(54) Title: METHOD FOR STABLE TRANSFORMATION OF PLANTS

(57) Abstract

The present invention relates to a method for producing stably transformed plant material using a specifically designed DNA/protein complex comprising in operable linkage to an expressible DNA a VirD2 protein. The invention further relates to the said DNA/protein complex itself and to the plant material transformed therewith.
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cleavage reaction. If the said substrate involves not the whole but only part of the T-DNA border sequence, it is to be ensured that the said partial sequence still comprises those parts of the T-DNA border sequence that encompasses the recognition and cleavage site of the VirD2 protein.

The chimeric recombinant DNA construct as described above is preferably a single stranded DNA construct. Also comprised within the scope of the invention is a double-stranded molecule with a single-stranded overhang which is a substrate for VirD2 or a chimeric recombinant DNA construct negatively supercoiled (form I) containing border sequences as the preferred substrate for VirD1/VirD2 catalyzed cleavage.

Upon *in vitro* cleavage of the T-DNA border sequence or of functional parts thereof, using the VirD2 protein, the said VirD2 protein remains covalently attached to the cleaved DNA forming the DNA/protein complex according to the invention.

The present invention thus further relates to a method of preparing a DNA/protein complex as described before, comprising:

(a) preparing a chimeric recombinant DNA construct that comprises in operable linkage to an expressible DNA at least one T-DNA border sequence or functional parts thereof, which serves as a substrate in the VirD2 cleavage reaction;

(b) *in vitro* cleaving of the DNA substrate prepared according to step (a) by means of VirD2 protein, which may be accompanied by further Vir proteins such as, for example VirD1 and/or VirE2 and/or any other DNA binding protein, which is able to protect the DNA from nuclease attack.

The substrate to be used in the VirD2 cleavage reaction may contain one or more T-DNA border sequences or one or more functional parts of T-DNA border sequences, which may be of the same or of different specificities. This means, that the substrate may involve one or more DNA sequences with left border specificity or one or more DNA sequences with right border specificity or combinations of DNA sequences with left and right border specificities.

The substrate may involve further DNA sequences such as, for example, overdrive sequences.
METHOD FOR STABLE TRANSFORMATION OF PLANTS

The present invention is in the field of plant genetic engineering. In particular, it relates to a method for producing stably transformed plant material using a specifically designed DNA/protein complex. The invention further relates to the said DNA/protein complex itself and to the plant material transformed therewith.

Techniques for the transformation of monocotyledonous plants involve the use of, for example, particle gun transformation, microinjection and direct gene transfer into both intact cells and protoplasts. However, there is room for improvement in efficiency of all of these monocot transformation techniques.

The Agrobacterium plant transformation system is widely used for the stable transformation of higher plants. In this system genes to be transferred are carried by the T-DNA, a well-defined region of the Agrobacterium Ti plasmid. The Ti plasmid also contains a virulence (vir) region, which encodes proteins involved in the transformation via Agrobacterium of plant cells. At least one of these proteins, VirD2 is involved in targeting to the plant nucleus and integration into the plant genome [Tinland et al, Proc Natl Acad Sci USA 89: 7442-7446, 1992; Mayerhofer et al, EMBO J 10: 697-704 (1991)].

It is, therefore, the main object of the invention to provide a method for producing stably transformed plant material, including phenotypically normal looking and preferably fertile plants, which method does not involve Agrobacterium transformation.

This object could be achieved within the scope of the present invention by using the properties of proteins produced by the vir region such as, for example, VirD1, VirD2 and VirE2, but especially that of the VirD2 proteins, in non-Agrobacterium plant transformation. In particular, a specifically adapted DNA/protein complex is provided comprising a chimeric recombinant DNA, which may comprise, for example, an expressible DNA operably linked to suitable plant expression signals involving promoter and termination sequences and covalently associated therewith a VirD2 protein.

This DNA/protein complex may be obtained by first providing a recombinant DNA construct that comprises in operable linkage to the elements already mentioned above at least one T-DNA border sequence or functional parts thereof as a substrate in the VirD2
Preferred within the invention are DNA substrates comprising either a left or a right border element or functional parts thereof or a combination of left and right border elements or of functional parts thereof, that in addition may be accompanied by one or more overdrive sequences. Also preferred are substrates comprising at least two T-DNA border elements of the same specificity, that is right or left border elements that in addition may be accompanied by one or more overdrive sequences.

The DNA substrates according to the invention preferably involve at least those parts obtainable from T-DNA border sequences that comprise in addition to the cleavage site at least the following core sequence according to SEQ ID NO 1:

\[ \text{ApTpCpKpG} \]

with K being either guanosine or thymidine/uridine; but more preferably the core sequence according to SEQ ID NO 2:

\[ \text{TpApTpCpTpG} \]

the said core sequences immediately preceding the said VirD2 cleavage site;

In a preferred embodiment of the invention the DNA substrate involves a DNA sequence that comprises between 6 and approximately 50 nucleotides, preferably between 10 and approximately 40 nucleotides and which can be described by the following general formula according to SEQ ID NO 3:

\[ (\text{Np})_n \text{ApTpCpKpGp} \uparrow N_m \]

but preferably by the formula according to SEQ ID NO 4:

\[ (\text{Np})_n \text{TpApTpCpTpGp} \uparrow N_m \]

wherein N may be any of the nucleotides selected from the group consisting of adenosine, guanosine, cytidine and thymidine or uridine, K is either guanosine or thymidine/uridine, n is an integer between 0 and 42, preferably between 0 and 25, more preferably between 0
and 14 and m is an integer between 2 and 10, preferably between 3 and 10, more preferably between 4 and 8 and wherein the wedge indicates the position of the cleavage site.

In a further preferred embodiment of the invention the DNA substrate involves a DNA sequence that comprises between 10 and approximately 50 nucleotides, but preferably between 14 and 29 nucleotides and which can be described by the following general formula according to SEQ ID NO 5:

$$(Np)_nRBPpHpYpApTpCpCpKpGp^\triangledown YpMp(N)_m$$

wherein N may be any of the nucleotides selected from the group consisting of adenosine, guanosine, cytidine or thymidine/uridine; B is guanosine or cytidine or thymidine/uridine; H is adenosine or cytidine or thymidine/uridine; M is adenosine or cytidine; R is guanosine or adenosine; Y is thymidine/uridine or cytidine; K is either guanosine or thymidine/uridine; and n is an integer between 0 and 40, but preferably between 2 and 17; and m is an integer between 2 and 10, preferably between 3 and 10, more preferably between 4 and 8 and wherein the wedge indicates the position of the cleavage site.

More preferred is a DNA substrate involving a DNA sequence that comprises between 17 and 25 nucleotides and which can be described by the following general formula according to SEQ ID NO 6:


wherein N may be any of the nucleotides selected from the group consisting of adenosine, guanosine, cytidine and thymidine/uridine; B is guanosine or cytidine or thymidine/uridine; H is adenosine or cytidine or thymidine/uridine; M is adenosine or cytidine; R is guanosine or adenosine; Y is thymidine/uridine or cytidine; and n is an integer between 0 and 12, preferably between 0 and 8; including any DNA sequences that are structurally and/or functionally homologue thereto.

Especially preferred are the following DNA sequences according to SEQ ID NOS 7 to 13:


including any DNA sequences that are structurally and/or functionally homologue thereto.

As used in the present application, substantial sequence homology means close structural relationship between sequences of nucleotides. For example, substantially homologous DNA sequences may be 60% homologous, preferably 80% and most preferably 90% or 95% homologous, or more. Homology also includes a relationship wherein one or several subsequences of nucleotides or amino acids are missing, or subsequences with additional nucleotides or amino acids are interdispersed.

The term "homology" as used herein not only embraces structural homology but also functional homology.

Upon transformation of the DNA/protein complex according to the invention into plant material, the transformation frequency and also the quality of the integrated DNA can be improved considerably. This is especially true with regard to stable transformation events, which occur more frequently as compared to conventional, non-protein associated DNA constructs.

The present invention thus further comprises a method of transforming plant material comprising

(a) preparing a chimeric recombinant DNA construct that comprises in operable linkage to an expressible DNA at least one T-DNA border sequence or functional parts thereof, which serves as a substrate in the VirD2 cleavage reaction, as defined hereinbefore;
(b) \textit{in vitro} cleaving of the DNA substrate prepared according to step (a) by means of VirD2 protein, which may be accompanied by further Vir proteins such as, for example
VirD1 and/or VirE2 and/or any other DNA binding protein, which is able to protect the DNA from nuclease attack;
(c) introducing the thus cleaved DNA comprising at least the VirD2 protein covalently bound to the 5’ end of the cleavage site into the plant material to be transformed by methods known in the art.

The DNA/VirD2 protein complex according to the invention may be accompanied by further Vir proteins, [which, for example, are capable of catalyzing physical changes in the complex] such as, for example, VirE2, which is known to bind to ssDNA, and/or VirD1. VirE2 can be purified by methods known in the art such as those described in Christie et al [J Bacteriol 170(6): 2659-2667 (1988)]. The purification of the VirD1 protein can be achieved according to the method provided in the following examples.

It is advantageous in this connection to obtain the VirD2 protein and optionally further Vir proteins that may be involved in the cleavage reaction, in amounts and in a purity that is suitable for carrying out the method according to the invention. This can be achieved by overproducing the Vir proteins in a suitable host organism and purifying them in a multiple-step procedure.

It is thus a further object of the present invention to provide a method for obtaining VirD2 or VirD1 protein in high amounts and purity, which method comprises

(a) introducing the VirD2 or VirD1 gene in one of the known overproducing plasmids and incorporating said plasmid into a suitable production organism;

(b) isolating the VirD2 or VirD1 protein from the production organism or from the supernatant by high speed centrifugation;

(c) after several washing, centrifugation and solubilization steps involving dialysis, subjecting the resulting VirD2 fraction to affinity and/or ion-exchange chromatography;

(d) removing VirD2 degradation products using gel filtration; and

(e) optionally further purifying the VirD2 protein by size exclusion chromatography.

As mentioned before, the main object of the present invention is a DNA/protein complex
comprising operably linked to an expressible DNA at least one T-DNA border sequence or functional parts thereof and covalently associated therewith a VirD2 protein, which complex can be suitably used in a process for transforming DNA into plant material. The DNA/VirD2 protein complex may contain non-covalently associated further Vir proteins such as, for example, VirD1 and/or VirE2.

The DNA to be used in the process according to the invention for transforming plant material may be either of homologous or heterologous origin with respect to the plant material involved or it may be of synthetic origin or both.

The coding DNA sequence can be constructed exclusively from genomic DNA, from cDNA or from synthetic DNA. Another possibility is the construction of a hybrid DNA sequence consisting of both cDNA and genomic DNA and/or synthetic DNA.

The cDNA may originate from the same gene as the genomic DNA, or alternatively both the cDNA and the genomic DNA may originate from different genes. In any case, however, both the genomic DNA and/or the cDNA may each be prepared individually from the same or from different genes.

Synthetic DNA is to be understood as comprising DNA sequences that have been been prepared entirely or at least partially by chemical means. Synthetic DNA sequences may be suitably used, for example, for modifying native DNA sequences in terms of codon usage, expression efficiency, etc. Examples of synthetic genes include the PAT gene and the endotoxin genes of Bacillus thuringiensis.

If the DNA sequence to be transformed into the recipient plant material contains portions of more than one gene, these genes may originate from one and the same organism, from several organisms that belong to more than one strain, one variety or one species of the same genus, or from organisms that belong to more than one genus of the same or of another taxonomic unit (kingdom).

Chimaeric recombinant DNA molecules that comprise an expressible DNA, but especially a structural gene, preferably a heterologous structural gene operably linked with expression signals active in plant cells, such as promoter and termination sequences, as well as, optionally, with further coding and/or non-coding sequences of the 5' and/or 3' region may also be preferably used within the transformation process as part of the
DNA/protein complex according to the present invention.

Especially suitable for use in the process according to the invention are all those structural genes which upon expression lead to a protective effect in the transformed plant cells, also in the tissues developing therefrom and especially in the regenerated plants, for example increased resistance to pathogens (for example to phytopathogenic fungi, bacteria, viruses, etc.); resistance to chemicals [for example to herbicides (e.g. triazines, sulfonylureas, imidazolinones, triazole pyrimidines, bialaphos, glyphosate, etc.), insecticides or other biocides]; resistance to adverse environmental factors (for example to heat, cold, wind, adverse soil conditions, moisture, dryness, etc.).

Within the scope of this invention, special mention is to be made of structural genes that are associated with the control of plant pathogens and parasites.

Resistance to insects can be conferred, for example, by a gene coding for a polypeptide that is toxic to insects and/or their larvae, for example the crystalline protein of Bacillus thuringiensis [B.t.]. Especially suitable are synthetic B.t. genes such as those disclosed in, for example, Koziel M.G. et al, Bio/Technology 11: 194-200 (1993).

A second class of proteins mediating resistance to insects comprises the protease inhibitors. Protease inhibitors are a normal constituent of plant storage structures and are therefore normally located in vacuoles or protein bodies. It has been demonstrated that a Bowman-Birk protease inhibitor isolated from soybeans and purified inhibits the intestinal protease of Tenebrio larvae. The gene that codes for the trypsin inhibitor from the cowpea is described in Hilder et al (1987).

The majority of insects, for example, have a cuticular skeleton in which chitin micelles in lamellar layers are embedded in a base substance. A great many phytopathogenic fungi also contain chitin as an integral part of their hypha and spore structures, for example Basidiomycetes (smut and rust fungi), Ascomycetes and Fungi imperfecti (including Alternaria and Bipolaris, Exerophilum turicum, Colletotricum, Gleocercospora and Cercospora). Chitinase is capable of inhibiting the mycelial growth of certain pathogens both in vitro and in vivo. A plant organ or tissue that is capable of expressing chitinase constitutively or in response to the penetration of a pathogen can therefore protect itself from attack by a large number of different fungi.
A further gene, which encodes an enzyme which presumably plays a central role in the plant’s defence mechanism against pathogens is the β-1,3-glucanase gene, that may thus also be used for protecting plants against a fungal attack, alone or in combination with a chitinase gene.

A further class of genes that may be used within the scope of this invention are those coding for the so-called lytic peptides. These are natural or synthetic peptides having anti-pathogenic activity which are capable of penetrating, lysing or otherwise damaging the cell membrane of pathogens. Representatives of such lytic peptides that may be used within the scope of the present invention are known both from animal sources [including insects] and from plant and microbial sources and include, for example, the defensins, cecropins, thionins and mellitins of mammals, and the defensins, magainins, attacins, dipterins, sapecins, caerulins and xenopsins of insects, and hybrids thereof. The amino acid sequences of various lytic peptides are shown in the following publications: WO 89/11291; WO 86/04356; WO 88/05826; US 4,810,777; WO 89/04371.

Lytic peptides in the broadest sense of the term are also to be understood as being compounds whose ability to penetrate, lyse or damage cell membranes is based on enzymatic activity, for example lysozymes and phospholipases.

Moreover, reciprocal use of expression and exogenous application may also be envisaged, the lytic peptides especially being suitable for the latter purpose, in conjunction with the auxiliaries and/or additives customarily used for this purpose.

Another class of genes that may be used in the scope of the present invention are those coding for phospholipid transfer proteins disclosed, for example, in WO 92/20801.

A further class of genes that may be used within the scope of the present invention comprises genes which encode pathogenesis-related proteins [PRPs] such as PR-1A, PR-1B, PR-1C, PR-R major, PR-R minor, PR-P, PR-Q, PR-2, PR-2’, PR-2”, PR-N, PR-O, PR-O’, PR-4, SAR8.2a-e, cucumber chitinase/lysozyme, cucumber basic peroxidase, tobacco basic glucanase and tobacco basic chitinase/lysozyme, tobacco acidic chitinase/lysozyme. Examples of the above genes and proteins including chimeric genetic constructs comprising the said genes are provided in EP-A 392,225.

The DNA sequence according to the invention can also be used for the production of
desirable and useful compounds in the plant cell as such or as part of a unit of higher organisation, for example a tissue, callus, organ, embryo or a whole plant.

Genes that may also be used within the scope of the present invention include, for example, those which lead to increased or decreased formation of reserve or stored substances in leaves, seeds, tubers, roots, stems, etc. or in the protein bodies of seeds. The desirable substances that can be produced by transgenic plants include, for example, proteins, carbohydrates, amino acids, vitamins, alkaloids, flavins, perfumes, colourings, fats, etc..

There may also be associated with the DNA sequence according to the invention structural genes that code for pharmaceutically acceptable active substances, for example hormones, immunomodulators and other physiologically active substances.

The genes that can come into consideration within the scope of this invention therefore include, but are not limited to, for example, plant-specific genes, such as the zein gene from maize, the avenin gene from oats, the glutelin gene from rice, etc., mammal-specific genes, such as the insulin gene, the somatostatin gene, the interleukin genes, the t-PA gene, etc., or genes of microbial origin, such as the NPT II gene, etc. and synthetic genes, such as the insulin gene, etc..

Apart from naturally occurring structural genes that code for a useful and desirable property, within the scope of this invention it is also possible to use genes that have been modified previously in a specific manner using chemical or genetic engineering methods.

Furthermore, the broad concept of the present invention also includes genes that are produced entirely or partially by chemical synthesis. Genes or DNA sequences that may be used within the scope of the present invention are therefore both homologous and heterologous gene(s) or DNA and also synthetic gene(s) or DNA according to the definition given within the scope of the present invention. The insulin gene may be mentioned at this point as an example of a synthetic gene.

In order to ensure the expression of the said structural genes in the plant cell, it is advantageous for the coding gene sequences first to be linked in operable manner to expression sequences capable of functioning in plant cells.

The hybrid gene constructions within the scope of the present invention therefore
comprise, in addition to the DNA sequence according to the invention, one or more structural gene(s) and, in operable linkage therewith, expression signals which include both promoter and terminator sequences and other regulatory sequences of the 3' and 5' untranslated regions.

Any promoter and any terminator capable of bringing about an induction of the expression of a coding DNA sequence (structural gene) may be used as a constituent of the hybrid gene sequence. The said expression signals may promote continuous and stable expression of the gene. Especially suitable are expression signals originating from genes of plants or plant viruses. Examples of suitable promoters and terminators are those of the Cauliflower Mosaic Virus genes (CaMV) or homologous DNA sequences that still have the characteristic properties of the mentioned expression signals. Also suitable are bacterial expression signals, especially the expression signals of the nopaline synthase genes (nos) or the opine synthase genes (ocs) from the Ti-plasmids of Agrobacterium tumefaciens. Also to be mentioned here are, for example, ubiquitine promoters, actin promoters, histone promoters and tubulin promoters.

Within the scope of this invention, preference is given to the 35S and 19S expression signals of the CaMV genome or their homologues which can be isolated from the said genome using molecular biological methods, as described, for example, in Maniatis et al (1982), and linked to the coding DNA sequence.

Within the scope of this invention, homologues of the 35S and 19S expression signals are to be understood as being sequences that, despite slight sequence differences, are substantially homologous to the starting sequences and still fulfill the same function as those starting sequences.

The expression signals may also comprise tissue-preferential or tissue specific promoters. The term tissue-preferential promoter is used to indicate that a given expression signal will promote a higher level of transcription of an associated expressible DNA, or of expression of the product the said DNA as indicated by any conventional RNA or protein assay, or that a given DNA sequence will demonstrate some differential effect; i.e., that the transcription of the associated DNA sequences or the expression of a gene product is greater in some tissue than in all other tissues of the plant. For example, the tissue-preferential promoter may direct higher expression of an associated gene product in leaves, stems, roots and/or pollen than in seed. One example of a tissue-preferential
promoter, which may be suitably used within the scope of the present invention, is a pith-preferred promoter isolated from a maize TrpA gene.

The term tissue-specific promoter is used to indicate that a given regulatory DNA sequences will promote transcription of an associated expressible DNA sequence entirely in one or more tissues of a plant, or in one type of tissue, e.g. green tissue, while essentially no transcription of that associated coding DNA sequence will occur in all other tissues or types of tissues of the plant. Numerous promoters whose expression are known to vary in a tissue specific manner are known in the art. One such example is the maize phenol pyruvate carboxylase [PEPC], which is green tissue-specific [Hudspeth RL and Grula JW, 1989]. Other green tissue-specific promoters include chlorophyll a/b binding protein promoters and RUBISCO small subunit promoters. Further to be mentioned here are, for example, pollen-specific promoters such as those obtainable from a plant calcium-dependent phosphate kinase [CDPK] gene.

A developmentally regulated promoter can also be used. Of course, in the present invention, any promoter which is functional in the desired host plant can be used to direct the expression of an associated gene.

It is often advantageous to incorporate a leader sequence between the promoter sequence and the adjacent coding DNA sequence, the length of the leader sequence being so selected that the distance between the promoter and the DNA sequence according to the invention is the optimum distance for expression of the associated structural gene.

Further regulatory DNA sequences that may be used for the construction of chimaeric genes include, for example, sequences that are capable of regulating the transcription of an associated DNA sequence in plant tissues in the sense of induction or repression.

There are, for example, certain plant genes that are known to be induced by various internal and external factors, such as plant hormones, heat shock, chemicals, pathogens, oxygen deficiency, light, stress, etc..

Another class of genes that are inducible in plants comprises the light-regulated genes, especially the nuclear-coded gene of the small subunit of ribulose-1,5-biphosphate carboxylase (RUBISCO). Morelli et al, Nature 315: 200-204 (1985) have shown that the 5'-flanking sequence of a RUBISCO gene from the pea is capable of transferring light-inducibility to a reporter gene, provided the latter is linked in chimaeric form to that
sequence. It has also been possible to extend this observation to other light-induced genes, for example the chlorophyll-a/b-binding protein.

A further group of regulatable DNA sequences comprises chemically regulatable sequences that are present, for example, in the PR (pathogenesis-related) protein genes of tobacco and are inducible by means of chemical regulators such as those described in EP-A 332,104.

The regulatable DNA sequences mentioned by way of example above may be of both natural and synthetic origin, or they may comprise a mixture of natural and synthetic DNA sequences.

Within the DNA/protein complex according to the invention, a chimeric recombinant DNA construct comprising an expressible DNA as described above involving, for example, one or more protein encoding DNA sequences, promoter and termination sequences and optionally further regulatory sequences of the 3` and 5` untranslated regions, is covalently associated with a VirD2 protein.


Possible methods for the direct transfer of the DNA/protein complex according to the
invention into a plant cell comprise, for example, the treatment of protoplasts using procedures that modify the plasma membrane, for example, polyethylene glycol treatment, heat shock treatment or electroporation, or a combination of those procedures [Shillito et al, Bio Technology, 3: 1099-1103 (1985)].

In the electroporation technique, plant protoplasts together with the DNA/protein complex according to the invention are subjected to electrical pulses of high field strength. This results in a reversible increase in the permeability of biomembranes and thus allows the insertion of the DNA/protein complex according to the invention. Electroporated plant protoplasts renew their cell wall, divide and form callus tissue. Selection of the transformed plant cells can take place with the aid of the above-described phenotypic markers.

A further method for the direct introduction of the DNA/protein complex according to the invention into plant cells, which is based on purely chemical procedures and which enables the transformation to be carried out very efficiently and rapidly, is described in Negrutiu et al, Mol Gen Genet 199: 330-337 (1985).

Also suitable for the transformation of plant material is direct gene transfer using co-transformation [Schocher RJ et al, Bio/Technology, 4: 1093-1096 (1986)].

Co-transformation is a method that is based on the simultaneous taking up and integration of various DNA molecules (non-selectable and selectable genes) into the plant genome and that therefore allows the detection of cells that have been transformed with non-selectable genes.

Further means for inserting the DNA/protein complex according to the invention directly into a plant cell comprise using purely physical procedures, for example by microinjection using finely drawn micropipettes [Neuhaus et al (1987)] or by bombarding the cells with microprojectiles that are coated with the transforming DNA ["Microprojectile Bombardment"; Wang Y-C et al, Plant Mol. Biol. 11: 433-439 (1988)] or are accelerated through a DNA containing solution in the direction of the cells to be transformed by a pressure impact thereby being finely atomized into a fog with the solution as a result of the pressure impact [EP-A-434,616].

Microprojectile bombardment has been advanced as an effective transformation technique
for cells, including cells of plants. In Sanford et al (1987) it was reported that microprojectile bombardment was effective to deliver nucleic acid into the cytoplasm of plant cells of Allium cepa (onion). Christou et al (1988) reported the stable transformation of soybean callus with a kanamycin resistance gene via microprojectile bombardment. Christou et al reported penetration at approximately 0.1 % to 5 % of cells. Christou further reported observable levels of NPTII enzyme activity and resistance in the transformed calli of up to 400 mg/l of kanamycin. McCabe et al (1988) report the stable transformation of Glycine max (soybean) using microprojectile bombardment. McCabe et al further report the recovery of a transformed R₁ plant from an R₀ chimaeric plant.

The transformation of maize plants, including elite maize plants, by microprojectile bombardment can be carried out according to the general protocol described for example in EP-A 478 502, the disclosure of which is incorporated herein by reference.

The list of possible transformation methods given above by way of example is not claimed to be complete and is not intended to limit the subject of the invention in any way.

The present invention therefore also comprises transgenic plant material, selected from the group consisting of protoplasts, cells, calli, tissues, organs, seeds, embryos, ovules, zygotes, etc. and especially, whole and preferably phenotypically normal plants, that has been transformed by means of the processes described above and comprises the recombinant DNA according to the invention in expressible form, and processes for the production of the said transgenic plant material.


Transformation of the plant cells includes separating transformed cells from those that have not been transformed. One convenient method for such separation or selection is to incorporate into the material to be inserted into the transformed cell a gene for a selection marker. As a result only those cells that have been successfully transformed will contain
the marker gene. The translation product of the marker gene will then confer a phenotypic trait that will make selection possible. Usually the phenotypic trait is the ability to survive in the presence of some chemical agent, such as an antibiotic, e.g., kanamycin, G418, paromomycin, etc., which is placed in a selection media.


An example of a gene useful primarily as a screenable marker in tissue culture for identification of plant cells containing genetically engineered vectors is a gene that encodes an enzyme producing a chromogenic product. One example is the gene coding for production of beta-glucuronidase (GUS). This enzyme is widely used and its preparation and use is described in Jefferson, Plant Molecular Biology Reporter 5: 387-405 (1987).

Once the transformed plant cells have been cultured on the selection media, surviving cells are selected for further study and manipulation. Selection methods and materials are well known to those of skill in the art, allowing one to choose surviving cells with a high degree of predictability that the chosen cells will have been successfully transformed with exogenous DNA.

Positive clones are regenerated following procedures well-known in the art. Subsequently transformed plants are evaluated for the presence of the desired properties and/or the extent to which the desired properties are expressed. A first evaluation may include, for example, the level of bacterial/fungal resistance of the transformed plants, stable heritability of the desired properties, field trials and the like.

Further comprised within the scope of the present invention are transgenic plants, in particular transgenic fertile plants transformed by means of the aforesaid described process of the invention and their asexual and/or sexual progeny, which still display the new and desirable property or properties due to the transformation of the mother plant.
The transgenic plant according to the invention may be a dicotyledonous or a monocotyledonous plant. Preferred are monocotyledonous plants of the Graminaceae family involving Lolium, Zea, Triticum, Triticale, Sorghum, Saccharum, Bromus, Oryzae, Avena, Hordeum, Secale and Setaria plants.

Especially preferred are transgenic maize, wheat and barley plants.

Among the dicotyledonous plants rape seed and sunflower are especially preferred herein.

The expression 'progeny' is understood to embrace both, "asexually" and "sexually" generated progeny of transgenic plants. This definition is also meant to include all mutants and variants obtainable by means of known processes, such as for example cell fusion or mutant selection and which still exhibit the characteristic properties of the initial transformed plant, together with all crossing and fusion products of the transformed plant material.

Another object of the invention concerns the proliferation material of transgenic plants.

The proliferation material of transgenic plants is defined relative to the invention as any plant material that may be propagated sexually or asexually in vivo or in vitro. Particularly preferred within the scope of the present invention are protoplasts, cells, calli, tissues, organs, seeds, embryos, pollen, egg cells, zygotes, together with any other propagating material obtained from transgenic plants.

Parts of plants, such as for example flowers, stems, fruits, leaves, roots originating in transgenic plants or their progeny previously transformed by means of the process of the invention and therefore consisting at least in part of transgenic cells, are also an object of the present invention.

It is a further object of the invention to provide a method for obtaining VirD2 protein in high amounts and purity, which method comprises

(a) introducing the VirD2 gene in one of the known overproducing plasmids and incorporating said plasmid into a suitable production organism;

(b) isolating the VirD2 protein from the production organism or from the supernatant by
high speed centrifugation;

(c) after several washing, centrifugation and solubilization steps involving dialysis, subjecting the resulting VirD2 fraction to affinity and/or ion-exchange chromatography;

(d) removing VirD2 degradation products using gel filtration; and

(e) optionally further purifying the VirD2 protein by size exclusion chromatography.

In a specific embodiment of the invention the VirD2 is purified by a 4-step procedure to near homogeneity. Initially, the protein is found in the insoluble pellet obtained by highspeed centrifugation of lysed cells. Probably, upon overproduction of VirD2 in E. coli, the protein is deposited in inclusion bodies. Following extensive washing of the insoluble protein under high salt conditions, VirD2 is solubilized in 6 M urea. Stepwise dialysis against buffers of decreasing urea concentration resulted in a VirD2 fraction soluble under physiological conditions. The following purification steps involved affinity chromatography on heparin-Sepharose and ion-exchange chromatography on DEAE-Sephacel. A final gel filtration on Superose 12 using FPLC removed VirD2 degradation products that co-purify on the first two columns, resulting in a 93 % pure VirD2 fraction. N-terminal microsequencing of the overproduced and purified VirD2 protein revealed an amino acid sequence that is in agreement with the proposed start of the pTiC58 VirD2 gene. A solid phase immuno assay using Vir2-specific antiserum revealed identical sizes for VirD2 gene products encoded by a gene under its original translational control and those obtained from cells containing pPS11, the overexpression plasmid.

The method according to the invention can be advantageously used to increase the transformation efficiency of non-Agrobacterium mediated transformation processes, in that, for example, less transforming DNA is needed as compared to the conventional techniques. In addition the quality of the integrated DNA can be improved by the precision of the integration process, and possible rearrangements which are likely to happen to naked DNA can be avoided.

REFERENCE EXAMPLES

General recombinant DNA techniques
Since many of the recombinant DNA techniques employed in this invention are a matter of routine for the person skilled in the art, it is better to give a short description of these generally used techniques here rather than to describe them every time they occur. Except where there is a specific indication to the contrary, all these procedures are described in Maniatis et al (1982) reference.

A. Cleaving with restriction endonucleases

A reaction batch typically contains about 50 to 500 µg/ml of DNA in the buffer solution recommended by the manufacturer, New England Biolabs, Beverly, MA. 2 to 5 Units of endonucleases are added for each µg of DNA and the reaction batch is incubated for from one to three hours at the temperature recommended by the manufacturer. The reaction is terminated by heating at 65°C for 10 minutes or by extraction with phenol, followed by precipitation of the DNA with ethanol. This technique is also described on pages 104 to 106 of the Maniatis et al (1982) reference.

B. Treatment of DNA with polymerase in order to produce blunt ends

50 to 500 µg/ml of DNA fragments are added to a reaction batch in the buffer recommended by the manufacturer, New England Biolabs. The reaction batch contains all four deoxynucleotide triphosphates in concentrations of 0.2 mM. The reaction takes place over a period of 30 minutes at 15°C and is then terminated by heating at 65°C for 10 minutes. For fragments obtained by cleaving with restriction endonucleases that produce 5'-projecting ends, such as EcoRI and BamHI, the large fragment, or Klenow fragment, of DNA polymerase I is used. For fragments obtained by means of endonucleases that produce 3'-projecting ends, such as PstI and SacI, the T4 DNA polymerase is used. The use of these two enzymes is described on pages 113 to 121 of the Maniatis et al (1982) reference.

C. Agarose gel electrophoresis and purification of DNA fragments from gels

Agarose gel electrophoresis is carried out in a horizontal apparatus, as described on pages 150 to 163 of the Maniatis et al reference. The buffer used is the tris-borate buffer described therein. The DNA fragments are stained using 0.5 µg/ml of ethidium bromide which is either present in the gel of tank buffer during electrophoresis or is added after
electrophoresis. The DNA is made visible by illumination with long-wave ultraviolet light. If the fragments are to be separated from the gel, an agarose is used that gels at low temperature and is obtainable from Sigma Chemical, St. Louis, Missouri. After the electrophoresis, the desired fragment is cut out, placed in a plastics test tube, heated at 65°C for about 15 minutes, extracted three times with phenol and precipitated twice with ethanol. This procedure is slightly different from that described by Maniatis et al (1982) on page 170.

As an alternative, the DNA can be isolated from the agarose with the aid of the GeneClean kit (Bio 101 Inc., La Jolla, CA, USA).

D. Addition of synthetic linker fragments to DNA ends

If it is desired to add a new endonuclease cleavage site to the end of a DNA molecule, the molecule is optionally first treated with DNA-polymerase in order to produce blunt ends, as described in the section above. About 0.1 to 1.0 μg of this fragment is added to about 10 ng of phosphorylated linker DNA, obtained from New England Biolabs, in a volume of 20 to 30 μl with 2μl of T4 DNA ligase from New England Biolabs, and 1 mM ATP in the buffer recommended by the manufacturer. After incubation overnight at 15°C, the reaction is terminated by heating at 65°C for 10 minutes.

The reaction batch is diluted to about 100 μl in a buffer appropriate for the restriction endonuclease that cleaves the synthetic linker sequence. About 50 to 200 units of this endonuclease are added. The mixture is incubated for 2 to 6 hours at the appropriate temperature, then the fragment is subjected to agarose gel electrophoresis and purified as described above. The resulting fragment will then have ends with endings that were produced by cleaving with the restriction endonuclease. These are usually cohesive, so that the resulting fragment can then readily be linked to other fragments having the same cohesive ends.

E. Removal of 5'-terminal phosphates from DNA fragments

During the plasmid cloning steps, treatment of the plasmid with phosphatase reduces the recircularisation of the vector (discussed on page 13 of the Maniatis et al reference). After cleavage of the DNA with the correct restriction endonuclease, one unit of calf intestinal alkaline phosphatase obtained from Boehringer-Mannheim, Mannheim, is
added. The DNA is incubated at 37°C for one hour and then extracted twice with phenol and precipitated with ethanol.

F. Linking of DNA fragments

If fragments having complementary cohesive ends are to be linked to one another, about 100 ng of each fragment are incubated in a reaction mixture of 20 to 40 µl containing about 0.2 unit of T4 DNA ligase from New England Biolabs in the buffer recommended by the manufacturer. Incubation is carried out for 1 to 20 hours at 15°C. If DNA fragments having blunt ends are to be linked, they are incubated as above except that the amount of T4 DNA ligase is increased to 2 to 4 units.

G. Transformation of DNA into E. coli

*Escherichia coli* strain SCS1 (obtained from Stratagene, LaJolla, CA), a high transformation variant of DH1 [Hanahan D, J Mol Biol 166: 557-580 (1983)] is used predominantly as host for plasmids. DNA is introduced into *E. coli* using the calcium chloride method, as described by Maniatis *et al* (1982), pages 250 and 251 or via electroporation.

H. Screening of E. coli for plasmids

After transformation, the resulting colonies of *E. coli* are tested for the presence of the desired plasmid by means of a rapid plasmid isolation process. Two customary processes are described on pages 366 to 369 of the Maniatis *et al* (1982) reference.

I. Large-scale isolation of plasmid DNA

Processes for the isolation of plasmids from *E. coli* on a large scale are described on pages 88 to 94 of the Maniatis *et al* (1982) reference.

J. Cloning in M13 phage vectors

In the following description it is to be understood that the double-stranded replicative form of the phage M13 derivatives is used for routine processes, such as cleaving with restriction endonuclease, linking etc..
Unless there is a specific indication to the contrary, enzymes can be obtained from Boehringer, New England Biolabs or BRL. They are used in accordance with the manufacturer’s instructions unless otherwise indicated.

K. Oligonucleotide labelling

Oligo-deoxyribonucleotides are labeled either at their 3'-ends using [α-32P]ddATP (110 TBq/mmol) and terminal transferase (Amersham) or at their 5'-ends using [γ-32P]ATP (110 TBq/mmol) and phage T4 polynucleotide kinase (31). Standard molecular cloning techniques are performed as described in Sambrook et al [Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab, Cold Spring Harbor, NY (1989)].

L. Southern blot analysis

The extracted DNA is first treated with restriction enzymes, then subjected to electrophoresis in a 0.8 % to 1 % agarose gel, transferred to a nitrocellulose membrane [Southern E.M. (1975)] and hybridized with the DNA to be detected which has previously been subjected to nick-translation (DNA-specific activities of 5 x 10^8 to 10 x 10^8 cpm/μg). The filters are washed three times for 1 hour each time with an aqueous solution of 0.03M sodium citrate and 0.3M sodium chloride at 65°C. The hybridized DNA is made visible by blackening an X-ray film over a period of 24 to 48 hours.

Example 1: VirD2 Purification

1.1 Cloning procedure and protein expression

1.1.1 Cloning procedure 1
In order to express to high levels the VirD2 gene [from pTiA6] in E.coli, the reading frame of VirD2 is placed under the control of the strong translation and expression signals of phage T7 gene 10 in plasmid pET3a [Studier et al, in: Methods of Enzymology Vol 185: 60-89 (1990)]. The VirD2 coding sequence is amplified by Polymerase Chain Reaction [PCR] with primers p1 [5’GGGCTCGAGCATATGGCCGGATCGCGCTC3’, SEQ ID NO: 14] and p2 [5’CCCGAGCTCGATCCTAGGTCCCCCGCGCC3’,
SEQ ID NO: 15] using plasmid pVD43 [Rossi et al, Mol Gen Genet, (1993)] as template. The 1.3 kb reaction product is isolated by gel electrophoresis and inserted in plasmid pTZ19U [commercially available from BioRad] at the SmaI site resulting in plasmid pTZ19VirD2. The sequence of the 5' end of the gene is confirmed by dideoxy chain termination sequencing, which can be done, for example, by using Sequenase from USB [United States Biochemical] according to the instructions provided by the supplier. A NdeI-Sall restriction fragment from pTZ19VirD2 is ligated together with a Sall-EcoRI restriction fragment from plasmid pVD43 into plasmid pET3a digested with NdeI and EcoRI giving plasmid pET3aVirD2. Recombinant clones are selected on the basis of the presence of the 1.3 kb insert by digestion with NdeI and EcoRI.

1.1.2 Protein Expression
Plasmid pET3aVirD2 is introduced by electroporation into E. coli strain BL21(DE3) [Studier et al, in: Methods of Enzymology Vol 185: 60-89 (1990)]. Cultures are grown in LB medium containing 100 μg/ml ampicillin at 37°C with shaking. At an optical density of A600 ~ 0.5-1, expression of VirD2 is induced by the addition of IPTG [Isopropyl β-D thiogalactopyranoside] to a final concentration of 1mM. Shaking is continued for 5 hours. The cells are then pelleted by centrifugation at 4,000xg at 4°C for 30 min. The cell pellet is stored at -80°C until further use.

1.1.3 Cloning procedure 2
To obtain VirD2 protein in amounts suitable for biochemical analysis the original translational initiation signal preceding the VirD2 structural gene of plasmid pTiC58 is replaced by that of phage T7 gene 10. The gene is placed under control of the lacI-regulated tac promoter of expression vector pMS119HE resulting in the VirD2 overproducing plasmid pPS11. Following induction of transcription with IPTG as described below nearly 10 % of SDS-soluble E. coli cell extracts consisted of VirD2.

To construct the VirD2 overexpression plasmid, the 1,410 bp SacI-BamHI fragment of pVIR97.89 [Alt-Moerbe et al, (1986) EMBO J. 5, 1129-1135] carrying the VirD2 reading frame except for the first five codons is inserted between the NdeI and BamHI sites of the polylinker sequence of the T7 promoter ph10 / gene 10 SD expression plasmid pT7-7 [described in Sano & Cantor, (1990) Proc. Natl. Acad. Sci. USA 87, 142-146]. Synthetic oligodeoxyribonucleotides are applied to restore the original 5'-end of the gene and to link the SacI cohesive end of the VirD2 fragment to the NdeI-end of the vector molecule (OL1: 5'-TATGCCCAGATCGAGCT-3'(SEQ ID NO: 36); OL2:
5'-CGATCGGGCA-3' (SEQ ID NO: 37) complementary to part of OL1). To place the manipulated VirD2 gene under control of the more convenient IPTG-inducible tac promoter, the manipulated gene, together with the T7 gene 10 Shine-Dalgano sequence, is inserted as an XbaI-HindIII fragment in the multi-cloning site of pMS119HE [Balzer et al, (1992) Nucleic Acids Res. 20, 1851-1858] resulting in pPS11.

1.1.4 Cloning procedure 3
In order to express to high levels the VirD2 gene in *E. coli*, the reading frame of VirD2 is placed under the control of the strong expression signals of phage T7 gene 10 in plasmid pET3a (Studier et al, 1990). The VirD2 coding sequence is amplified by Polymerase Chain Reaction with primers pr1J (5'GGGCTGAGCATATGCCCCGATCGCGCTC3', SEQ ID NO: 14) and pr2J (5'TGATTACGAATTCTATCCGTCCTCTGCTCTAGGTCCCCC3', SEQ ID NO: 16)

using plasmid pVD43 (Rossi et al, 1993) as template. The 1.3 kb amplified fragment is cloned into the Ndel-EcoRI sites of pET3a resulting in plasmid pFSvirD2.

1.1.5 Protein expression
Plasmid pFSvirD2 is introduced by electroporation into *E. coli* strain BL21(DE3) [Studier et al, 1990]. Cultures are grown in LB medium containing 100 μg/ml ampicillin at 37°C with shaking. At A<sub>600</sub> $\sim$ 0.5-1, expression is induced by the addition of IPTG [Isopropyl β-D thiogalactopyranoside] to a final concentration of 1 mM. Shaking is continued for 5 hours. The cells are then pelleted by centrifugation at 4,000 x g at 4°C for 30 min.

1.2 VirD2 Purification

1.2.1 VirD2 Purification - Procedure 1
Cultures (4 x 1.2 l) of SCSI(pPSII) are grown in YT medium containing 100 μg/ml ampicillin, sodium salt, at 37°C with shaking. At A<sub>600</sub> = 0.7 transcription of VirD2 is induced by addition of IPTG to a final concentration of 1 mM. Shaking is continued for 5 h, at the end A<sub>600</sub> reached 3.8. Cells are harvested by centrifugation (4,000 x g, 10 min), cells (1 g of wet weight) are resuspended in 5 ml of 0.1 M spermidine tris(hydrochloride) /
0.2 M NaCl / 2 mM EDTA and frozen in liquid nitrogen. After the cells are harvested all procedures are performed at 0-4°C and contact of protein solutions with metal surfaces is strictly avoided. Fraction I, crude extract: Frozen cells (24.4 g in 120 ml are thawed and lysed by addition of 10% (w/v) sucrose / 100 mM Tris-HCl, pH 7.6 / 5 M NaCl / 10% (w/v) Brij-58 / 100 mg/ml lysozyme to final concentrations of 4.5%, 45 mM, 1.3 M, 0.225%, 0.67 mg/ml, respectively. The total volume of the lysis mixture is 600 ml. After 1 h at 0°C the highly viscous lysate is centrifuged at 100,000 x g for 90 min. The pellets, containing VirD2 protein in form of inclusion bodies, are washed thoroughly with 80 ml 1 M NaCl 100 mM Tris-HCl, pH 7.6 and centrifuged at 100,000 x g for 60 min. To solubilize VirD2, pellets are eluted for 2 h with 90 ml buffer A [20 mM Tris-HCl, pH 8.5 / 1 mM EDTA / 0.1% Brij-58 / 10% (w/v) glycerol] + 6 M urea + 1 M NaCl and centrifuged again at 100,000 x g for 60 min. To the supernatant (90 ml) 33.6 g (NH₄)₂SO₄ is added (60% saturation) and precipitated proteins are collected by centrifugation at 100,000 x g for 30 min. The sediment is solubilized in 100 ml buffer A + 6 M urea + 0.5 M NaCl. The protein solution is dialyzed for 10 h each against six changes of buffer A + 0.5 M NaCl (1 l) containing 5, 4, 3, 2, 1 and 0.5 M urea. In a final step proteins are dialyzed for 10 h against 1 l of buffer A + 0.2 M NaCl (Fraction I, 140 ml). Fraction II, heparin-Sepharose® CL-6B: Fraction I is diluted with buffer A containing 0.1 M NaCl to a final volume of 300 ml and applied at 90 ml/h to a heparin-Sepharose® CL-6B column (2.6 x 24 cm) equilibrated with buffer B [20 mM Tris-HCl, pH 7.6 / 1 mM dithiothreitol / 1 mM EDTA / 0.01% Brij-58 / 10% (w/v) glycerol] containing 0.1 M NaCl. The column is washed with 250 ml buffer B / 0.1 M NaCl and proteins are eluted with a 1.0 l gradient from 0.1 to 1 M NaCl in buffer B at a flow rate of 90 ml/h. VirD2 eluted at about 0.375 M NaCl (fraction II, 175 ml). Fraction III, DEAE-Sepharcel®: Fraction II is applied at a flow rate of 60 ml/h to a DEAE-Sepharcel® column (2.6 x 10 cm) equilibrated with buffer B + 50 mM NaCl. The column is washed with 250 ml of the same buffer. The first two fractions of the flowthrough containing VirD2 essentially free from degradation products are pooled and concentrated by dialysis against 20% polyethylene glycol 20,000 in buffer B + 100 mM NaCl (fraction III, 2.5 ml). Fraction IV, Superose® 12: 0.1 ml- aliquots of fraction III are applied to a Superose® 12 gel filtration column (1 x 30 cm) equilibrated with buffer B + 0.5 M NaCl using the Pharmacia FPLC equipment. The column is run with the same buffer at a flow rate of 0.5 ml/min. VirD2-containing fractions are pooled, concentrated by dialysis against 20% polyethylene glycol 20,000 in buffer B + 0.1 M NaCl and finally dialyzed against 50% glycerol in buffer B + 0.25 M NaCl (Fraction IV, 1.8 ml). The protein is stored at -20°C without detectable loss of activity for at least 8 months.
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<th>Recovery [%]</th>
<th>Purity [%]</th>
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</table>

1.2.2 VirD2 Purification - Procedure 2

The cells (1 litre culture) are resuspended in 50 ml ice cold lysis buffer [50 mM Tris-HCl pH 8.5, 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF [phenylmethansulfonylfluorid], 10 mM β-mercapto ethanol (β-ME), 0.5 mg/ml lysozyme, 0.1% Brij-35 (polyethylen glycol-dodecylether]. After 1 hour incubation on ice, the lysis mixture is centrifuged at 12,000xg for 30 min at 4°C. The pellet is resuspended in the same volume of lysis buffer without lysozyme, incubated 30 min on ice and centrifuged at 12,000xg for 30 min. VirD2 is solubilized by resuspending the pellet in solubilization buffer [50 mM Tris-HCl pH 8.5, 1mM EDTA, 0.5 M NaCl, 0.1% Brij-35, 6M urea, 10 mM βME].

After 1 hour incubation, insoluble material is removed by centrifugation [12,000xg, 45 min] and the supernatant dialysed over night against 50 mM Tris-HCl pH 8.5, 0.1 M NaCl, 1 mM EDTA, 10 mM βME, 0.05% Brij-35. Precipitated material is removed by centrifugation [12,000xg, 30 min] and the supernatant diluted two fold and applied to a heparin column [available, for example, from BioRad] equilibrated with buffer A [25 mM Tris-HCl pH 8.5]. The protein solution is applied at a flow rate of 2 ml/min. The column is washed with 20 ml buffer A and bound proteins are eluted with a linear gradient from 0 to 1 M NaCl in buffer A at a flow rate of 1 ml/min. VirD2 elutes at about 400 mM NaCl. Fractions [2 ml] containing VirD2 are individually concentrated to 200 μl by ultrafiltration using, for example, a Centrion 30 device available from Amicon. Glycerol is added to 50% final concentration and fractions are stored at -20°C.

If necessary, additional purification steps can be performed in order to obtain homogenous VirD2 preparations. Some minor contaminants that may interfere with subsequent in vitro reactions can be removed, for example, by size exclusion chromatography.
1.2.3 VirD2 Purification - Procedure 3

The cells (1 litre culture) are resuspended in 50 ml ice cold lysis buffer (buffer A) [50 mM Tris-HCl pH 8.5, 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF (Phenyl-methanesulfonyl-fluoride), 10 mM β-mercaptoethanol (β-ME), 0.5 mg/ml lysozyme, 0.1% Tween-20]. After 1 hour incubation on ice, the lysis mixture is centrifuged at 12,000xg for 30 min at 4°C. The pellet is resuspended in the same volume of buffer A without lysozyme, incubated 30 min on ice and centrifuged at 12,000xg for 30 min. VirD2 is solubilized by resuspending the pellet in 50 ml buffer B (25 mM NaOAc pH 5, 8M urea, 10 mM β-ME, 0.1 mM PMSF). After 1 hour incubation, insoluble material is removed by centrifugation (12,000xg, 45 min) and the supernatant is loaded at a flow rate of 1ml/min onto an ion exchange column (EconoPac S, BioRad) equilibrated with buffer B. The proteins are eluted with a NaCl gradient (from 0 to 1 M) in buffer B. VirD2 peak fractions are pooled and dialysed overnight against 1 liter of buffer C (50 mM Tris-HCl pH 8.5, 0.15 M NaCl, 10 mM β-ME, 0.05% Tween-20, 0.1 mM PMSF, 5 mM MgCl₂). Precipitated material is removed by centrifugation [12,000xg, 30 min] and the supernatant diluted two fold and applied to a heparin column [BioRad] equilibrated with buffer C. The protein solution is applied at a flow rate of 2 ml/min. The column is washed with 20 ml buffer C and bound proteins are eluted with a linear gradient from 0.15 to 1 M NaCl in buffer C at a flow rate of 1 ml/min. VirD2 peak fraction, eluting at about 400 mM NaCl, is dialysed overnight against buffer C. Insoluble material is removed by centrifugation [12,000xg, 30 min]. Aliquots are supplemented with 10% glycerol, frozen on dry ice and stored at -80°C.

In cases where higher purity is required, the heparin-purified protein is further purified on reverse-phase HPLC column as follows:

VirD2 samples (100μg, 200μl) are injected onto a C18 reverse phase column (Vydac) equilibrated with 0.1% trifluoroacetic acid (TFA). The bound protein is eluted (flow rate 0.5ml/min) with a linear acetonitrile gradient (from 0 to 70% in 1 hour) in 0.1% TFA. The VirD2 peak fraction, eluting at 48% acetonitrile is lyophilised in a SpeedVac apparatus. VirD2 is resuspended in buffer D [20mM Tris-HCl, pH 8.5, 5mM MgCl₂, 50mM NaCl, 0.05% Tween-20, 10% (v/v) glycerol], frozen on dry ice and stored at -80°C.

1.3 Enzymatic activity test

VirD2 containing fractions are checked for activity using the oligonucleotide cleavage
assay described in Pansegrau et al [Proc Nat Acad Sci USA, (1993b)]. A 17-mer oligonucleotide homologous to the pTIA6 right border sequence [5'GGTATATATCC-TGCCAG3', SEQ ID NO: 17] is used as a substrate. The 13-mer reaction product is analyzed by electrophoresis on 19% acrylamide gels containing 8 M urea.

Example 2: Site specific Cleavage of Oligonucleotides by VirD2

Oligodeoxyribonucleotides (10 pmol) labeled at their 5'-ends, are incubated with VirD2 (2.4 µg, 100 pmol) in a total volume of 30 µl TNM (20 mM Tris-Cl, pH 8.8 / 50 mM NaCl / 5 mM MgCl₂), for 3 h at 37°C. Reactions are stopped by addition of formamide [30 % (w/v), final concentration] and analyzed. Products are analyzed on a 20 % (w/v) polyacrylamide gel containing 8 M urea [Pansegrau et al, Proc Natl Acad Sci USA 90: 2925-2929 (1993)]. Reaction products are quantified by autoradiography of gels with the storage phosphor technology [Johnston et al, Electrophoresis 11: 355-360 (1990)].

Where appropriate, the cleavage reaction is followed by trypsin digestion in presence of 0.01 % SDS. The following oligonucleotides are used as substrates: pTiC58 RB (right border), d(p*CCATATATCCCTG\textsuperscript{+}TCAA) (SEQ ID NO: 18); pTiA6 RB, d(p*GGTATATATCCCTG\textsuperscript{+}CCAG) (SEQ ID NO: 19); RP4 oriT, d(p*TTCACCTATCCCTG\textsuperscript{+}CCCG) (SEQ ID NO: 20). Positions of the cleavage sites are indicated by wedges. The radioactive label is symbolized by an asterisk.

Following incubation of VirD2 with 17-mer oligonucleotides \textsuperscript{32}P-labeled at the 5' end in presence of Mg\textsuperscript{2+} ions, potential cleavage products are separated on a denaturing polyacrylamide gel. Under these conditions a product with the electrophoretic mobility of a 13-mer is observable irrespective whether an oligonucleotide with a pTiC58, a pTiA6 or an RP4 sequence is offered. There is no apparent difference in cleavage efficiency between oligonucleotides with the sequence of the left or of the right border of pTiC58.

Occurrence of a 13-mer indicates that VirD2 mediates cleavage at the same site within the pTiC58 border sequences that is found by analysis of T-DNA produced \textit{in vivo} [Dürenberger et al, Proc. Natl. Acad. Sci. USA 86, 9154-9158 (1989)]. Cleavage at an equivalent site is also found using a VirD2 protein obtained from pTiA6.

Incubation of the reaction products with protease (trypsin) did not alter the electrophoretic mobility of the 5'-end labeled product, indicating, that the 3' end produced by VirD2-catalyzed cleavage is not covalently associated with protein. The reaction is
dependent on the presence of Mg$^{2+}$-ions.

**Example 3:** VirD2 covalently attaches to the 5'-end of cleaved oligonucleotides.

VirD2 has been found to be covalently associated with the 5'-ends of the T-DNA single strands that are produced in agrobacteria induced by plant phenolic compounds like acetosyringone. To find out whether covalent attachment of VirD2 to the DNA 5'-end can be mimicked by the oligonucleotide cleavage reaction *in vitro*, 3'-labeled oligonucleotide is incubated in presence of Mg$^{2+}$ ions with VirD2 and the products are separated on a sequencing gel. Oligonucleotide cleavage under these conditions resulted in a labeled species that did not enter the sequencing gel, indicating that the 3'-terminal oligonucleotide moiety is tightly associated with high molecular weight material. Treatment of the material with a variety of proteases resulted in labeled species with distinct electrophoretic mobilities reflecting the covalent association of the 5'-end of cleaved oligonucleotides with different peptide species generated by proteases of different specificity.

**Example 4:** VirD2 transfers a covalently attached oligonucleotide moiety to a preformed border sequence 3'-end

Integration of the T-DNA into the plant genome requires reversion of the cleavage reaction. To detect specific joining of oligonucleotides by VirD2 *in vitro*, a preformed border 3'-terminus is synthesized as an 13-mer oligonucleotide and incubated in various amounts together with VirD2 protein and a 3'-end labeled 30-mer oligonucleotide carrying the pTiCS8 right border cleavage site. Specific transfer of the 3'-terminal moiety of the 30-mer to the 13-mer by VirD2 resulted in a 22-mer detectable on polyacrylamide gels by the 3'-label. This demonstrates that the 13-mer participates in the oligonucleotide cleavage reactions presumably by reaction with free VirD2-d (pTCAAACAC)dA* (SEQ ID NO: 21) that probably exists under the conditions employed. Quantification of reaction products revealed that (i) the labeled 3'-terminal moiety is efficiently transferred to the preformed 3'-terminus even at moderate molar ratios [13-mer]/[30-mer]; (ii) the efficiency of oligonucleotide cleavage drops with increasing concentration of 13-mer, indicating that a thermodynamic equilibrium is shifted by addition of a reaction product to the joined oligonucleotide form.

**Example 5:** Site specific Cleavage of large Oligonucleotides by VirD2
M13 phages are constructed that contain the pTi plasmid border sequence and single-stranded substrates longer than the oligonucleotides reported in Example 2. The procedure is the following:

Starting out from plasmid pTZ containing the pTiA6 right border sequence (pTZ18RB), a PCR fragment containing as well the border sequence is amplified using a pair of primers (UP-40: 5' GTTTTCCCAGTCACGAC3' (SEQ ID NO: 22) and UP-112: 5' CACTC-ATTAGGCACCACCGGC3' (SEQ ID NO: 23)). The PCR product is 249 bp long. The DNA is gel purified, phenol extracted and precipitated. Two to 4 % of this dsDNA are used in a second amplification step using only the UP-40 primer. Therefore, the next amplification step (40 cycles) is linear and produces single-stranded molecules corresponding to only one of the strands of the input dsDNA. This single-stranded DNA is expected to be processed by VirD2. The ssDNA product is gel purified, phenol extracted and precipitated. Hundred ng are labeled with $^{32}$P-ATP and polynucleotide kinase using the supplier’s conditions. After the labeling reaction, the DNA is phenol extracted, precipitated and resuspended in 100 μl of water.

The in vitro cleavage is performed in the conditions previously described, using 1 ng ssDNA per assay. A kinetic analysis revealed that a few minutes incubation of the VirD2 preparation with the substrate produces a well defined band, shorter than the input DNA (as expected).

**Example 6: Site specific Cleavage of Chimeric Gene Construct comprising an GUS Expression Cassette**

6.1 Procedure 1

6.1.2 Substrate preparation

VirD2 substrates are single stranded DNA molecules containing the pTiA6 Right Border sequence. Two complementary oligonucleotides (top strand: 5' AATTCTGGCAGG-ATATATACTGGTATTATGAC3' (SEQ ID NO: 24) and bottom strand: 5' AA-ATTACAACCGTATATCCTGCGAG3' (SEQ ID NO: 25)) are annealed and ligated into pTZ19U digested with EcoRI and KpnI to produce pZ19URB. A EcoRI fragment from pGUS23 [Puchta et al, Mol Cell Biol 12: 3372-3379 (1992)] containing the GUS gene is introduced into pZ19URB to produce pZ19URBGUS. A EcoRI/HindIll fragment from pZ19URBGUS is subcloned into M13mp18 and M13mp19 to obtain pM18RBGUS.
and pM19RBGUS respectively. Control M13 vectors are constructed by insertion of the EcoRI fragment of plasmid pGUS23 into M13mp18 cleaved at the EcoRI site giving pM18GUS(+) (GUS coding strand is present in the viral (+) strand) and pM18GUS(-) (GUS coding strand is present in the viral (-) strand). These two vectors lack the pTiA6 border sequence. Single stranded DNA is prepared as described in [Sambrook et al, (1989)] and are further purified using a Qiagen DNA [QIAGEN Inc., CA, USA] purification column according to the manufacturer instructions.

6.1.2 In vitro reaction
The VirD2 protein is allowed to react with single stranded DNA vector containing the pTiA6 right border sequence, using the vectors without the border sequence as controls. Reaction conditions are similar to those described in Pansegrau et al (1993b). Reaction buffer is 20 mM Tris-HCl, pH 8.5, 5 mM MgCl₂, 1 mM EDTA, 50 mM NaCl. Ten pmole VirD2 are reacted with 1 pmole single-stranded DNA substrate. Cleavage is allowed to proceed for one hour at 37°C Upon cleavage, the protein becomes linked to the 5' end of the DNA that is thereby linearized.

6.1.3 Quantification of the reaction
Quantification of the ssDNA cleavage is checked by 3' end labelling aliquots of the reaction mixture with Terminal Transferase (Boehringer) using a³²P-ddATP as label. Incorporation of the ³²P label in ethanol-precipitable is a measure of the extent of ssDNA cleavage by VirD2.

6.1.4 Isolation of reaction products
Free VirD2 is removed from the reaction mixture by repeated dilution with reaction buffer and ultrafiltration with an Centricon 100 device.

6.2 Procedure 2

6.2.1 Substrate preparation
VirD2 substrates are single stranded DNA molecules containing the pTiA6 Right Border and Overdrive sequence (Peralta et al, 1986). A 108 bp EcoRI-Sall fragment from pTD20 (5' aattcgcagTGGCAGGATAATACCCTTTGTAATTTgagctgtgtg ³²P label in ethanol-precipitable is a measure of the extent of ssDNA cleavage by VirD2. aatTAAGTCGCTGTGATAGTTTGTGTiTTGagctgtgtg 3', SEQ ID NO: 26) (Right border and Overdrive sequence are in upper case letters) is cloned into the EcoRI-Sall
sites of M13mp19 resulting in pM13RB.

6.2.2 In vivo reaction
a) Transient expression vectors:
An EcoRI fragment from pGUS23 (Puchta et al, 1992) containing the GUS gene is introduced into M13mp19RB at the EcoRI site to produce pM13RBGUS. Control M13 vectors are constructed by insertion of the EcoRI fragment of plasmid pGUS23 into M13mp19 cleaved at the EcoRI site giving pM13GUS. This vector lacks the pTiA6 border sequence.

A further plasmid (pM13PstGUS) is constructed, which is almost identical to pM13RBGUS, except that the GUS gene, oriented in the opposite direction, is flanked by two PstI sites. This vector can be used to quantify the extent of VirD2 processing using a primer extension technique (see below). It can also be used to produce linear single-stranded DNA by digestion with the PstI restriction endonuclease.

b) Stable transformation vector:
An EcoRI fragment containing the kanamycin resistance gene from plasmid pH28 (Paszkowski et al, 1988) is cloned into the EcoRI site of pM13RB resulting in pM13RBKan.

c) Transformation protocol:
The transformation is carried out with a spray-type particle gun a described in EP-A 434,616. The target tissue used are tobacco SR1 leave pieces of 0.5 to 2.0 cm. The plant material is preferably submitted to plasmolysis [20% maltose treatment] before shooting.

6.2.3 DNA preparation
Single-stranded DNA is prepared as described in (Sambrook et al, 1989) and further purified by CsCl gradient centrifugation.

6.2.4 In vitro reaction
Enzymatic activity test
The VirD2 fractions are checked for activity using the oligonucleotide cleavage assay described in Pansegrau et al (1993b). For example, a 17-mer oligonucleotide homologous to the pTiA6 right border sequence (S’GGTATATATCCTGCGG3’, SEQ ID NO: 27) is used as substrate. The 13-mer cleavage products are analysed by electrophoresis on 20%
acrylamide gels containing 8 M urea.

T-complex formation
The VirD2 protein is allowed to react with single-stranded DNA containing the pTiA6 right border sequence, using vectors lacking the border sequence as controls.

As an example:
5 micrograms of purified VirD2 are allowed to react with 1 microgram of single-stranded DNA. Reaction (20 μl) is performed in the standard reaction buffer (20 mM Tris-HCl, pH 8.5, 5 mM MgCl₂, 50 mM NaCl) at 37°C for 15 minutes. The reaction mixture is used as such either immediately or is frozen on dry ice and thawed immediately before use.

6.2.4 Quantification of the reaction
Quantification of the ss DNA cleavage is done by performing a primer extension reaction, using a linear ss DNA (obtained by digestion of pM13PstGUS with PstI).
After the reaction of VirD2 with its linear ssDNA substrate is completed, the reaction mixture is digested with Proteinase K, in order to remove the protein covalently linked to the 5' end of the cutting site. After phenol extraction, the DNA is precipitated with ethanol and used in a primer extension reaction using the Sequenase kit (USB) according to the instructions concerning the sequencing of single-stranded templates. The primer used is located about 100 nucleotides 3' from the VirD2 cutting site. It is annealed and extended using (α-35S)-labelled dATP and unlabelled dCTP, dGTP and dTTP. The labelling reaction (the extension) is allowed to proceed at 37°C for 10 minutes. The extension reaction is terminated either at the cut introduced by VirD2 or at the extremity of the linear molecule in case VirD2 did not process it. Therefore the ratio between prematurely terminated extension products and full length products is a measure of the extent of cutting by VirD2. An normal sequencing reaction is performed in parallel on non-processed DNA with the same primer. One can then identify the cut site by comparison with the sequencing ladder.

6.2.5 Isolation of reaction products
Free VirD2 can be removed from the reaction mixture by repeated dilution with reaction buffer and ultrafiltration with an Centricon 100 device.

Example 7: VirD1 Purification
7.1) Molecular cloning procedure

To allow effective translational initiation in *E. coli* the original SD sequence preceding the VirD1 gene is replaced by that of phage T7 gene 10. Two CGG triplets of a cluster of rare Arg codons are altered by site directed mutagenesis. This prevents termination of translation at this position and thus production of truncated VirD1 molecules. The modified VirD1 gene is placed under control of the LacI-regulated tac promotor in the expression vector pMS119HE resulting in the VirD1 overproducing plasmid pPS20. Following chemical induction of gene expression by IPTG addition to the culture medium approx. 8% of SDS-soluble cell protein consists of VirD1.

To construct the VirD1 overexpression plasmid, the 464 bp MaeIII-SaeI fragment of pVir97.89 [Alt-Moerbe *et al.*, (1986)] carrying the VirD1 reading frame except for the first ten codons is inserted in the NdeI and SaeI sites of the poly linker of the T7 promotor F10 / gene 10 SD expression plasmid pT7-7 [Sano & Cantor, (1990)]. Using synthetic oligodeoxiribonucleotides the SaeI cohesive end of the VirD1 fragment is linked to the NdeI-end of the vector and the original 5'-end of the gene is restored.

Sequence of synthetic oligodeoxiribonucleotides for molecular cloning of VirD1:

\[
\begin{align*}
\text{NdeI} & \quad \text{MaeIII} \\
5' & - \text{TATGTCGCAAAGGCAGTACGCCCACCTCAA} - 3' \quad \text{(SEQ ID NO: 28)} \\
3' & - \text{ACACCGTTCGTCATCCGGGTGATCTCTCTG} - 5' \quad \text{(SEQ ID NO: 29)}
\end{align*}
\]

The rare CGG Arg codons are changed by site directed mutagenesis following the procedure of Sayers *et al.* (1988). The sequence CGG CGG CGG (nucleotides 1048-1056 of Atuvird, accession number M33673) is mutated to CGC CGG CGT. The manipulated VirD1 gene with the changed codons and the T7 gene 10 Shine-Dalgano sequence is inserted as an XbaI-SaeI fragment in the multi-cloning site of pMS119HE [Balzer *et al.*, 1992] resulting in pPS20.

7.2) VirD1 purification procedure

Cultures (4 x 1.2 l) of SCS1(pPS20) are grown in YT medium [Sambrook *et al.*, (1989) Cold Spring Harbor, New York] containing 0.1% casaminoacids and 100 μg/ml
ampicillin, sodium salt, at 37°C with shaking. At A_{600}= 0.5 transcription of VirD1 is induced by addition of IPTG to a final concentration of 1 mM. Shaking is continued for 4 h, A600 reaches 2.8. Cells are harvested by centrifugation (16,000 x g, 10 min), resuspended in 0.1 M spermidine tris(hydrochloride) / 0.2 M NaCl / 2 mM EDTA / pH 7.6 to a final concentration of 150 A_{600}/ml and frozen in liquid nitrogen. All subsequent procedures are performed at 0-4°C, contact of the protein solution to metal surfaces is strictly avoided.

**Fraction I, crude extract.** Frozen cells (29 g in 90 ml) are thawed and lysed in 4.5 % (wt/vol) sucrose / 70 mM Tris-HCl (pH 7.6) / 1 M NaCl / 0.25 % (wt/vol) Brij-58 / 0.75 mg/ml lysozyme. The total volume of the lysis mixture is 460 ml. After 1 hr incubation at 0°C the inclusion bodies, containing the VirD1 protein, are pelleted by centrifugation (100,000 x g, 1 hr). The pellets are washed thoroughly with 3 M urea / 1 M NaCl / 1 mM EDTA / 0.5 mg/ml sodium deoxycholate. This washing step is repeated. To solubilize VirD1, pellets are eluted with 150 ml buffer A [20 mM Tris-HCl (pH 8.7) / 500 mM NaCl / 1 mM EDTA / 0.05 % (wt/vol) Brij-58 / 10 % (wt/vol) glycerol] / 8 M urea / 10 mM DTT overnight and centrifuged (100,000 x g, 1 hr). To the supernatant (NH4)2SO4 is added to 65 % saturation and precipitated proteins are collected by centrifugation at 100,000 x g for 30 min. The sediment is solubilized in 250 ml buffer A / 8 M urea / 5 mM DTT. The protein solution is dialyzed for 6 hr each against six changes of buffer A (2 liter) containing 5, 4, 3, 2, 1, 0.5 M urea then against four changes of buffer B [20 mM Tris-HCl (pH 7.6) / 1 mM EDTA / 1 mM DTT / 0.01 % (wt/vol) Brij-58 / 10 % (wt/vol) glycerol] (2 liter) containing 250, 125, 65, 0 mM NaCl. The dialyzed solution is centrifuged (100,000 x g) and the pellet discarded (fraction I, 240 ml).

**Fraction II, DEAE-Sepharose.** Fraction I is loaded on a DEAE-Sepharose column (2.6 x 20 cm) equilibrated with buffer B at a flow rate of 54 ml/hr. The column is washed with 200 ml buffer B and the VirD1 containing fractions of the flowthrough are pooled (fraction II, 360 ml).

**Fraction III, phosphocellulose-P11.** Fraction II is applied at a flow rate of 55 ml/hr to a phosphocellulose P11 column (2.6 x 24 cm) equilibrated with buffer C [30 mM Tris-phosphate (pH 7.0) / 1 mM EDTA / 1 mM DTT / 10 % (wt/vol) glycerol]. The column is washed with 150 ml of buffer C and 50 ml of buffer C / 50 mM NaCl. Then proteins are eluted with a 1.4-1 gradient from 50 to 750 mM NaCl in buffer C. VirD1 elutes at about 375 mM NaCl. Pooled fractions are concentrated by dialysis against buffer B / 20 % (wt/vol) polyethylene glycol 20,000 and finally dialyzed against buffer B / 50 % (wt/vol) glycerol (fraction III, 5.7 ml). The protein is stored at -20°C without detectable loss of activity for at least 6 months.
A typical purification example:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Step</th>
<th>Protein [mg]</th>
<th>Recovery [%]</th>
<th>Purity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Crude extract</td>
<td>312,0</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>II</td>
<td>DEAE-Sephacel</td>
<td>72,0</td>
<td>71</td>
<td>65</td>
</tr>
<tr>
<td>III</td>
<td>Phosphocellulose P11</td>
<td>5,7</td>
<td>8</td>
<td>96</td>
</tr>
</tbody>
</table>

Example 8: Specific cleavage reaction of formI DNA by VirD1 and VirD2 in vitro.

8.1 Substrates
pPS100: form I DNA pBR329 Ω [BamHI-Sall, right T border of pTiC58, 43 bp], 3918 bp
pPS101: form I DNA pPS100 Ω [PstI-AatII, left T border of pTiC58, 43 bp], 3284 bp
pPS110: form I DNA pPS100 Ω [NheI-BamHI, overdrive of pTiA6, 40 bp], 3812 bp
pPS111: form I DNA pPS101 Ω [NheI-BamHI, overdrive of pTiA6, 40 bp], 3178 bp

Coordinates of the restriction sites in pBR329 (4151 bp) [Covarrubias, L. & Bolivar, F., (1982)]:

NheI (460), BamHI (606), SalI (882), PstI (2755), AatII (3432)

Sequence of T border and overdrive oligodeoxyribonucleotides inserted into pBR329 for construction of substrate plasmids:
pS124.1: form I DNA containing two right borders and one overdrive element of pTiA6
[Alt-Moerbe et al., (1990)]

8.2) Cleavage reaction
Form I substrate DNA (0.7 μg) is incubated with VirD1 (750 ng, 47 pmol) and VirD2 (550 ng, 11 pmol) in a total volume of 20 μl TNM (20 mM Tris-HCl (pH 8.8) / 50 mM NaCl / 5 mM MgCl$_2$), for 40 min at 37 C. Cleavage products are analyzed on vertical 0.7 % agarose gels after proteinase K digestion in the presence of 1 % SDS. The cleavage reaction requires form I DNA, is T border specific, VirD1-, VirD2- and Mg$^{2+}$-dependent.

Reaction products are quantified by scanning with a FluorImager 575 (Molecular Dynamics). The yield of specifically cleaved DNA is 35-95 % of the input form I DNA, depending on the substrate. Substrates pPS100 and pPS110 containing one border element are cleaved to 35 %, pPS101 and pPS111 to 70 %, pS124.1 to 95%. The higher yield of cleaved plasmid DNA from substrates carrying two border sequences is due to a higher probability for cleavage to occur at one of the nick-sites.
Specificity and nick-site were determined by electrophoresis of linearized reaction products on alkaline agarose gels and runoff sequencing. The position of the cleavage site is identical to that found in vivo [Dürrnerberger et al., (1989)] or with single-stranded DNA substrates and VirD2 in vitro [Pansegrau et al., (1993)]. Likewise, cleavage results in covalent attachment of VirD2 protein to the 5-terminus of the broken DNA strand.

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Alt-Moerbe et al., (1986), EMBO J. 5, 1129-1135
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Sano & Cantor, (1990), Proc. Natl. Acad. Sci. USA 87, 142-146
Sayers et al., (1988), Nucleic Acids Res. 16, 791-802
Studier et al., (1990), Methods Enzymol 185, 60-89
SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Method for Stable Transformation of Plants

(iii) NUMBER OF SEQUENCES: 37

(iv) COMPUTER READABLE FORM:
   (A) MEDIUM TYPE: Floppy disk
   (B) COMPUTER: IBM PC compatible
   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
   (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
   APPLICATION NUMBER: EP 93113028.0

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 6 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATCCKG

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 7 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TATCCCTG

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 58 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 59 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 62 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO
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NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN RBHYATCCKG YMNININNNN
   60
NN
   62

(2) INFORMATION FOR SEQ ID NO: 6:

   (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 29 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
NNNNNNNNNN NNBHYAYMTA TOCTGYNR
   29

(2) INFORMATION FOR SEQ ID NO: 7:

   (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 17 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
CCAATATATC CTGTCA
   17

(2) INFORMATION FOR SEQ ID NO: 8:

   (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 17 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGTATATAC CTGOCAG

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 17 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTCACCTATC CTGCCCG

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 27 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AAATTACAAC GGTATATAC CTGOCAG

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 35 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: both
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCTCAAATTA CAACGGTATA TATCTTGCCA GTCA

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GATCTCTCTTA GGTTTACCG CCAATATATC CTGTCAAACA CTG

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xiii) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCAATTGTT TACACCACAA TATATCTGC CACCAGCGA CGT

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xiv) SEQUENCE DESCRIPTION: SEQ ID NO: 14:
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(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCCGAGCTCG GATCCCTAGG TCCCCCGCG CCC

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TGATTACGAA TTCTATCGG TCTTCTGCTG TCTTACGTC CCCC

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGTATATATC CTGCCAG
(2) INFORMATION FOR SEQ ID NO: 18:

(i)  SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 17 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 18:
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(2) INFORMATION FOR SEQ ID NO: 19:

(i)  SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 17 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 19:
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(2) INFORMATION FOR SEQ ID NO: 20:

(i)  SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 17 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 20:
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(2) INFORMATION FOR SEQ ID NO: 21:
(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 9 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

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(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 17 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GTTTCCAG TCAGCAC

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CACTCATTAG GCCACCCAGG C

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:
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(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:
AAATTACAAC GGATATATAC CTGCGAG

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:
AATTCAGCT GGCAGGATAT ATACGTTGT AATTTGAGCT CGTGTAATA AGTGCCTGTG
TATGTTTGTT TGATCGGGC CGCAAGCTTT CTAGGAGTC CCTGGAG

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GGTATATATC CTGCCAG

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 29 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

TATGTCCGAA GGCAGTAGGC CCACCTCAGA

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 32 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GTCTCTTGAG GTGGCGCTAC TGCCCTGCCA GA

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 43 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:
GATCCTCTTA GGTTCACCG CCAATATATC CTGCAACA CTG

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:
TCGACAGTGT TTGACAGGAT ATATGGCGG GTAACCTAA GAG

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:
GCAATTTGTT TACACCGAA TATATCCTGC CACCGCGGA CGT

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

CGGCTGGTGG CAGGATAT TGTGGTGA ACAAATTGCT GCA

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 40 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CTAGCATCAA ACAAACATAC AGACCGACT ATTCACAOGG

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 40 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GATCCCGTGT GAATAAGTG CTTGGTATGT TTGTTTGATG

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 16 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:
TATGCCGGAT CGAGCT

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:
CGATCGGSCA
Patent claims

1. A substantially pure DNA/protein complex comprising a chimeric recombinant DNA construct covalently associated with a VirD2 protein.

2. A DNA/protein complex according to claim 1, wherein the chimeric DNA construct comprises an expressible DNA sequence under the control of plant expression signals.

3. A DNA/protein complex according to claim 2, wherein the said expressible DNA sequence encodes for a structural gene.

4. A DNA/protein complex according to claim 3, wherein the said structural gene upon expression leads to a protective effect in the transformed plant material, for example increased resistance to pathogens (for example to phytopathogenic fungi, bacteria, viruses, etc.); resistance to chemicals (for example to herbicides (e.g. triazines, sulfonylureas, imidazolinones, triazole pyrimidines, bialaphos, glyphosate, etc.), insecticides or other biocides); resistance to adverse environmental factors (for example to heat, cold, wind, adverse soil conditions, moisture, dryness, etc.).

5. A DNA/protein complex according to claim 1, wherein the said plant expression signals are promoter and termination sequences functional in plant cells.

6. A DNA/protein complex according to claim 5, wherein the said promoter sequences are of plant origin.

7. A DNA/protein complex according to claim 5, wherein the said promoter sequences are of viral origin.

8. A DNA/protein complex according to any one of claims 6 or 7, wherein the said promoter sequences are those selected from the group consisting of CaMV 35 S promoter, CaMV 19S promoter, pith-specific promoters, pollenspecific promoters.
9. A DNA/protein complex according to claim 5, wherein the said promoter is an inducible promoter.

10. A DNA/protein complex according to any one of claims 1 to 9 comprising in non covalent association further Vir proteins.

11. A DNA/protein complex according to claim 10, wherein the said Vir protein is VirD1 and/or VirE2.

12. A method of preparing a DNA/protein complex according to any one of claims 1 to 9, comprising:

(a) preparing a chimeric recombinant DNA construct that comprises in operable linkage to an expressible DNA at least one T-DNA border sequence or functional parts thereof, which serves as a substrate in the VirD2 cleavage reaction;

(b) in vitro cleaving of the DNA substrate prepared according to step (a) by means of VirD2 protein, which optionally may be accompanied by further Vir proteins such as, for example VirD1 and/or VirE2 and/or any other DNA binding protein, which is able to protect the DNA from nuclease attack.

13. A method of preparing a DNA/protein complex according to any one of claims 10 to 11, comprising:

(a) preparing a chimeric recombinant DNA construct that comprises in operable linkage to an expressible DNA at least one T-DNA border sequence or functional parts thereof, which serves as a substrate in the VirD2 cleavage reaction;

(b) in vitro cleaving of the DNA substrate prepared according to step (a) by means of VirD2, VirD1 and/or VirE2 protein.

14. A method according to claim 12, wherein the substrate prepared according to step (a) is a single-stranded DNA molecule.

15. A method according to claim 12, wherein the substrate prepared according to step (a) is a double-stranded DNA molecule with a single stranded overhang.
16. A method according to claim 13, wherein the substrate prepared according to step (a) is a negatively supercoiled double-stranded DNA, which is cleaved \textit{in vitro} by the combined action of VirD1 and VirD2 proteins.

17. A method according to any of claims 12 to 16, wherein the parts obtainable from T-DNA border sequences comprise in addition to the VirD2 cleavage site at least the following core sequence according to SEQ ID NO 1:

\[ \text{ApTpCpKpG} \]

with K being either guanosin or thymidine/uridine; which core sequence immediately precedes the said cleavage site.

18. A method according to claim 17, wherein the core sequence is

\[ \text{TpApTpCpTpG} \]

19. A method according to any of claims 12 or 16, wherein the DNA border sequence comprises between 6 and approximately 50 nucleotides, preferably between 10 and approximately 40 nucleotides and which can be described by the following general formula according to SEQ ID NO 3:

\[ (Np)_n\text{ApTpCpKpGp}^\nabla N_m \]

wherein N may be any of the nucleotides selected from the group consisting of adenosine, guanosine, cytidine and thymidine or uridine, K is either guanosine or thymidine/uridine, n is an integer between 0 and 42, preferably between 0 and 25, more preferably between 0 and 14 and m is an integer between 2 and 10, preferably between 3 and 10, more preferably between 4 and 8 and wherein the wedge indicates the position of the cleavage site.

20. A method according to any of claims 12 or 16, wherein the DNA border sequence comprises between 10 and approximately 50 nucleotides, but preferably between 14 and 29 nucleotides and which can be described by the following general formula according to SEQ ID NO 5:

\[ (Np)_n\text{RpBpHpYpApTpCpKpGp}^\nabla YpM_p(N)_m \]
wherein N may be any of the nucleotides selected from the group consisting of adenosine, guanosine, cytidine or thymidine/uridine; B is guanosine or cytidine or thymidine/uridine; H is adenosine or cytidine or thymidine/uridine; M is adenosine or cytidine; R is guanosine or adenosine; Y is thymidine/uridine or cytidine; K is either guanosine or thymidine/uridine; and \( n \) is an integer between 0 and 40, but preferably between 2 and 17; and \( m \) is an integer between 2 and 10, preferably between 3 and 10, more preferably between 4 and 8 and wherein the wedge indicates the position of the cleavage site.

21. A method of transforming plant material with a DNA construct encompassing at least one Ti plasmid border region or functional parts thereof comprising

(a) preparing a chimeric recombinant DNA construct that comprises in operable linkage to an expressible DNA at least one T-DNA border sequence or functional parts thereof, which serves as a substrate in the VirD2 cleavage reaction;
(b) \textit{in vitro} cleaving of the DNA substrate prepared according to step (a) by means of VirD2 protein, which may be accompanied by further Vir proteins such as, for example VirD1 and/or VirE2; and
(c) introducing the thus cleaved DNA comprising at least the VirD2 protein covalently bound to the 5' end of the cleavage site into the plant material to be transformed by methods known in the art.

22. A method according to claim 21, wherein the transformation is achieved by a method selected from the group consisting of microinjection, electroporation of protoplasts or intact cells, direct gene transfer and ballistic particle acceleration.

23. A method according to claim 21, wherein the plant material to be transformed is a monocotyledonous plant.

24. A method according to claim 23, wherein the plant is selected from the group consisting of maize, wheat and barley.

25. A transformed plant including the progeny thereof, comprising a DNA/protein complex according to any one of claims 1 to 11.

26. A plant according to claim 25 which is maize, wheat and barley.
27. A method for obtaining VirD2 protein in high amounts and purity, which method comprises

(a) introducing the virD2 gene in one of the known overproducing plasmids and incorporating said plasmid into a suitable production organism;

(b) isolating the VirD2 protein from the production organism or from the supernatant by high speed centrifugation;

(c) after several washing, centrifugation and solubilization steps involving dialysis, subjecting the resulting VirD2 fraction to affinity and/or ion-exchange chromatography;

(d) removing VirD2 degradation products using gel filtration; and

(e) optionally further purifying the VirD2 protein by size exclusion chromatography.

28. A method for obtaining VirD1 protein in high amounts and purity, which method comprises

(a) replacement of rare codons from the VirD1 coding sequence by site-directed mutagenesis;
(b) introducing the virD1 gene in one of the known overproducing plasmids and incorporating said plasmid into a suitable production organism;
(c) isolating the VirD1 protein from the production organism or from the supernatant by high speed centrifugation; and
(d) after several washing, centrifugation and solubilization steps involving dialysis, subjecting the resulting VirD1 fraction to affinity and/or ion-exchange chromatography.