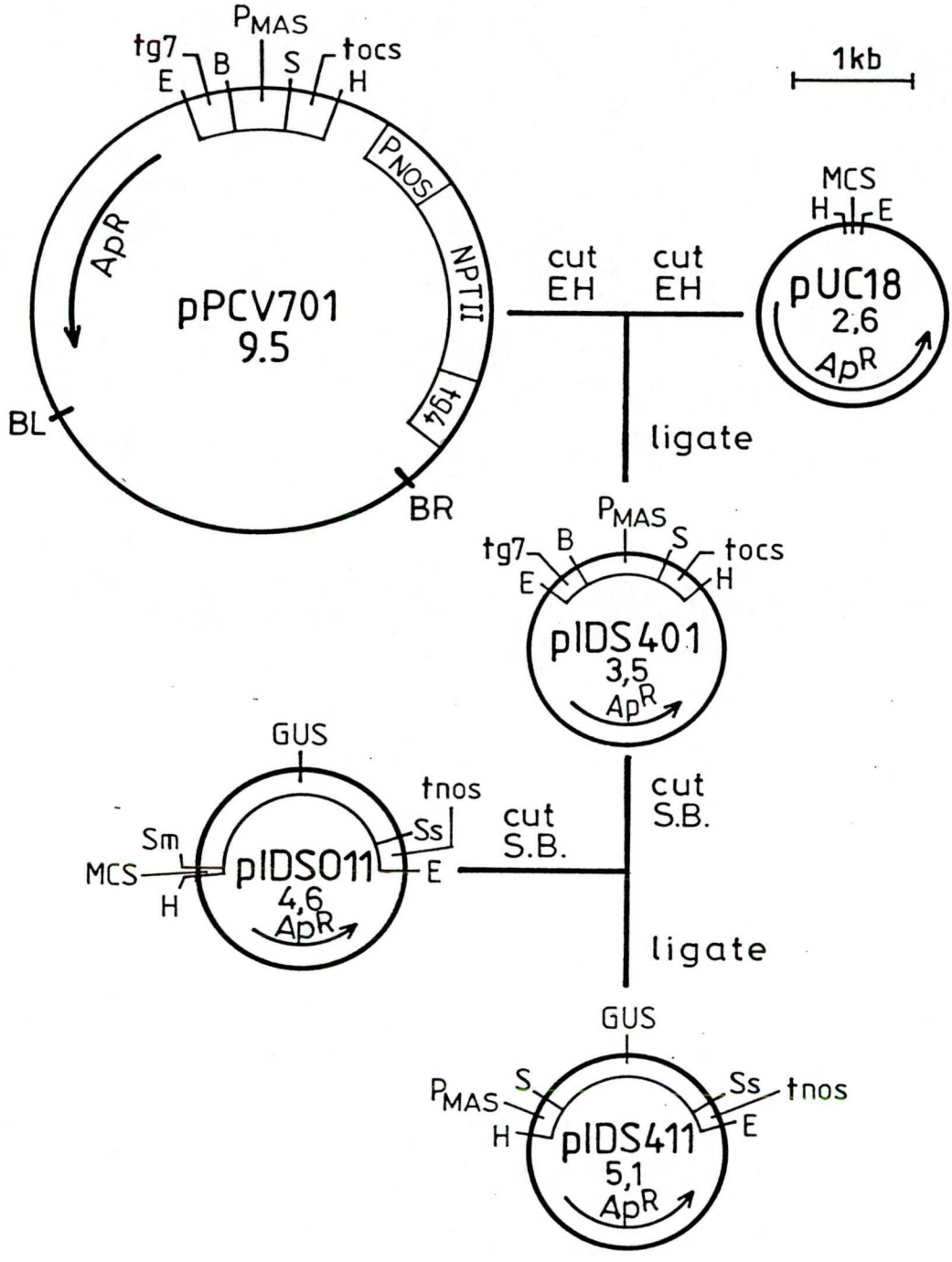


Fig. 8. **Strategy in constructing the transient expression vector pIDS 411.**

Abbreviations.

Promoters: PMAS- mannopine synthase, PNOS- nopaline synthase. Termination signals: tg4- gene #4, tg7- gene #7, tocs- octopine synthase (all from *Agrobacterium* Ti plasmids). Restriction endonuclease recognition sites used for cloning. MCS- multiple cloning site (polylinker), E- Eco RI, B- Bam HI, S- Sal I, H- Hind III, Ss- Sst I. BL and BR indicate the left and right borders of the binary vector. ApR marks the dominant gene conferring resistance towards ampicilin/carbenicilin.

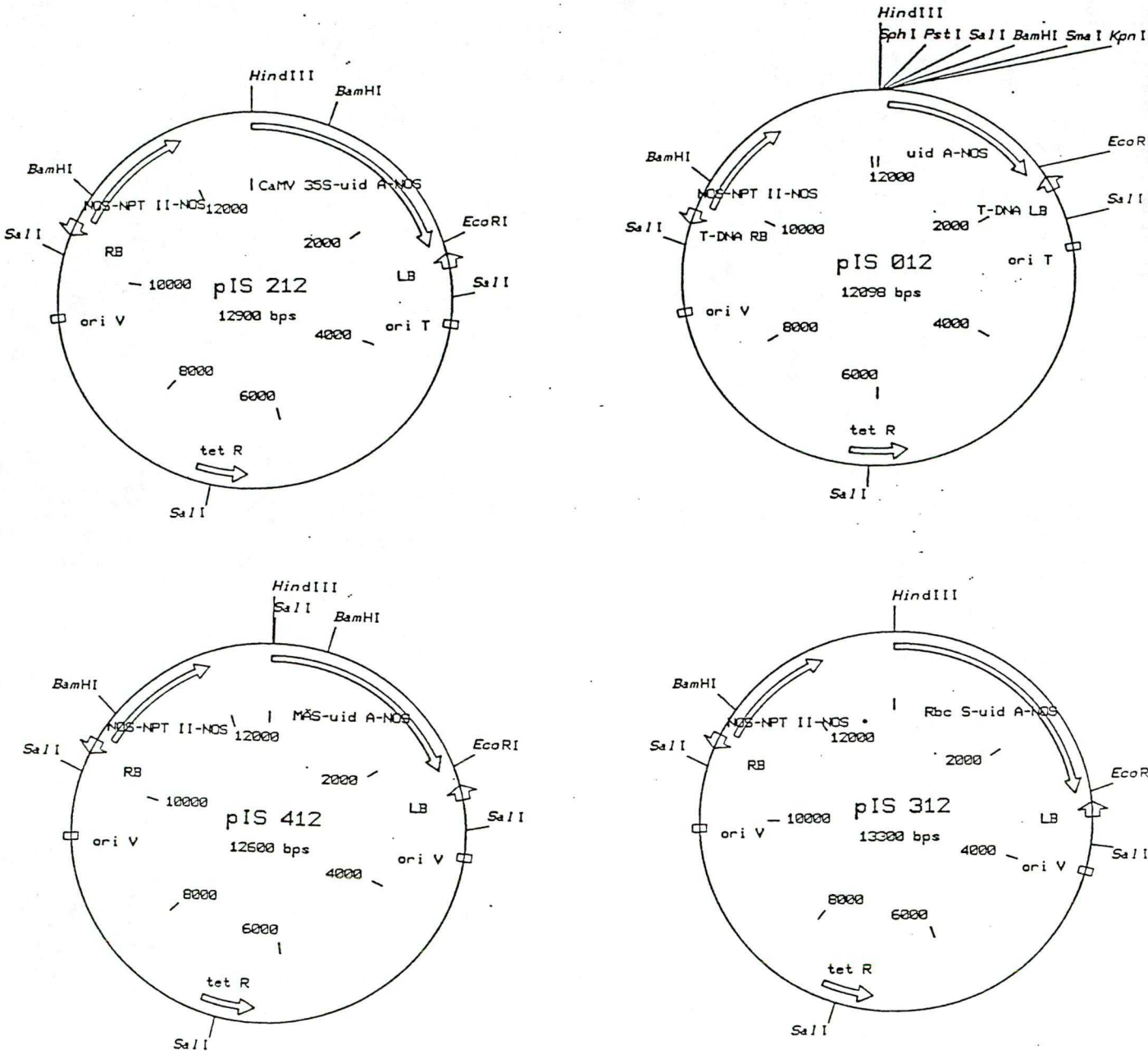


Fig. 9. Genetic maps of the binary vectors used in experiments to study stable transformation of plants and gene expression.

Abbreviations.

The abbreviation are as in Fig. 1. Also: ori T- origin of conjugal transfer, ori V- origin of replication of the broad host range plasmid pRK 2, ori pBR 322-origin of replication of the *E. coli* plasmid pBR 322, Tet R- dominant gene conferring resistance to tetracyclin, NOS- NPT II- NOS- chimeric gene conferring resistance towards kanamycin both in bacteria and plants.

Table 2.

1. Essential parameters of the vectors for transient gene expression.

Original Binary Vector	Promoter	Reporter gene	Size k.b.	E.C./P ratio*	compact vector	Size k.b.p.	E.C./P ratio*
pBI 101.1	-	GUS	12	-	PIDS OII	4.6	-
pBI 121.1	CaMV 35	- GUS	12.8	0.23	PIDS 2II	5.4	0.52
pBI 131.1	rbcS	GUS	13	0.23	PIDS 3II	5.6	0.54
pBI 701	mas	-	9.5	-	PIDS 4II	5.1	0.48

E. C. Ratio- the ratio between the effective sequence and the overall plasmid lenght.

4.2. TRANSIENT EXPRESSION OF THE *uid A* GENE UNDER THE CONTROL OF DIFFERENT PROMOTERS IN PLANT PROTOPLASTS

Tobacco SR1 axenic plants were maintained on MS medium lacking hormones. Healthy young plants (1 month after transfer to a new medium) were used for protoplast isolation. 10^6 purified protoplasts were treated with 40 μg of the appropriate plasmid DNA and the uptake was promoted by adding PEG 4000 at a final concentration of 20 %. The protoplasts were washed and cultivated in K3 medium. Samples were taken at daily intervals and the GUS activity was measured. Fig. 10. shows a typical time- course of the changes of activity in protoplasts transformed with transient expression vectors. During the early period of tobacco protoplast culture, we found the promoter of *mas* gene to be the most active. 2 days after DNA uptake there is a peak in function of this promoter. The activity of CaMV 35S promoter gradually increased in time. The rbcS promoter provided a low level of expression during the whole studied period.

An another way of quantification of gene expression in transient expression is the histochemical staining of immobilized protoplast-derived cells for GUS activity. Fig. 11. shows protoplasts expressing the *uid A* gene during the second day after transformation. By this approach the number of transformed cells with high expression can be determined as well as the promoter activity can be concluded from the staining intensity. In agreement with the fluorigenic assay the *mas* promoter was found to be the most efficient.

To study the influence of the plasmid size on the expression activity of the gene and thus the transformation efficiency we compared the specific GUS activity of protoplast lysates obtained after two days of cultivation following the uptake of different vectors containing the *uid A* gene under the control of the CaMV 35S and *mas* promoters. Plasmids had similar expression cassettes (Table 3.). Based on GUS activity data it is evident that smaller variants of both transformation vectors provided higher efficiency.

4.3. TRANSFORMATION AND REGENERATION OF TRANSGENIC PLANTS CONTAINING FUNCTIONAL MODIFIED T-DNAs EXPRESSING THE NPTII AND *uid A* GENES.

4.3.1. Tobacco.

Several transformants containing different constructs were grown as *in vitro* shoot cultures, propagated and several ones potted into soil to obtain seeds. GUS assays performed at early stages of regeneration demonstrated the high variability in expression between different putative transformed clones (Fig. 12.).

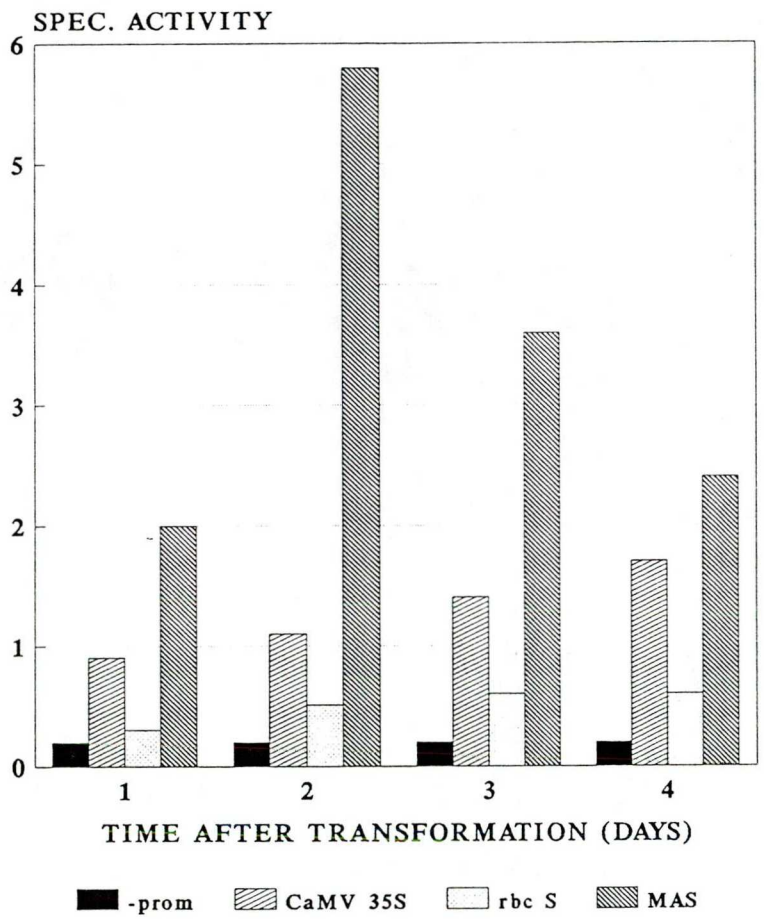


Fig. 10. Transient expression of the *uid A* gene under the control of CaMV 35S, *rbcS* and *mas* promoters after PEG-mediated DNA uptake in leaf protoplasts of tobacco.

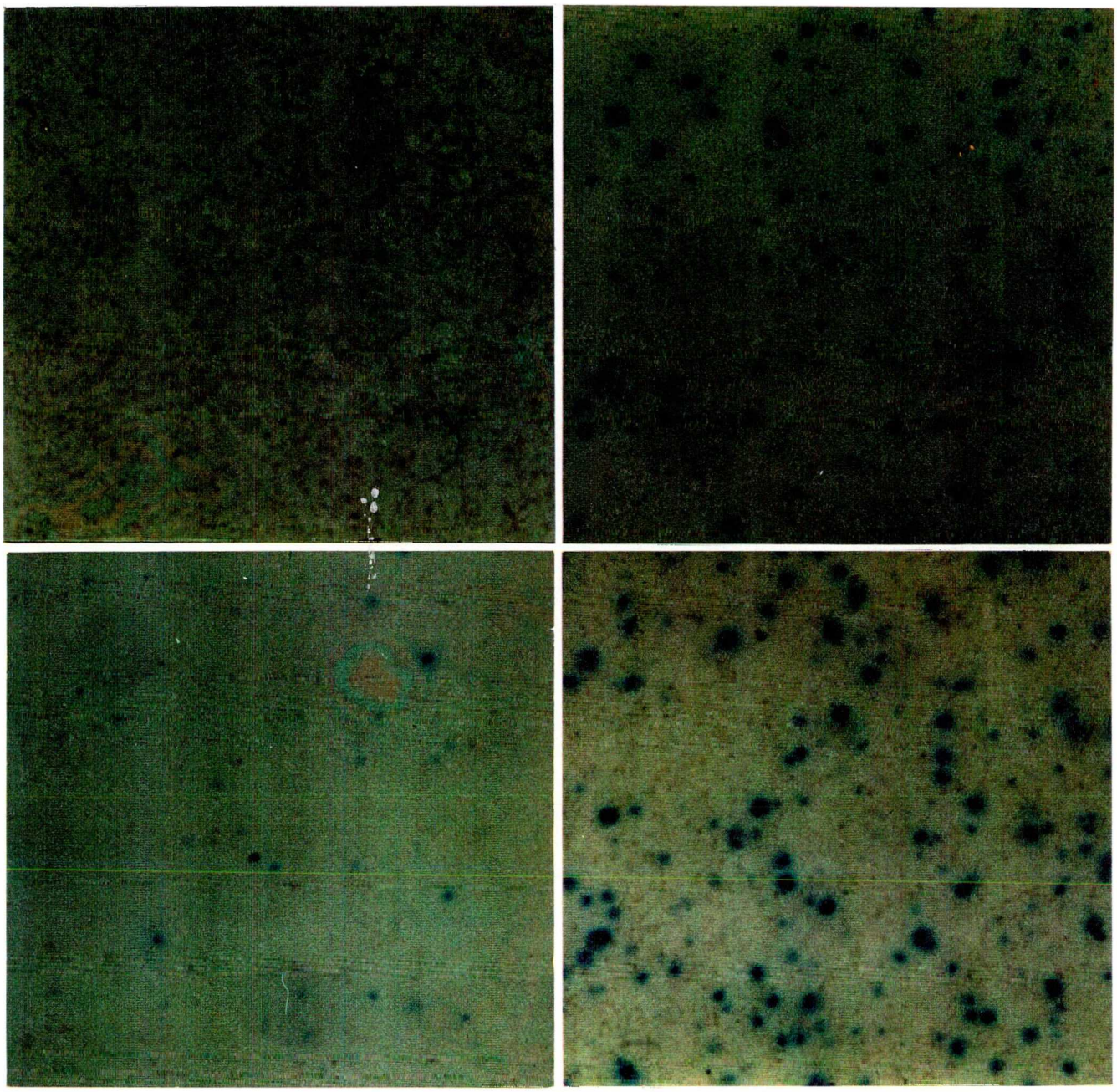


Fig. 11. Histochemical detection of GUS activity in tobacco leaf protoplasts immobilized on nitrocellulose filters two days after PEG- mediated plasmid DNA uptake.

a. promoterless construct used as negative control;
b. CaMV 35S promoter;
c. *rbcS* promoter;
d. *mas* promoter.

Table 3. Vector- and promoter- dependent expression of the *uid A* gene under the control of CaMV 35S (pIDS 211, pIS 212) and *mas* (pIDS 411, pIS 412) promoters. pIDS 011 is a promoterless construct used as a negative control.

PLASMID/promoter	SIZE k.b.	QUANTITY, ug	ACTIVITY
pIDS 211/35S	5.4	40	1.5 +/- 0.4
pIDS 411/mas	5.1	40	5.8 +/- 0.8
pIS 212/35S	12.9	40	0.6 +/- 0.3
pIS 412/mas	12.6	40	1.6 +/- 0.4
pIDS 011	4.6	40	0.2 +/- 0.3

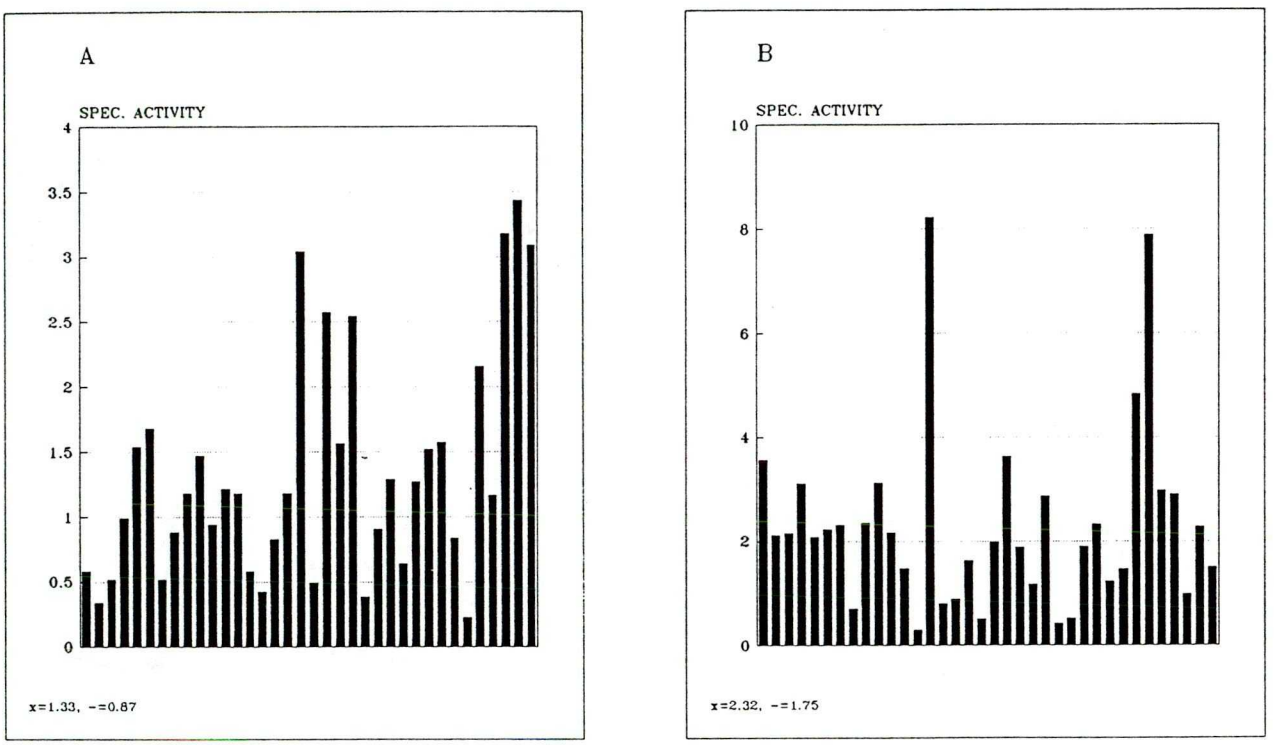


Fig. 12. GUS activity in individual Km resistant calli from SR 1 tobacco transformed with: A. pIS 212 (CaMV 35S promoter) and B. pIS 412 (*mas* promoter). Only calli with a specific activity of more than 0.1 units are scored as GUS positives.

The average enzyme activity was 2.32 units for the *mas* promoter and 1.33 for the CaMV 35S. The variability amongst transformants containing the *mas* promoter was higher. Two of the lines exhibited a very high GUS level- approximately 8 units.

4.3.2. Rapeseed.

The transformation protocol described in Materials and Methods has made possible to produce sufficient number of transformants also with winter cultivars. The sampling of individual calli for GUS activity demonstrated a much higher expression of the *mas* promoter than in the case of CaMV 35S promoter (Fig. 13.). Although the variations in the cultivar Arabella are more pronounced (S. D. 2.253 versus S. D. 1.438 in Santana), in our opinion this is not due to differences in the cultivars, rather fluctuations in activity between the different lines.

4.3.3. Alfalfa.

In contrast to rapeseed, in alfalfa callus tissues we could not detect significant differences between the two promoters (CaMV 35S, *mas*) based on GUS activity. Rather, the variation in promoter function was more pronounced in the case of *mas* promoter (Fig. 14).

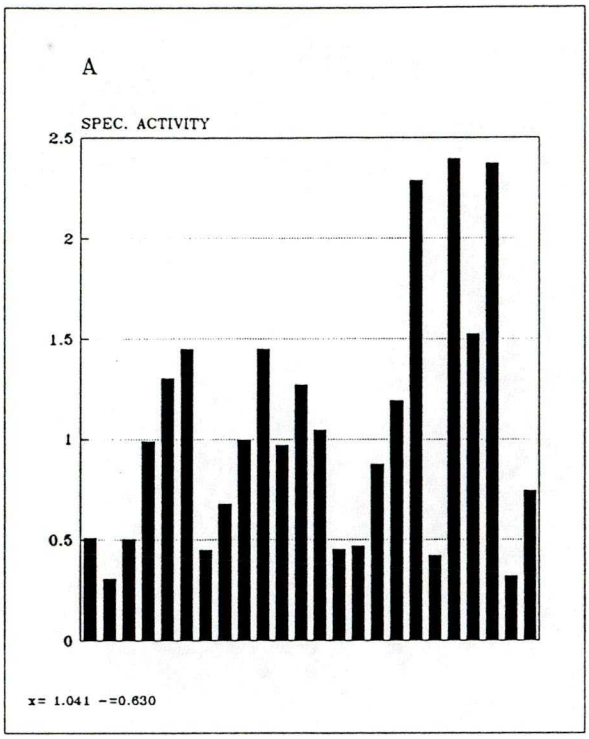
4.4. EVIDENCES FOR THE TRANSGENIC NATURE OF THE REGENERATED PLANTS.

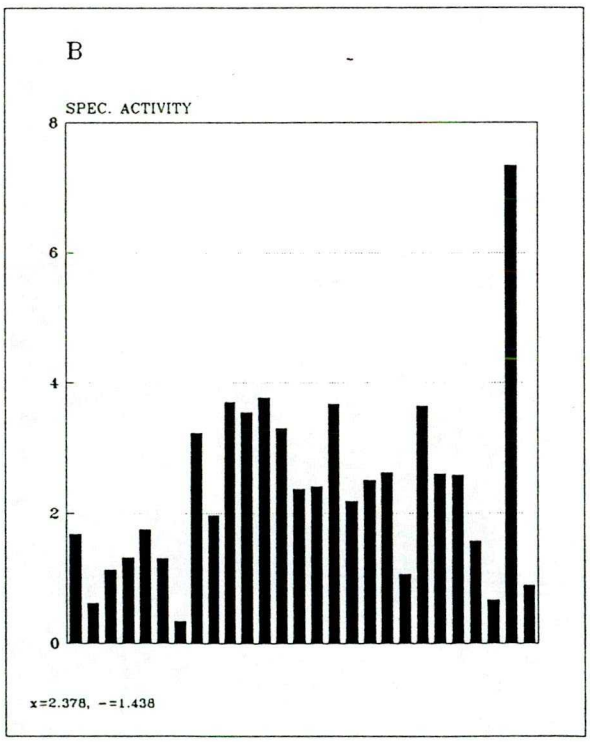
Table 4. summarizes the production of transgenic plants using *Agrobacterium* mediated transformation. As shown by the data, we have succeeded to produce plants that expressed the resistance marker and the GUS reporter gene in all three crop species studied.

4.4.1. DNA- DNA hybridization.

To confirm the presence of the *uid A* gene in the genome of the transformed plants, total plant DNA was isolated from randomly selected plants, purified and digested with restriction endonucleases. The fragments were separated according to size in agarose gel and immobilized on nitrocellulose membranes.

Fig. 15. shows, that after digestion with Eco RI and Hind III restriction endonucleases and Southern analysis only one hybridizing band can be seen on the autoradiograms. The enzymes used had no recognition site within the introduced DNA. From the observed hybridization pattern we cans conclude that in all analyzed plants the integration has occurred at one position and the introduced sequence can be considered as a single copy gene.





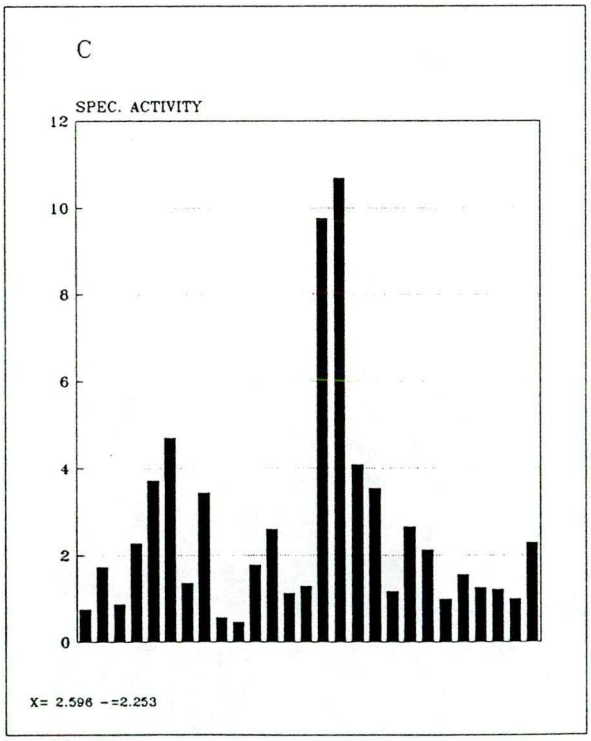
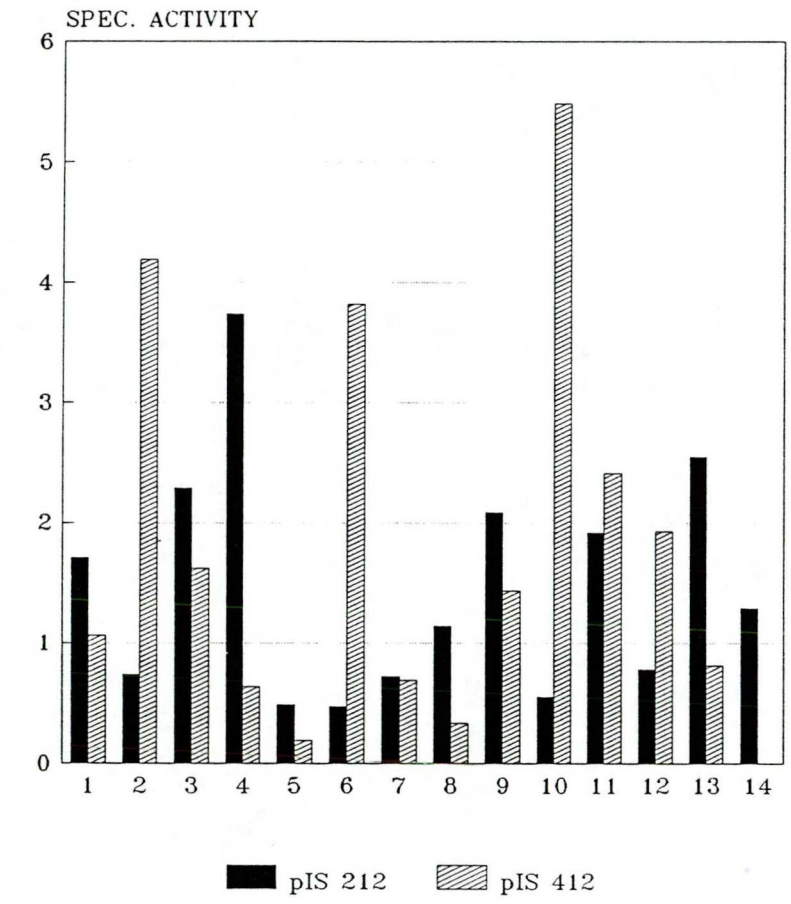


Fig. 13. GUS activity in individual Km resistant calli from rapeseed transformed with: A.- pPCV 701 GUS (CaMV 35S promoter) and B. and C.- pIS 412 (*mas* promoter). Only calli with a specific activity of more than 0.1 units are scored as GUS positives.



212 x= 1.463 -= 0.970
412 x= 1.893 -= 1.650

Fig. 14. **ß- glucuronidase activity in km resistant alfalfa calli.**

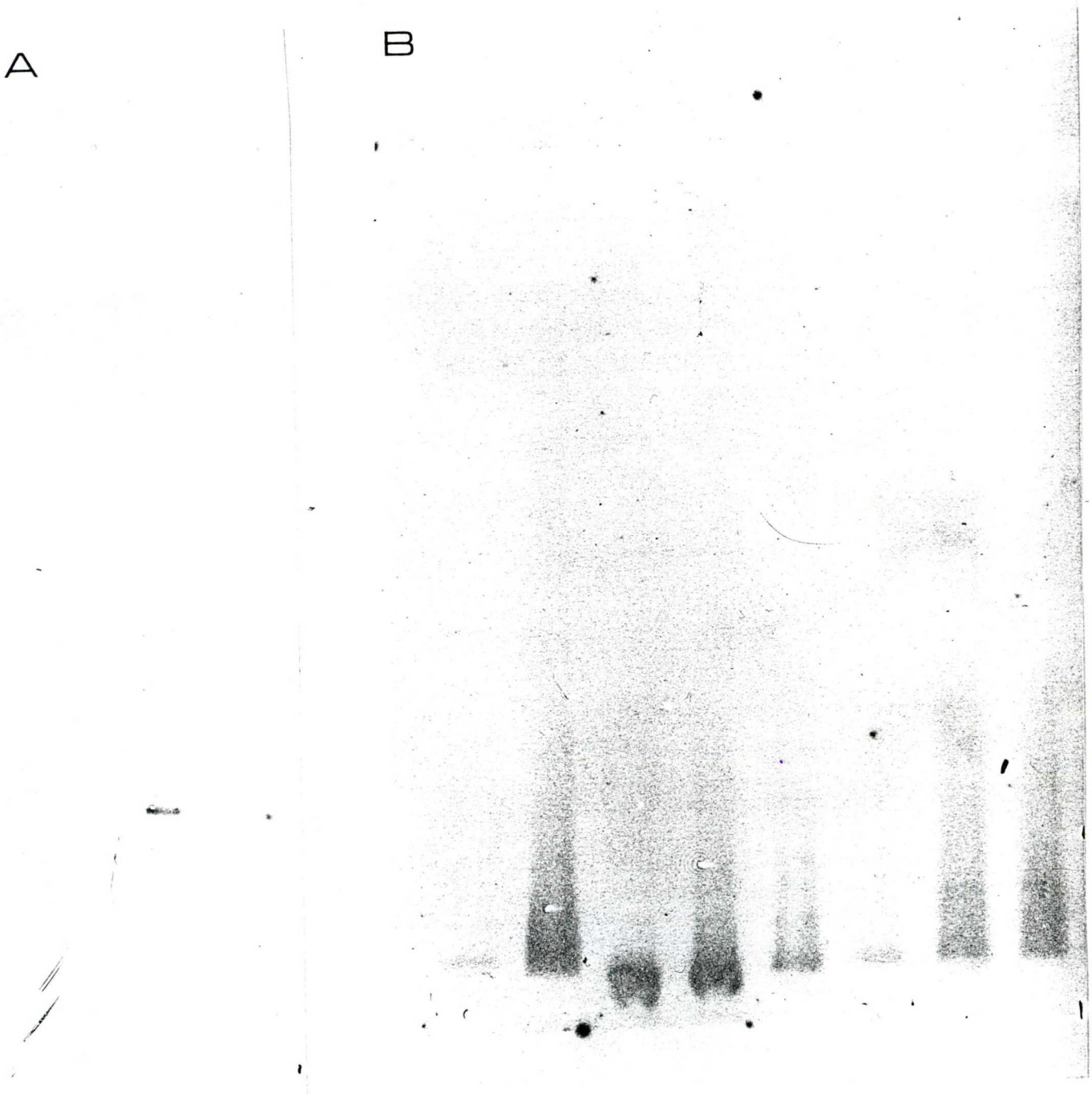


Fig. 15. Detection of the *uid A* gene in genomic DNA from:

A. Rapeseed.
B. Tobacco.

4.4.2. Callus induction assay of the primary transformants in the presence of selective inhibitor.

One of the most reliable assays for the resistance or sensitivity of a plant is the induction of callus tissues in young explants of the primary transformants on medium containing kanamycin. In our experiments all GUS- positive plants were able to form callus and organized structures on selective medium (data not shown).

4.4.3. Expression of the NPT II gene in extracts of transformed plants.

Samples were taken from different transgenic plants and the expression of the bacterial NPT II or *neo* gene was determined to confirm the presence of integrated selective marker gene.

In all of selected transformed plants a high level of expression of NPTII gene was detected as shown by (Fig. 16.).

4.4.4. Expression of the *uid A* gene.

A fast screening for GUS activity of putative transformants was done during the early stages of morphogenesis (tobacco) and callus formation (alfalfa and rapeseed) of clones growing on selective medium. The activity of the non- selectable marker is also a proof for transformation. As it was already mentioned, samples were taken and assayed using the fluorigenic method (Figs.12.- 14.).

4.4.5. Mendelian inheritance of the transferred genes in the progeny of the primary transformants.

Greenhouse- grown tobacco and rapeseed plants were selfed and tested for the inheritance of the transferred genes in the progenies. Because of the difficulties in characterization of rapeseed plants several complementary methods were used:

1. Germination of seeds and development of plantlets on sterile tap water or K3.5 medium containing Km
2. Test for formation of callus tissues on hypocotil segments on K3.2 medium containing Km.
3. GUS assay of plantlets.
4. Normal growth of plantlets on hormone free K3 medium containing 100 mg/ l Km.

The fate of individual plantlets and explants was traced and the results of GUS assay and callus tests were correlated. Km resistant and sensitive plantlets were assayed for GUS. In all the cases the assays demonstrated a tight linkage between the two genes. The ratio between the sensitive and resistant plantlets and calli and the presence of GUS activity indicated a Mendelian inheritance of linked genes (Table 4).

Fig. 16. Expression of the *neo* gene in tissues of transgenic rapeseed

For analysis of segregation among progenies of tobacco transformants, seeds were sown on MS medium containing 100 mg/ml Km. After 3- 4 days plantlets appeared. The ones containing the NPT II gene continued their development, while the homozygous wild type stopped growing at the primary leaf- stage. Histochemical staining for detection of GUS activity was also used (Data not shown) The inheritance ratio of sensitive vs. resistant seedlings demonstrated a single copy integration of the foreign gene into the plant genome (Table 5.).

The alfalfa genotype used in the present experiments is known for its poor seed-setting capability (Bocsa I, Nagy B, personal communication). Up to now we couldn't obtain seeds and we have not any data on the inheritance of the transferred genes.

4.5. EXPRESSION OF THE *uid A* GENE DURING CULTURE OF LEAF PROTOPLASTS FROM TRANSGENIC TOBACCO PLANTS.

Protoplasts were isolated from expanded leaves of *in vitro* grown tobacco plants containing the reporter gene under the control of the CaMV 35S, *rbcS* and *mas* promoters. The activity was monitored at daily intervals. Samples from leaves were kept frozen and assayed for GUS activity for reference about the initial activity. The CaMV 35S promoter had decreasing activity during first two days, with a subsequent increase. The increase in activity was at the time when most of the protoplasts started first division. The promoter of the small subunit of ribuloso- bisphosphate carboxylase decreased its activity during the whole cultivation time. *Mas* promoter became more and more active during the course of cultivation (Fig.17.).

4.6. INFLUENCE OF PLANT GROWTH HORMONES ON CAMV 35S AND *mas* PROMOTERS

Microcallus suspensions from transgenic alfalfa plants were treated with various growth hormones for three days and analyzed for GUS activity using the fluorigenic method.

Table 6. demonstrates that the CaMV 35S promoter has a very low sensitivity to hormone influence. In contrast, the *mas* promoter was induced by auxins, different levels of 2,4-D and NAA was found to be necessary to obtain the same quantitative expression. ABA and GA 3 are clearly inhibited *mas* promoter activity.

Table 4. **Production of transgenic plants by *A. tumefaciens* transformation.**

	pIS 212	PPCV 701GUS	pIS 312	pIS 412
TOBACCO	~100/65/32	N.U.	~100/33*/12	~100/51/45
HANNA	N.U.	~100/35/5	N.U.	N.U.
ARABELLA	N.U.	N.U.	N.U.	~100/27/3
SANTANA	N.U.	~100/27/3	N.U.	~100/22/3
M. varia A2	~200/18/7	N.U.	~300/7/0	~300/15/4

Number of explants/ GUS positive calli/ GUS positive plants. N. U.- not used in the experiments. * GUS activity was assayed in leaves of plantlets.

Table 5. **Inheritance of the Km resistance trait in T1 progeny of transformed tobacco and rapeseed plants.**

	km.R	km.S	Ratio
Rapeseed Hanna	17	5	3.4:1
Santana	163	63	2.58:1
Arabella	167	41	4.07:1
SR1 212 2	314	118	2.66:1
312 7	95	29	3.27:1
412 5	222	69	3.21:1

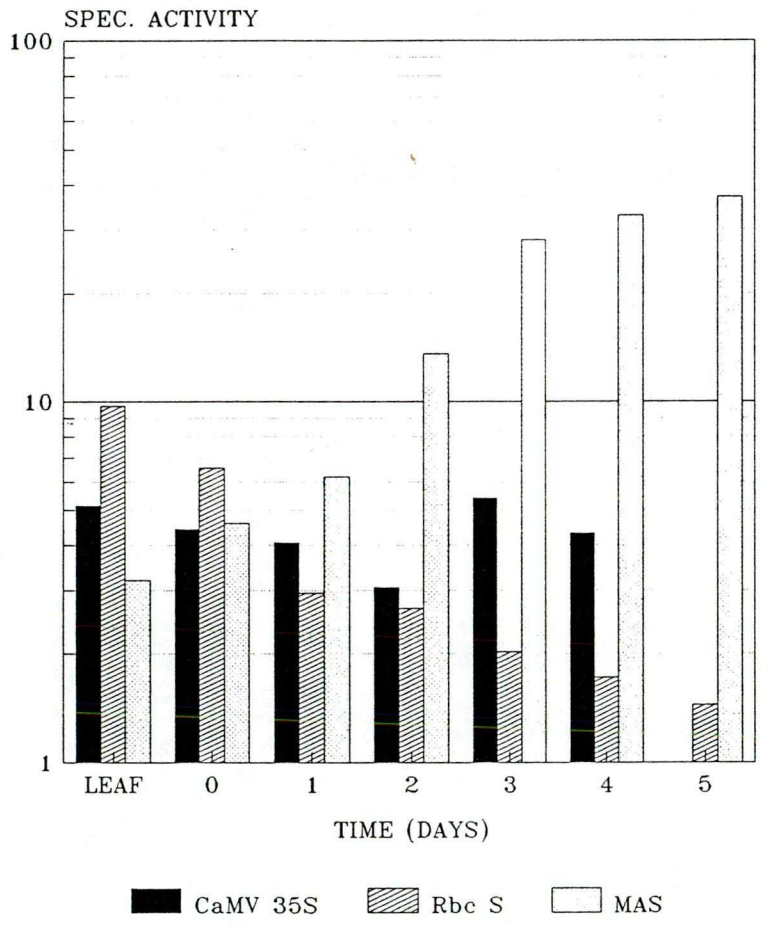


Fig. 17. **Expression of *uid A* gene driven by CaMV 35S , *rbcS* and *mas* promoter during cultivation of leaf protoplasts from transgenic plants.**

Table 6. **Influence of plant growth hormones on promoter activity analyzed in cell suspensions of alfalfa.**

HORMONE	CONC.ug/l	CaMV 35S	MAS
H-	=	1.245 +/- 0.12	7.407 +/- 0.58
2,4- D	0.1	1.405 +/- 0.11	8.330 +/- 0.44
	0.5	1.262 +/- 0.35	8.300 +/- 1.6
	1	1.277 +/- 0.19	10.03 +/- 1.32
	5	1.265 +/- 0.17	15.33 +/- 0.72
	10	1.315 +/- 0.11	12.12 +/- 0.45
NAA	0.1	1.195 +/- 0.36	6.920 +/- 1.1
	0.5	1.052 +/- 0.22	7.550 +/- 0.66
	1	1.390 +/- 0.23	9.480 +/- 0.83
	5	1.195 +/- 0.40	10.36 +/- 1.44
	10	1.437 +/- 0.23	11.44 +/- 0.63
BA	0.1	1.490 +/- 0.26	7.765 +/- 0.62
	0.5	1.602 +/- 0.43	8.910 +/- 0.31
	1	1.63 +/- 0.49	6.620 +/- 0.38
ABA	0.1	1.237 +/- 0.14	7.140 +/- 0.69
	0.5	1.191 +/- 0.40	4.047 +/- 0.33
	1	1.305 +/-0.23	3.905 +/- 0.41
GA 3	0.1	1.430 +/-0.13	7.285 +/- 0.28
	0.5	1.560 +/-0.16	5.110 +/-0.26
	1	1.085 +/-0.11	4.486 +/- 0.35

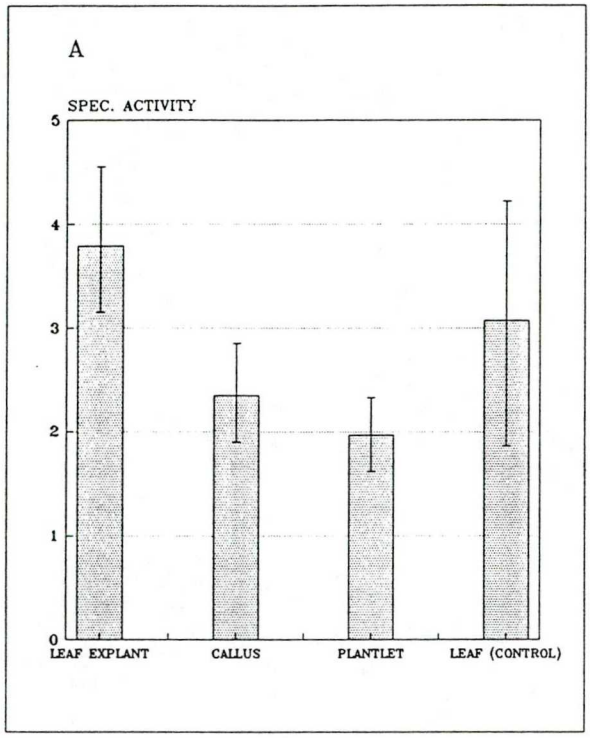
4.7. CHANGES IN THE EXPRESSION OF THE *uid A* GENE UNDER THE CONTROL OF DIFFERENT PROMOTERS DURING INDUCTION OF CALLUS TISSUES AND MORPHOGENESIS

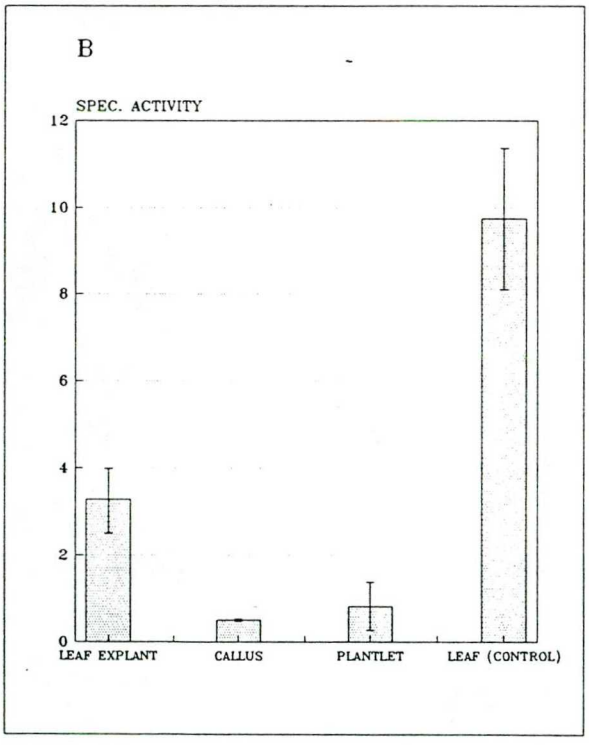
Tobacco leaf explants were plated on MS.4. medium. Several leaf samples were frozen at -80 ^{o}C to have reference for the initial state of the explant. Three weeks later, regions of the leaf explant without callus formation, calli that appeared on the cut- edge of explants and developing plantlets were sampled and the expression of the reporter GUS enzyme was measured. All the measurements were carried out three times. As it can be seen on picture 18.A., the CaMV 35S promoter provided lower GUS level in calli and shootlets than in leaf explants and intact young leaves. When the transcriptional activity of the *uid A* gene was directed by the *rbcS* promoter very low activity was observed in callus, slightly higher in plantlets and rather high in the leaf explant. As expected the highest GUS activity was seen in intact leaves (Fig.18.B.). *Mas* promoter had a high activity in the leaf explant with callus tissue and in callus. A decreased activity was detected during organogenesis (Fig.18.C.)

The histochemical localization of GUS activity (Fig.19.) confirmed these results. Strong indigo staining can be seen both in the enlarged, but not dividing cells of the explant and the rapidly dividing callus cells at the upper right of the explant, but it can not be detected in the organized structures represented by shoots at various developmental stages.

Seeds from *B. napus* plants transformed with binary vectors containing the *uid A* gene under the transcriptional control of either the CaMV 35S or the *mas* promoters were sterilized and sown on K3 medium lacking growth hormones and containing 100 mg/ l Km. The hypocotyls of resistant seedlings were cut into 5- 7 mm long pieces and plated for regeneration. After several steps, while the produced calli were on regeneration medium, samples were taken for monitoring the changes in gene expression during morphogenesis (Fig. 20. A. and B.). Non-morphogenic callus refers to callus regions without organized structures.

The CaMV 35S promoter directed the highest expression in the morphogenic callus. In non- morphogenic one the GUS activity is similar to that in detected in the emerging shoot. Often a leaf appeared first that was followed by the shootlet development in a process similar to the activation of a lateral bud. This leaf is marked as "first Leaf" at Fig. 20. The activity of the *mas* promoter was lower in this leaf than in the surrounding callus and the emerging shootlet (Fig. 20.B.). The structure of the morphogenic callus is rather complex, with trachear elements, parenchima- like cells and meristematic regions.





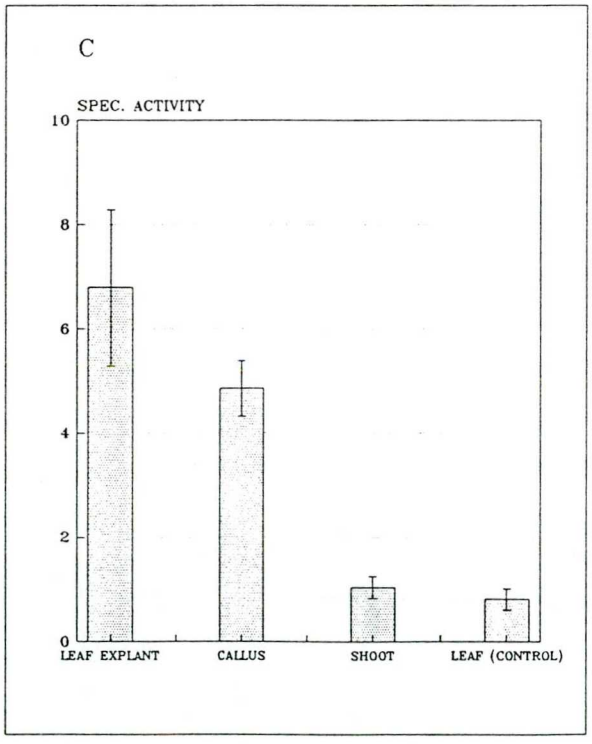


Fig. 18. GUS activity during in vitro morphogenesis in tobacco calli containing different chimeric genes:

A. pIS 212 (CaMV 35S promoter)
B. pIS 312 (*rbc S* promoter)
C. pIS 412 (*mas* promoter)

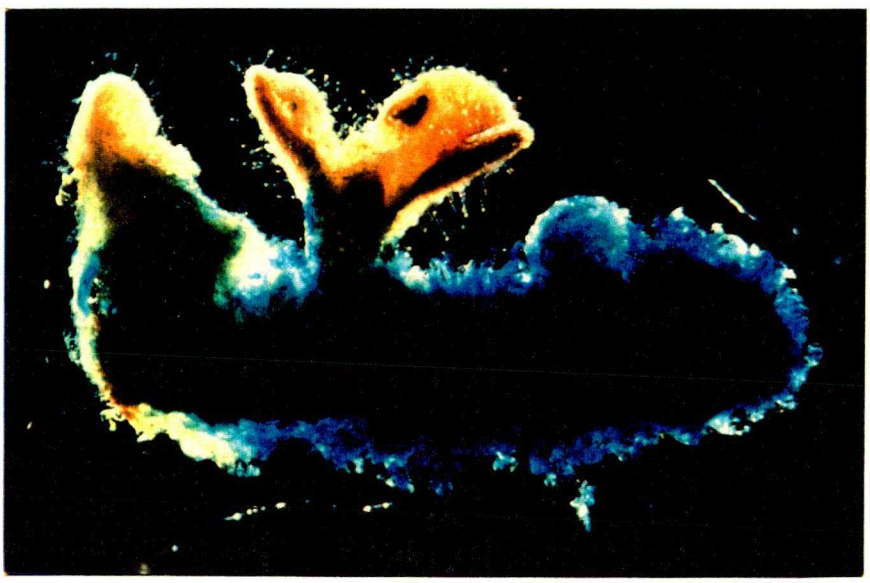


Fig. 19. **Histochemical localization of GUS expression directed by the *mas* promoter during morphogenesis of tobacco.**

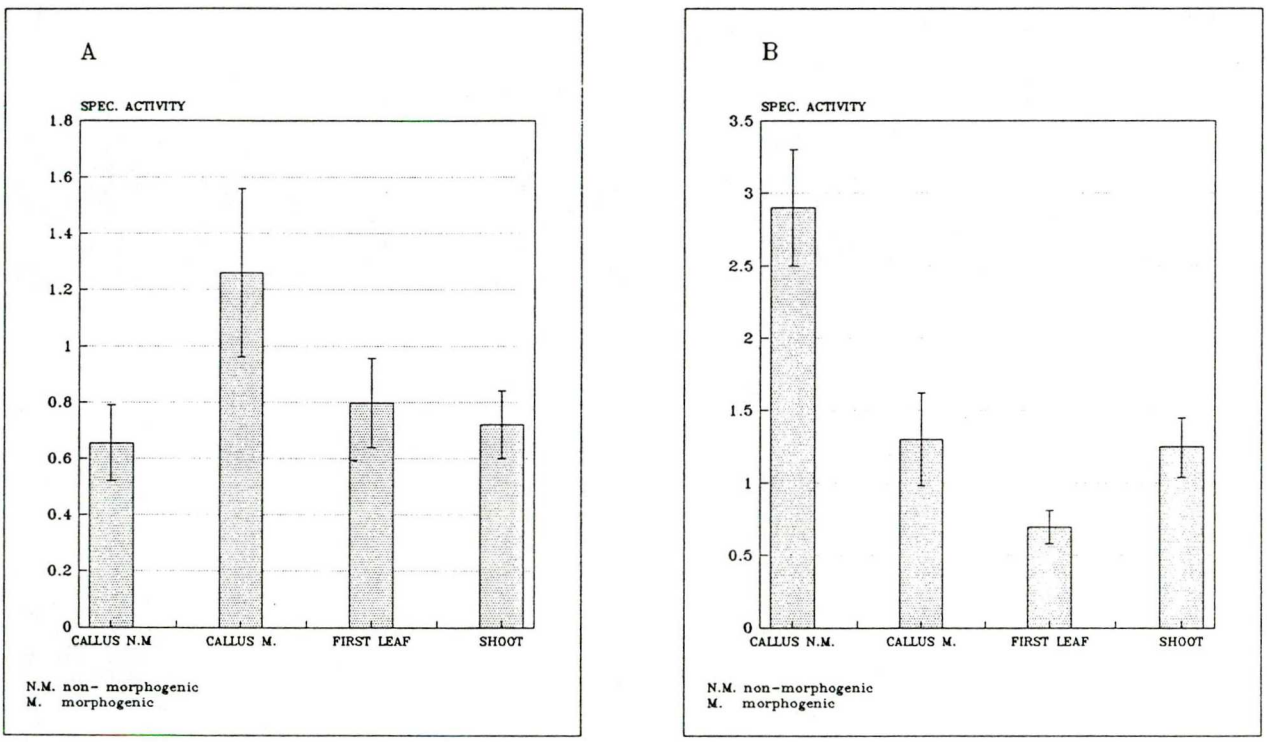


Fig. 20. Expression of the *uid* A gene under the control of CaMV 35S (A) and *mas* (B) promoters during morphogenesis of *Brassica napus*

Histochemical staining of thin sections revealed that the apparent activity is localized in the meristematic regions and the shootlets in the case of the CaMV 35S promoter and in the callus in the case of the *mas* promoter (data not shown). Since the two analyzed promoter showed an opposite trends in activity pattern in morphogenic and non-morphogenic tissues they might serve as an important molecular markers to monitor organ differentiation.

Young, fast growing roots from transgenic alfalfa plants containing the GUS gene under the control of the CaMV 35S and *mas* promoters were plated onto callus induction medium. Two weeks later they were transferred on embryo induction medium solidified with agar. Soon a population of embryos at different stages was formed as well as different structure of callus tissues could be detected. Three basic types of callus were formed: a compact green callus never producing embryos; a bright yellow- gold friable one, which can produce zones of meristematic growth and embryos. They are referred as "compact", "friable" and "embryogenic" respectively. Tissue samples were taken and the activity of the promoters was evaluated from the results of fluorimetric assays. As it can be seen on Fig. 21.A., the CaMV 35S promoter has a high activity in compact and embryogenic calli, slightly lower in friable one. Although lower, the activity during somatic embryogenesis did not show considerable differences amongst the different stages. Rather, the deviation from the mean values was smaller during the early stages (globular and elongated globular). When plantlets were formed, the GUS expression was lower. In contrast, the decrease of activity of the *mas* promoter during somatic embryogenesis was drastic, with a tenfold drop at the globular stage (Fig. 21.B.). Histochemical staining revealed a developmentally regulated, stage- dependent expression of the *uid A* gene under the control of both promoters. We were able to trace the changes in transcriptional activity of the CaMV 35S promoter during the whole process.

First signs of structural organization is reflected by appearance of GUS active zone in parallel with the induction of callus from roots, are surrounded by the mass of non- differentiated cells. The groups of small cells with high GUS activity (Fig. 22.A.). Later on, during the embryo induction, they gave rise to globular structure, also displaying strong GUS staining (Fig. 22.B.). The formation of bipolar structure, the elongated globular embryos corresponds to the changes in the pattern of expression of the gene. Stronger staining was detected at the extremities, and the epidermal cells were lacking GUS activity (Fig. 22.C.). The trend was even more obvious during the torpedo stage. A cross section through the middle of a "torpedo" demonstrates that the specific staining is located in the procambial tissue, while the ground meristem has an unspecific GUS activity at the sites of contact between the parental callus and the embryo (Fig. 22.D.).

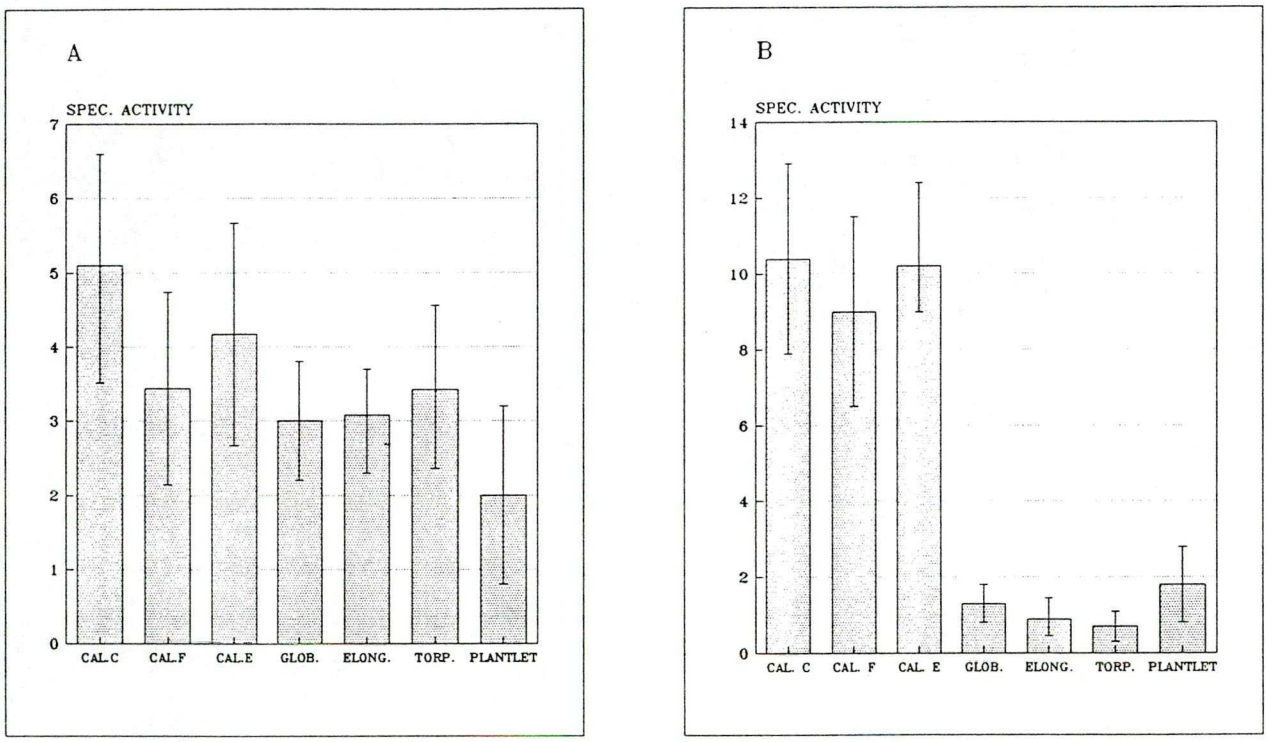


Fig.21. Changes in GUS activity during somatic embryogenesis in transgenic A2 calli.

A. transformed with pIS 212,
B. transformed with pIS 412.

Abbreviations:
Cal. C- compact callus; Cal. F- friable callus; Cal. E.- embryogenic callus, Glob.- globula; Elong- elongated globular; Torp.- torpedo stage.

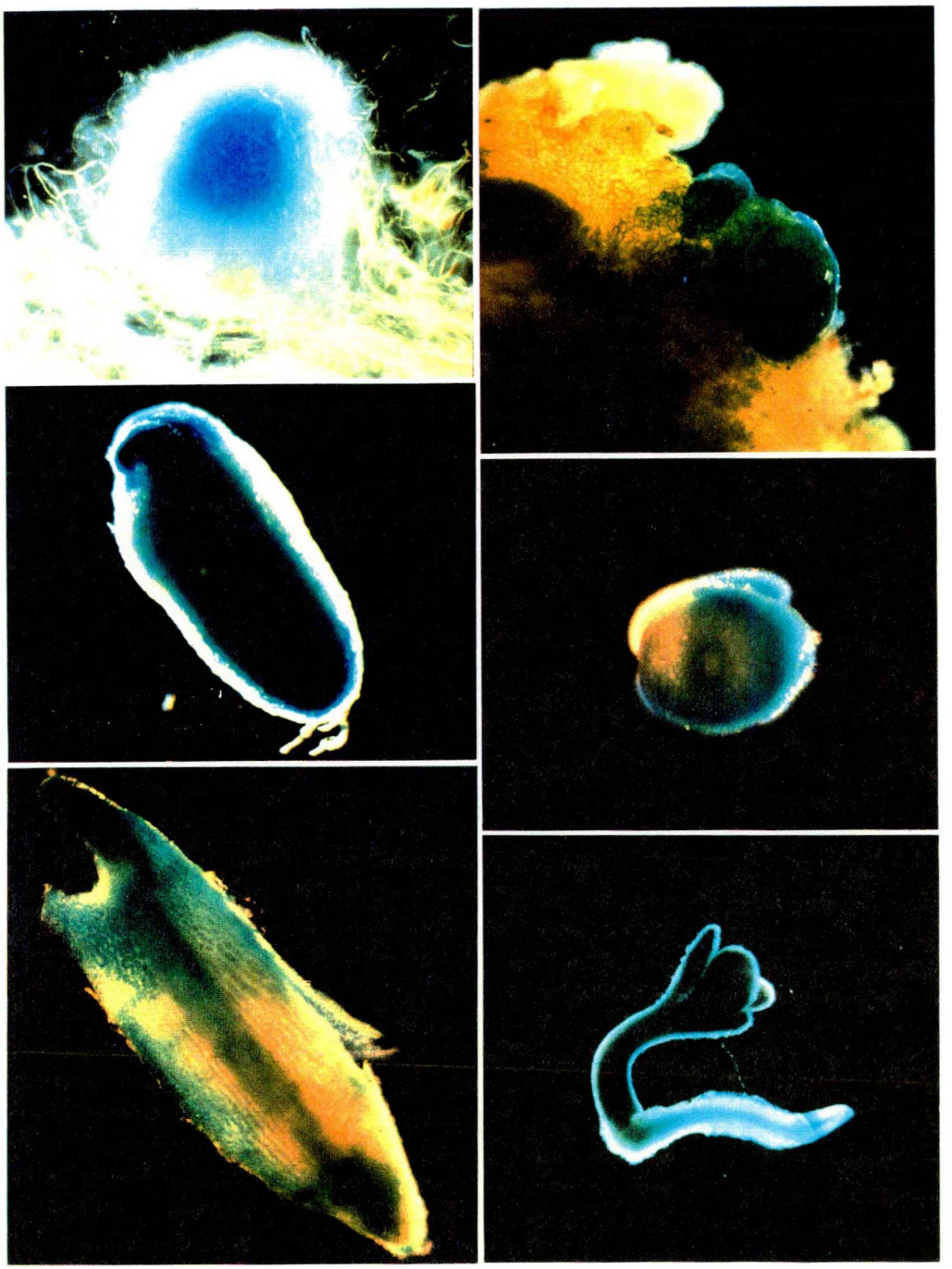


Fig. 22. Detection of apparent GUS activity during somatic embryogenesis of alfalfa transformed with a construct containing the CaMV 35S promoter.

A. Center of meristematic growth,
B. Globular, stage embryo
C. Elongated globular, stage embryo
D. Cross- section of a torpedo- stage embryo,
E. Longitudinal section of an embryo at similar stage,
F. Embryo at cotyledonous stage.

A longitudinal section demonstrates an enhanced polarity in the distribution of activity, the plumular part having both stronger staining and a larger area expressing the *uid A* gene in a detectable manner (Fig. 22.E.). At the cotyledon stage the polarization was even greater with the root having activity only in the procambium and the plumular part (hypocotyl and cotyledon) stained in all tissues, (Fig. 22.F.).

The *mas* promoter has high activity in calli, and almost no detectable activity was found in all the stages of somatic embryogenesis.

Table 7 summarize the changes of expression during later stages of somatic embryogenesis. The CaMV 35S promoter was more active in the plumular region of embryos at all the stages. When plantlets were formed, the highest activity was detected in the leaves, while the activity along the plant axis remained almost constant. The mannopine synthase promoter had also higher activity in the upper segments of somatic embryos. In plantlets its activity was lower in the shoot.

4.8. DIFFERENTIAL PROMOTER ACTIVITIES DURING PLANT DEVELOPMENT.

In order to assesses the *uid A* gene activity under the control of the CaMV 35S, *rbcS* and *mas* promoters we measured the activity of GUS in different plant parts during plant development. We have selected various ontogenic stages having different hormone balance. As it was already pointed out, plant growth regulators are potential factors affecting the transcriptional activity of the analyzed promoters. Since the three species studied have different patterns of development, they were sampled at different relevant stages.

For detection of tissue- and organ- specific expression of the *uid A* gene longitudinal and cross sections of stem and other organs were incubated in X- gluc buffer for 12- 15 h and the resulting tissue- specific staining scored under binocular microscope at 10- 50 fold magnitude.

4.8.1. Expression in tobacco.

Km resistant tobacco plantlets expressing the *uid A* gene under the control of the CaMV 35S, *mas* and *rbc S* promoters were sampled at the 7- 8 day after germination and the activity of GUS measured (Table 8.). CaMV 35S promoter had the strongest activity in roots, much lower in the hypocotyl. Cotyledons had intermediate activity. *Mas* promoter had higher activity in cotyledons and hypocotyl, lower in roots. As it could be expected, *rbc S* promoter had high activity in cotyledons, while its activity in other plantlet parts was low.

Table 7. Expression of the *uid A* gene under the control of CaMV 35S and *mas* promoters in transgenic alfalfa somatic embryos

DEVEL. STAGE	ORGAN	CaMV 35S	MAS
TORPEDO	PLUMULA	5.18 +/- 1	0.94 +/- 0.2
	RADICULA	3.1 +/- 0.4	0.51 +/- 0.1
COTYLEDONS STAGE	PLUMULA	3.05 +/- 0.5	1.5 +/- 0.3
	RADICULA	0.95 +/- 0.2	0.8 +/- 0.2
PLANTLETS	SHOOT	2.85 +/- 0.5	1.52 +/- 0.4
	PETIOL	2.80 +/- 0.6	2.0 +/- 0.5
	YOUNG LEAF	6.11 +/- 1.2	2.31 +/- 0.8
	ROOT	2.32 +/- 0.6	2.35 +/- 0.4

Table 8. Expression of GUS on 7-8 days old seedlings of tobacco carrying various promoters. Seeds were germinated at $16^h/8^h$. (light/dark period)

	CaMV 35S	MAS	Rbc S
COTYLEDONS	1.22 +/- 0.2	2.67 +/- 0.8	2.1 +/- 0.7
HYPOCOTYL	0.75 +/- 0.1	2.13 +/- 0.1	0.2 +/- 0.04
ROOT	2.48 +/- 0.2	1.15 +/- 0.3	0.18 +/- 0.07

Table 9. GUS activity in transgenic SR1 plants before flowering.

		CaMV 35S	MAS	Rbc S
PLANT AXIS	APICAL BUD	1.04 +/- 0.35	0.15 +/- 0.05	0.08 +/- 0.02
	STEM UPPER	0.51 +/- 0.02	0.06 +/- 0.01	0.17 +/- 0.02
	STEM MID.	1.98 +/- 0.6	N.M	N.M
	STEM BOTTOM	3.29 +/- 0.35	0.44 +/- 0.1	0.3 +/- 0.15
	HYPOCOTYL	2.57 +/- 0.7	1.22 +/- 0.5	0.21 +/- 0.05
	ROOT UPPER	1.15 +/- 0.2	0.41 +/- 0.1	0.06 +/- 0.01
	ROOT LOWER	2.40 +/- 1.1	0.39 +/- 0.2	0.16 +/- 0.05
LAST LEAF	PROXIMAL	1.38 +/- 0.02	0.03	0.21 +/- 0.01
	DISTAL	1.75 +/- 0.04	0.05	0.51 +/- 0.01
LEAF,YOUNG	PETIOL	4.50 +/- 1.2	0.62 +/- 0.17	0.82 +/- 0.1
	BASE	0.60 +/- 0.3	0.55 +/- 0.17	1.36 +/- 0.15
	TIP	2.43 +/- 1.1	1.11 +/- 0.3	1.18 +/- 0.15
LEAF, BOTTOM	PETIOL	3.29 +/- 1.1	1.05 +/- 0.35	0.89 +/- 0.28
	BASE	3.07 +/- 1.2	0.81 +/- 0.2	2.76 +/- 1.05
	TIP	3.82 +/- 1.05	1.88 +/- 0.24	2.11 +/- 0.8

Plantlets were planted in pots and sampled before flowering, when their height was about 80 cm- 1 m (Table. 9.). Along the plant axis a gradient of CaMV 35S promoter was seen, with a maximum value in the lower part of the foliated stem and the root tip. The activity in leaves was dependent on the age and the area sampled tissues. In young leaves the highest activity was detected in petioles, the body of the leaf having an increase of activity towards the tip. Elder leaves in the lower segments had high, uniform activity. In the plants we studied the *mas* promoter had lower overall activity also with a maximum in the basal part. The youngest leaves (3-5 mm long) had very low activity, at the level of the control, untransformed plants. The activity of the *rbc S* promoter was high in leaves, the younger ones having lower activity. Stem had also some activity, although very low.

Cross sections below the apex and at the bottom revealed differences in the cellular localisation of activity of the promoters. In the upper parts, the CaMV 35S promoter was active in the protoxylem and adjacent cells of the pith parenchima. Very low activity was detected in the phloem. Similar distribution was seen in the lower parts of the stem (Fig. 23. A. and C.). Contrastly, the *mas* promoter had detectable activity only in the phloem (Fig. 23. B. and D. The greenish colour at B. is an artefact).

Longitudinal sections from flower buds showed that the *mas* promoter confers activity to the vascular cambial tissue in the receptacle and the pedicel and in the intercalar meristem zones, responsible for the elongation of the pedicel. CaMV 35S promoter has activity in all actively dividing tissues: the vascular bundle with its cambial components, the intercallar meristems, the carpel and the veins of the youngest leaves surrounding the bud. (Fig. 24.).

4.8.2. Expression in rapeseed.

Seedlings were cultivated in the dark and in 16/ 8 hours light/ dark photoperiod. The CaMV 35S promoter conferred high expression to the root, especially under normal illumination. In cotyledons the activity was lower. In contrast to it, *mas* promoter had highest activity in cotyledons. The lack of light and the consequent etiolation negatively influenced its activity (Table 10.).

Young plants, grown in the greenhouse were used to determine the activity of the promoters during vegetative growth of both winter and spring cultivars. The growth of the plants was slow and a considerable amount of leaves was accumulated. A distinct gradient of expression along the plant axis was seen in the case of the CaMV 35S promoter. The maximum was at the lower portion of roots. High expression was detected in cotyledons, which were still greenish in appearance at that stage. The expression in basal, fully expanded leaves was higher than in growing ones.

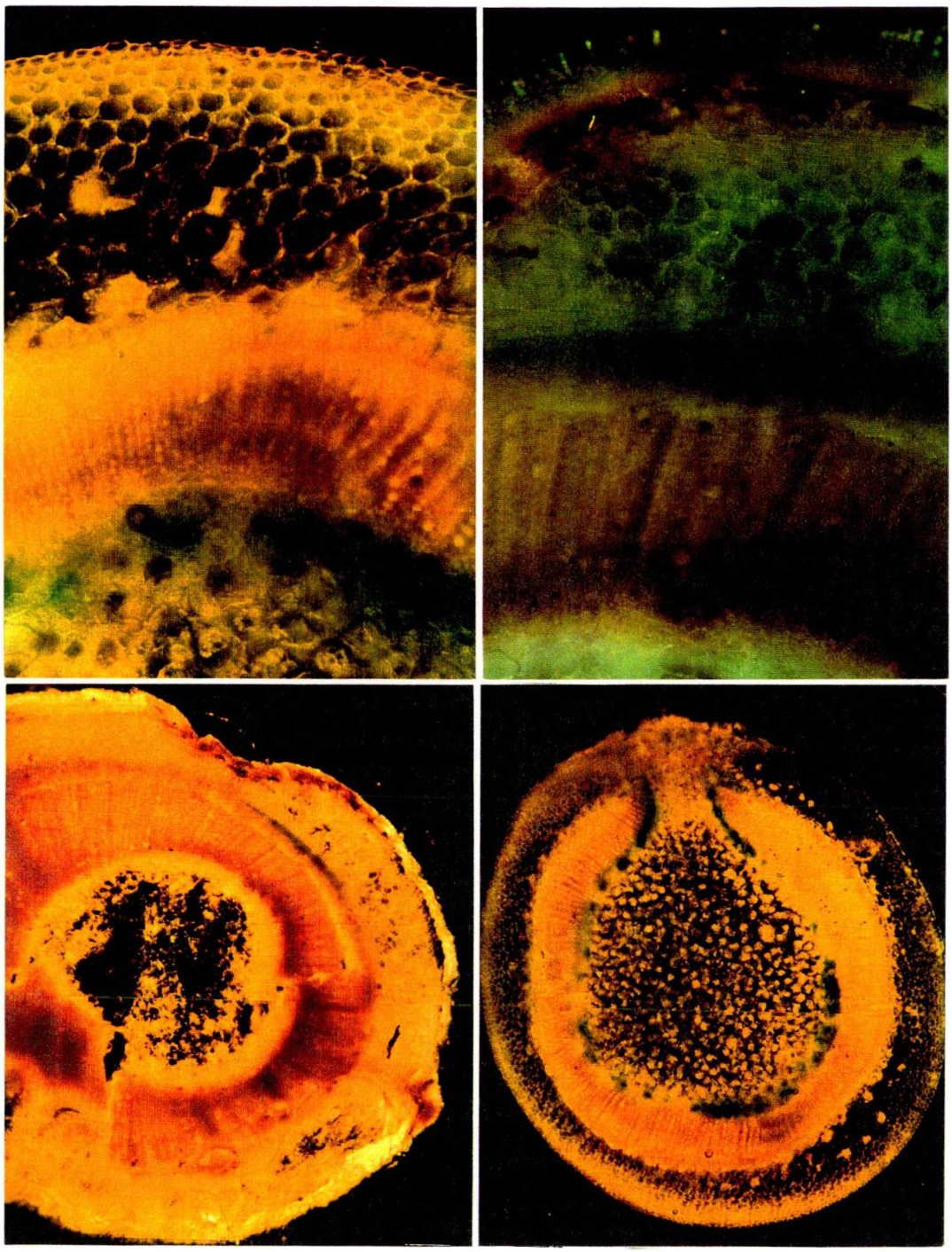


Fig. 23. GUS activity in stem cross-sections of tobacco plants transformed with constructs containing the *uid* A gene under the control of:

A. CaMV 35S promoter, upper segment
B. *mas* promoter, upper segment
C. CaMV 35S promoter, above ground
D. *mas* promoter, above ground

Fig. 24. Longitudinal sections of transgenic tobacco bearing the CaMV 35S (A) and *mas* (B) promoters. Sections were fixed and stained with X-gluc

Table 10. Expression of β-glucuronidase in 6-8 days old seedlings of *Brassica napus*. Seeds were germinated either on darkness or at 16/8 hours photoperiod

	CaMV 35S promoter		MAS promoter	
	LIGHT	DARK	LIGHT	DARK
COTYLEDONS	1.34 +/- 0.3	1.12 +/- 0.2	9.53 +/- 2.4	3.87 +/- 1.1
HYPOCOTYL	3.92 +/- 0.4	3.55 +/- 0.6	4.25 +/- 2.6	1.89 +/- 0.5
ROOT	5.92 +/- 0.9	3.28 +/- 0.6	2.70 +/- 1.4	1.25 +/- 0.4

Table 11. Expression of β-glucuronidase in young rapeseed plants at the rosette stage

		CaMV 35S	MAS
PLANT AXIS	APICAL BUD	0.85 +/- 0.1	1.65 +/- 0.2
	STEM	1.38 +/- 0.4	2.20 +/- 0.6
	HYPOCOTYL	1.48 +/- 0.4	3.12 +/- 1.2
	ROOT UPPER	1.45 +/- 0.5	2.22 +/- 0.9
	ROOT LOWER	2.15 +/- 1.2	2.10 +/- 0.8
LAST LEAF		2.05 +/- 0.2	1.70 +/- 0.4
YOUNG LEAF	PETIOL	3.30 +/- 1.8	2.41 +/- 0.7
	BASE	2.20 +/- 0.4	2.46 +/- 0.7
	TIP	2.14 +/- 0.2	1.7 +/- 0.4
FULLY EXPANDED LEAF	PETIOL	4.33 +/- 1.6	3.43 +/- 1.6
	BASE	4.85 +/- 2.3	3.72 +/- 1.9
	TIP	3.17 +/- 0.9	4.50 +/- 0.4
COTYLEDONS		5.58 +/- 1.5	6.91 +/- 1.9

Table 12. **Expression of β-glucuronidase in plants after vernalization (80 cm)**

		CaMV 35S	MAS S / A
PLANT AXIS	APICAL BUD	1.08 +/- 0.05	0.31 +/- 0.03 / 0.38 +/- 0.1
	UNDER INFLOR.	0.86 +/- 0.15	0.55 +/- 0.1 / 0.30 +/- 0.05
	BRACK 70 CM	1.60 +/- 0.25	0.79 +/- 0.1 / 0.44 +/- 0.02
	BRACK 40 CM	2.20 +/- 0.2	0.95 +/- 0.1 / 0.66 +/- 0.02
	STEM 15 cm	3.08 +/- 0.4	1.33 +/- 0.4 / 0.66 +/- 0.05
	HYPOCOTYL	2.84 +/- 0.6	1.41 +/- 0.3 / 0.96 +/- 0.15
	ROOT	4.84 +/- 0.9	1.95 +/- 0.7
LAST LEAF		1.1 +/- 0.1	1.1 +/- 0.02
LEAF ON THE BRACK	PETIOLE	1.71 +/- 0.2	1.04 +/- 0.2
	BASE	1.33 +/- 0.3	1.4 +/- 0.4
	TIP	1.44 +/- 0.1	1.54 +/- 0.1
LEAF BOTTOM	PETIOLE	2.67 +/- 0.35	3.97 +/- 1.1
	BASE	2.33 +/- 0.3	3.52 +/- 0.8
	TIP	3.18 +/- 0.5	3.16 +/- 0.8

Mas promoter had increasing activity from the apical bud towards the hypocotyl, lower in the roots. Its activity was also highest in the cotyledons. Fully expanded leaves had also higher activity than younger ones (Table 11.)

In contrast to tobacco, rapeseed, especially the winter cultivars need a vernalization step before they set flowers. Plants were kept at 3- 5 ^{o}C in dim light for 4- 5 weeks. Upon transfer to the greenhouse, they grew rapidly and reached a height of 70- 90 cm in 10- 12 days. This process is involving an increased production of hormones. Accordingly, we were expecting changes in the expression of the studied promoters. In the plant axis both promoters showed lowered activity. The transcriptional activity of the *mas* promoter clearly demonstrated strong suppression by some factor. The cultivar Arabella produced well developed burgeons at the leaf axles. This correlated with lower activity of the *mas* promoter all along the stem of representative plants from this cultivar. Concerning the activity in leaves, both promoters had lower expression in young leaves on the brack. CaMV 35S had also lowered activity in fully expanded leaves, while *mas* promoter showed no difference in the expression, compared with the rosette stage (Table 12.).

Histochemical staining demonstrated, that in upper segments of rapeseed stem, the CaMV 35S promoter has strong expression in the collenchima, the phloem-bound parenchima cells and the cambium. Rarely pith cells were stained (Fig.25.A.). In sections from the bottom of the plant the apparent activity was concentrated in the phloem (Fig.25.C.). The *mas* promoter has high activity in the phloem, the cambium and in isolated cells of parenchima and collenchima, the overall activity being much lower than that of the CaMV 35S promoter (Fig.25.B.). In lower sections of the stem the detectable activity was concentrated in the phloem, but also in the protoxylem (Fig. 25.D.)

In the apical region detectable 35S promoter activity was found in parenchima, the colenchima and in lesser extend the vascular tissue. Higher activity was detected at the branching sites (Fig. 26.A.). *Mas* promoter drove low detectable activity in the vascular tissue, much higher in the intercallar meristems at the branching sites (Fig. 26.B). CaMV 35S promoter has high activity in the meristematic regions of lateral buds (Fig. 26.C.).

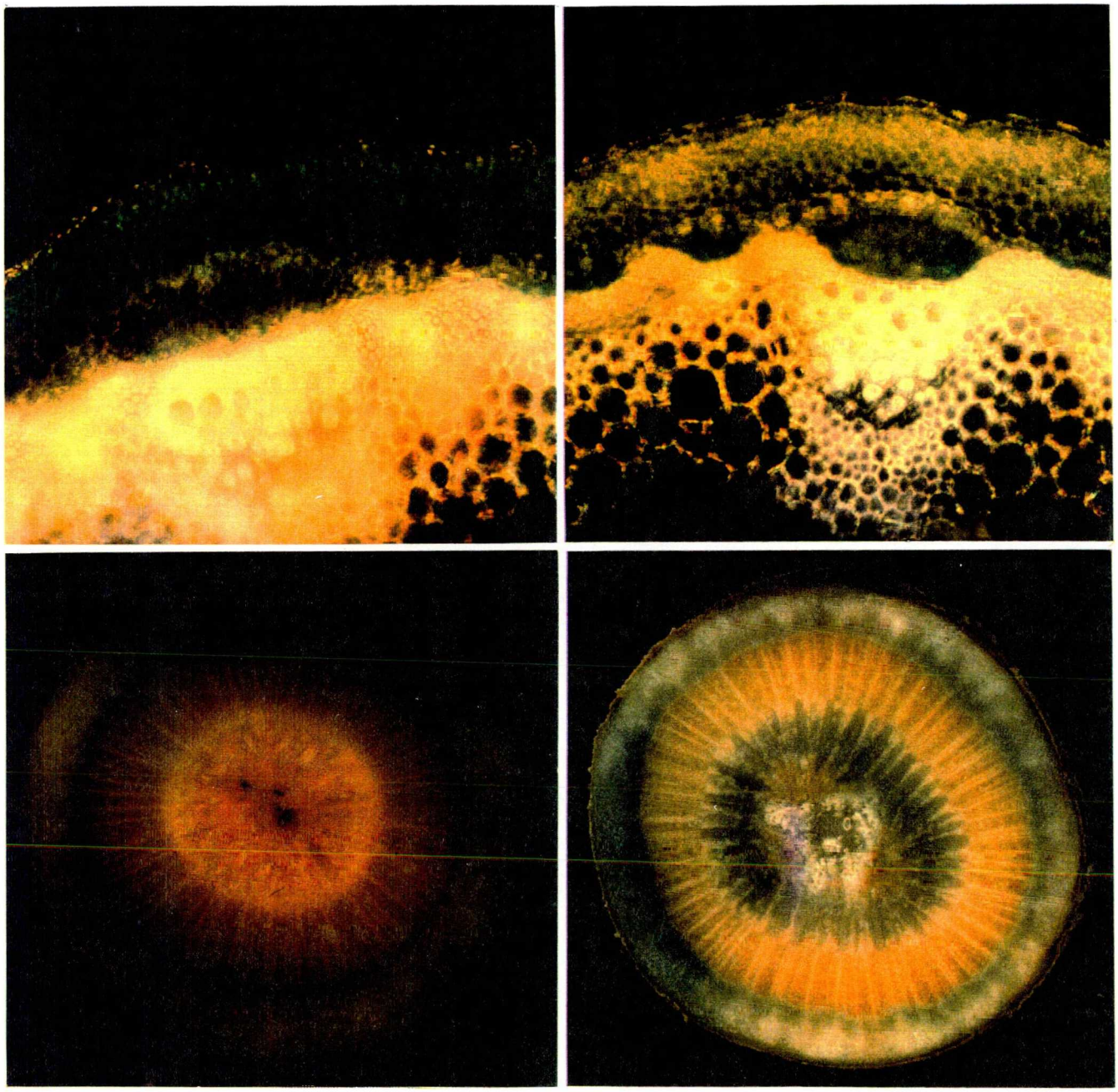


Fig. 25. **GUS activity in stem cross-sections of rapeseed plants transformed with constructs containing the *uid* A gene controlled by** CaMV 35S promoter **(A and C) and the *mas*** promoter **(A,B)**

A and B upper segment
C and D above ground

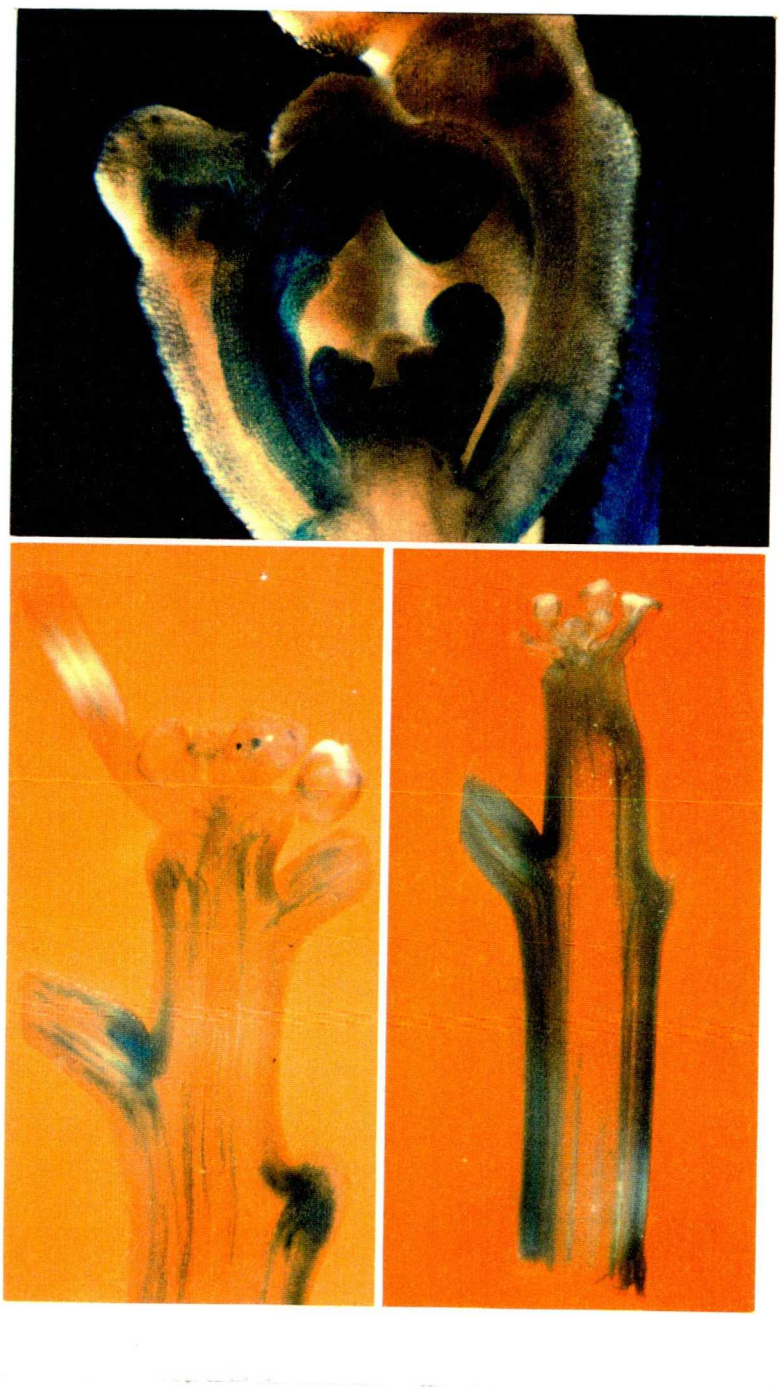


Fig. 26. GUS activity in shoot apex and in lateral buds of flowering rapeseed plants

A. Lateral bud a plant with the CaMV 35S promoter
B. Plant transformed with construct containing CaMV 35S promoter
C. Idem, transformed with *mas* promoter

4.8.3. Activity of the CaMV 35S promoter in alfalfa:

Since we were not able to obtain seeds from our transformants, somatic embryos (torpedo and cotyledons stages) and axenic somatic embryo- derived plantlets were used for the early development stages and the results are reported in the previous paragraph (Table 7.). Developed plants were potted and grown in the greenhouse. Unfortunately, only plants containing the construct with CaMV 35S promoter survived the transfer. A period of hardening was required in order to improve the viability. Plants were left to grow for 6- 7 weeks before handling. To stimulate growth they were cut 4- 5 cm above the ground. This induced a rapid development of the lateral buds and formation and elongation of stems. About two months later the growth was slowed down and at the leaf axles lateral buds developed into bouquets of juvenile in appearance leaves, referred as nodal leaves. Samples were taken from different parts of fast growing spruces and ones with the growth rate already slowed down. The overall activity of the promoter was lower in elder spruces, especially in the upper section of the stem and lower leaves, which were decaying at the time of sampling (Table 13.).

Staining of cross- sections of fast- growing spruces and ones with low growth rate showed that the differences are mostly quantitative and no significant changes in the spatial pattern of expression could be detected (Fig. 27. A. and B.). High expression was found in the cortical and fascial colenchima, the fascicular cambium and the protoxylem. Lower expression was detected in the parenchima and the phloem. In old spruces the overall activity was lower and the activity in the protoxylem was not detectable. In roots of axenically grown plants the activity was strong in the apical meristem and the procambium (Fig. 27.C.). Later the expression was restricted inside the perycycle, as it is shown on Fig. 26.D. demonstrating a lateral root growing through the cortex. The lateral root primordia also showed a high degree of staining

Table 13. GUS activity in transgenic alfalfa plants containing the CaMV 35S promoter

		GROWING SPRUCE	ELDER SPRUCE
PLANT AXIS	APEX	3.57 +/- 1.0	0.7 +/- 0.3
	STEM UPPER	3.50 +/- 1.6	1.33 +/- 0.4
	STEM MID.	2.55 +/- 0.6	1.81 +/- 0.4
	STEM BOTTOM	1.66 +/- 0.5	1.43 +/- 0.4
	STOLON	N.M.	0.84
	ROOT UPPER	1.50 +/- 0.6	1.2 +/- 0.5
	ROOT LOWER	2.61 +/- 0.5	2.23 +/- 0.6
UPPER	NODUS	1.72 +/- 0.2	1.11 +/- 0.15
	PETIOL	3.477 +/- 1.1	2.69 +/- 1.3
	LEAF	5.82 +/- 1.0	2.92 +/- 0.9
	NODAL LEAVES	--	2.34 +/- 0.7
LOWER	NODUS	3.10 +/- 0.5	0.53 +/- 0.1
	PETIOL	2.25 +/- 1.2	2.1 +/- 1.0
	LEAF	4.92 +/- 1.2	1.975 +/- 0.95
	NODAL LEAVES	3.28 +/- 0.4	2.73 +/- 0.5

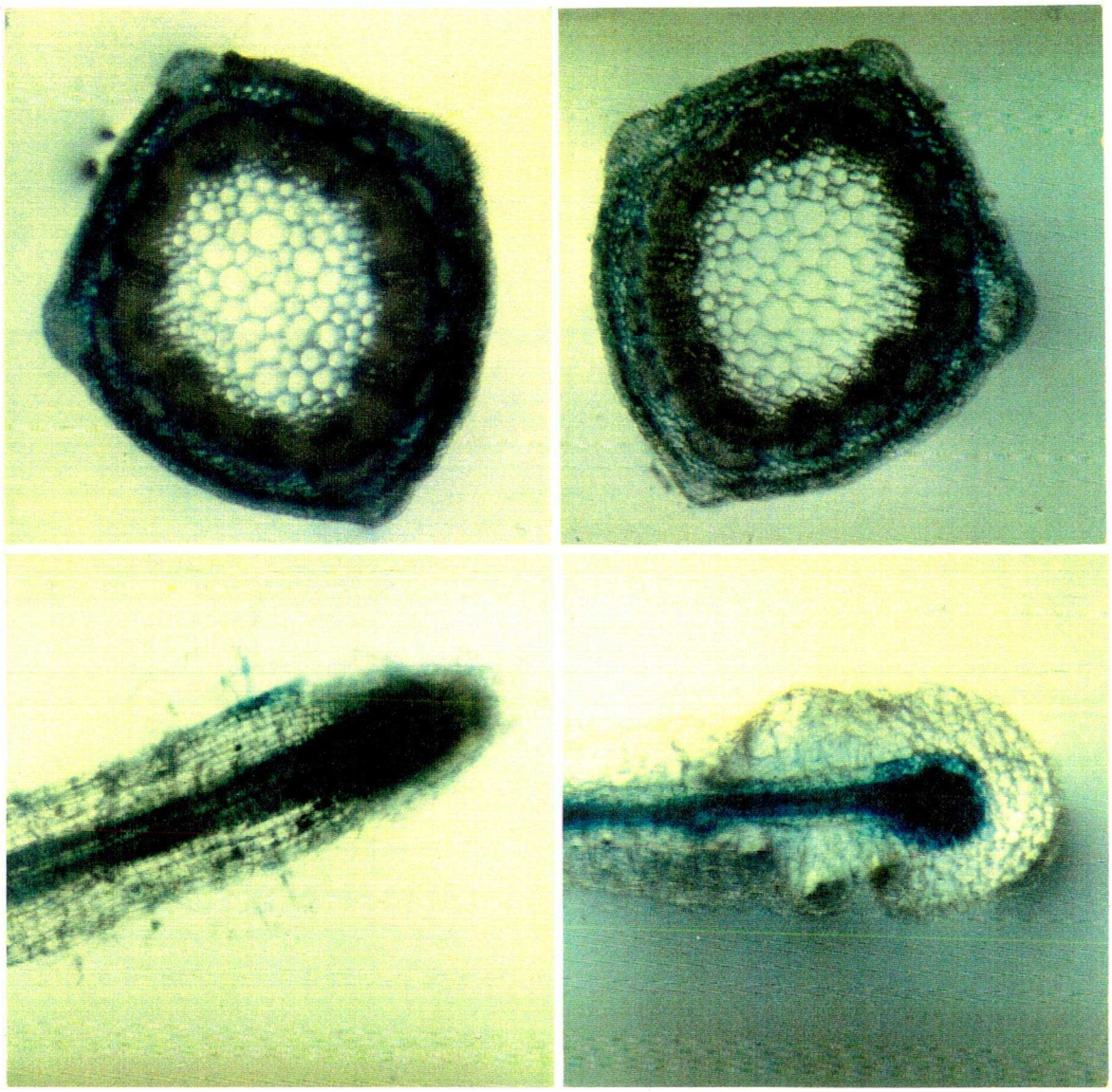


Fig. 27. GUS activity in transgenic alfalfa containing CaMV 35S *uid* A construct

A. Cross-section of a stem in rapid growth
B. Cross-section of a stem with hindered growth
C. Cross-section of a root at the embranchement
D. root tip

5. DISCUSSION

5.1. PRODUCTION OF TRANSGENIC PLANTS AND EVIDENCES FOR THE INTEGRATION OF THE FOREIGN GENES INTO THE PLANT GENOME.

In the present work we have used the *Agrobacterium*- mediated processes method for gene transfer to alter the genome of three crop species. Since our aim was to analyse the transcriptional activity of specific promoters during the ontogenesis of these plants, we constructed nine gene fusion vectors carrying the bacterial *uid A* gene under the control of three different 5' regulatory elements from viral, bacterial and plant sources. After construction of plasmid molecules by routine cloning protocols, the transformation vectors were tested for their ability to direct transcription of specific RNA and protein by transient expression experiments.

Transient expression experiments provide an opportunity to make a rapid assessment of the expression of a promoter, but there are certain limitations of the system. First, the protoplast is not a "normal" plant cell. The lack of 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). Nor a leaf or "mesophyll" protoplast population is homogeneous. Beside the parenchima cells (which already are morphological and physiologically different- spongy parenchima an palisade parenchima) are the non- photosynthetic cells from the vascular tissue, the perivascular parenchima, the epidermal cells, the trichoma cells and the stomatal guard cells, the later ones having also photosynthetic activity. All these cells have a different competence for plasmid DNA uptake and expression of the transferred genes and respond in a different way to the culture conditions. The successful uptake of plasmid DNA and the subsequent expression of the introduced genes is affected by many parameters. Some of them are of technical character: purity and viability of protoplasts, of plasmid DNA, of reagents, the physiological state of the plant source (Masson and Paszkowsky, in press). One of the parameters we wanted to analyze was the size of the introduced DNA. Table 4. 2 demonstrates the strong inverse relation between the expression and the size of the plasmid. The experiments were carried with two different promoters, in several repetitions. Our results suggest two main reasons for the lower expression in protoplasts transformed with larger plasmids: 1. A larger plasmid is more susceptible to enzymatic attack both before uptake and during transport through cell membrane and in the cytoplasm.

Some reports indicated high amounts of DNA- degrading enzymes are present in protoplast suspensions (Zilberstein, personal communication) and recombinations can occur during introduction of plasmids in the cell. 2. Equimolar quantities of a large and a small plasmid contain different quantities of "active DNA"- sequences carrying genetic information that can be expressed by the plant transcriptional and translational mechanisms. These two reasons cause a non-linear dependence of the expression on the size.

Nevertheless, transient expression experiments can provide useful information on the activity of some promoters. We used transient expression of the *uid A* gene to assess the functional integrity of the expression vectors and for pilot studies on the expression of the nominally constitutive CaMV 35S and *mas* promoters and the *rbcS* promoter, specifically expressed in photosynthetically active cells. Our results indicated a different temporal pattern of expression of the genes. The CaMV 35S promoter has increasing activity during the whole period of cultivation. The *rbcS* promoter conferred low activity of the reporter gene. The *mas* promoter has an increasing activity during first two days. Later the activity slowly decreased. The very high activity of the *mas* promoter is consistent with the results of Langridge et al. (1989), who demonstrated high activity in protoplasts obtained from transgenic plants. Similar results were obtained with *Nicotiana africana* suspension protoplasts (Pestenacz, Csitari, Menczel, Stefanov, unpublished), tomato leaf protoplasts (Stefanov et al., 1991), alfalfa A2 suspension protoplasts (Stefanov, unpublished) and *Brassica napus* hypocotyl protoplasts (Pauk et al., 1991). In all experiments the *mas* promoter had highest activity. The level of expression was dependent on the system used and the genotype of the donor plant (Stefanov et al., 1991).

Histochemical staining of protoplasts immobilized on support two days after DNA uptake showed not only difference in the quantity of stained protoplasts, but also in the intensity of staining (Fig.11/B.).

An interesting observation was the heterogeneity in the expression of individual protoplasts. Since it was postulated for years, that the transient expression is not affected by position effect, other explanations should exist. Amongst them the different competence of the cells for DNA uptake, differences in the response to culture conditions, uneven repaire of the DNA between the protoplasts, partial inactivation of the introduced genes due to minor rearrangements during the transformation process and differences in the accumulation of functional enzyme, due to delayed transcription, are the most probable causes of such heterogeneity. The use of protoplasts derived from synchronized cell suspensions with known duration of the phases of the cell- cycle and the engineering of methods for precise measurement of the expression should answer some of the questions.

We wanted also to compare the activity of the promoters obtained when protoplasts were transformed using PEG- mediated transfer methods and when protoplast derived from transgenic plants carrying the studied construct were cultivated. A comparison between figures 4.4. and 4.23. immediately reveals the striking differences in the timing and strength in the expression of all the promoters. The only promoter having quantitative differences is CaMV 35S. Supposedly peaks in its activity are seen when the majority of the cells are at the stage of start of the cell division and this happens at different time in transformed and not handled protoplasts. *Rbc S* promoter had a low activity in transient expression experiments. This result can be expected, since the high sugar and hormone concentration in the medium and the stress can be responsible for depressing its activity. The same factors are the cause for the decrease of activity of this promoter during cultivation of transgenic protoplasts. Most unexpected and controversial findings were linked to the activity pattern of the *mas* promoter. During cultivation of protoplasts which had been transformed using PEG, the maximum in activity was observed two days after transformation with subsequent decrease. In protoplasts derived from transgenic plants, its activity was increasing during the whole cultivation period. Langridge and coworkers (1989) experienced a 500 fold increase in the promoter activity during protoplast isolation, by using bacterial luciferase as reporter gene. In our experiments the enhancement was only tenfold, but the choice of reporter gene and differences between plants might be the cause.

More disturbing is the fact that expression data from transient expression and stable integrated genes did not match and no explanation could be find up to now. These results, together with the above mentioned discrepancies, increase further comprehensive investigations. The derivatives of the binary plant transformation vector pBIN 19 (Bevan, 1984), among which are the vectors of the pBI type (Jefferson, 1987), are known to be unstable in some strains of *Agrobacterium* (J. Jones, personal communication) and although the selection for the plasmid in the bacterium is effective, some rearrangements might occur in the T- DNA, thus alters the properties of the genes introduced into the plant genome. For these reasons we preferred to reclone the expression cassettes to the more stable vector pGA 482 (An, 1987), which contains also the right border overdrive sequence enhancing the precision of the transfer (Fedoroff, 1988). It contains suitable restriction sites and although the main bacterial selection (Tetracicline resistance) is prone to failures (Hooykaas, 1988), the additional Km R selection extended the possibilities for proper selection of transconjugant *Agrobacterium*- s.

Tobacco is the most widely used laboratory plant for tissue culture experiments. It has a very well defined response to external stimuli and the ratio of auxins and cytokinins in the culture milieu can be manipulated to shift the cells towards unorganized growth or organogenesis (Tran than Van et al., 1974; Thorpe and Murashige, 1968). The possibility to induce direct organogenesis from explants is important for diminishing the culture time and thus to avoid undesirable somaclonal variations. These features have made tobacco an often used model for transformation and foreign genes expression experiments (Rogers et al., 1987, Deblaere et al., 1987; An, 1988).

The use of *Agrobacterium* strains harbouring disarmed Ti-helper plasmids ensured not only regeneration of large quantities of transgenic plants, but also that the tissue and cell-specific promoter activity is not distributed by higher auxin or cytokinin levels in the transgenic cells and plants.

It has been demonstrated that even phenotypically normal and fertile transgenic tobacco plants can have higher than the natural auxin level when the gene transfer was done using wild type Ti- plasmids (Szabo, Csiszár and Stefanov, unpublished). This can lead to some misinterpretation of the results, especially if someone works with hormone-sensitive promoters.

In our experiments statistically every explants gave rise to Km resistant shoots. It means, the transformation frequency was about 100 %. Only one shoot per explant was further used, in order to avoid siblings (regenerants obtained from one transformation event). Not every kanamycin resistant shoots or calli were found to be GUS positive.

The previously published transformation and regeneration protocols have emphasized the limitation in the regeneration potential especially in the case of winter types of rapeseed (De Block et al., 1989). The majority of the previous attempts was based on production of shoots directly from the incubated explants, which decreased the risk of unwanted somaclonal variations and reduced the time of *in vitro* culture. In our experiments we found beneficial to induce first the explants to form callus before organ differentiation. Since not all the cells with competence for transformation are also competent for dedifferentiation and further development towards plant regeneration, this multistep culture procedure can be a way to increase the chances for activation of potentially 'regenerable' cells. Another prerequisite for the successful regeneration was the use of $AgNO_3$ during the shoot induction (De Block et al., 1989).

The most probable mode of action of the silver ions is the inhibition of ethylene action and they were used in regeneration protocols for several plant species (Purnhauser et al., 1987; De Block, 1988; De Block et al., 1989). In pilot experiments, calli grown on various media lacking Ag- ions were unable to produce shoots.

To summarize, the key elements of our regeneration protocol are as follows:

1. Callus induction in the presence of 2,4-D, NAA and cytokinin for 2-3 weeks (K3.2 medium).
2. Preconditioning with reduced concentration of 2,4-D for 2-3 weeks (K3.3 medium)
3. Shoot induction with cytokinin (BA 1 $mg.l^{-1}$) and weak auxin (NAA at 0.1 m $g.l^{-1}$). The $AgNO_3$ in this induction medium was an essential for formation of shoots. Induction of wound-stress by cutting the calli with a sharp razor also boosted the formation of shoots.

4. Excision of the plantlets from the primary calli and planting on hormone- free medium with reduced sucrose (K3.5) where they grow vigorously for up to 3 weeks. After that period they should be transferred to the next medium, otherwise their development is hindered. The use of kanamycin in this medium ensured the effective elimination of non-transformed regenerants (which can appear in *Brassica* cultures with rather high frequency, especially if the primary calli are too volumous).
5. For induction of root formation we cut the bottom of the plantlets and plant them in medium containing IBA.

With the help of the described regeneration system plants were successfully obtained from Km resistant tissues both in winter and spring cultivars with at least 7 % frequency. Thus, the critical stage in establishment of the transgenic plants was not the transformation itself (production of transgenic calli), but the plant regeneration. The regenerated plants were fertile and seeds could be obtained after self-pollination.

The inductive role of auxins, especially 2,4- D in triggering somatic embryogenesis in alfalfa has been widely documented (Saunders and Bingham, 1975; Walker and Sato, 1981; Walker et al, 1979; Dudits et al, 1991). Although the presence of cytokinins seems to be facultative for the formation of embryos, Walker and coworkers (1979) suggested they are required for better control of embryo formation.

Several other factors are crucial for production of healthy somatic embryos and plants. The duration of embryo induction appears to be important and genotype dependent. In the case of A2 the optimum induction period was found to be 25- 30 days. The embryos developed asynchronously. We do not know if it was the result of different sensitivity of the cells towards the 2,4- D treatment, or was it a question of shift in the timing for competence for regeneration. Previous results (Denchev et al, 1991a; Denchev et al, 1991b) demonstrated that changes in osmolarity during the development of embryos can influence the output of healthy embryos able to produce plants. The use of PEG 6000 at a concentration of 1 % yielded the highest quantity of normal embryos in pilot experiments.

Some authors (Atanassov and Brown, 1984) reported high frequency of abnormalities, such as fused cotyledons, vitrification, recallusing and multiple embryo formation, supposedly because a process similar to the precocious germination of zygotic embryos. Exposure of the embryos to ABA advances them through maturation, diminishing the quantity of abnormalities, especially when combined with PEG treatment. Embryos arrested at torpedo stage by ABA treatment need an external stimulus to reasume their development. Since the use of auxins or cytokinins would lead to recalussing GA3 was used to break this kind of "dormancy" and to induce conversion to plants. Regenerants showing normal phenotype were used for further experiments.

The presence of the alien genes was proven by Southern analysis in the case of tobacco and rapeseed. Simultaneous and specific expression of both the *neo* (NPT II), conferring resistance against aminoglicosides, and the non-selectable maker *uid A* (GUS), genes in the primary transformants and their progeny provided further evidences for the presence of the introduced genes.

5.2. CHARACTERISTIC PROMOTER FUNCTION DURING *IN VITRO* DIFFERENTIATION

In vitro culture systems provide unique experimental approaches to study cell growth and differentiation under artificial conditions. The long history of plant tissue culture demonstrated clearly that the morphogenesis and development are to a great extend controlled by external factors, especially plant hormones (Reviewed by Ammirato, 1983; Flick et al, 1983). In recent years, empirical observations were complemented with molecular approaches to study and understand the basic processes underlying specific *in vitro* tissue culture responses to environmental factors.

Among the available techniques, identification and purification of specific proteins (Sung and Okimoto, 1981) and cloning of genes with differential expression (Sung and Okimoto, 1983) during callus and embryo induction were successfully applied. In our study we wanted to extend the available tools with the new methods based on the use of reporter genes introduced in the genome of transgenic plants. According our present knowledge, this approach has been used in the same time with our work published recently in studies on carrot embryogenesis (Fujii and Uchimia, 1991).
Since plant growth regulators are the most crucial factors affecting *in vitro* behaviour of plant tissues, we wanted to compare the response of the CaMV 35S and *mas* promoters to different concentrations of various hormones. The fluorimetric measurement of enzymatic activity of lysates from hormone- treated cells confirmed the results of Langridge et al, 1989, that auxins enhance the activity of *mas* promoter, while ABA and GA3 inhibits its function. The influence of cytokinins, namely BA, was concentration- dependant. The CaMV 35S promoter seems to be insensitive to hormones, at least in the concentration range studied. It may be proposed that differences in expression of this promoter *in planta* are not caused by the internal levels of hormones, but other factors could be responsible for the observed expression patterns.

We were not able to establish a clear pattern of differential expression of the CaMV 35S promoter during *in vitro* cultivation in any of the three species studied. At the same time, its use coupled with histochemical staining turned out to be a valuable tool, clearly discriminating between different cell types and tissues. Structural changes occurring at different stages of somatic embryogenesis of alfalfa were successfully monitored with the help of this promoter (Fig. 22.). Under the influence of 2,4- D the cell division cycle in the primary explant is reactivated thus leading to callus formation and to the production of somatic embryos (Dudits et al, 1991; Dudits et al, in press). In parallel with the lack of proliferation of large cells with huge vacuols, the formation of clusters of small cells with dence cytoplasm lacking vacuoles can occur. As it is shown at Fig 22.A. the CaMV 35S promoter is preferentially active in these cell clusters, and in the globules arising from them (Fig. 22.B.). With the development of the somatic embryos, the expression pattern of the promoter is becoming more and more similar to that is seen in fully grown plants. The polarity in expression can be obderved at the stage of elongated globula (Fig. 22.C.), and the concentration of the activity in the procambium at the torpedo stage (Fig. 22.D and E). Differences in the activity of the promoter between the plumular and radicular parts seen in quantitative measurement were confirmed with histochemical staining (Fig. 22.F).

Langridge et al, 1998 demonstrated a decrease in the activity of the *mas* promoter during the transition from unorganised growth to morphogenesis. Our results are consistent with this observation and its validity can be extended to all three species we investigated.
Both quantitative (Figs 18., 20. and 21.) and histochemical (Fig 19.) analyses demonstrated the consistency of this trend.

5.3. EXPRESSION OF THE *UID A* GENE DURING ONTOGENESIS OF TRANSGENIC PLANTS.

An important question concerning the use of transgenic plants, but also of general scientific interest is the tissue- specific expression of a given gene in the plant body at different development stages. Here the use of gene fusions is one of the most powerful methods for precise quantitative analysis and tissue specific detection. We were interested in monitoring the changes in expression of the *uid A* gene during different ontogenic stages of the investigated plants.

Rbc S promoter was assayed only in tobacco and its pattern of expression was consistent with previous results (Nagy et al, 1985; Jefferson et al, 1987). Since tobacco was the source for the isolation of this promoter, its behavior can be analyzed in host plant, therefore, it was used as a control to compare the activity of the heterologous promoters. We were not able to produce alfalfa plants having significant level of expression of this promoter. The reason is still unclear.

During early stages of plant development, the CaMV 35S promoter has the highest activity in the roots of both tobacco and rapeseed. The difference is, that in cotyledons of rapeseed the activity is lower than in hypocotyl, while in tobacco the minimum activity was detected in hypocotyl. The cotyledons are a site of storage and processing of proteins, polysaccharides, and in the case of rapeseed- oils, needed as sources of energy and building materials during the development of seedlings. *Brassica* cotyledons are a much more massive source of metabolytes than tobacco's, with strong enzymatic activity. Experiments carried out with seedlings grown in the dark and at day- night cycle demonstrated that in spite the remarkable differences in morphology between the two groups of seedlings, lower expression in the root was the only difference in terms of CaMV 35S promoter activity. This surprising finding may be related to the lower growth rate of roots under these conditions, which can lead to a lower expression of this promoter. In a very unexpected way, the somatic embryos of alfalfa had lower activity of the CaMV 35S promoter in the roots, than in the upper parts. We still do not know what is the reason for this distribution.

Histochemical staining confirmed the quantitative results and demonstrated a distribution of the activity similar to that in tobacco zygotic embryos (see Fig. 22 and Benfey et al, 1990). Unfortunately the authors of the above mentioned article do not give quantitative data, so the comparison with our results would be a bit speculative. Based on them, we can only state, that the behaviour of CaMV 35S promoter in zygotic embryos of rapeseed and tobacco differ from that in somatic alfalfa embryos. Is the pattern in the later ones characteristic also for zygotic alfalfa embryos, or does it reflect differences between somatic and sexual embryos is still unclear. To analyze this problem requires to produce transgenic alfalfa zygotic embryos and to compare the transcriptional activities of the promoter.

In contrast to CaMV 35S promoter, *mas* promoter had high activity in the upper part of seedlings, somatic embryos and plantlets at cotyledon stage derived from them in all the three studied species. As it was reported earlier (Langridge et al, 1989), *mas* promoter is sensitive to hormones. Cultivation of *Brassica* seedlings in the dark resulted in etiolated plantlets. This fast growth is hormone- stimulated, the main regulator can be gibberellins. As already discussed in section 5.2 GA_3 has inhibiting effect on the activity of this promoter. Experiments with rapeseed seedlings grown in the dark confirmed these results and demonstrated the validity of the rule for *in vivo* systems. Interestingly Langridge and coworkers (1989) measured highest activity in the roots of seedlings, while Leung et al. (1991) reported lower expression of the *mas 1* promoter in the root. Since the former group used bacterial luciferase, requiring the use of both *mas* promoters, as reporter enzyme, this can explain the discrepancies. The use of simpler reporter systems can diminish the possibility of unwanted artifacts.

From anatomical point of view, a seedling is much simpler than a fully grown plant. The very few cell and tissue types present in an embryo or seedling evolve in intricate systems of tissues with defined and varied functions, which form organs and the entire plant body. These complicated arrays are an obstacle when expression of genes is studied, especially if the available techniques do not allow discrimination between tissues and cells in a given explant. The use of gene fusions is one of the most advanced methods for a fine mapping of gene expression in individual plants, organs and cells.

Unfortunately quite a few publications are dedicated to the study of fully grown plants. In the present work we made an attempt to investigate in more details the discrete repartition of promoter- directed gene activity in plants, both with quantitative and histochemical methods.

In rapeseed and tobacco, no drastic differences in the expression of both promoters could be seen during the growth of the plants. A gradient of expression towards the lower part and the hypocotyl is the most consistent trend. Results about expression in roots should be interpreted with caution, because the possible interactions of the plant with soil microorganisms. Contamination of the samples with species from the soil microflora, having ß- glucuronidase- like activities or modifying the substrate, can also produce misleading results. High expression of both promoters was observed in the fully grown leaves at the bottom, especially the petiols. After vernalisation of rapeseed, the expression of *mas* promoter in the plant axis and the leaves was lower than at the rosette stage. In our opinion the main cause is the production of gibberellins, which are the main substances controlling the bolting process. GA_3 was shown to inhibit the activity of *mas* promoter (Table 6.). An interesting observation was the low activity of this promoter in the stem of plants from the cultivar Arabella. Under the conditions of our greenhouse these plants produced well- developed burgeons at the leaf base. We do not know is the low activity of the promoter the result of inhibition by some substance, or the content of auxins in this cultivar is lower and that is the reason for the low expression.

We compared the tissue specificity of detectable ß- glucuronidase activity, directed by the CaMV 35S and *mas* promoters in mature tobacco and rapeseed plants. Whenever possible we also compared our results with these of other laboratories working in the same field. Interpretation of results from histochemical staining is not a straightforward and trivial matter, especially when tissues from mature plants are used. At the same time it is the only way to detect differences between cell types. In our view, results from histochemical and fluorimetric determination have to be used in order to have a more complete picture of the expression of foreign genes in plants. In contrast to the results of the fluorigenic measurements, the histochemical staining revealed rather complex and different patterns of detectable expression of the two promoters.

Jefferson and co- workers (1987) detected non- uniform activity of the CaMV 35S promoter in tobacco stem cross- sections, the activity being connected with protoxylem, phloem and cambium. In our experiments, protoxylem and closely related to it pith- parenchima cells stained positive, while cambium had very feint activity. In *Brassica* cambium and phloem and parenchima cells bound to it.Very high expression was found in collenchima cells.In lower parts of the plant, protoxylem in tobacco and phloem in rapeseed showed detectable activity. Histochemical staining of explants bearing the *uid A* gene under the control of *mas* promoter showed similar patterns of expression in *Brassica napus* and *Nicotiana tabacum* stems.

Interestingly, in lower parts of rapeseed plant activity was detected in protoxylem too. Our results, compared with works from other laboratories demonstrated certain common features in the action of the promoters: close connection of the activity with cells related to the vascular system, or having high metabolic or mitotic activity; uneven distribution in cells of the same type and differences in the tissue specificity amongst the two species. As it was already mentioned both promoters consist of blocks with different tissue and development specificity.

In contrast to tobacco and rapeseed, alfalfa has a system of stems, giving a bushy phenotype to the plant. Another important feature is the fast regeneration after removal of rather important parts of the stem, as it is during grassing by herbivores. The speed of this regeneration is depends largely on the available carbohydrates, stored in the trunk (upper part of the root- hypocotyl). After a vigorous regeneration and stem elongation, the growth rate is slowed down and the metabolytes are reoriented towards flowers or branches. The study of the expression of the chimeric genes during fast growth and at later stages demonstrated totally different patterns of expression of the CaMV 35S promoter. Highest activity was detected in the upper segments of the plant. In contrast, elder spruces had highest activity in the lower part, but this redistribution was caused by the lower activity in the top of the plant. At the node in the upper part of the plant the activity was much lower. Cross-sections of stems showed, that no redistribution of the activity occurred, rather the same types of cells expressed the gene at a lower level. These results suggest a linkage between cell proliferation and CaMV 35S promoter activity. This proposal can be in agreement with published results from Nagata et al. 1987.

6. SUMMARY

The aim of this work was to compare the expression of chimeric genes containing one plant promoter and two heterologous viral or bacterial promoters, in three different plant systems, having different habitues, morphology and different requirement for *in vitro* development, different *in vitro* morphogenesis patterns. We have compared the expression during different developmental stages and during *in vitro* morphogenesis and embryogenesis.

Binary plasmid vectors, containing the promoters fused to the *uid A* reporter gene were introduced into the genome of the plants and the transcriptional activity was monitored using both quantitative and histochemical methods. The process required the establishment of new transformation and regeneration protocols and modification of previously published ones.

Results presented here provide evidence for the non- constitutive and different expression pattern of two nominally constitutive promoters: the promoter of the 35S RNA transcript of CaMV and the *mas* promoter from plasmid of *A. tumefaciens*.

- CaMV 35S is not influenced by plant hormones, while *mas* is activated by auxin and up to some concentration by cytokinins. Abscisic acid and gibberellins lowered the activity of this promoter in a concentration- dependent manner.
- *In vitro* morphogenesis has a negative effect on *mas* promoter, while procedure of protoplast isolation and callus formation enhanced the expression of this promoter.
- Both promoters have distinctive patterns of expression during early seedling or embryogenesis stage of plant development.
- Studies on mature plants revealed a multicomponent expression pattern that can reflect the complexity of the plant body.
- Physiological state of a given explant influenced gene expression to a considerable extent.
- Both promoters were active in cells and tissues with characteristic metabolitic and mitotic activities. The expression of CaMV 35S promoter was correlated with cell proliferation in *planta*, but not in callus.
- Differences in tissue specificity of the studied promoters were recognized between species and in different plant parts.

They may reflect different affinity of *trans*- acting elements, binding to the different subdomains of promoters. Only the modular model for the structure of the promoters may explain these intricate patterns of expression.

The CaMV 35S promoter was found to be extremly active in meristematic centres that can provide the basis for somatic embryo development.

As a conclusion we would like to suggest, that gene fusions can be successfully used to study gene expression in relation to various questions of plant developmental biology, but a system for control and safeguarding against artifacts should be ingeneered for every different purposes.

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8. APPENDIXES

8.1. MEDIA AND ANTIBIOTICS.

LB medium (Maniatis et al. 1982)

Bacto triptone	10 g/l
Bacto yeast extract	5 g/l
NaCl	10 g/l
Bacto Agar	1.6- 2 %
pH 7.0 - 7.2	

ABM medium, modified from G. An (1987)

20 X AB salts:	
NH4Cl	20 g/l
MgSO4.7H2O	6 g/l
KCl	3 g/l
$CaCl_2$	0.2 g/l
$FeSO_4$	50 mg/l
20 X AB buffer:	
K_2HPO_4	60 g/l
NaH_2PO_4	23 g/l
pH 7.0	

Mannitol solution: 10 g/l mannitol

1.8- 2 % bacto agar

When liquid ABM madium is used, 0.1 % bacto yeast extract must be added to supplement it with trace elements.

The three solutions are autoclaved separately and when cooled to 40- 45 oC, mixed in the appropriate ratio.

MGL medium (An, 1987):

Bacto triptone	5 g/l
NaCl	5 g/l
Mannitol	5 g/l
Yeast extract	2.5 g/l
Monosodium glutamate	1.16 g/l
KH2PO4	0.25 g.l
MgSO4.7H2O	0.1 g/l
Biotin	1 mg/l
pH 7.0	

Lactose medium (Bernaerts and De Ley, 1963):

Lactose	10 g/l
Yeast extract	1 g/l
Bacto agar	1.8- 2 %
pH 7.0	

Table 14. shows the antibiotics used for selection of transformed bacteria and plants and to kill the bacteria after plant transformation

Name	Abbreviation	Concentration of stock solution	Final concentration	
			Microbiological medium	Plant medium
Carbenicillin	Cb	200 mgl/H2O	100 ul	200, 300 ul
Claforan	Cf	100 mgl/H2O	-	200 ul
Kanamycin	Km	100 mgl/H2O	25-50 ul	100 ul
Tetracyclin	Tc	10 mgl/ETOH	5-10 ul	-
Rifampycin	Rf	10 mgl/MeOH	20 ul	-

8.2. EXPERIMENTAL PROTOCOLS

8.2.1. NPTII TEST OF PLANT LYSATES (DOTS)

(Mc Donell et al. 1987. Pl. Mol. Biol. Reporter.3/4. pp380-386.)

SOLUTIONS:

Extraction buffer 2X:

Glycerol	2 ml
0.5M Tris pH 6.8	2.5 ml
β-mercaptoEtOH	1 ml
SDS 10%0.	2 ml
H_2O	4.3 ml

Reaction buffer 5X:

Tris	2.02 gr
$MgCl_2$	2.14 gr
NH_4Cl	5.35 gr
H_2O	up to 30ml

Titrate to pH 7.1 with 1M Maleic acid (pure cristaline) to pH 7.1 (about 10-20 ml). Raise the volume to 50 ml and check again the pH.

Assay mixture:

Reaction buffer 1X	0.98 ml
ATP 10 mM stock	1 µl
ATP 10 µCi/µl stock	0.5 µl
Kanamycin 22 mM stock	3 µl
NaF or KF 1M stock	10 µl

Soaking solution:

ATP	20 mM
Pyrophosphate	100 mM

PROCEDURE:

1. Grind 200-400 ug tissue in 500 ul Extraction buffer on iceif possible in the cold room. Use a small glass pestle fitting in an eppendorf tube.
2. Spin in an eppendorf centrifuge for 5 min. in the cold room.

3. Transfer into a new tube and keep on ice. Run a Bradford assay to know the total protein content. It is good to have 20-30 ug per reaction. If you have a small sample number you can filter ther them thru a glass paper filter. It will retain some impurities.
4. Mix in an eppendorf tube 15 ul sample extract and 15 ul Assay mixture. Incubate 30 min. at 37oC. Spin again for 5 min in the cold room.
5. Meantime prepare a piece of the appropriate size of Watman P81 (cellulose phosphate) paper. The distance between the spot targets should be about 2 cm. Deep it in soaking solution and dry to lower the background.
6. Centrifuge the tubes for 5 min. A pellet will form.
7. Spot 20 ul of each sample at the targets (marked with apencil) on the paper. Try to have smaller spots - pipeting at small portions.
8. After the spots have dried wash the paper in 10 mM phosphate buffer pH 7.5 at 80oC for 2 min.
9. Wash 3-4 times at room temperature.
10. Expose to a X-ray film for overnight or longer.

8.2.2. NPTII TEST OF PLANT LYSATES (ELFO)

F. Nagy et al. 1988. Plant Mol. Biol. Manual, B4. See also A. Reinaerts et al. 1988. idem A9. Modified.

SOLUTIONS:

Solutions for preparation of nondenaturing polyacrilamide gel- 10% according one of the published receipes. The receipt below is working well in our laboratory.

STOCK SOLUTIONS:

Monomer solution (Acrylamide/bis- acrylamide 30: 0.8)

Acrylamide	30 g
Bis- acrylamide	0. 8 g

Deyonised water up to 100 ml.

Filther through a 45 μm filter.

Ammonium persulfate (APS):

Dissolve 0.15 g APS in 10 ml H2O just before the experiment.

TEMED is used as sullpied. Store in a dark bottle in the refrigerator.

1 M Tris solutions with the pH adjusted.

A. Bottom (separation) gel

Monomer solution	10 ml
1 M Tris, pH 8.7	11.2 ml
H_2O	6.3 ml
1.5 % APS	1.5 ml
TEMED	15 µl
Total vol. 30 ml.	

B. Upper (stacking) gel

Monomer solution	1.67
1 M Tris, pH 6.8	1.25 ml
H_2O	6.6 ml
1.5 % APS	0.5 ml
TEMED	5 l

Running buffer:

Tris	3.03 gr
Glicin	14.41gr
H_2O	to 1 l

The pH should be 8.4 to 8.6.

Homogenisation buffer:

Tris-HCl	50 mM
β-merkaptoEtOH	5 %
Glycerol	10 %
SDS	0.1%

Assay buffer: (per liter)

Tris	8 gr
$MgCl_2.2H_2O$	4 gr
or $MgCl_2$.6H2	13 gr
NH_4Cl	21.4gr
H_2O	to 1 l

adjust the pH to 7.1 with 3-4 gr Maleic acid.

Assay buffer 2X :
The same as above, but dissolved in 0.5 l.

Agarose gel for the NPTII reaction:

Prepare separately:

A.	2X Assay buffer	2o ml
	Kanamycine 20 mg/ml	40 μl
	*ATP 10 μCi/μl	20 μl
B.	2% agarose in H2 boiled	20 ml
	Washing solution	2 l 10 mM Phosphate buffer pH 7.5

PROCEDURE:

1. Prepare the gel before starting the assay itself.
2. Grind 100 ug material in 100 ul Homogenisation buffer in an eppendorf tube on ice.
3. Spin at 4oC for 10 min. and transfer to a new tube. Run a Bradford assay.
4. Load 100 ug protein from each sample on the gel and run at 100 V, 4oC overnight. Load a positive and negative controll and a 0.005% Bromphenolblue marker (in Homogenisation buffer). The run is sufficient when the marker is near the lower edge of the gel.
5. Remove carefully the gel, wash twice in destiled water for 10 min. and equilibrate in Assay buffer for 30 min.
6. Place it on a convenient support (e.g. ELISA- plate cover, Finntip- box cover or other plastic disposable tray. Even a glass plate will do the job, but be carefull with the washing after the end of the experiment).
7. Mix the components of the Assay agarose gel (A. and B. and pour on the AA gel. Incubate 45 min. at room temperature.
8. Cut a sheet of Watman P 81 phosphocellulose paper to be slightly larger than the gel and overlay on it. Place two sheets of Watman 3 MM paper and a stack of paper towels. Blot it with a 0.5 kg. weight for 2-4 h. Longer incubation will increase the background.
9. Wash the P81 paper two times at 80oC for 15 min.
10. Dry tyhe paper at room temperature and expose to a X-ray film from 1 h. to several days.

8.2.3. FLUOROGENIC ASSAY FOR B- GLUCURONIDASE

(R.A. Jefferson, (1987), Plant Mol. Biol. Reporter, V.5 No 4, pp 387- 405.) with modifications.

BUFFERS AND SOLUTIONS:

GUS Extraction Buffer:

50 mM	$NaPO_4$, pH7.0
10 mM	dithiothreitol (DTT)
1 mM	Na_2EDTA
0.1 %	Sodium Lauryl Sarcosine
0.1 %	Trion X- 100

GUS assay buffer:

1 mM MUG in Extraction Buffer.

Dissolve 22 mg MUG in 50 ml extraction buffer. Store at +4 ^oC for several days (up to 10).

Stop Solution:

2 M	Na_2CO_3.

Stock Solution for Calibration of the Fluorimeter.

1 mM	Methylumbelliferone.

Dissolve 19.8 mg Na^+ methyl- umbelliferone in 100 ml double distiled water. Store in the dark at +4 oC.

Bradford Solution for Protein Determination. Use a BIO- RAD Protein Determination Kit for fast and reproducible results.

STEPS IN THE PROCEDURE:

This is an inexpensive, accurate and easy modification of the basic protocol, which is especially useful when dealing with numerous samples. It is recommended to keep the samples cool (0- 4 ^oC) during the handling.

1. Take a sample from the tissue to be analysed (100 mg are more than enough) and place it into an eppendorf tube. It is useful to know the fresh weight of the sample. Add 200 l Extraction Buffer.

2. Grind the tissue with a small glass pestle, fitting in the eppendorf tube. Dilute with 200 ul of the same buffer.
3. Spin the samples for 3- 5 min in a cooled eppendorf centrifuge and carefully take 100 l from the clear phase. Store the remaining at -70 $_0$C for back-up. Divide the sampled quantity in three wells of a microtiter plate and in a test tube with 1 ml Bradford Reagent (25 l each). If more samples are to be assayed put the samples in three rows corresponding to different incubation times.
4. Put 100 l Stop Solution in each well of the first row. This is time 0 min. Prewarm the samples and the Assay Buffer in a 37 $_0$C thermostate. Cover the samples to avoid evaporation.
5. Pipette 75 l Assay Buffer in each well. Work with a steady tempo, it is important for the reproducibility of the results. Do not allow the samples or the Assay Buffer to cool.
6. After a defined period of time (usually 30 min or 1 hour) Pipette 100 l of stop solution in the second row and continue the incubation. Be careful to respect the tempo.
7. After 30 more minutes or 1 hour add 100 l stop solution to the third row and thus stop the assay.
8. Calibrate the fluorimeter with 1 M and 100 nM MU in 10 times diluted stop solution (0.2 M Na_2C0_3). The exitation wavelength is 365 nm, the emission 455 nm.
9. Transfer the stopped samples to a fluorometric cuvette and fill it up to 1 ml. Read the relative fluorescence of the time course for each extract and determine the concentration of MU. Presumably it is proportional to the quantity of enzyme in the extract and reflects the transcriptional activity of the promoter. Now you have three time points for each reaction and are able to make a rough kinetic analysis.
10. Determine the protein concentration in the lysate using the sample in the Bradford Reagent. Convert the values to nanomoles MU produced per minute per milligram protein. Other denominators as nmoles MU/ min/ per fresh weight, per g DNA, per protoplast and so on can be used as well.

8.2.4. HISTOCHEMICAL ASSAYS FOR B- GLUCURONIDASE

(R.A. Jefferson, (1987). Plant Mol. Biol. Reporter, V 5, No 4, pp 387- 405; I.A. Castle & R.O. Morris, 1990, Plant Mol. Biol. Reporter, V 8, No 1, pp 28- 39.) with modifications.

BUFFERS AND SOLUTIONS:

0.1 M Phosphate Buffer, pH 7.0.

X- Gluc buffer:

0.1 M	Phosphate Buffer
10 mM	Na_2EDTA
5 mM	$K_3[Fe(CN_6)]$
5 mM	$K_3[Fe(CN_6)]$

Store at 4 ^{o}C in the dark. Dissolve 5 mg X- Gluc in 100 l dimethyl formamide. Add it to 10 ml buffer. Use within 2- 3 weeks.

FIXING Solution:

0.1 M	Phosphate Buffer
0.3 %	formaldehyde
0.3 M	mannitol
70 % Ethanol.	

EXPERIMENTAL PROTOCOLS

8.2.4.1. STAINING OF PLANTLETS:

Immerse the plantlets in X- Gluc buffer in a suitable recipient (microtiter plate, small petri dish, cell culture plate etc.). Incubate at room temperature to avoid unspecific heat- shock induction of some promoters.

8.2.4.2. STAINING OF STEM SEGMENTS:

1. Cut 0.1- 0.5 mm tick segments by hand, using a hand- held microtome or a 'Vibratome'. Work fast to avoid the drying of the samples. Deep the samples in ice- cold fixing solution for 10- 15 min. The fixing step is required to avoid wound- induction of the promoter. If only a 'yes- no' answer is needed it is better to ommit this step, because it is lowering the expression. In that case fix the material *after* staining.
2. Wash several times with ice- cold phosphate buffer and place in X- Gluc buffer. Incubate at 37 ^{o}C.
3. Wash again with phosphate buffer.
4. Clear in 70 % EtOH.

8.2.4.3. STAINING OF PROTOPLASTS:

1. Make several dilutions of the protoplast sample to be analysed. Adjust the volume to 2- 3 ml with osmoticum. For transient assay with a strong promoter (e.g. CaMV 35S or MAS promoters) 100, 500, 1000 protoplasts are usually sufficient.
2. Immobilise the protoplasts on a 24 mm cellulose filter.
3. Place the filter on a 24 mm Whatman GF/C glass filter imbibed with 0.2- 0.3 ml X- Gluc buffer with 0.3 M mannitol. Incubate at 37 ^{o}C. Take care not to dry the samples.
4. Fix the material by placing the filter on a GF/C with fixing solution

8.2.5. DETECTION OF ß- GLUCURONIDASE ACTIVITY AFTER PAGE SEPARATION OF PROTEINS

(B. Dewald, J. T. Dulaney and O Touster, (1973) Methods of Enzymology, vol ,pp 82- 102.; I. Nagy, personal comunication) With modifications.

The solutions and buffers are already decribed in APPENDICES 7.2.2. and 7.2.3.

PROCEDURE:

1. Prepare a 7.5 % acrylamide gel
2. Make protein extraction of the samples and determine the protein concentration. Bring them to equal protein concentration with extraction buffer and add half avolume of glycerol with 0.05 % Bromphenol blue.
3. Run PAGE at 100 V for 12 h. at 4o C.
4. Wash the gel 3- 4 times in cold GUS extraction buffer.
5. Incubate in assay buffer for 30 min at 0- 4o C.
6. Dry the gel between filter papers and incubate at 37o C for 10- 60 min (control the reaction kinetics with a UV- light transiluminator).
7. Deep the gel in 0.2 M Na2CO3. Examine and photograph it under UV light.

8.2.6. PLASMID MINIPREP

SOLUTIONS

Lysis solution I:

glucose	50 mM
EDTA	10 mM
Tris, pH 8	25 mM

Lysis solution II:

SDS	1%
NaOH	0.2 N

Sodium acetate solution, pH 4.8, 3 M

Ethanol (EtOH), absolute and 70 % with water..

TE buffer	
Tris- HCL, pH 8,	10 mM
EDTA	1 mM

STEPS IN THE PROCEDURE

1. Transfer 1.5 ml of a dance overnight culture into an eppendorf tube. Spin for 30 sec in an eppendorf centrifuge.
2. Discard the supernatant and resuspend the cells in 0.1 ml ice- cold lysis solution I. Incubate for 10 min at room temperature.
3. Add 0.2 ml freshly prepared lysis solution II, tapp gently with a finger to mix and incubate for 10 min on ice.
4. Add 150 l sod. acet. sol. and vortex for 2 min at maximum speed. Incubate on ice for 5 min.
5. Spin for 2 min to pellet cell debris and chromosomal DNA.
6. Transfer supernatant to a new tube and mix with 0.9 ml absolute EtOH. Store at -20o C for 10- 15min.
7. Spin for 10 min in a cooled centrifuge. Discard the supernatant.
8. Wash twice with 1 ml of ice- cold 70 % Et OH.
9. Discard **all** the supernatant solution and resuspend the pellet in TE buffer. The DNA is ready for restriction enzyme analysis.

8.2.7. *AGROBACTERIUM* QUICK SCREEN MINIPREP

SOLUTIONS

Lysis solution I:

glucose	50 mM
EDTA	10 mM
Tris, pH 8	25 mM
lysozyme (add fresh)	4 mg/ml

Lysis solution II:

SDS	1 %
NaOH	0.2 N

Sodium acetate solution I, pH 4.8, 3 M

Sodium acetate solution II,pH 7, 0.3 M

Phenol, pure, freshly destilated or white cristals.

EtOH absolute and 70 % with water..

STEPS IN THE PROCEDURE

1. Transfer 1.5 ml of a dance overnight culture into an eppendorf tube. Spin for 30 sec in an eppendorf centrifuge.
2. Discard the supernatant and resuspend the cells in 0.1 ml ice- cold lysis solution I. Incubate for 10 min at room temperature.
3. Add 0.2 ml freshly prepared lysis solution II, vortex gently to mix and incubate for 10 min at room temperature.
4. Add 50 l phenol equilibrated with two volumes lys. sol. II and vortex. It will become very viscous.
5. Add 150 l of sod. acet.sol I and shake well to mix. Incubate at -20 o C for 15 min.
6. Spin the tube for 3 min . Quickly pour the supernatant into a new eppendorf tube. Fill it with ice- cold absolute ethanol and incubate at -80o C for 15 min. Alternatively store at -20o C for two or more hours.
7. Spin for 10 min. in the cold. Discard the supernatant.
8. Add 0.5 ml of ice- cold sod. acet. sol. II and dissolve the pellet. Fill the tube with ice- cold absolute EtOH. Repeat the freesin gprocedure of 6.
9. Repeat step 7. Drain away as much liquid as you can.
10. Wash twice with 1 ml of ice- cold 70 % Et OH.

11. Discard **all** the supernatant solution and resuspend the pellet in TE buffer. The DNA is ready for restriction enzyme analysis.

8.2.8. PLANT DNA ISOLATION

SOLUTIONS:

Extraction buffer

CsCl	0.5 M
EDTA	0.1 M
Tris pH 8	0.1 M
β-merkaptoEtOH	5 mM

Sarkosyl solution 10 %

"Juice" CsCl/H2O in a -1/1 ratio

Destaining solution
i-propanol saturated with water saturated CsCl

Etidium bromide stock 10mg/ml

PROCEDURE:

1. Freese young plant material in liquid nitrogen. Grind 1-3 gr in a mortar with dry ice and quarts sand (the later is required for a more complete disruption of the cells) until you obtain a very fine powder.
2. Transfer to a cold blue-capped tube and add Extraction buffer. For the first 1-2 gr add 8ml, for every additonal gr- 4 ml buffer. Mix very carefully on ice. Add 1/10 volume of Sarkosyl solution and mix again. Let on ice for 1 hour with gentle shaking.
3. Transfer to a 35 ml centrifuge tube and spin at 10-12 Krpm for 20 min.
4. Pour the supernatant into a blue-capped tube and add 0.9gr CsCl for every ml extract. It is importent to have the DNA band lower in the tube after centrifugation.
5. Transfer to a UC tube and add Et Br to a final concentration of 0.6 mg/ml. Spin at 60 Krpm for 20 h.
6. Repeat once more the UC.
7. Extract the EtBr with saturated i-propanol, dilute four times with water and precipitate. The DNA is ready for use.