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Biotechnology in Plant Production I

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1 INTRODUCTION TO THE STUDY OF BIOTECHNOLOGY IN PLANT PRODUCTION

The aim of this subject is the study and application of actual technological possibilities (biotechnological methods, procedures and techniques) aimed at the rationalization and intensification of plant production – production of economically important products.

Plant biotechnologies make it possible to address the following priority goals in a targeted manner:

1. Targeted creation of new plant genotypes using modern methods of cell and molecular breeding (tissue culture techniques and genetic engineering) and genetic engineering of plants is studied in *Biotechnology in Plant Production I*:
Creation of genotypes with pre-programmed characteristics:
a) Production performance (harvest)
b) Production quality (nutritional, technological)
c) Plant resistance against adverse biotic and abiotic environmental factors
d) Minimization of material and energy inputs during crop cultivation and plant cultivation
e) Creation of genetically modified plants (GMP) for special biotechnological applications (production of special bioproducts usable in the food, pharmaceutical and energy industries)

2. Development and application of predictive methods for the detection and identification of molecular markers (at the level of DNA and proteins) using the genetic markers of plants is studied in *Biotechnology in Plant Production II*:
a) Diagnosis of genes, metabolites and storage substances determining biologically and economically significant properties of plant products
b) Nutritional quality (content and fractional composition of proteins, AA – composition, digestibility, presence of antinutritional substances: antimetabolites, toxins, allergens, etc.)
c) Technological quality (content and fractional composition of stock proteins)
d) Other properties

Definition of the term BIOTECHNOLOGY
BIOTECHNOLOGY: BIOS Þ alive, TECHNOLOGY Þ work process
a) In narrower sense:
Integrated use of biochemistry, microbiology and engineering to ensure the technological application capabilities of microorganisms, tissue culture cells and their components.
b) In broader sense, which best expresses biotechnology in plant production:
Technologies that use the metabolic and biosynthetic capabilities of plants or their components to produce or modify products, breeding plants for their specific use.

Biotechnologies are classified into five basic categories by colour based on their purpose or impact:
1. **Red biotechnology** – biotechnologies applied in medicine, such as the production of vaccines, antibiotics, molecular diagnostic methods, genetic manipulations, gene therapies, pharmacogenomics and others.
2. **White biotechnology** – includes the applications of biotechnology related to industry, such as the use of microorganisms for the production of chemical substances, construction and production of new materials, such as plastics and textiles, and production of new sustainable energy sources, such as biofuels and biogas.

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3. **Gray biotechnology** – includes biotechnologies that are directly related to the environment. They are mainly used in two industries: contaminant removal and conservation of biodiversity.
4. **Green biotechnology** – focuses on agriculture as a means to produce food and feed. It also includes the creation of new economically important plant species, production of biofertilizers, biopesticides, plant cloning and in vitro cultivation. It also includes the production of genetically modified plants resistant to herbicides, pests, diseases and the production of genetically modified plants with improved nutritional properties as well as plants that can be used as biological factories capable of producing various substances of medical and industrial interest, while these substances can be easily isolated, purified and produced in large enough quantities.
5**. Blue biotechnology** – uses marine resources to produce the products and materials for industrial use. Hydrocolloids and gels are used in food production, health protection and treatment, diagnosis of diseases, and also in research, e.g. the use of marker molecules originating from marine organisms.

Some dedicated biotechnology applications are marked with additional colours, such as gold (bioinformatics, nanotechnology), yellow (food biotechnology, nutrition), brown (arid region and desert biotechnology) or black (bioterrorism, biological warfare, biological warfare against crops, etc.).

Plant biotechnology currently includes 3 main areas:
Tissue cultures of plants – techniques to grow a whole plant in laboratory conditions from a minimum amount of plant parts (root, stem, leaf...), and even from a single plant cell.
- Advantages – rapid production of homogeneous plant material.

Genetic engineering of plants – selective transfer of an important and useful gene from one organism to another in order to create improved plants.
- Advantages – possible gene transfer between the species and different organisms.

Breeding supported by plant molecular markers – a technique that uses molecular markers to select economically important genes.
- Advantages – speeding up the plant breeding process with the required properties.

History of Genetically Modified Plants:
1983 Preparation of the first genetically modified plant (petunia) using the Ti plasmid
1985 First transgenic plants resistant to insects, viruses and bacteria grown in field tests
1986 Approval of the release of first transgenic plant (in the USA), discovery of PCR reaction
1994 Preparation of genetically modified tomato (FlavrSavr), development of agrobiotechnologies
1996 Beginning of commercial cultivation of genetically modified plants
1997 Sequencing of the *E.coli* genome, cloning of Dolly the Sheep
2000 Sequencing (first plant genome) of the *Arabidopsis thaliana* genome
2000 Preparation of genetically modified Golden Rice 1 (Golden Rice)
2002 Sequencing of the rice genome
2006 Beginning of commercial cultivation of GMP plants in SR
2009 Global cultivation of GMP on an area of 134 million ha
2010 Planting of genetically modified potatoes "Amflora" (amylopectin potato) in Germany and the Czech Republic, planting of GM corn in Slovakia on an area of 1500 ha
2019 Worldwide cultivation of GMP in 29 countries on an area of 190.4 million ha
2019 The US FDA concluded the consultations on the first food from a plant modified by a new method of genome editing (CRISP/Cas)

2 GENETIC TRANSFORMATIONS OF PLANTS

Genetically modified plants (GM plants) are plants whose genetic information has been altered by genetic engineering methods. The essential prerequisite for the production of genetically modified organisms is knowledge of the structure and function of DNA, which is the basis of heredity. Since the discovery of DNA, it has taken 20 years for scientists to find out how genetic information is realised in the model bacterium *Escherichia coli* - *via* the central dogma of molecular biology: DNA – RNA – protein. Subsequently, methods and techniques for the targeted modification of DNA were developed. The basic principle of GM plant preparation is that the transferred gene is isolated from the donor DNA and transferred into the host plant with the help of a vector.

The process of genetic transformation of plants, also called transgenesis, consists of two basic steps:

- transfer and integration of the transferred genes into the plant DNA
- a plant regeneration process based on the totipotency of the cells (regeneration of the transformed cells into intact plants)

Advantages of plant transgenesis:

- the transfer of genes from organisms (e.g. bacteria) that do not usually occur in nature
- the transfer of genes even from distant plant species, which would not be possible through conventional breeding
- a more rapid change in the quality parameters of plants than is possible with conventional breeding – the transfer of specific traits

Disadvantages associated with transgenesis:

- problems with stable expression of the introduced genes in the progeny of the transgenic plants
- public concern about the possible transfer of antibiotic resistance genes to bacteria living in the soil or in the digestive tract of animals and humans and their impact on the environment

The transfer of foreign DNA into the plant genome is carried out by **direct** methods based on the physical or chemical properties of the plant cells and by **indirect** (biological) methods based on the interaction between the bacterium and the plant cell.

Of the direct methods, several procedures have been tested more or less successfully under laboratory conditions. However, the most commonly used indirect methods are protoplast transformation and biolistic transformation. Indirect methods use the mechanism by which the soil bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* infect plant cells in nature.

The success of the transformation depends not only on the ability to integrate foreign DNA into the plant genome but also on the ability of the transformed cell to regenerate under in vitro conditions. Regeneration is based on totipotency, i.e. the ability of the plant cell to regenerate into a whole plant. It is achieved by regeneration on sterile culture media in the presence of the phytohormones cytokinins and auxins. A combination of these substances leads to the control of regeneration. However, not every plant species can regenerate well under *in vitro* conditions. It is then problematic to obtain transgenic plants from such plant species.

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2.1 Direct gene transfer methods

2.1.1 Transformation of protoplasts

The method is based on the property of protoplasts (cells with entirely or partially removed cell walls mechanically or enzymatically), which eliminates the problem of overcoming the natural barrier – the cell wall. DNA integration takes place

- chemically with polyethylene glycol or $CaCl_2$
- using electrical impulses (electroporation)
- microinjection (incorporation of DNA into the cell nucleus using a special syringe)

2.1.2 Biolistic transformation

The protoplast transformation method is based on removing all or part of the protoplast cell wall mechanically or enzymatically, eliminating the problem of overcoming a solid cell wall. DNA transfer is performed chemically with polyethylene glycol or $CaCl_2$, by electroporation with electrical pulses, or by direct microinjection into the nucleus.

Biolistic transformation or bombardment is the most commonly used direct method. It is preferably used to transform monocotyledonous plants that are intact for infection by *A. tumefaciens*. The advantage is that the method is applicable to many plant tissues, such as embryogenic cultures, callus, leaf explants, microspores, immature pollen grains, *etc*. The biolistic transformation consists of the following steps:

1. preparation of microprojectiles
2. transfer of DNA into a plant cell by bombardment with microprojectiles
3. integration of DNA into the plant genome
4. regeneration of transformed cells under *in vitro* conditions.

In the first step, the microprojectiles consist of metal particles coated with the transferred DNA. The metal used is usually gold particles with a size of about 0.6 μm to 1 μm, but palladium, rhodium, platinum, tungsten or iridium can also be used. The expression cassette transferred into the plant genome (Figure 2.1) can be part of a standard cloning vector or in linearised form. Biolistic transformation enables the production of transgenic plants for commercial use, as other (unintended) DNA sequences in the transgenic plants may not be acceptable to the public.

The actual transfer of the foreign DNA is carried out with the help of a so-called **gene gun** or even **a special biolistic device** (Figure 2.2), which contains a compressed helium gas that gives the microarrays a rapid acceleration (Figure 2.2). This allows the microprojectiles to break through the cell wall and enter the cell. Subsequently, some DNA is released from the metal particles, enters the nucleus and is integrated into the chromosome (Figure 2.3). In many cases, the microprojectiles deliver the target genes into the cell where they can be transiently expressed ("transient expression"), but plant nucleases gradually degrade them. Transient expression is used to rapidly monitor the efficiency of DNA transfer or the functional expression of the transferred gene.

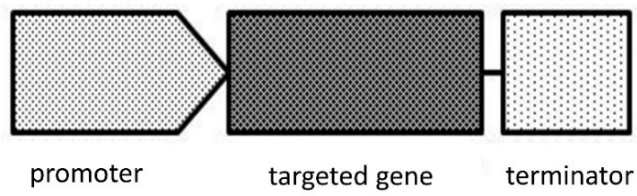

Figure 2.1 Expression cassette consists of a plant promoter, which controls the expression of the introduced gene, and a terminator. The promoter can be constitutive, inducible, or tissue-specific.

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Figure 2.2 Helios® Gene Gun System (BioRad) and PDS-100/HeTM biolistic system (BioRad) (source: http://www.bio-rad.com/en-cn/product/helios-gene-gun-system; http://www.tavernarakislab.gr/extimages/he1_l.jpg)

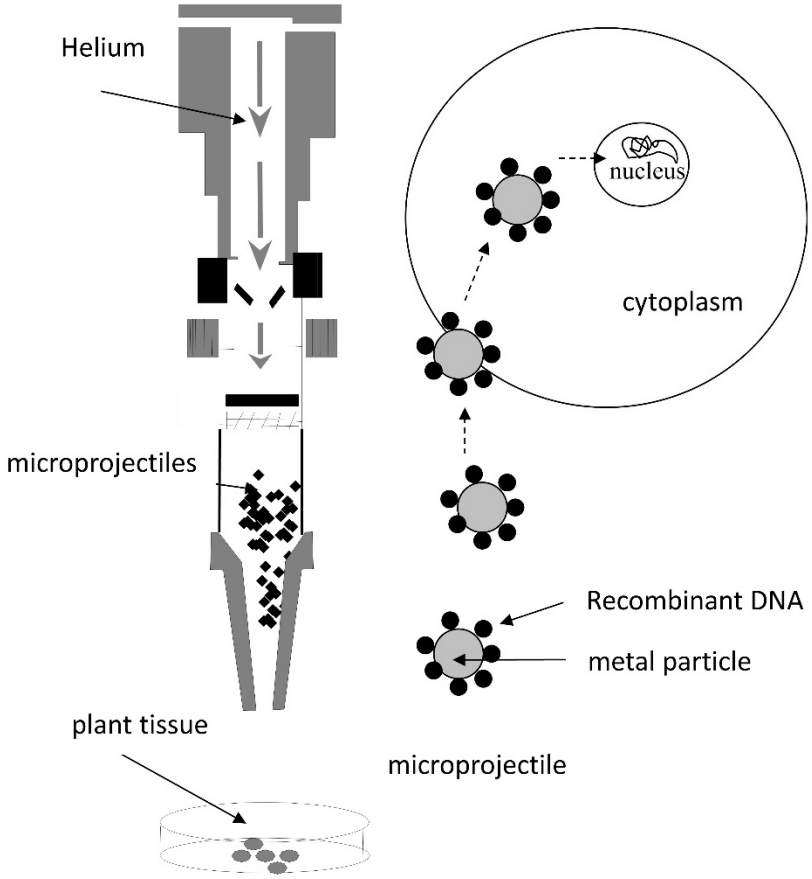

Figure 2.3 Biolistic transformation (bombardment) of plants

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2.2 Indirect methods of gene transfer

The indirect method is also called the biological method because it uses the property of the soil pathogen *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* to attack wounded parts of dicotyledonous plants. During infection, *Agrobacterium* transfers its own DNA (called T-DNA) into the plant chromosome and stably incorporates it. The advantages of this method are its simplicity, low cost and high efficiency. The disadvantage is host specificity, as *Agrobacterium* preferentially attacks dicotyledonous plants in nature. Transformation with *A. tumefaciens* is one of the most commonly used methods for producing transgenic plants.

2.2.1 Agrobacterium tumefaciens

Bacteria of the genus *Agrobacterium* are gram-negative soil bacteria that live mainly saprophytically. However, only a few of these strains can grow parasitically on plants and cause hairy root disease (*A. rhizogenes*) or crown gall disease (*A. tumefaciens, A. rubi,* and *A. vitis*). Both diseases are characterised by neoplastic growth at the site of infection, which results from the cells' genetic modification.

In nature, the injured plant cell is a port of entry for infection. For protection, the plant accumulates **phenolic substances** in the subepidermal layers of these cells. Some of these substances, such as **acetosyringone** (AS), are essential for *A. tumefaciens*. AS induces bacterial virulence genes that play a key role in infection. The presence of AS is detected by a two-component receptor system consisting of the ***vir*A** and ***vir*G** genes, which are expressed at low levels under normal conditions. In the presence of an inducer, their expression increases sharply, and other *vir* genes are activated. The attraction of agrobacteria to injured plant cells consists of two steps:

1. **attraction** of agrobacteria to injured plant cells by bacterial and plant extracellular molecules, which have not yet been studied in detail
2. **adhesion** of agrobacteria to plant cells is promoted by the presence of bacterial cyclic 1,2-β-D-glucans, which form **a biofilm**. Their synthesis is ensured by the expression of the bacterial *chv*A, *chvB* and *exo*C genes.

Agrobacterium never enters the plant cell; however, for tumour growth, it must ensure the expression of oncogenes in the plant host cell by transferring a specific segment of its DNA, called T-DNA ("transferred" DNA). **The T-DNA** region is located on the Ti plasmid ("tumour-inducing plasmid") (Figure 2.4).

There are two groups of genes in the **T-DNA region**:

1. genes involved in the **production of auxins** (*iaa*M, *iaa*H) and **cytokinins** (*ipt*Z) cause tumour formation.

2. the gene encoding octopine synthetase, an enzyme involved in the synthesis of opines. Opines are a source of carbon and energy and create the conditions for bacterial reproduction. The bacterial genes located in the T-DNA region are controlled by eukaryotic promoters allowing them to function in the plant cell.

Activating the **VirA/VirG** two-component system also activates the *vir*D1 and *vir*D2 genes. The **VirD1** and **VirD2** proteins recognise 25 bp long border sequences, referred to as LB ("left border") and RB ("right border"), respectively (Figure 2.5). At this point, the lower T-DNA strand is truncated from both sides. Once the T-DNA strand is excised, a **T-complex** is formed consisting of a **VirE2** protein (which envelops the strand and protects it from damage during transport) and a **VirD2** protein that remains covalently bound to the 5' end of the released strand, giving the resulting T-DNA complex a polar character.

Transfer of the T-complex into the plant cell occurs via the bacterial secretion system T4SS, consisting of **11 VirB** proteins and the **VirD4** protein. The estimated size of the T-DNA complex is about 50,000 kDa, far exceeding the size of the nuclear pore (60 kDa). This means

that the transfer of the T-complex into the nucleus requires active transport. Agrobacterium uses the plant **ViP1** protein for this purpose.

ViP1, normally located in the cytoplasm, is directed into the nucleus after phosphorylation by the MPK3 kinase, where it activates the expression of defence proteins. The T-complex uses the phosphorylated plant ViP1 protein for active transport into the nucleus. Once the T-complex has been transported into the nucleus, the ViP1 protein is degraded by the bacterial protein **VirF**, which is part of the T-complex. To integrate the T-DNA into the plant genome, *Agrobacterium* exploits a plant mechanism by which the plant cell repairs its damaged DNA, called DNA double-strand break (DSB) repair. DSB repair can be performed either by **homologous recombination** (HR) or by **non-homologous end joining** (NHEJ), but HR requires some sequence homology with T-DNA for integration to be efficient. It is more likely that NHEJ is used for T-DNA integration because end joining is achieved by re-ligation of gaps.

Moreover, the plant cell uses NHEJ mainly to repair damaged DNA ends. Before integration, the T-DNA strand is freed from the protein complex that accompanied and protected the strand during its transport. The T-DNA enters the nucleus as a single strand. Whether synthesising the second strand occurs before integration or during splicing is not entirely clear. It is possible that it is a multi-step process.

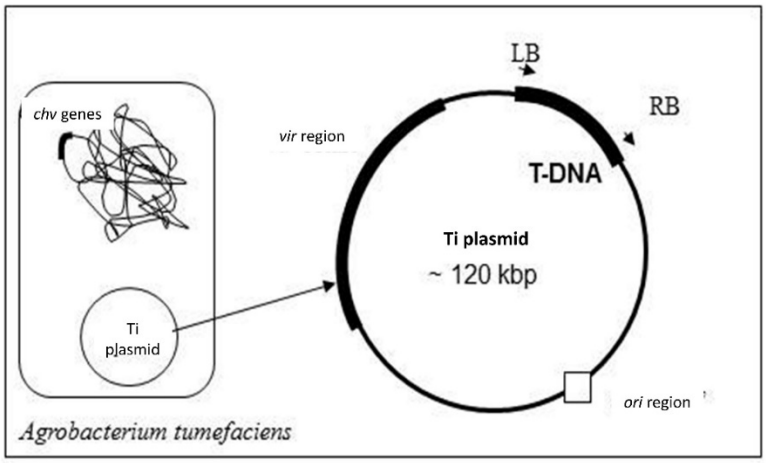

Figure 2.4 *Agrobacterium tumefaciens*

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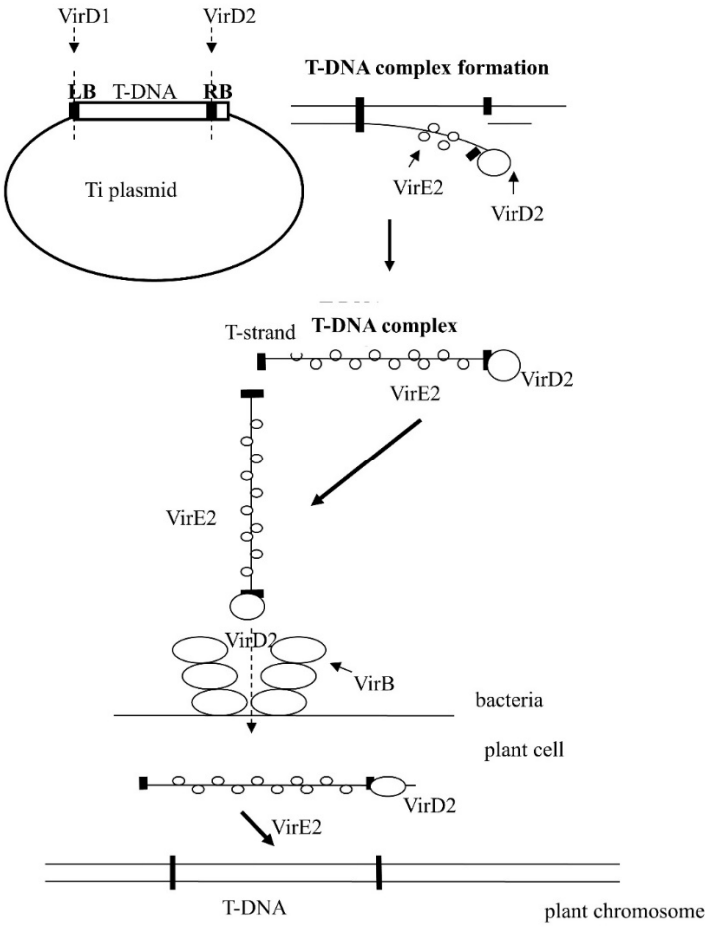

Figure 2.5 Mechanism of T-DNA transfer and integration into the plant genome. The process consists of several steps: (1) attraction of agrobacteria to plant cells, (2) activation of *vir* genes, (3) excision of T-DNA, (4) formation of the T – complex, (5) transfer of the T – complex to the cytoplasm, (6) targeting of the T-complex to the nucleus, (7) active transport to the nucleus, and (8) integration into the plant genomic DNA.

2.2.2 *Agrobacterium tumefaciens* and genetic engineering

The first information about *A. tumefaciens* came from 1897, when it was first isolated. Gradually, the bacterium was found to cause tumour-like diseases. However, a breakthrough did not occur until 1977, when the mechanism by which *Agrobacterium* can cause disease was first described. Genetic engineering has used this mechanism to transfer targeted genes, so-called transgenes, into the genome of plants. In a first step, all disease-causing genes were removed from the T-DNA region, leaving only the genes essential for T-DNA transfer. A so-called **disarmed Ti plasmid** was prepared and modified to introduce targeted transgenes into the T-DNA region. Currently, **binary vectors** consisting of two plasmids are used for transformation (Figure 2.6). The first is the disarmed Ti plasmid, in which the T-DNA region has been removed, but the *vir* and *ori* regions have been retained. The second is a smaller vector containing RB and LB sequences with transgenes. This smaller vector has its own *ori* region, which allows it to replicate independently (Figure 2.6).

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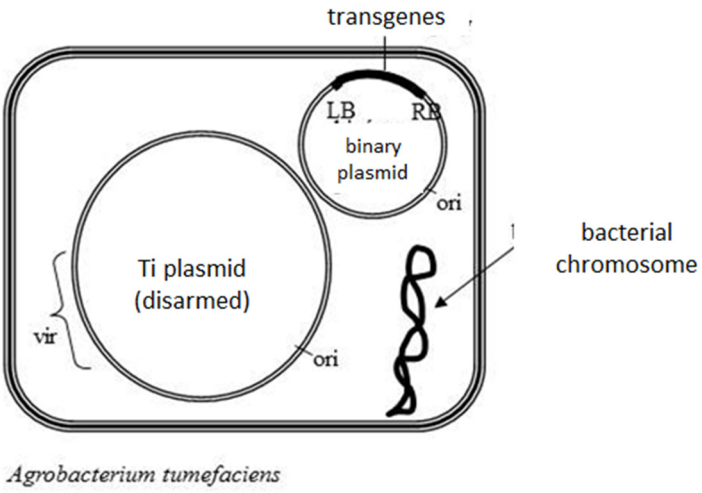

Figure 2.6 *Agrobacteirum tumefaciens* with binary vector

The T-DNA region of the plant transformation vector (Figure 2.7, Figure 2.8) must contain **a multiple cloning site** ('MCS') and a selection marker gene. The MCS is a unique cleavage site for the selected restriction endonucleases. We use the MCS as a cloning site to place targeted genes in the T-DNA region.

A targeted gene is a gene that is introduced into the genome, for example, to improve some quality characteristics of a plant or to increase resistance to various diseases.

Selectable marker genes allow us to select transgenic cells because only a small proportion of cells can take up the foreign DNA and be transformed. They are an integral part of vectors for plant transformation, as they give the transformed cells a selection advantage. Positive selection is based on the expression of genes that enable the transgenic cell/tissue to regenerate:

- in the presence of a toxic substrate to the non-transformed tissue.
- in the absence of an external substrate (e.g. isopentyltransferase, the presence of which endogenously alters the content of plant hormones and thus promotes shoot formation).

Selection marker genes encoding enzymes are divided into three primary groups according to their specificity for a particular type of substrate:

I. Antibiotic resistance genes (kanamycin, hygromycin, gentamicin, streptomycin, and others)

II. Herbicide resistance genes (phosphinothricin, glyphosate, bromoxynil, atrazine, 2,4-D, and others)

III. genes involved in plant metabolic pathways (4-methyltryptophan, threonine, adenine, and others.)

The most commonly used selection markers are genes encoding antibiotic resistance (kanamycin, hygromycin, gentamicin, *etc*.) and herbicides (phosphinothricin, glyphosate, *etc.*). The presence of such substances in the regeneration medium is toxic to non-transformed cells, whereas transformed cells can degrade and thus detoxify them. A classic example of a selection marker gene is the gene encoding neomycin phosphotransferase II (*npt*II). This gene was isolated from the Tn5 transposon of *Escherichia coli* K12. The enzyme neomycin phosphotransferase II inactivates aminoglycoside antibiotics such as neomycin, paromomycin, kanamycin or geneticin by phosphorylation.

The functionality of the *npt*II gene in plants is ensured by plant regulatory sequences.

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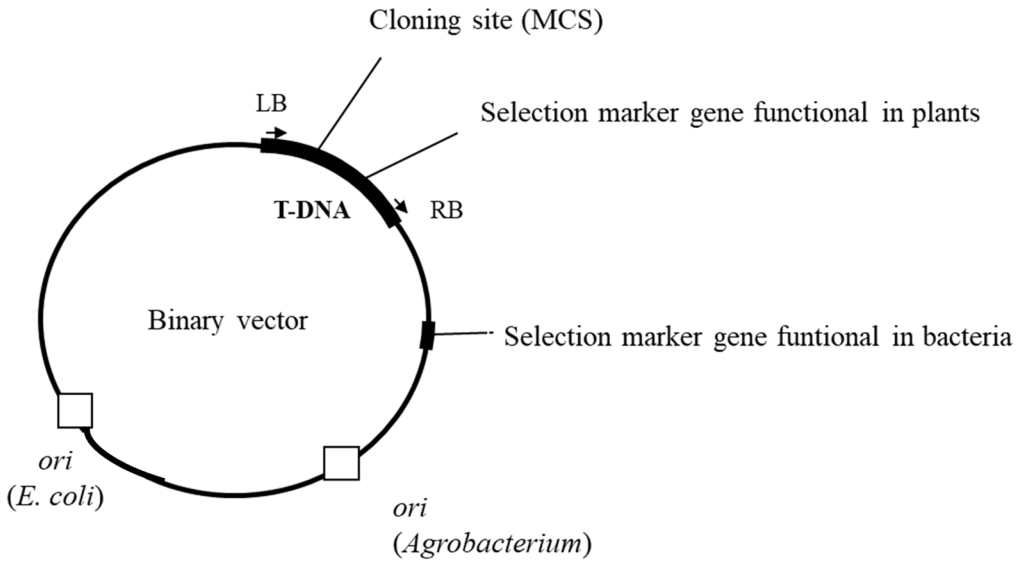

Figure 2.7 Standard binary vector

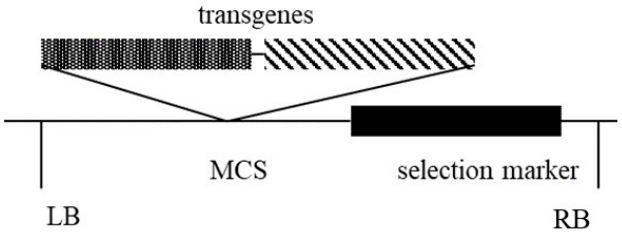

Figure 2.8 T-DNA region of the binary vector

A prerequisite for successful transformation is the integration and functionality of the inserted gene in plant cells. **Reporter genes** encode products that can either be detected directly or catalyse specific reactions whose products are easily detectable. The ideal reporter gene should be unique and non-toxic to the plant cell.

The β-glucuronidase (*uid*A or ***gus*) gene** was isolated from the bacterium *E. coli* K12. Glucuronidase (GUS) hydrolytically cleaves a wide range of -glucuronides. Currently, the *gus* gene is one of the most widely used reporter genes, as it fulfils all the necessary criteria for use in plants:

- easy quantification,
- high sensitivity,
- sufficient specificity of the enzymatic reaction with minimal interference with normal cellular metabolism.

Qualitative detection of GUS activity is performed histochemically with the substrate X-Gluc (5-bromo-4-chloro-3-indolylglucuronide). The enzymatic reaction of -glucuronidase with the X-Gluc substrate results in a blue-coloured product. Different substrates enable them to quantify the activity of GUS spectrophotometrically or fluorimetrically.

The green fluorescent (*gfp*) gene, encoding the green fluorescent protein (GFP), was isolated from the marine organism *Aequorea victoria*. The advantage of this reporter gene is that detecting the GFP protein does not require the presence of a substrate (it is a spontaneously

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fluorescent protein) and is easily detectable under UV or blue light without destroying the plant material.

Outside the T-DNA region, there are a selection marker gene with function in bacteria, *vir* genes and *ori* regions that allow replication of the binary vector in *A. tumefaciens* and *E. coli*. This is because the manipulation of the binary vector (cloning of DNA sequences) takes place in *E. coli* and is only then transferred to *A. tumefaciens*.

All individual steps in preparing the binary vector (cloning) are carried out in *E. coli*. After the final preparation of the binary vector, it is transferred to *A. tumefaciens* either directly by electroporation (using electrical pulses) or indirectly by conjugation with an auxiliary plasmid that ensures the transfer of the binary vector to *A. tumefaciens* since the binary vector itself is not capable of direct conjugation. *A. tumefaciens* containing the binary vector is ready for plant transformation.

2.2.3 Plant transformation using *Agrobacterium tumefaciens*

Plant transformation using *A. tumefaciens* is carried out under the following conditions:

- *In vitro* - co-cultivation of plant cells with a bacterial suspension, followed by selection and regeneration of the transformed cells to obtain *in vitro* transgenic plants (Figure 2.9).
- *In planta* (specifically for *Arabidopsis*) – by briefly immersing *Arabidopsis* flower buds in a bacterial suspension, obtaining transgenic material when the seeds have matured.

The process of plant transformation by *A. tumefaciens* under *in vitro* conditions can be divided into the following steps:

Wounding of the tissue
↓
Co-cultivation of the wounded tissue with *A. tumefaciens*/binary vector
↓
Removal of *Agrobacterium* residues
↓
Selection of the transformed cells
↓
in vitro regeneration
↓
Transgenic plants

Wounding tissue is essential in plant transformation, as wounded plant parts produce phenolic substances that attract agrobacteria. Wounding is achieved by cutting with a scalpel. As a rule, dicotyledonous plants produce sufficient phenolic substances to allow infection. If the plant does not produce sufficient amounts of phenolic compounds or is not part of the *Agrobacterium* host range (monocotyledonous plants), acetosyringone as an inducer of *vir* genes is added to the co-cultivation medium as a substitute. After wounding, such a plant tissue is subjected to infection by **co-cultivation** with a bacterial inoculum containing *A. tumefaciens* cells carrying the corresponding binary vector. The bacterial inoculum is prepared from an overnight culture of agrobacteria(A_{620nm}=1). The duration of the co-cultivation varies (from 10 min to 48 h). After plant cell transformation, the continued presence of *Agrobacterium* is undesirable, as remnants of *A. tumefaciens* may outgrow the plant tissue and prevent its regeneration process. Antibiotics (e.g. cefotaxin, timentin) are used **to prevent *Agrobacterium* overgrowth** and are added to the regeneration medium. These antibiotics must not be toxic to the plant and must not interfere with the regeneration process.

The selection of the transformed tissue depends on the selection marker gene that we used to produce the transformation vector. The selection must be sufficient to kill non-transformed cells, but at the same time, the presence of the antibiotic (at a particular concentration) must not inhibit the regeneration process. The antibiotic kanamycin (*npt*II selection marker gene) is most commonly used for selection. The concentration of the antibiotic is determined experimentally for each plant tissue. The lowest experimentally determined value at which the non-transformed tissue dies is chosen.

Regeneration of the transgenic plants is the final step in the transformation process. The transformation's success depends on the plant tissue's ability to regenerate into intact plants. Without mastering the regeneration protocol, obtaining transgenic plants is impossible. The age (young tissue is preferable) and the type of tissue (leaf, nodal or hypocotyl segments are most commonly used) play an important role. The ability to regenerate is genotype-dependent. Some varieties regenerate well under *in vitro* conditions, while others are recalcitrant.

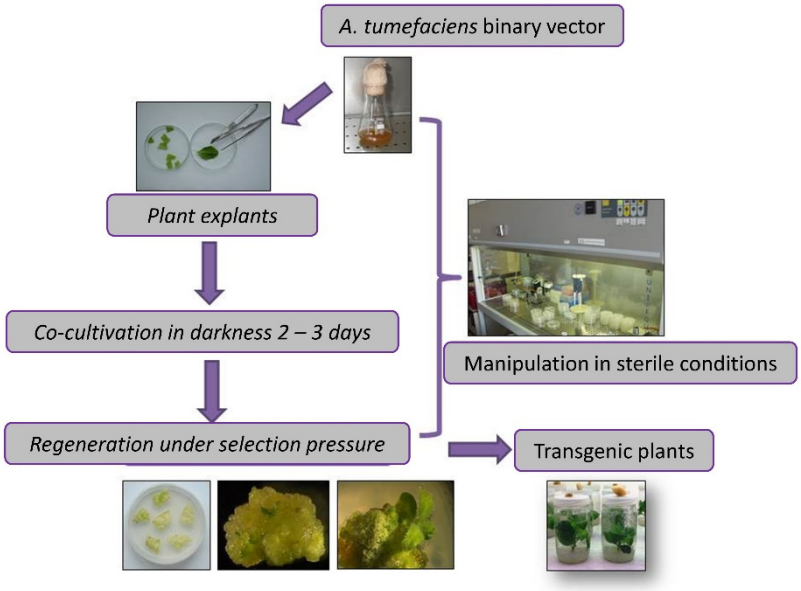

Figure 2.9 Schematic representation of the plant transformation process with *A. tumefaciens*.

2.3 Introduced transgenes and their heredity

Analyses of transgenic plants have shown that T-DNA can be integrated into any of the chromosomes that make up the genome. Within each chromosome, T-DNA can be integrated at different chromosomal positions. One or more copies of T-DNA can be integrated into each locus. Insertion can occur at one or more loci but never at both alleles of the same locus. According to Mendelian inheritance rules, the inserted T-DNA becomes a natural part of the plant DNA and is inherited as a dominant gene (Figures 2.10 and 2.11). A segregation test can be used to determine the number of T-DNA loci. For a single T-DNA locus, the segregation ratio is 3:1; for two independent T-DNA loci 15:1; and for three independent T-DNA loci 63:1. *Example*: transgenic tobacco plant with one T-DNA locus. Using *npt*II as a selection marker gene means integrating one copy of the *npt*II gene, which is inherited as the dominant gene.

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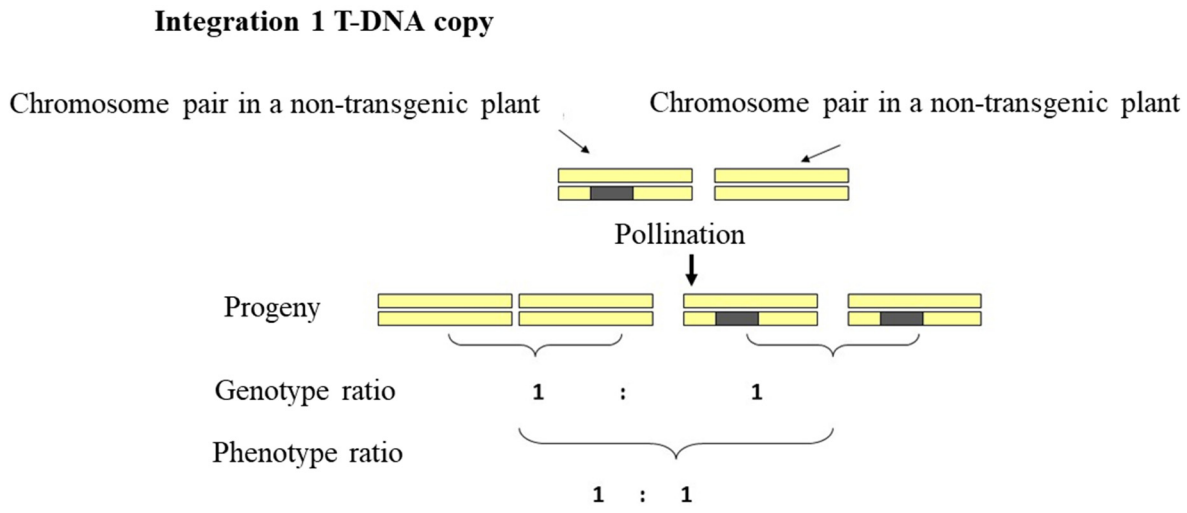

Figure 2.10 Progeny of a transgenic plant after cross-pollination

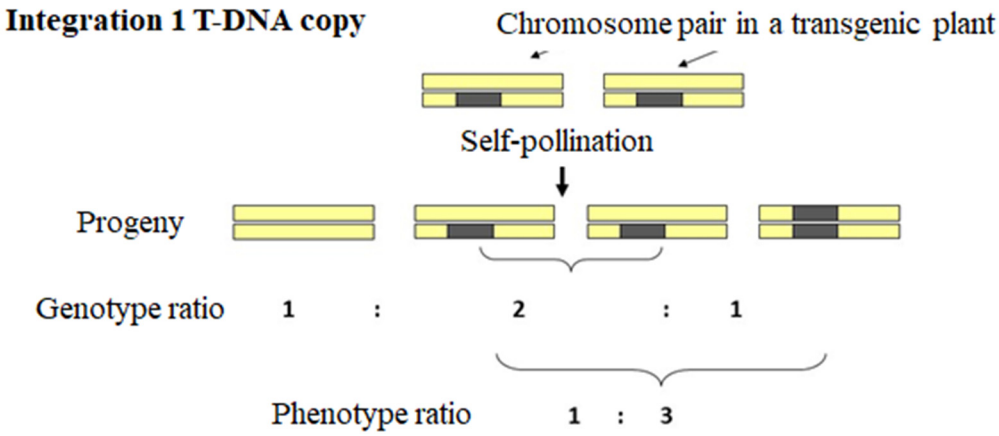

Figure 2.11 Progeny of a transgenic plant after self-pollination

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3 TRANSGENE EXPRESSION IN PLANTS AND METHODS OF DETECTION

3.1 The basic characteristic of gene expression and regulation in eukaryotic cells

Gene expression is the process by which information encoded in genes is transferred into the synthesis of gene products, usually proteins and functional RNAs such as transfer RNAs. Regulation of gene expression involves several mechanisms used by living cells to control the formation of the final gene product. In unicellular organisms, regulation of gene expression is adapted to the needs of cellular metabolism to obtain nutrients in a changing environment. In multicellular eukaryotes, gene regulation enables the differentiation of cell types. In plants, this leads to the developing of individual tissues that function in a coordinated manner in each organism. When preparing genetically modified organisms by recombinant DNA techniques, it is essential to consider whether the transgene is expressed in a prokaryotic or an eukaryotic organism (Figure 3.1).

Differences in gene expression in eukaryotic organisms compared to prokaryotic organisms can be summarised as follows:

- RNA transcription occurs in the nucleus of the eukaryotic cell
- The primary mRNA contains introns that are removed before the mRNA leaves the nucleus
- The eukaryotic mRNA contains a polyA sequence at the 3'- end
- After removing the intron sequences, the mRNAs move from the nucleus to the cytoplasm, where translation occurs at the ribosomes.

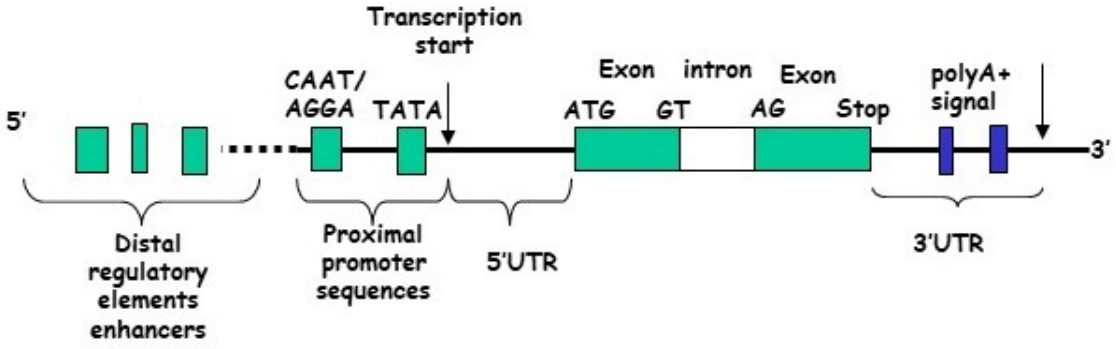

Fig. 3.1 The structure of a typical plant gene

3.2 Plant promoters

A promoter is a part of a gene sequence that contains information about when and where (in which plant tissue) a gene is to be transcribed in mRNA form (Figure 3.2). The promoter is located upstream of the transcription start site (TSS), where the start of transcription is designated +1.

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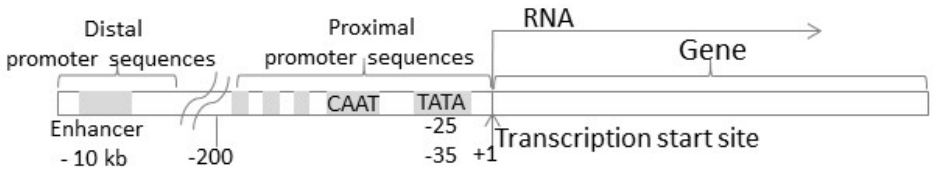

Figure 3.2 Schematic representation of the promoter structure

The promoter consists of ***proximal and distal parts*** containing *cis*-transcriptional elements (boxes, motifs). These are nucleotide sequences consisting of 2 to 10 nucleotides that bind to specific protein transcription factors. The best-known promoter element is the TATA box, a conserved sequence (-30 bp from TSS) that binds the RNA polymerase complex (RNA polymerase and protein regulators) (Figures 3.2 and 3.3).
The CAAT box (-75 bp from TSS) belongs to the proximal elements and regulates the frequency of transcription initiation. In addition to these two sequence motifs, plant promoters contain dozens of other *cis*-regulatory elements responsible for the overall gene expression profile. Their binding to the preinitiation complex occurs *via* the corresponding protein transcription factors (Figure 3.3).

Distal promoter sequences also regulate and modulate gene expression. They are crucial for genes that are developmentally regulated or controlled by external stimuli. Sequences within 500 bp of the TSS have been shown to affect a low level of tissue-specific expression. Conversely, DNA sequences further away can increase gene expression. Some distal promoter sequences do not function when separated from the original gene, while others function regardless of their position and orientation.
Enhancers and silencers are usually located at some distance from the promoter. They activate or repress expression *via* protein factors that bind to them. Subsequently, binding occurs between *enhancer*, *protein factor*, *preinitiation complex,* and *RNA polymerase II*.

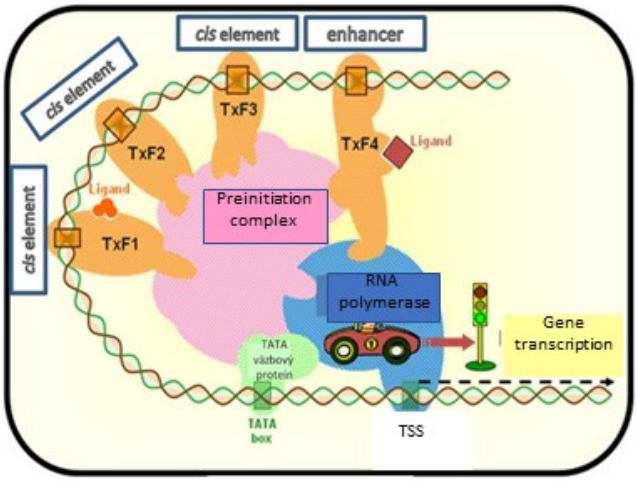

Figure 3.3 Binding of the RNA polymerase complex to the TATA box of the promoter

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The complex also includes *cis*-regulatory elements that bind *via* protein factors (TxF1, TxF2...) to the preinitiation complex. Similarly, distal *cis*-elements, enhancers, and silencers bind to the preinitiation complex *via* their protein factors.

Promoters are among the most important tools in biotechnological processes as transcription of a transgene is the first step in gene regulation. The correct choice of the promoter can influence whether the transgene expression in the transgenic organism is:

- high
- low
- constitutive
- inducible
- organ/tissue-specific

The ***CaMV35S*** promoter belongs to the best-known constitutive promoters controlling the high expression of transgenes in transgenic plants. It functions mainly in dicotyledonous and in some monocotyledonous plants. The ***Ubi*** promoter isolated from corn achieves strong expression mainly in monocotyledonous plants.

Inducible promoters are also increasingly used for specific biotechnological applications because they trigger gene expression in response to various inducers. An example is the ***GLP*** ("germin-like" protein) promoter isolated from *Tamarix hispida*, inducible by abiotic stress such as drought, increased by soil salinity, low temperatures, *etc*.

Organ/tissue-specific promoters include promoters that can ensure transgene expression only in specific organs or tissues. These include, for example, promoters active in plant roots that may have biotechnological applications in projects related to soil bioremediation, protection of plants from drought, root pathogens, *etc*. One of the first root-specific promoters that have been used in plant transgenesis is the ***rolD*** promoter of the *Agrobacterium rhizogenes* gene. The second example of tissue/organ-specific promoter is seed-specific ***α-globulin*** promoter isolated from cotton, which is thought to drive robust expression of storage proteins in native organisms. Such a type of promoter can be used, for example, to improve the nutritional quality of seeds. The first generation of transgenic crops mainly used constitutive promoters. Transgenic crops with constitutive expression of transgenes are now grown in agriculture. However, increasing attention is being focused on inducible and organ/tissue-specific expression of transgenes in plants, which is less energy – and nutrient-demanding for the plants themselves and, in many cases, acceptable from a biosafety point of view.

3.3 Other factors affecting gene expression in plants

In addition to the promoter, other genetic elements can also influence the resulting transgene expression: ***Enhancers, introns, 5'- and 3'-untranslated sequences (5'-UTR, 3'-UTR), matrix-binding sequences (MARs), insulators,*** *etc*., and therefore must be taken into account when producing transgenic plants.

The 5'-untranslated region (5'-UTR) sequence is located between the transcription start, and the translation start sites (Figure 3.4). After gene transcription, a so-called cap (7'-methylguanosine triphosphate bound to the first dNTP) is added to the 5'-end of the UTR, which protects the mRNA from degradation and at the same time plays a role in binding the mRNA to the ribosome. Generally, genes with strong expression usually have short 5'-UTRs without significant secondary structure. mRNA formation that is tissue or developmentally regulated usually contains long 5'-UTR regions. The 5'-UTR also influences the rate of translation.

In the case of 5'-UTR, for optimal translation of chimeric genes in plants, it is necessary to check for the presence of conserved nucleotides at positions -3 (A, G) and +4 (G) near the translation ATG start (Figure 3.4). This sequence motif is also called the Kozak sequence. If the divergence of these nucleotides is substantial, the expected transgenic protein may not be produced in the transgenic plants.

Figure 3.4 Illustration of the Kozak sequence at positions -3 and +4, which is essential for optimal translation of chimeric genes in plants

3'-untranslated sequences (3'-UTR, terminators)
The 3'-untranslated sequence is located downstream of the triplet encoding the translation stop and extends upstream of the transcription stop. The so-called polyA signal (AAUAAA) is essential for transcription termination. During post-transcriptional processing, polyA (150-250 nucleotides) is added to the 3' transcript (Figure 3.5). The poly(A) sequence provides the mRNA export to the cytoplasm. Later, 3'-UTRs were shown to play an essential role in mRNA stabilization and translation efficiency. The length of 3'-UTRs in plants typically ranges from several tens to hundreds of nucleotides. In the preparation of conventional expression units, the ***35S, nos,*** **and** ***ocs terminators*** are most commonly used. The *nos* (nopaline) and *ocs* (octopine) terminators were isolated from expression units found in the T-DNA Ti plasmid of *A. tumefaciens*.

Figure 3.5 Illustration of 3'UTR. During post-transcriptional editing for mRNA at the site of the polyadenylation signal, the polyA sequence is attached.

Introns
Introns are non-coding sequences that occur in open reading frames (ORFs). Plant introns range in size from tens to thousands of nucleotides. In the preparation of chimeric genes, introns are sometimes intentionally incorporated into the transcribed portion of the gene, providing increased transcript stability. It has been particularly confirmed for transgenes in the transformation of monocotyledonous plants. In dicotyledonous plants, the results are more variable. Introns can also harm expression – mainly if they originate from distant species.

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Localization signal

Proteins expressed in the cell may remain in the cytosol or be transported to different cell structures such as the cell membrane, ER /Golgi apparatus, nucleus, chloroplasts, mitochondria, or vacuoles. A prerequisite for transport is the presence of a localization signal sequence, usually referred to as a signal peptide/leading sequence. The length of this sequence at the protein level is generally between 7 and 30 amino acids (Figure 3.6). It is usually located at the N-terminus but can also occur at the C-terminus of the protein, possibly as an intra-protein domain. Once the protein is transported to its target place, this sequence is cleaved from the protein. When producing chimeric expression units, it is necessary to consider the localization of the transgenic protein and, if necessary, to append the DNA sequence for the corresponding localization signal to the transgene.

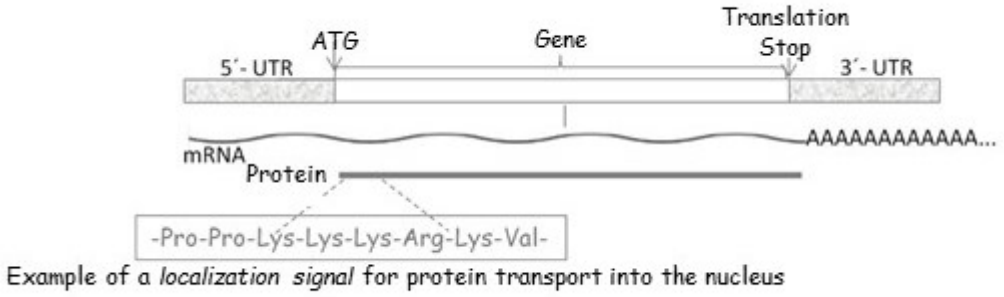

Figure 3.6 Example of a localization signal for transporting a protein into the nucleus, located at the N-terminus of the protein sequence.

3.4 Preparation of transgenic expression units in cloning or cassette vectors

Transgenes are introduced into plants in the form of expression units that contain the gene itself and the regulatory elements required for transgene expression. ***A basic transgene expression unit*** has ***promoter*** active in plants, ***transgene***, and ***terminator***. When multiple genes are introduced into a plant, each contains its regulatory elements. The fusion of genes with regulatory sequences is achieved by cloning or in cassette vectors (Figures 3.7, 3.8).

When a cloning vector is used for the expression unit preparation, the DNA fragments corresponding to the promoter, gene, terminator, and vector are digested with appropriate restriction endonucleases and then ligated with the vector. Then ligation mixture is introduced into competent *Escherichia coli* cells using genetic transformation. If the cloning vector contains the *lac*Z system, white colonies grow on X-Gal and IPTG plates after the fragment(s) is inserted into the polylinker and subsequent transformation. The colonies remain blue if the insert(s) is not incorporated into the polylinker. In contrast to cloning vectors, the regulatory sequences (promoter and polyA sequence) are part of the vector for cassette vectors.

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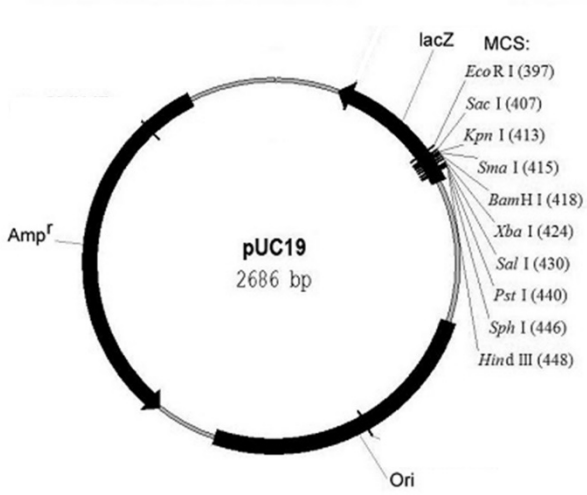

Figure 3.7 An example of the cloning vector pUC19 designed to clone (amplify) a DNA fragment. MCS – polylinker for cloning DNA fragments. *Ori* – the beginning of replication in bacteria. Amp^R – ampicillin resistance gene.

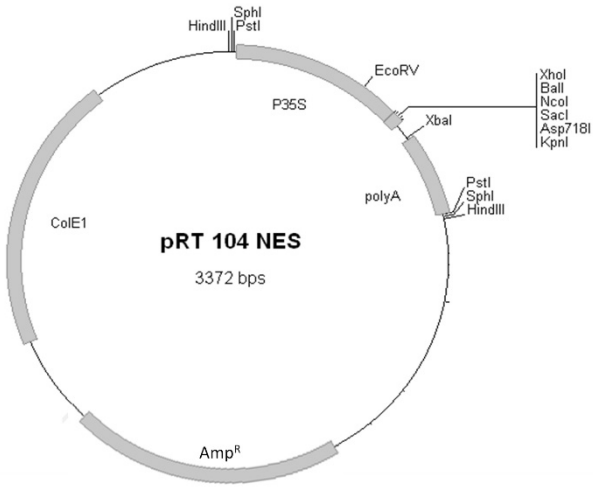

Figure 3.8 Example of the pRT104 cassette vector. The plasmid already contains the *CaMV 35S* promoter (P35S) and the *35S* terminator (poly A). The polylinker is designed to insert the transgene sequence.

3.4.1 Introduction of transgenic expression units into plants using transformation vectors

The type, size, and properties of the vector used to transform plants depend on the transformation method. For example, a classical cloning plasmid containing an expression unit can be used directly to transform plant tissue by bombardment. On the other hand, binary vectors (Figure 3.9) and cointegrating vectors are employed for transformation with agrobacteria. The prepared expression unit(s) is(are) ligated into the polylinker (MCS) T-DNA of the binary plant vector and introduced into *E. coli* using genetic transformation. Subsequently, the binary vector is transferred from *E. coli* to agrobacteria by conjugation. Agrobacteria containing vector construct are used for the transformation of the plant tissue.

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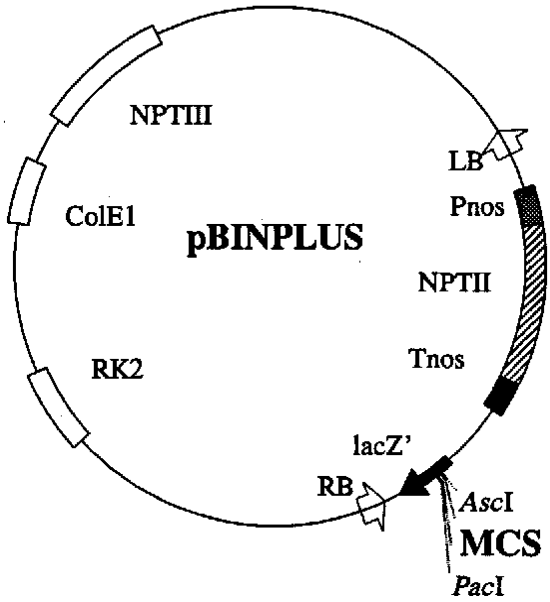

Figure 3.9 Schematic representation of the binary vector pBINPLUS (van Engelen et al. 1995) used to transform plants with agrobacteria. The prepared expression unit for plant transformation is ligated into the MCS polylinker. An expression unit that confers kanamycin resistance (Pnos-NPTII-Tnos) to the transformed tissue is pre-inserted into the T-DNA that is bounded by the right (RB) and left (LB) borders. The cellular apparatus of agrobacteria primarily control the transfer and insertion of T-DNA. In addition to T-DNA, the binary vector pBINPLUS contains a replication start for *E. coli* (ColE1), a replication start for agrobacteria (RK2), and a kanamycin resistance gene expressed in prokaryotic cells.

3.5 Analysis of transgenic plants

After regeneration of transgenic plants under *in vitro* conditions, the transgenic plants are mostly phenotypically indistinguishable from non-transgenic plants. For this reason, the transgenic nature of the primary transformants and the level of transgene protein production in the target tissues are monitored by appropriate molecular and biochemical analyses. The basic list of analyses of transgenic plants includes:
(1) Selection assay – transgenic individuals, grow in the presence of a selection agent, unlike non-transgenic individuals
(2) PCR – detects the presence of transgene(s) in plants
(3) Southern hybridization – determines the number of independent T-DNA integrations into the plant genome
(4) RT-PCR – determines whether a transgene product is formed in the transgenic organism at the transcriptional level
(5) Northern hybridization – determines the level of transgene transcript production
(6) Western blot analysis – confirms transgene protein production in plants
(7) Biochemical determination of transgene protein/enzyme activity – verifies the functionality of the transgene protein

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3.5.1 Selection test

When transgenic plants carry a selection marker gene, they can grow on culture media in the presence of a specific selection agent that is toxic to non-transgenic individuals. An example is the kanamycin resistance gene, often introduced into plants as a selection marker gene. The product of this gene, neomycin phosphotransferase, degrades the toxic kanamycin to a harmless product that poses no threat to transgenic individuals.

3.5.2 PCR analysis

The availability of the DNA sequences of the inserted transgenes and their regulatory elements is a prerequisite for this type of analysis. The DNA sequences of expression units are used to design transgene-specific primers and calculate the expected size of the PCR product. ***Polymerase chain reaction*** (PCR) is an *in vitro* technique based on generating a large number of copies of a DNA molecule. It is based on a cyclic alternation of thermal denaturation and re-denaturation of DNA (typically 25 to 35 cycles). In the PCR method, specific DNA sequences of the transgene are amplified with short single-stranded oligonucleotides (primers). The primers must be designed to avoid nonspecific amplification of homologous sequences of endogenous genes in the plant. In the analysis of transgenic plants, genomic DNA isolated from plant tissue serves as a template. PCR is performed in a thermal cycler and involves the following steps:

(a) Denaturation of the DNA at high temperature (90 – 94 ºC);

(b) Annealing of the primers to single-stranded DNA at a specific temperature;

(c) Extension of the newly synthesized DNA strand by a thermostable DNA polymerase.

The authenticity of the PCR product is verified electrophoretically in the agarose gel. The size of the product must match the number of base pairs between the designed "forward" and "reverse" primers on the DNA strand (Figure 3.10). Its size is determined by comparison with a marker (a mixture of fragments with precisely known base pair sizes).

```
841  gtcggcatcc ggtcagtggc agtgaagggc gaaca gttcc tgattaacca caaac cgttc
901  tactttactg gctttggtcg tcatgaagat gcggacttgc gtggcaaagg attcgataac
961  gtgctgatgg tgcacgacca cgcattaatg gactggattg gggccaactc ctaccgtacc
1021 tcgcattacc cttacgctga agagatgctc gactgggcag atgaacatgg catcgtggtg
1081 attgatgaaa ctgctgctgt cggcttttcg ctctctttag gcattggttt cgaagcgggc
1141 aacaagccga aagaactgta cagcgaagag gcagtcaacg gggaaactca gcaagcgcac
1201 ttacaggcga ttaaagagct gatagcgcgt gacaaaaacc acccaagcgt ggtgatgtgg
1261 agtattgcca acgaaccgga tacccgtccg caaggtgcac gggaatattt cgcgccactg
```

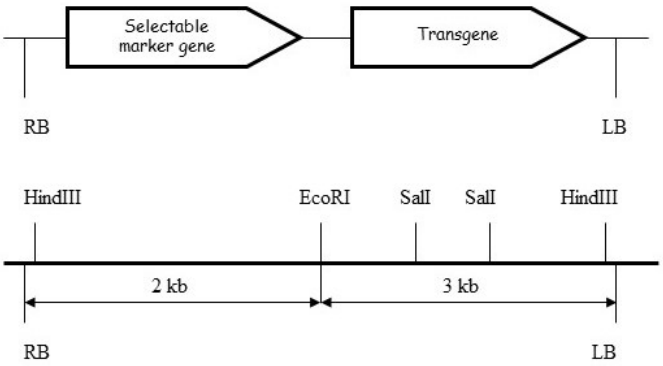
Figure 3.10 Example of part of the sequence of the β-1,3-glucuronidase gene. The nucleotides used to design the "forward" and "reverse" primers are *in bold*. The expected size of the amplified fragment corresponds to the number of nucleotides between the two primers (445 bp). In the case of a positive PCR reaction, a fragment of 445 bp is detected on the agarose gel.

In transgenic plants, the PCR method can detect the presence of the transgene in the genome but not its copy number. Currently, PCR is the most widely used method for detecting and identifying genetically modified organisms. PCR tests are designed to detect the inserted genetic material: the promoter, the gene itself, the terminator, or the marker gene. PCR can also be used for general GMO screening. For this, primers recognizing DNA sequences common to

most GMOs (*CaMV*35S promoter, *nos* promoter, *nos* terminator of *A. tumefaciens*, etc.). are often used. If GMO screening requires more detailed analyses of inserted genes and the DNA sequences are not available (e.g., because they are under patent protection), biotech companies producing GMOs must provide specific tests to identify the relevant transgene(s).

3.5.3 Southern hybridization

Southern hybridization (named after Edward M. Southern) is a widely used method to detect the presence of a transgene in the genome of the analyzed transgenic plant and its copy number or arrangement. In a first step, the isolated genomic DNA is digested into smaller fragments using suitable restriction endonucleases. The strategy for selecting the appropriate restriction endonuclease for DNA analysis is shown in Figure 3.11. The restricted DNA fragments are separated by agarose gel electrophoresis. The negatively charged DNA molecules move to a positively charged electrode due to the different sizes of the base pairs. The DNA is subsequently denaturated in the gel in an alkaline environment to obtain single-stranded DNA accessible to the labeled sample. In the next step, the DNA fragments are transfered from the gel to a material capable of binding the denatured DNA (usually a nylon membrane) and immobilized (e.g., by exposing the membrane to UV light or 120ºC). The DNA molecules are then hybridized with a probe labeled with a radioactive (phosphorus, ^{32}P) or non-radioactive (e.g., digoxigenin) probe. The hybridization process is based on the property of nucleic acids to pair with a complementary nucleotide sequence, the probe. After removing the remnants of the unbound probe, the sites where the probe was bound by autoradiography (β-emissions from the probe cause blackening of the X-ray film), are detected. In this way, it is possible to determine the number of fragments and their size (Figure 3.12).



Restriction of DNA	*EcoR*I	*EcoR*I	*Sal*I
Probe (fragment)	*Hind*III-*Eco*RI (selection gene, 2 kb)	*Eco*RI-*Hind*III (transgene, 3 kb)	*Sal*I-*Sal*I (transgene, 1 kb)
Expected fragments	> 2 kb	> 3 kb	1 kb (internal)

Figure 3.11 Ilustration of a strategy for selecting an appropriate restriction endonuclease and probe for Southern analysis. RB, LB – right and left T-DNA borders of the binary vector used to transform plants with *A. tumefaciens*.

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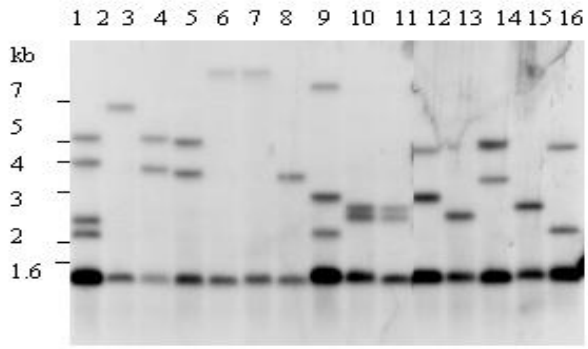

Figure 3.12 Example of an autoradiogram of a Southern blot of DNA digested with the appropriate restriction endonuclease. The blot was hybridized to a radiolabeled fragment encoding a *gus* reporter gene.

3.5.4 RT-PCR (Reverse Transcription PCR)

This method is used for rapid screening of transcription of transgenes into the mRNA form. In the first step, total RNA is isolated from the tissue of the transgenic plants. The mRNA is then transcribed into cDNA form using reverse transcriptase, followed by conventional PCR. RT-PCR products are similarly detected by gel electrophoresis to PCR products. Although transcription itself is not technically demanding, working with RNA is considered more challenging because the action of ribonucleases rapidly degrades RNA acids. These are common contaminants of samples, instruments, and chemicals. To prevent RNA degradation, tools and solutions are necessary to treat with RNase inhibitors (e.g., diethylpyrocarbonate DEPC).

3.5.5 Northern hybridization

Northern hybridization is a method that allows us to determine whether a successfully inserted gene is transcriptionally active and whether the corresponding mRNA is produced and to estimate its relative abundance. The principle of the method is similar to Southern hybridization, but instead of DNA molecules, we work with RNA. Using formaldehyde gel electrophoresis, total RNAs isolated from transgenic plants are separated under denaturing conditions. After the transfer and fixation of RNA on the membrane, we hybridize the RNA molecules with a radiolabeled probe (phosphorus, ^{32}P). We use a DNA fragment (without intron) of the corresponding transgene as the probe. Detection is performed by autoradiography (Figure 3.13).

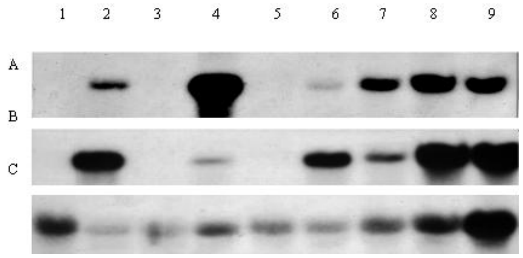

Figure 3.13 Example of Northern blot autoradiograph showing total RNA isolated from leaves of untransformed potato (lane 1) and leaves of individual transformed potato (lane 2-9). The blot was hybridized with radiolabeled DNA fragments encoding tobacco glucanase (A), cucumber chitinase (B), and potato tubulin (C).

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3.5.6 *In situ* hybridization

In situ hybridization (ISH) localizes specific RNA or DNA sequences directly in the plant tissue or on a chromosome. In this method, the plant tissue or chromosome is fixed to a slide, treated with chemicals to increase cell permeability, DNA denatured, and hybridized with a labeled probe. The probe may be radioactively labeled and detected by autoradiography, fluorescently labeled (FISH), and immunochemically detected. The hybridization signal is observed with an optical microscope (radiolabeling of the probe) or a fluorescence microscope (fluorescent labeling of the probe). This technique is suitable for detecting the expression of a transgene when its promoter is, for example, developmentally specific, active only in a particular tissue type, or inducible (e.g., by wounding).

3.5.7 Western blot analysis

Western blot analysis is a rapid and sensitive method for detecting and characterizing proteins. It allows us to verify the functionality of the incorporated transgene at the protein level. With this technique, proteins are identified using specific monoclonal antibodies. The first methodological step involves protein extraction. Proteins are electrophoretically separated in a polyacrylamide gel under denaturing conditions (PAGE with SDS) and transferred to a nitrocellulose membrane. Nonspecific protein binding sites are blocked with appropriate agents on the membrane. This way treated membrane is immersed in a solution containing the primary antibodies, washed, and immersed in a solution containing secondary antibodies (commercially available) that bind to the primary antibodies (Figure 3.14). In the presence of an appropriate substrate, these secondary antibodies are detected histochemically or fluorescently.
Primary antibodies can be produced by cloning the transgene into an expression vector and subsequent transformation into bacteria. Once an expression of the transgene has been trigged in bacteria, we obtain a protein that we purify and inoculate to animals (mice, rabbits, goats). These begin to produce monoclonal antibodies, which we use in experiments after purification. When the primary antibodies are prepared in rabbits, secondary antibodies are produced in goats, e.g., against rabbit immunoglobulins. The secondary antibodies are commercially available.

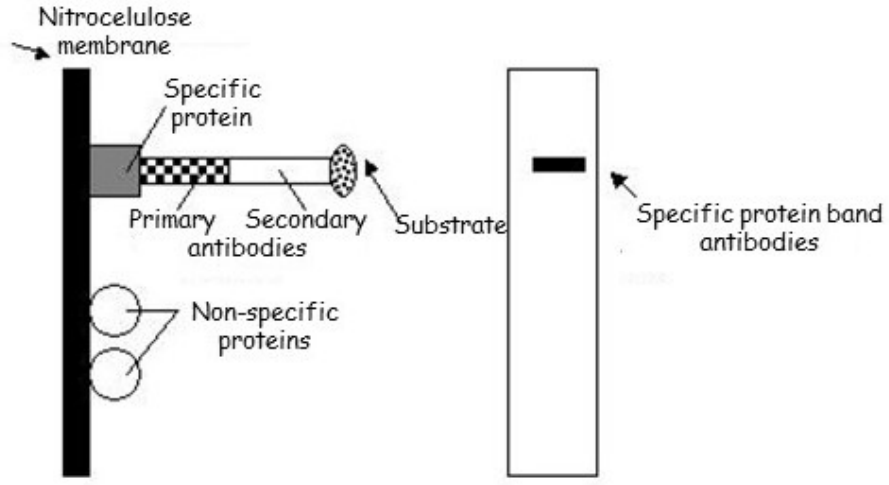

Figure 3.14 Schematic representation of Western blotting. A polyacrylamide gel with a nitrocellulose membrane and filter paper (Whatman 3MM) sandwiched between two porous layers is immersed in an electrophoresis buffer. The proteins are electrophoretically transferred

from the gel to the nitrocellulose membrane. Next, the nitrocellulose membrane containing the transferred proteins is incubated with primary antibodies that specifically recognize the proteins of interest. Secondary antibodies are bound to the primary antibodies (after applying a solution containing these antibodies). The secondary antibodies form a complex with, *e.g*., horseradish peroxidase, which can be detected fluorescently or histochemically in a suitable substrate.

3.5.8 Biochemical determination of the activity of transgenic proteins/enzymes

This method verifies that the transgenic protein is functional in the transgenic plant. This analysis is possible if the transgenic protein has enzymatic activity and cleaves a suitable substrate.
An example might be a transgenic chitinase that, when produced in the plant, should result in increased chitinase activity compared to non-transgenic plants, which can be measured according to the substrate chosen for the chitinase, e.g., spectrophotometrically or fluorimetrically.
Another well-known assay example is the β-glucuronidase transgene, also known as ***the reporter gene***. Its product, β-glucuronidase, cleaves the substrate X-Gluc (5-Bromo-4-chloro-3-indolyl-beta-D-glucuronide) to form a blue product (Figure 3.15). After such analysis, the functional transgene product can detect the transgenic plant directly. The reporter gene is used in plant transgenesis mainly to characterize isolated plant promoters. Plants are transformed with a promoter of an unknown expression profile linked to the reporter gene. After confirming the transgenic character (*e.g*., PCR), histochemical evidence can determine which tissue types or under which physiological conditions the promoter is active.

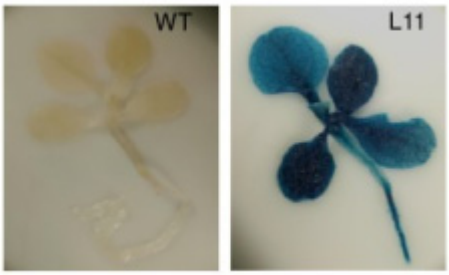

Figure 3.15 Histochemical detection of the protein GUS in transgenic tissues of *Arabidopsis thaliana*. The non-transgenic tissue in the presence of substrate remains almost colorless after removing chlorophyll. Transgenic tissue with a functional GUS protein cleaves X-Gluc and forms a blue product, as shown by the blue staining of the tissue

3.6 Possible problems associated with the transfer of transgenes into plants

Improving specific plant traits through transgenic technologies has brought new possibilities in plant breeding. Still, on the other side, it has also revealed a problem related to the expression of transgenes in plants. Although transgenes can be successfully introduced into the genome of plants, the level of their expression in individual transgenic plants ***is highly variable*** and can be partially or entirely suppressed.

Because integrating a transgene into the plant genome is a random process, a transgene inserted into the coding sequence of another significant plant gene will have its function disrupted. It is improbable that a new plant will be regenerated from such a cell. If the T-DNA

is inserted into an untranscribed region of genomic DNA (hypermethylated or condensed chromatin region) and the transgene or selection marker gene is not expressed, we cannot expect the transformant to regenerate.

When regenerated transgenic plants are analyzed, significant differences in expression of the transgene of interest (e.g., the chitinase gene), even though the same promoter drives the transgene of interest, are observed. Differences in transgene expression between transformants are due to a difference in chromosomal positioning known as the position effect. One approach to counteract the influence of the position effect is to clone ***MARs (matrix attachment regions)*** elements at the borders of the expression units. MARs cause the introduced T-DNA to become a domain-independent of the surrounding chromatin.

For analyses of transgenic plants, the plants with one copy of the transgene are preferred. Integration of a high number of transgene copies and subsequent overproduction of the transgene mRNA transcript often results in suppression of gene expression, known as gene silencing. Silencing of expression of multiple or all transgene copies occurs either at the transcriptional level or the post-transcriptional level. In the case where silencing of transgene expression occurs at the DNA level, changes occur in the nucleus. The triggers are:

- Interactions between transgenes and endogenous genes
- Transgene – transgene (in multiple copies)
- Promoter – promoter (in multiple copies)

These are interactions of homologous gene sequences or promoters. Subsequently, DNA methylation occurs, and a condensed chromatin structure is formed with the help of chromatin components.

Silencing of transgene expression can also occur at the mRNA level, whereby homologous mRNA molecules are degraded in the cytoplasm. This mechanism was probably originally used to defend against viruses. Later, it became clear that plants could also use it to suppress transgene expression.

The triggers are:

- a large number of mRNA transcripts due to a higher copy number of the transgene
- a strong promoter fused to the transgene
- the presence of an inverse arrangement of two copies of the transgene

As a result, double-stranded RNA (dsRNA) is formed, which subsequently serves as a trigger for RNA degradation. Transgenes that are arranged inversely directly produce dsRNA. In the case of high transgene expression, RNA-dependent RNA polymerase is activated, which synthesizes an antisense transcript. Subsequently, dsRNA is produced, which is sequentially fragmented, and these fragments (siRNA) form a multiprotein complex with proteins that can trigger methylation of the corresponding gene sequences.

This mechanism of suppression of (trans)gene expression, first described in transgenic plants, has gradually found biotechnological applications. For example, in the case where suppressing the expression of a gene improves the quality of a crop. It can be achieved by transforming the plant with a construct that has designed DNA in the expression unit downstream of the promoter so that, after transcription, mRNA complementary to the mRNA of the endogenous gene (antisense mRNA) is produced, resulting in dsRNA that blocks the production of the protein. The given strategy to suppress gene expression is called "antisense". It was first used in tomatoes, where suppression of polygalacturonase expression resulted in the slowing of ripening and softening of the fruit.

4 TRANSGENIC PLANTS AS A TOOL FOR CROP IMPROVEMENT

Plant breeders' ultimate goal is to develop plant varieties with desired agronomic characteristics. In conventional breeding, this is achieved by crossing plants with the appropriate quality traits and selecting progeny with the desired combination of traits. The whole process is time-consuming, and there is little or no guarantee that a particular combination of genes will result from the crosses. In contrast, genetic engineering allows the direct transfer of one or a few genes of interest within or across species boundaries to obtain the plants with the desired agronomic trait. In some respects, this process is faster and more predictable than conventional breeding.
The first transgenic plants were produced as early as 1983; they have been gradually cultivated, first in experimental fields and later commercially. The first genetically modified product to reach the market was Calgene's FlavrSavr tomato in 1994, but it was not a commercial success. Since 1996, however, the cultivation of genetically modified crops, especially those resistant to herbicides and pests, has become part of agricultural practice worldwide. The total area under GM crops increased almost 100-fold between 1996 (1.7 million ha) and 2016 (185 million ha) and accounted for 12% of the world's total arable land in 2016. The largest growers of GM crops are the USA, Canada, Argentina, Brazil, China, and South Africa. Statistics from 2016 also state that the most commercially grown GM are soybeans, corn, cotton, and canola. Regarding the genetic modifications used, herbicide-resistant crops are grown on about 52% of the area, insect-resistant crops are grown on about 13% of the area, and crops with both genetic modifications occupy 32% of the area with transgenic crops.

4.1 Genetically modified herbicide-resistant plants

Successful cultivation of crops requires their protection from weeds. For this, herbicides are often used. Depending on weeds and their status, they are applied either preventively to the soil or directly to the plants. The basic characteristic of herbicides is that they usually act non-selectively, usually by inactivating a vital enzyme in the essential metabolic pathway. Since they are non-selective, they can also damage cultivated crops. Therefore, the focus is mainly on preventive weed control.

Currently, the cultivation of genetically engineered crops resistant to a new generation of herbicides is being introduced into agricultural practice to a greater or lesser extent (depending on the country's attitude towards GMO cultivation). The importance of this trend is that a new generation of herbicides can be used during the growing season of a crop without harming it. Genetic engineering involves the insertion of the gene for resistance to the corresponding herbicide into the genome of the genetically modified crop. Transgenes that confer resistance to individual herbicides have been isolated from various sources (plants, bacteria) and then transferred to specific crops using genetic transformation techniques. The first herbicide-resistant crop was a variety of soybean, engineered by Monsanto in 1996. Later this technology was applied to many other crops, including corn and sugar beets. In the following section, we will attempt to explain the nature of transgenic resistance to two different herbicides.

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4.1.1 Glyphosate resistance

Plants use the shikimate metabolic pathway to synthesise aromatic amino acids, flavonoids, lignins, and other substances with an aromatic ring. A key enzyme in this metabolic pathway is **EPSP synthase** (5-enolpyruvylshikimate-3-phosphate synthase). It can be inhibited by **glyphosate** [*N*-(phosphonomethyl)glycine] (Figure 4.1), the active ingredient in the herbicide Roundup. Glyphosate is a non-selective broad-spectrum herbicide and crop desiccant widely used by farmers to kill annual broadleaf weeds and grasses competing with crops.

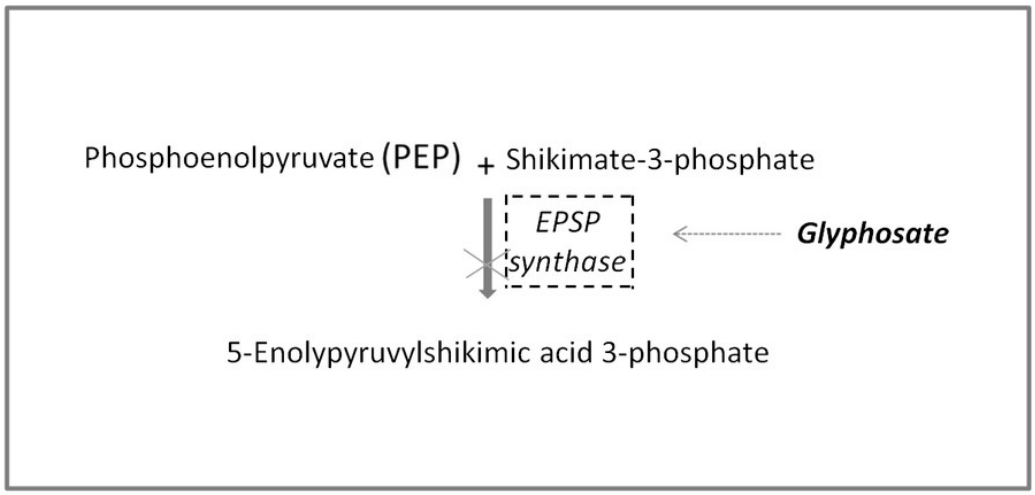

Figure 4.1 Illustration of the part of the shikimate biosynthetic pathway that leads to the synthesis of aromatic acids. Glyphosate inhibits the action of the EPSP synthase enzyme.

Glyphosate is applied to the leaf tissue by spraying, absorbed through the cuticle, and transported to the plant's root system through the phloem. Gradual inhibition of the shikimate synthesis pathway causes the plant to die. Glyphosate is rapidly degraded in the soil, mainly by microorganisms. Its penetration into groundwater is also minimal. However, due to the tremendous use of glyphosate in agriculture, releases to groundwater have been recorded in recent years. Since animals do not use the shikimate metabolic pathway, glyphosate does not affect them.
Nevertheless, some animal studies have shown that it can act as an endocrine disruptor. For this reason, it has been included in the group of potentially carcinogenic substances by the Agency for Research on Cancer (IARC). Part of the professional public cannot identify with this strict classification.

Initially, two strategies were proposed to produce glyphosate-resistant transgenic plants:

(a) Enhanced gene expression for EPSP synthase
Applying a strong constitutive promoter for transcription of *EPSP* synthase gene was expected to result in EPSP overproduction in transgenic plants. *EPSP* synthase genes isolated from plant species such as *Petunia hybrida* or *Arabidopsis thaliana* were used for cloning.
(b) Applying such gene for *EPSP* synthase for transgenesis, the product of which shows lower susceptibility to the herbicide glyphosate

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It was found that alteration of only one amino acid makes this enzyme insensitive to glyphosate. The first such mutant gene was isolated from *Salmonella typhimurium.*

Both strategies resulted in increased tolerance to glyphosate compared to non-transgenic plants, but the degree of tolerance was insufficient to allow these plants to be used in agricultural practice. Such transgenic plants sprayed with glyphosate showed symptoms of partial damage. The problem was that the transferred bacterial mutant EPSP synthase was not present in the chloroplasts, where aromatic amino acids were synthesised. Therefore, a different strategy was used to produce plant expression units, which involved cloning the sequences responsible for targeting the enzyme in chloroplasts and a strong constitutive promoter. However, the lack of resistance remained even after the correct localisation of the corresponding EPSP synthase in chloroplasts. In the case of bacterial EPSP synthase, it was demonstrated that a mutation in the gene sequence resulted in a decreased affinity for glyphosate but also decreased affinity for one of the enzyme substrates phosphoenolpyruvate (PEP). It led to the search for an isoform of the EPSP synthase enzyme that had a low affinity for glyphosate but retained a high affinity for the substrate PEP. It could be achieved either by further mutations in *the EPSP* synthase gene of *S. typhimurium* and subsequent selection of the gene with the appropriate properties or by finding a suitable gene in other organisms. Finally, the gene isolated from *Agrobacterium tumefaciens* strain CP4 was shown to fulfil the required criteria.

The prepared expression unit contained:

- the *EPSP* synthase gene from *A. tumefaciens* strain CP4
- a sequence for the localisation of the EPSP peptide in chloroplasts
- a strong constitutive *CaMV35S* promoter

When this expression unit was introduced into crops, it provided sufficient resistance to glyphosate, even under field conditions. Transformation of monocotyledonous plants uses a fusion of the *EPSP* synthase gene from *A. tumefaciens* strain CP4 with other promoters that function in monocotyledonous plants.

The first Roundup-ready ® soybean plants were tested by Monsanto in field trials in the United States in 1991. Ten years later, they accounted for 70% of soybean production in the United States. In addition to herbicide-resistant transgenic soybean, herbicide-resistant transgenic corn, cotton, and canola were produced.

4.1.2 Glufosinate resistance

The second most commonly used non-selective herbicide is glufosinate which kills many weed species but not as many as glyphosate.

This compound irreversibly inhibits glutamine synthetase (Figure 4.2), an enzyme necessary for glutamine production and ammonia detoxification. Application of glufosinate to plants leads to reduced glutamine and elevated ammonia levels in tissues, halting photosynthesis and resulting in plant death.

Glufosinate (phosphinothricin) was initially discovered as an antibiotic produced by *Streptomycetes fungi*. In general, organisms that produce antibiotics also produce substances that protect them from the toxic effects of antibiotics. The preservative is the enzyme PAT

(phosphinothricin acetyltransferase) encoded by *bar* gene, which acetylates phosphinothricin, thereby inactivating it. This knowledge led to developing a strategy to use suitable resistance in plants.

The *bar* gene for the synthesis of PAT, isolated from *S. fungus* and fused to an appropriate promoter, was introduced by genetic transformation into crops such as corn, oilseed rape and rice. These are currently sold under the brand name Liberty Link by Bayer. Trade names for the herbicide glufosinate are Liberty, Ignate, or Basta.

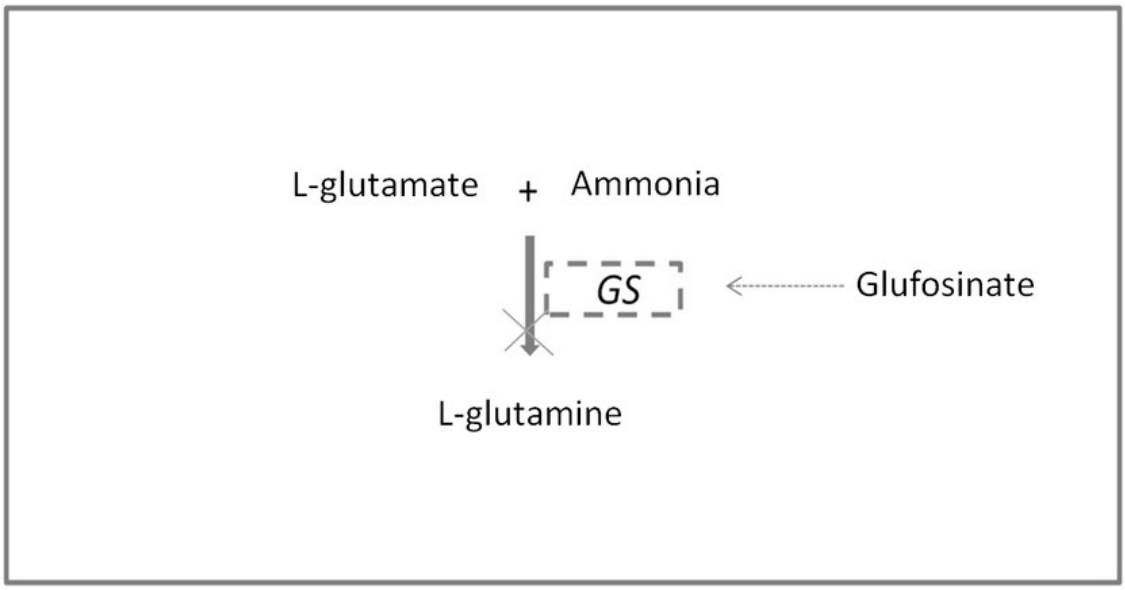

Figure 4.2 Plants detoxify ammonia in the metabolic pathway in the presence of L-glutamate, with which ammonia reacts in the presence of glutamine synthetase (GS). When plants are sprayed with glufosinate, the GS is inhibited, and no L-glutamine or derived amino acids are produced in the cell. However, introducing the *bar* gene encoding PAT (phosphinothricin acetyltransferase) into the plant genome abolishes the glufosinate effect.

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4.1.3 Herbicide-tolerant crops and their environmental applications

Currently, genetically modified – herbicide-resistant – crops have both supporters and opponents. The table 4.1 covers the main issues discussed by experts and the public.

Attitudes	
ANTI-GMO	GMO
	A transgenic seed can survive on farmland soil after harvest (e.g., herbicide-resistant crop) and germinate the following year. After applying another crop resistant to the same herbicide, such escaped plants would form undesirable 'weeds'. However, this problem can be solved by proper crop rotation and changing the herbicides used.
Risk of weeds becoming resistant to new types of herbicides.	This risk cannot be avoided entirely, and the problem occurs after a certain time using any herbicide. The solution may be to use a different type of herbicide that kills the resistant and 'classic' weeds. This problem can also be avoided by crop rotation and alternating herbicides.
Transfer herbicide resistance genes from resistant crops to related wild species by horizontal transfer.	Such a phenomenon has already occurred, but there are no concerns about a superweed; it is always possible to use some other type of herbicide
Overuse of the new types of herbicides	The increase is because the novel herbicides can be applied when GM crops are grown during the growing season. Higher yields offset the negative effect of their increased use.
Growth of agricultural area treated with herbicides	It is impossible to grow crops on a large scale today without weed control. The available herbicides are used on up to 97% of the cultivated area.
Growth in the use of one type of herbicide	It refers primarily to Monsanto's glyphosate. Although Monsanto is the leading company bringing herbicide-resistant crops to market, other biotechnology companies are now promoting their products.
Impact of herbicide-resistant crops and new herbicides on the environment	A rigorous approval process precedes the marketing of new biotechnology products by the relevant authorities, which assess any potential risks to human health or the environment. Toxicity is assessed both before

	the products are placed on the market and during their use.
Impact of herbicide-resistant crops on biodiversity	In cultivated fields the main object is to have only a cultivated crop. For this reason, herbicides are used in agricultural practice.
The use of new herbicides in the cultivation of herbicide-resistant crops and their impact on the increase of pesticide residues in food	When a novel herbicide is introduced to the market, all risks associated with its use in practice are rigorously assessed. The vast majority of herbicides associated with the cultivation of herbicide-resistant crops are considered to be more environmentally safe than the herbicides commonly used in the past.

Table 4.1 Attidudes towards GMOs

4.2 Genetically modified crops resistant to insect pests

Pest insects cause enormous damage to crops and also to ornamental plants. They can damage plants during germination, vegetative growth and generative reproduction - fruit and seed production. Insect destroys above-ground organs such as leaves, stems, and the tissues of generative organs; and the root system and storage organs such as tubers. During crop storage, damage can also occur (insect eggs laid in the seeds).

The current options for plant protection against insect pests are as follows:

- reduction of insect populations by sowing seeds when the insects do not lay eggs
- biological control, e.g., by application of insect predators or pheromone-impregnated plates
- chemical control, e.g., by application of synthetic chemical insecticides
- genetic resistance acquired through traditional breeding
- genetic resistance acquired through genetic engineered strategy

Although the application of insecticides is the most commonly used approach to crop protection, it has its limitations because:

- repeated application of insecticides shortens the life of the plant
- spraying is sometimes challenging to reach the damaged part of the plant
- insecticides also kill beneficial non-target insects
- insects can develop resistance to insecticides
- insecticides damage the environment

Conventional plant breeding is one of the long-used methods for acquiring resistance to plant pests. The difficulties associated with this process are related to:

- the search for new sources of plant resistance
- the long period needed to acquire resistance

- the possible adaptation of insects to the genetically modified crop

Recombinant DNA technologies have made tremendous progress in producing transgenic plants with various genes encoding insecticidal proteins that confer resistance to pests.

4.2.1 Insecticidal proteins in plants

One of the sources of genes that can make plants resistant to insects is the bacterium *Bacillus thuringiensis* ssp. kurstaki, which produces Cry toxins. One of the characteristic features of this bacterium is that it forms spores under conditions unfavourable for its growth. The spores have a crystalline structure consisting of a small number of proteins (usually two or three) with insecticidal activity. Originally, extracts from this bacterium were massively applied to plants as an effective biopesticide that kills pests because of its ability to interact with the cell membrane of the insect intestine, where it acts as a cation channel (disrupting the osmotic balance in the cell). The particular Cry toxins have binding sites that are often specific for individual species or orders of insect organisms, but none of them can bind to mammalian cells. They are formed in an inactive form as pro-toxins and only become active in the insect gastrointestinal tract due to the pH of the environment.

In pest control, Cry toxins can be applied directly to plants as pure proteins. The bacteria grow in bioreactors where spores are produced. These are part of biopesticides such as the commercially known Biobit XL. However, they have a time-limited efficiency that depends on the weather.

Another way to increase resistance is to exploit the expression of genes for *Bt* toxins in transgenic plants, which have been in the pipeline since the 1990s. The first transgenic tobacco plants contained the *Cry*1A gene from *B. thuringiensis* under the control of the constitutive *CaMV35S* promoter. However, these plants were found to have low transgene expression and weak protection against insect pests. A synthetic *Cry* gene that was introduced into chloroplasts is now being used to achieve optimal plant protection. The advantage is that the chloroplasts are not present in the pollen, preventing the spread of resistance in nature. For commercial production was approved transgenic insect-resistant Bt cotton (*Gossypium* sp.) that is resistant to the bollworm (*Pectinophora gossypiella*) through recombinant production of Cry1AC. This pest eats not-engineered cotton from the inside and destroys the entire cotton crop. In India, people have had good experience with growing Bt cotton. Instead of using tons of insecticides, Bt cotton was grown on 12 million hectares, making India the world leader in exports. Similar good experiences have been made with the cultivation of Bt corn, which has become resistant to the corn borer (*Ostrinia nubilalis*) from the Lepidoptera order. The larvae of this butterfly mechanically damage the plants, and in addition, the damaged areas become susceptible to other, mainly fungal, diseases. Transgenic corn that produces the Cry toxin has higher yields and usually does not require the use of additional insecticides.

Benefits arising from the use of transgenic plants with Bt pest resistance:

- reduction of pesticide application
- weather-independent control efficiency
- protection of all parts of the plant
- bio-degradability of Bt toxin
- non-toxicity of Bt toxin to humans

The potential problem of developing resistance to the Bt toxin in other insect populations is being discussed. Research has shown that resistance does not develop faster than resistance to older types of pesticides. Preventive measures to slow the emergence of resistance include

growing crops containing more *Cry* genes. The onset of resistance is predicted to take twice as long with two *Cry* genes. Other measures to prevent the onset of resistance include growing non-transgenic crops on 5% of the area sown with the transgenic equivalent. In addition, buffer strips of non-transgenic crops prevent the unwanted spread of pollen.

Nevertheless, crossing transgenic and non-transgenic crops cannot be avoided entirely. For this reason, the cultivation of Bt cotton has been banned in some US states.

An alternative to Cry proteins is an application of ***vegetative insecticidal proteins*** (VIPs), also derived from *B. thuringiensis* spores. They have a similar effect to the better-known Cry proteins but have different binding sites and a faster onset of action. Genes for Cry proteins are often introduced into plants together with genes for VIP proteins. Furthermore, the transgenic plants with genes expressing ***inhibitors of the insect digestive enzymes proteases and amylases*** are currently under research.

4.3 Genetically modified virus-resistant plants

The most numerous groups of viruses that infect plants are RNA viruses. Only a few plant viruses are DNA viruses, including, for example, cauliflower mosaic virus (CaMV), from which the *CaMV35S* promoter was derived and which is frequently used in transgenesis. Plant viruses have a simple structure. The viral particle of a plant virus consists of the one or two coat proteins (CP) that protect the RNA or DNA genome. The role of coat proteins is to release the genetic information in the form of RNA (DNA in the case of DNA viruses) once it enters the plant cell. Subsequently, viral genes are expressed in the cell, and the viral RNA or DNA is replicated, resulting in new virus particles.

The development of the strategy for producing transgenic virus-resistant plants was based on the observation that plants infected with a strain of virus that causes only mild symptoms on the plant are subsequently protected against a more virulent type of virus. It is known as cross-protection. The protection was thought to be caused by some components produced by the virus. The question remained about how this protection works since plants have no immune system. One hypothesis is that the first stage of infection (unpacking of the virus after entering the cell) is blocked because the viral coat protein is already present in the cell.

The first transgenic plants produced based on such a strategy were tobacco plants expressing the coat protein of tobacco mosaic virus (TMV). Plants exposed to the virus showed no disease symptoms, suggesting that they were resistant to infection with the virus. However, following exposure to a high dose of the virus, disease symptoms occurred, but to a lesser extent than in non-transformed plants. The principle of this method has been successfully tested with different viruses and on different crops. One of the most compelling cases is papaya (*Carica papaya*), grown in the Hawaiian Islands. In the 1990s, a disease caused by the *papaya ringspot virus* (PRSV) spread in the main growing areas and caused a significant decline in production. The problem was solved by developing a resistant genotype carrying the gene for the virus's coat protein. This variety, named Rainbow, now accounts for 80% of papaya production in the Hawaiian Islands. Despite some speculation that the viral coat protein could cause allergic reactions in susceptible individuals, these concerns did not come true. However, the spread of GM pollen to non-transgenic individuals remains a significant concern in this case. In addition to the abovementioned examples, transgenic varieties of pumpkin (*Cucurbita pepo*) and potato (*Solanum tuberosum*) with such resistance are currently available.

4.4 Genetic manipulation for improved nutritional quality in rice – Golden Rice

In developing countries, rice is the most important staple food. Rice plants possess the whole machinery to synthesise β-carotene, and while this machinery is fully active in leaves, parts of it are turned off in the grain. Dependence on rice as the predominant food source leads to childhood blindness. Therefore, a large European project was launched to ensure the production of ß-carotene in rice grains. The following genes were transferred into the rice genome:

- a gene for phytoene synthase (*psy*) isolated from daffodil (*Narcissus*) that was fused to a promoter that allows expression in the endosperm
- a bacterial gene for carotene desaturase (*crtI*) from *Erwinia uredovora* under the control of the *CaMV 35S* promoter

The transfer of these genes into the rice genome has resulted in transgenic rice with a yellow endosperm colour, giving it the name Golden Rice (Figure 4.3). The first variety of Golden Rice appeared in Swiss laboratories in the year 2000. However, this variety did not provide the required amount of ß-carotene that a child should consume daily. Therefore, British scientists have successfully modified a new variety of Golden Rice 2, which contains many times more ß-carotene than before (it is about 37 mg/g of rice, originally 1.6 mg/g). It was achieved by replacing the gene from daffodil with an analogue from corn. Clinical and nutritional studies preceded possible commercialisation. Until 2018, Golden Rice 2 was not marketed due to intense pressure from environmental organisations. On the other hand, the scientific community has strongly supported its cultivation, as 113 Nobel laureates signed the memorandum for its support.

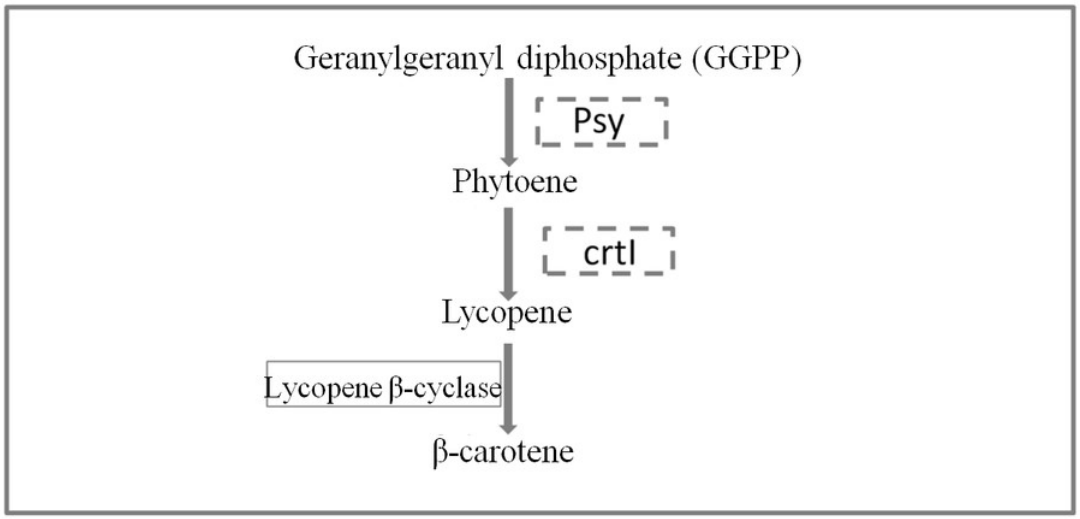

Figure 4.3 Illustration of the pathway of β-carotene synthesis from precursor (GGDP) using transgenic phytoene synthase (*psy*) and transgenic carotene desaturase *(crtI*) in rice endosperm.

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4.5 Genetic manipulation for quality characteristics improvement in crops - Tomato FlavrSavr with a prolonged shelf life

As mentioned earlier, the FlavrSavr tomato was the first genetically modified product (1994). Its genetic modification resulted in a slower ripening of the fruit and thus a longer shelf life after harvest. There are several reasons why this particular crop was chosen for genetic modification to change the quality of the product.

The reasons are as follows:

- tomato belongs to the Solanaceae family and undergoes a relatively easy genetic transformation
- the market for tomatoes is large, so extending the shelf life of the fruit after harvest would be economically efficient
- genes associated with slowing or stopping fruit ripening have been already identified

Ripening of unripe tomato fruit is associated with the following processes*:*

- increased ethylene formation, increased respiration, synthesis of carotenoids – lycopene, β-carotene, softening of the fruit, conversion of starch into sugar and a consequent change in taste
- The cell wall of the fruit consists of cellulose fibres, pectins, hemicelluloses and proteins
  As the fruit softens, the specific bonds between these components are broken down. The cleavage is carried out by cellulases, which degrade cellulose, polygalacturonases (PG) and pectin methylesterases (PME), which degrade pectin molecules. These enzymes are considered key to the ripening and softening of the fruit.

Calgene has proposed to reduce the expression of polygalacturonase (PG) by so-called "antisense" technology. The isolated tomato gene for polygalacturonase was fused to a promoter active in fruit to produce an 'antisense' mRNA after transcription in plants. A vector containing such an expression unit was used to transform the tomato. The delay in fruit softening was confirmed in transgenic tomato plants. Other biotechnology companies have also developed other methods to extend the shelf life of tomato fruit, for example, by reducing ethylene formation, which can be achieved by suppressing the expression of the individual components of the biosynthetic pathway leading to ethylene synthesis.

4.6 Other applications of plant transgenesis

Genetically modified plants may not only directly or indirectly serve to feed the world population but also produce therapeutically important proteins. Transgenic plants can be grown to produce cytokinins, hormones, enzymes, epidermal growth factors, interferons, vaccines, and antibodies. There are two ways to produce these substances. Genetic transformation by *A. tumefaciens* can result in stable integration of the relevant transgenes into the genome. The expression of these transgenes can be constitutive or inducible, depending on the promoter used in the expression units being prepared. For example, the production of antibodies in transgenic

plants could be ensured, cheaper and more accessible to a broad population. Similarly, the production of edible vaccines in plants eliminates the problem of their purification and the costs associated with their transportation and storage. This technology was proposed to produce HIV suppressor proteins in spinach or hepatitis B vaccines in potatoes.

The use of viral vectors, on the other hand, can result in transient expression of transgenes in plants. This way of plant transformation is also attractive from another point of view – a high yield of transgenic protein can be obtained quickly (the plants are used as bioreactors). Moreover, pharmaceutical products obtained in this way are not contaminated with human viruses, oncogenes or bacterial toxins.

Materials such as the polymer *PHB (polyhydroxybutyrate*) can also be produced in transgenic plants. The presence of such a polymer in transgenic cotton could improve the thermal and elastic properties of cotton fibres, and the material would be biodegradable at the same time. Another application of transgenesis is the production of modified oils in plants. Transgenic canola plants produce oils with a shorter carbon chain (lauric acid) due to transferring the gene for *acyl-ACP thioesterase* from rats to canola. The lauric acid dominated oils are helpful for the production of soaps and detergents.

In this chapter, we have focused only on describing the primary examples of the use of plant transgenesis, especially those that are already part of agricultural practice in the world, and we have mentioned only very briefly examples of some of the current trends since the treatment of this subject in this textbook is limited.

5 LEGISLATION

Genetic technologies include the activities of genetic engineering and modern biotechnology with the aim to create and use genetically modified organisms, including microorganisms, their parts and production. Their goal is to interfere with the natural genetic information of organisms. Recombinant DNA techniques therefore represent a set of work procedures that are necessary for the preparation, analysis and transformation of recombinant DNA. Recombinant DNA (rDNA) has been introduced in this context, whose primary structure is changed by incorporating a section of DNA from an unrelated organism. Thus, a genetically modified organism (GMO) is an organism whose genetic material has been changed in a way that does not occur naturally during sexual reproduction and natural recombination. It is a new and revolutionary research approach, which is applied both theoretically and practically in biology.

In practical terms, genetic technologies began to be widely implemented on the verge of the millennium especially in the pharmaceutical industry, agriculture and food industry. This fact led to the need to standardize the use of genetic modifications, both in the direction of establishing rules for the use of genetic methods and genetic techniques on living organisms in research and for the mass use of genetically modified organisms in everyday life, as well as by introducing normative protection against the misuse of genetic technologies to endanger humanity and the environment, especially biological diversity.

The issue of genetically modified organisms and the closely related biological safety gradually became the subject of regulatory efforts not only in the individual countries, but also in multinational structures and organizations, such as the European Union, FAO, OECD and others. In essence, the aim was to ensure the protection of human, animal and plant health, prevent damage to the environment and, based on free access to information, to convince the general public of the safety and acceptability of new biotechnologies.

The researchers themselves were the first ones to point out the risks of using the rDNA methods shortly after Cohen et al. (1973) published their principle. In 1975, they adopted the rules for the use of these techniques on their own initiative at a conference in California. A year later, the National Institute of Health (NIH – the largest grant agency covering public biomedical research in the USA) issued binding rules for the use of rDNA methods in projects funded by this institution. These rules (NIH Guidelines, 2001) were gradually adopted by all laboratories. The NIH Guidelines later became the basis for the creation of legislation that currently controls both the work and the use of GMOs.

From the point of view of the approach to the legal regulation of biological safety, the countries can be divided into three groups.

- The first group includes countries such as the USA, Canada, Argentina, Brazil, Chile and Australia in which no special laws have been developed for work with GMOs and the application of new biotechnologies. The individual institutions are governed by generally binding legislation.
- The second group of countries mainly includes the states of the European Union and the countries in the EU accession process. As part of the EU legislative process, the so-called directives (guidelines), which are binding as regards the resulting objective, have been implemented, but each member state can decide on the way in which the intended effect is achieved.
- The third group of countries is primarily represented by developing countries with economic problems that force them to take a pragmatic stance on the compromise between environmental protection and the necessity to tackle food shortages. In these countries, as a rule, the necessary capacities for a safe handling of genetically modified organisms do

not exist, and other applicable regulations that could represent the relevant legislation are often not available.

In 1995, at the second Conference of the Parties to the Convention on Biological Safety, a resolution was adopted on the need to develop a document addressing the issue of biological safety. The basic goal was to achieve the regulation of all activities related to the existence of GMOs, ranging from their development, through biotechnological use, to marketing.

The first meeting of experts was held in Aarhus, Denmark, in June 1996, in which representatives of 92 countries of the world, including Slovakia, were present. Despite intensive negotiations and the efforts of the majority of participants, it was not possible to reach a consensus on the wording of several parts of the draft at the three subsequent expert meetings in Montreal (May and October 1997, February 1998).

The entire process of drafting the Protocol was affected by interest groups and it culminated in the sixth meeting of experts in Cartagena, Colombia, in February 1999 and the subsequent First Extraordinary Conference of the Parties to the Convention on Biological Diversity. The final agreement on the wording of the draft Cartagena Protocol on Biosafety was only reached during the second part of the Extraordinary Conference of the contracting states of the Convention in Montreal in January 2000.

The main goal of the Biosafety Protocol is to contribute to ensuring the protection against the possible negative impacts of genetically modified organisms on the environment and human health. The Protocol applies to the handling, transport and use of GMOs, with the exception of those intended for pharmaceutical purposes. The Protocol determines the conditions and mechanisms of transporting GMOs across the state borders in some detail. It defines the exporter and importer as a legal or natural person who is subject to the jurisdiction of its own contracting party, that is, in the case of the exporter, the laws of the exporting country, and in the case of imports, the laws of the importing country. The Protocol was submitted for sign-off at the Fifth Conference of the Parties to the Convention on Biological Diversity in Nairobi, Kenya, in May 2000. The material is still subject to approval by the competent authorities in the individual countries, i.e. the governments, parliaments and heads of state.

In the USA, the safety of products made of transgenic organisms – both for food and pharmaceutical purposes – is guaranteed by the Food and Drug Administration (FDA), which issued the first permits for three transgenic products, namely Savr Flavr tomatoes, Bt-corn and RoundUp cotton, in 1994. The FDA also issues approvals for the use of drugs produced from transgenic organisms. Recombinant insulin – Humulin – was the first drug to be approved in this category, and in 1998 an approval was issued for the first human therapy using antisense RNA.

In 1990, two directives were issued in the countries of the European Union (EU): one for the handling of genetically modified microorganisms in closed systems (Council Directive 90/219/EEC on the contained use of genetically modified microorganisms), the other for the release of GMOs into the environment (Council Directive 90/220/EEC on the deliberate release into the environment of genetically modified organisms).

The EU directives and regulations are binding in their entirety, they have general validity, are directly applicable in all Member States, but the selection of relevant forms depends on the national authority in each country. The decisions are binding in their entirety for all parties concerned and do not require implementation into national laws.

The law regulates the rights and obligations of the users of genetic technologies and genetically modified organisms and the competence of state administration bodies.

In the Slovak Republic, Directive 90/219/EHS, 98/81/EC and 2001/18/EC was transformed into Act no. 151/2002 Coll. on the use of genetic technology and genetically modified organisms, which was passed by the National Council of the Slovak Republic on February 19, 2002, and the subsequent Decree of the Ministry of the Environment of the Slovak

Republic, which implements this Act (Decree No. 252/2002 Coll.). Act no. 151/2002 Coll. was amended by Act no. 587/2004 Coll., Act no. 77/2005 Coll., Act no. 100/2008 Coll., Act no. 515/2008 Coll., Act no. 117/2010 Coll. and Act no. 448/2012 Coll. In 2005, Decree No. 252/2002 Coll. was canceled and replaced by a new Decree of the Ministry of the Interior of the Slovak Republic no. 399/2005 Coll., which was amended in 2008 by Decree No. 312/2008 Coll. and Decree no. 86/2013 Coll. In 2019, the Decree of the Ministry of the Interior of the Slovak Republic No. 399/2005 Coll. was canceled and replaced by the Decree of the Ministry of the Interior of the Slovak Republic no. 274/2019 Coll. with effect from 15.9.2019.

In 2006, a new Act no. 184/2006 Coll. on the cultivation of genetically modified plants in agricultural production was passed and entered into force on 01.06.2006. It is implemented by Decree no. 69/2007 Coll. with effect from 14.2.2007. Act no. 184/2006 Coll. was later amended by Act no. 78/2008 Coll. with effect from 1.4.2008.

Imported GMOs intended for the production of food or feed can only be grown and sold in the EU with a permit, which is granted on the basis of a scientific risk assessment.

In 2013, the only GM crop grown in the EU was the MON 810 maize, which was authorized in 1998. Currently, its authorization is pending renewal. In 2013, it was still cultivated mainly in Spain on an area of 137 thous. ha, and to a lesser extent in Portugal, Czech Republic, Romania and Slovakia. Since 2017, GM corn has not been grown in Slovakia. In 2013, GMOs were banned in eight countries – Germany, Austria, Bulgaria, Luxembourg, Poland, Hungary, Greece and Italy. After April 2015, this list was expanded to include other countries. Eight applications for the cultivation of GMOs in the EU are currently being reviewed, including an application for the renewal of the MON 810 permit. The register of GMOs authorized in the EU can be found on the European Commission (URL8) website. In 2020, several foods, food additives and feeds made from genetically modified corn, soy, rapeseed and cotton were authorized in the EU.

Altogether 58 imported GMOs that are used in food or animal feed production are authorized to be sold in the EU. These mainly include corn, cotton, soybeans, rapeseed oil and sugar beet. A total of 58 other crops are awaiting authorization. In 2013, 36 million tonnes of soybean equivalent were used in the EU to feed livestock. Only 1.4 tons of this amount came from the EU, including traditional unmodified soy. These figures show that the EU is dependent on the imports from third countries in this area.

6 ADVANCED PLANT BIOTECHNOLOGY, GENOME EDITING AND OTHER NEW TECHNIQUES

Breeding for crop improvement is an important way of addressing the food shortage challenges posed by rapid population growth and it is also considered an important scientific issue. Plant domestication has taken place over thousands of years mainly through the accumulation of favourable mutations in natural variation, which is a long and completely uncontrollable process. In order to obtain the superior traits, new mutations have been introduced in the last century by chemical or radiation mutagenesis and a number of varieties with improved forms have been developed. However, identifying the mutation of interest is a lengthy and laborious process that has seriously slowed down the breeding rate. The methods and approaches to genetic plant breeding have expanded with the development of biotechnology in recent decades.

Conventional approaches to genetic engineering involve lengthy procedures. State-of-the-art OMICS approaches enhanced with the use of next-generation sequencing and the latest developments in genome editing tools have opened the way for targeted mutagenesis and new horizons for precision genome engineering.

The discovery of programmed sequence-specific nucleases (SSNs) has facilitated precise gene editing. The application of SSNs for accurate gene editing has been recognized in plant and animal systems as a breakthrough in genome engineering. Compared to transgenic strategies, which result in inadvertent gene insertions and sometimes random phenotypical characters, the gene editing approaches produce well-defined mutants, proving that gene editing is a powerful technique for plant breeding and functional genomics. In contrast to transgenic plants, genome-edited plants have the added benefit of site specificity. These improved plants can be proven useful in breeding programs and the subsequent species can be employed reliably with less concerns, and less extensive monitoring methods are needed in contrast to traditional genetically engineered plants.

Various genome editing tools, such as transcription activator-like nucleases (TALENs), zinc-finger nucleases (ZFNs) and meganucleases (MNs), have enabled the plant scientists to manipulate the desired genes in crop plants. These methods for targeting the DNA double-stranded breaks-inducing nucleases to specific genomic sites relied on protein-based systems with customizable DNA-binding specificities, such as meganucleases, zinc finger nucleases (ZFNs) and transcription activator–like effector nucleases (TALENs). In contrast to ZFN and TALEN methods, which use protein-DNA interactions for targeting, the RNA-guided nucleases (RGNs) use simple, base-pairing rules between an engineered RNA and the target DNA site. The ZFNs and TALENs are artificial fusion proteins composed of an engineered DNA binding domain fused to a nonspecific nuclease domain from the *FokI* restriction enzyme.

However, these approaches, also called first-generation genome editing tools, are expensive and laborious and they involve complex procedures for successful editing.

In contrast, CRISPR/Cas9 is an interesting, easy to design, cost-effective and versatile tool for precise and efficient genome editing in plants. In recent years, the CRISPR/Cas9 system has become a powerful tool for targeted mutagenesis, including single base substitution, multiplex gene editing, gene knockout and regulation of gene transcription in plants. Thus, CRISPR/Cas9-based genome editing has shown great potential for crop improvement, however, the regulation of crops with genome editing is still the inception phase. There are two main components of a CRISPR/Cas9 system: a single guide RNA (sgRNA) that identifies a specific DNA sequence and the Cas9 protein which produces DSBs at a targeted site.

Increasing agricultural productivity through modern breeding strategies is one of the main strategies for achieving global food security. A number of biotic and abiotic stresses affect

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the productivity and quality of crops, and therefore a primary need has arisen to develop crops with better adaptability, high productivity and resistance to these biotic/abiotic stresses.

CRISPR/Cas9 is the most powerful tool for crop improvement. In many crops it has been applied emphatically over the last five years against the abiotic and biotic stressors and to improve other agronomic traits. As a gene editing technology for site-direct mutagenesis, CRISPR/Cas9 has many excellent characteristics, including great target specificity, ease of execution and low cost, which are unattainable in conventional mutagenic strategies.

Remarkable progress has been made in the CRISPR/Cas9 toolbox to increase the targeted mutagenesis with increased efficiency via base editing, multiplex gene editing and generation of DNA-free plants. CRSIPR/Cas9 is a versatile tool for plant gene editing thanks to its sophisticated toolbox of Cas9 variants, such as the CRISPR/Cpf1 system and online accessible bioinformatics tools for designing highly precise delivery systems. The precise CRISPR/Cas9-based gene editing produces gene replacement, gene insertion and knockout mutations that are rapidly being used to increase yield, improve quality and enhance tolerance in crops to boost crop domestication and hybrid breeding.

In recent years, CRISPR has developed rapidly at an unprecedented rate, and has become popular for its high specificity and high efficiency to modify specific locus, paving the way for targeted gene editing and breeding in crops. The implementation of CRISPR/Cas9 gene editing technology results in huge cost reductions in the process to change the plant species, including the reduction in time, manpower and material resources. It also provides more diversified breeding strategies, including base editing.

The CRISPR/Cas9 technology has been used extensively in major crop plants such as wheat, rice, maize, cotton, soybean, tomato, and potato to cope with various abiotic stressors. The development of climate-smart and abiotic stress-tolerant crops via the CRISPR/Cas9 tool has modernized plant breeding programs.

An efficient genome editing method using the CRISPR/Cas9 ribonucleoproteins (RNPs) was used to bread wheat. Deep sequencing reveals that the chances of off-target mutations in wheat cells are much lower in the RNP-mediated genome editing than in CRISPR/Cas9 DNA editing. Because no foreign DNA is used in the CRISPR/Cas9 RNP-mediated genome editing, the resulting mutants are completely transgene-free. This method may be widely used in the production of genome-edited crop plants and has a good prospect of being commercialized.

Although CRISPR/Cas9-mediated gene editing has gained remarkable traction in crop improvement, there are certain challenges that need to be addressed to develop a more efficient system for plant gene editing. This includes assembling the pangenomes for crop improvement, programmed identification of candidate sites for gene editing via functional genomics, introduction of highly efficient delivery systems for gene editing, reducing the frequency of off-target editing, deciphering novel pathways for this reduction, and optimization of the Cas9 function. The major pitfalls of CRISPR/Cas9 include the inefficient delivery system for plant transformation because the current protocols are limited to certain tissues, genotypes and crop varieties. The packaging of Cas proteins into delivery vectors poses large barriers for an efficient delivery of CRISPR/Cas machinery. Recently, some novel cargo-vector systems have been introduced, which have a promising potential for efficient delivery systems. For example, carbon nanotubes have been utilized to transfer the CRISPR/Cas9 editing constructs into plant leaves.

The powerful gene editing capabilities of CRISPR have also enabled us to focus on the use of CRISPR in disease treatment and medical research. The emergence of CRISPR is an unprecedented therapeutic strategy with great potential in the treatment of diseases caused by genetic mutations carried by congenital abnormalities. In particular, the development of the base editor, which can achieve precise editing of a single specific base without causing double-strand breaks in DNA, provides a great strategy for correcting a series of congenital genetic

diseases caused by genetic mutations. In addition, CRISPR has also been widely used in medical research, including the construction of a series of animal models through gene knockout. The gene-based loss of function or gain of function CRISPR screening has been widely used in recent years, which directly leads to the acquisition of many potential drug targets that have not been reached by previous technologies.

Using innovative ideas from systems biology, synthetic biology, next-generation sequencing, and the latest developments in functional genomics integrated with advanced CRISPR/Cas9 tools will enable the development of intelligent crops with higher yields and improved traits. In the near future, CRISPR/Cas9 technology may be integrated with rapid breeding programs that will revolutionize global agriculture and promise to food security.

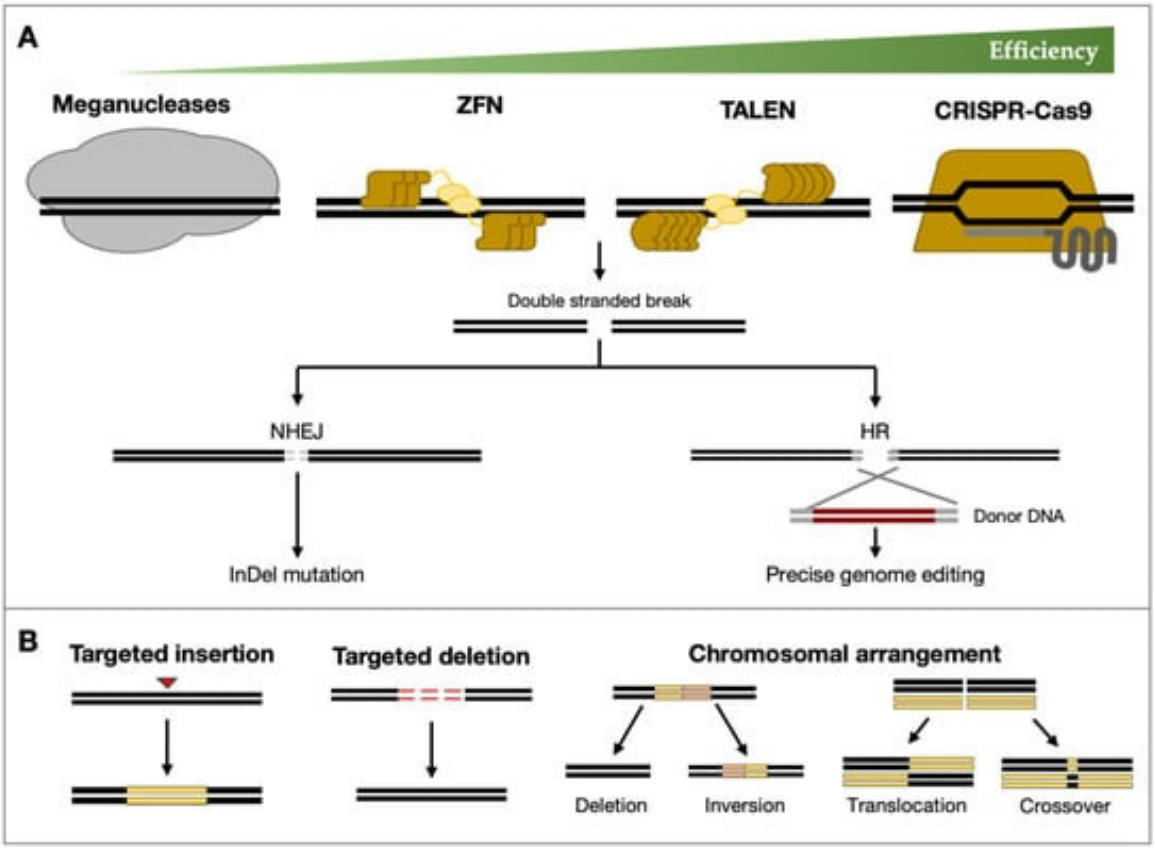

Figure 6.1 Different types of sequence-specific nucleases and types of editing (NHEJ-nonhomologous end joing, HR- Homology Repair) (https://doi.org/10.3390/plants11192625).

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