Title: TRANSFORMATION OF EUKARYOTIC CELLS BY MOBILIZABLE PLASMIDS

Abstract: The invention relates to the transfer of genetic material to eukaryotic cells by means of a process resembling conjugation, in particular by a system partially based on Agrobacterium tumefaciens-like transfer systems. In particular, transfer of genetic material into plant cells is disclosed using mobilisable, but non-conjugative plasmids by means of an Agrobacterium virulence system. The invention provides a method for transferring genetic material which is not a typical T-DNA surrounded by Agrobacterium T-borders from an Agrobacterium virulence system to an eukaryotic host cell, comprising providing said genetic material on a mobilisable plasmid, capable of forming a relaxosome, bringing said mobilisable plasmid in an Agrobacterium having at least the activity of the transfer genes of Agrobacterium not present on said mobilisable plasmid, whereby the necessary gene products providing the same or similar activity as a functional VirB operon are also present and cocultivating said Agrobacterium with said eukaryotic host cell.
Title: Transformation of eukaryotic cells by mobilizable plasmids

The invention relates to the transfer of genetic material to eukaryotic cells by means of a process resembling conjugation, in particular by a system partially based on Agrobacterium tumefaciens-like transfer systems. In particular transfer of genetic material into plant cells is disclosed using mobilisable, but non-conjugative plasmids by means of an Agrobacterium virulence system.

The Agrobacterium virulence system is routinely used for the transfer of genetic material into plants. Indications have been obtained that this system mediates the transfer of said genetic material by a process which resembles conjugation. Conjugation is a sophisticated process which requires a complex set of sequences and gene products present in bacteria in order to be successful.

Naturally, only genetic material which is surrounded by the Ti border repeats (T-DNA) is transferred by the Agrobacterium virulence system. The only exception is that the promiscuous IncQ plasmid can be transferred by the Agrobacterium system. The frequency of this transfer however is 100-fold less than that of the natural T-DNA. Moreover it depends on the presence of many, if not all of the activities of the Agrobacterium system.

The present invention now provides a new group of plasmids which can be transferred by the Agrobacterium virulence system at an efficiency at least similar to that of the natural T-DNA.

Thus the invention provides a new group of plasmids which comprise the mobilisation functions necessary for the transfer of genetic material to eukaryotic cells, but which need some but not all functions, determined by an
Agrobacterium virulence system i.e. that of A. tumefaciens or a related species. Thus in one embodiment the invention provides a method for transferring genetic material by means of an Agrobacterium virulence system to a eukaryotic host cell, providing said genetic material on a mobilisable plasmid, capable of forming a relaxosome, bringing said mobilisable plasmid in an Agrobacterium having at least the activity of the transfer genes of Agrobacterium not present on said mobilisable plasmid, whereby the necessary gene products providing the same or similar activity as a functional VirB operon are also present and cocultivating said Agrobacterium with said eukaryotic host cell. According to the present invention a mobilisable plasmid is defined as a plasmid that has (preferably in cis or optionally with some functions in trans) the capability of forming a relaxosome (in a suitable surrounding such as Agrobacterium) and being capable of being transferred by an Agrobacterium vir-like system into eukaryotic cells.

The necessary and desired functions will be discussed in detail in the detailed description. The genetic material to be transferred into the eukaryotic host cell (plant, yeast, fungi or animal) may be any genetic material of interest ranging from genes to antisense or cosuppressing sequences, etc. The field of providing cells with additional genetic material is by now well ploughed and candidate sequences are well within the skill of the art. Typically the transfer will occur by a conjugation-like system based on an Agrobacterium-like system. Any such system will suffice if it is capable of complementing the functions lacking on the mobilisable plasmid. Typically it will be necessary to provide for physical contact between the eukaryotic host cell and the Agrobacterium-like vehicle in order to effect transfer. Herein this is referred to as
cocultivation. Typical functions to be present on the mobilisable plasmids according to the invention include the origin of transfer or mobilisation, herein referred to as oriT. Thus in another embodiment the invention provides a method whereby said mobilisable plasmid comprises a functional oriT. Preferably the mobilisable plasmid also has the virD-like mobilization functions necessary for relaxosome formation and a virD-like coupling factor for connecting the relaxosome to the VirB transport channel but these latter functions may also be provided in trans. Also preferred is a method whereby the mobilisable plasmid comprises itself a functional VirB operon. Functional virD sequences and virB operons and other sequences encoding functional products are defined herein as sequences encoding products having at least one the same or similar relevant activity as e.g. the virD products, although their actual physical structures may differ. Preferred of course are derivatives of these functional products such as virD themselves or homologues thereof in different species. Derivatives may include functional fragments of e.g. virD.

It is of course preferable if the mobilisable plasmids according to the invention can be easily propagated and/or manipulated. The invention in a preferred embodiment thus provides a method whereby said mobilisable plasmid is derived from a group of mobilisable plasmids present in enterobacteria, which plasmids are non-self-conjugative, more preferably a method wherein said group of mobilisable plasmids comprises small plasmids which can be maintained in high copy number in enterobacteria, in particular wherein said group of enterobacteria comprises E.coli. The exemplified and preferred plasmid according to the invention is derived from the mobilisable plasmid CLoDF13.
The invention in another preferred embodiment provides a method wherein said mobilisable plasmid is produced and or multiplied in an enterobacterium, preferably E.coli. The invention of course also provides the plasmids according to the invention themselves and their uses.

Thus, in one embodiment the invention provides a mobilisable plasmid comprising genetic material to be transferred into a eukaryotic cell by Agrobacterium transfer, said mobilisable plasmid further comprising an oriT sequence, but whereby the mobilization functions and coupling factor are provided in trans from another replicon.

In yet another embodiment the invention provides a mobilisable plasmid comprising genetic material to be transferred into a eukaryotic cell by Agrobacterium transfer, said mobilisable plasmid further comprising a functional oriT and sequences encoding functional mobilisation products and a coupling factor. The requirements and desirabilities of the presence of the several functions in cis and/or in trans has been touched upon before and is discussed in greater detail in the detailed description. Although not necessarily so, it is preferred to have virB-like activity in trans, just as virD-like activities.

The plasmids according to the inventions can be put to uses according to the invention, in particular the use of transferring genetic material to cells, in particular the nucleus or cell organelles. The plasmids according to the invention typically are well suited for such sophisticated uses or can be manipulated to fit such uses.

Of course the preferred cell to be provided with additional genetic material according to the invention is a plant cell. The invention thus also includes plant cells and plants or parts of plants and/or offspring of plants or gametes of plants comprising plasmids or remains of plasmids or genetic material originating from plasmids according to the
invention. The invention will be described in more illustrative detail in the following detailed description. In still another preferred embodiment the invention provides a mobilisable plasmid comprising 2 oriT sequences preferably flanking a nucleic acid to be transferred thereby allowing transfer of the area between the 2 oriTs, separate from the rest of the mobilisable plasmid.

Detailed description.

The natural trans-kingdom genetic transfer from *Agrobacterium tumefaciens* to plants during tumorigenesis represents a sophisticated process of bacterial colonization (for review, see Hooykaas and Schilperoort, 1992). Such an infection relies on the transfer of a precise DNA fragment (the T-DNA) which is flanked by two 25-bp directly repeated sequences (the T-DNA borders). The T-DNA is part of a large bacterial tumor-inducing plasmid (the pTi) and is exported presumably as a DNA/protein complex (the T-complex) from the bacterial cell directly into the plant cell, where it integrates into the plant genome and expresses its onc-genes giving plant-cell divisions resulting in crown-gall tumor formation. *Agrobacterium*-mediated transformation has been shown as well for yeasts and fungi (Bundock et al., 1995; de Groot et al., 1998) and the mechanism of T-DNA transfer resembles the one previously observed in plants.

The genetic requirements for T-DNA transfer to plants have been extensively studied: a large set of vir-genes located adjacent to the T-DNA in the Ti plasmid are involved in this. Besides it requires the presence in *cis* of at least one of the 25-bp border repeats, the so-called right border (RB) (for reviews see Hooykaas and Beijersbergen, 1994; Sheng and Citovsky, 1996). Via the VirA protein the bacteria detect specific plant metabolites, such as acetosyringone (AS),
whereafter the VirG protein triggers the transcriptional activation of the remaining vir loci (Winans, 1992). This in turn leads to production of the VirD2 endonuclease, which assisted by the VirD1 protein makes site-specific nicks within the 25-bp border repeats of the T-DNA (Scheifele et al., 1995, Pansegrau an. Lanka, 1996). After border nicking VirD2 remains covalently linked to the 5’-end, of the T-DNA lower strand via a specific tyrosyl residue. Possibly by displacement synthesis starting from the free 3’OH end, the lower strand (T-strand) with the 5’ attached VirD2 protein is released and transferred to the plant via the pilus/pore structure made up of VirB proteins. Efficient transport to the plant cell nucleus of the T-complex is mediated by nuclear localization sequences (NLS) present in the C-terminal part of VirD2. The T-strand is believed to be co-operatively coated by VirE2, a single-stranded DNA binding protein that also possesses nuclear localization sequences (Zupan et al., 1996). VirE2 has been shown necessary for preserving the 3’-end of the T-DNA (Rossi et al., 1996), thus, the "packaging" function of VirE2 may provide protection against nuclease degradation in the plant cell. Otherwise VirE2 is also important for efficient nuclear delivery of the T-strand (Ziemienowicz et al, 1999).

Export of the T-complex from the Agrobacterium cell thus occurs via a mechanism that resembles bacterial conjugation. Conjugative plasmids encode sets of genes responsible for two distinct processes. Firstly, DNA processing by which the DNA is nicked at a specific site in the origin of transfer (oriT) sequence by a relaxase and auxiliary proteins, forming the so-called relaxosome. Secondly, transfer of a single-stranded DNA which is released by rolling circle replication, to the recipient via a multiprotein pilus/pore structure (Lanka and Wilkins 1995). Some plasmids carry the (mob) genes necessary for DNA processing at oriT, but lack the transfer (tra) genes
for building the transport pilus/pore. Such plasmids can be
mobilized by other, conjugative plasmids i.e. they can use
the transport structure of such conjugative plasmids for
their own transfer to the recipient. Whether such a
mobilizable plasmid is transferred by a conjugative plasmid
is determined to a large extent by the "coupling factor"
encoded by the conjugative plasmid (Cabezon et al., 1997). It
has been proposed that coupling proteins interact with the
relaxosome and mediate the transfer of the single-stranded
nucleoprotein complex to the mating machinery. They share
homology around two putative nucleotide-binding motifs and
therefore they may form the molecular motor allowing the
nucleoprotein complex to be transported to the recipient
cell. Besides the lack of information currently available on
certain steps, the similarities between T-DNA transfer and
bacterial conjugation have increased during the last few
years. Specifically, the Ti plasmid virulence machinery
mediates the transfer of the broad host range IncQ plasmid
RSF1010 to plant cells (Buchanan-Wollaston et al., 1987)
and between agrobacteria (Beijersbergen et al., 1992). This
DNA transfer has been shown to depend particularly on a
functional virB operon and virD4. The virB genes have been
shown to be essential for tumorigenesis (Berger and Christie,
1994) and their products have been described to be associated
with the bacterial envelope and to determine a pilus
structure (Beijersbergen et al, 1994; Fullner et al, 1996). The
VirD4 protein has all the characteristics of a coupling
protein. These findings match perfectly with the genetic
requirements for mobilization of small plasmids like RSF1010
among agrobacteria. However, pilus formation by conjugative
plasmids is dependent on the VirB-related conjugative
proteins, but not on the VirD4-like protein (Pansegrau et
al., 1996) as was found for the Vir-pilus (Fullner et al,
1996).
Our studies were focussed on the limited host range bacteriocinogenic plasmid CloDF13, which originates from *Enterobacter cloacae* (Tieze et al., 1969). It belongs to a group of mobilizable, but non-self-conjugative plasmids, with a small size that can be maintained at high copy number in enterobacteria (Nijkamp et al., 1986). We have cloned eukaryotic marker genes on CloDF13 and tested whether this plasmid could be transferred from *Agrobacterium* to yeast and to plant cells. Our results show that CloDF13 transfer is possible to eukaryotic cells and relies on a functional virB operon but is independent of the virD operon.

In summary, indications were obtained that the *Agrobacterium* virulence system mediates the transfer of genetic material to plant cells by a mechanism resembling conjugation. The transfer intermediate was found to be a ssDNA-protein complex, which was formed after the action of a Vir-encoded relaxase (VirD2) at a specific site, the border repeat (Lessl and Lanka, 1994). A transmembrane VirB pilus/pore protein complex turned out to be responsible for transport of the DNA across the bacterial membranes into the recipient. This VirB structure not only mediated transfer from Agrobacterium to plants, but also to fungi and other bacteria (Beijersbergen et al., 1992; Bundock et al., 1995; De Groot et al., 1998). Finally, the Vir-system mobilized the T-DNA of the Ti plasmid, but also the promiscuous IncQ plasmid to recipient cells, provided that the latter plasmid had intact mobilization functions and the oriT sequence. The frequency of IncQ plasmid mobilization, however, was 100-fold less than of the natural T-DNA (Bravo-Angel et al., 1999).

Although it was known that CloDF13 could be mobilized by different bacterial transfer systems from one bacteria to another bacteria (Cabezón, 1997), we provide for the first time evidence for the unexpected finding that the Vir-system can mediate efficient transfer to eukaryotic cells of the
limited host range, enterobacterial plasmid CloDF13 as well. Mobilization relies on the presence of the CloDF13 oriT sequence as well as its mobilization genes. From the Vir-system of the donor the virD operon including the VirD4 gene can be deleted without affecting CloDF-transfer to yeast indicating that they are functionally replaced by CloDF13 mobilization functions. This contrasts with the mobilization of the IncQ plasmid by the Vir-system, in which case presence of the VirD4 gene remains essential. Advantages of the use of CloDF13 derivatives as novel plant vectors are clear as they offer novel traits, i.e.: a) they are small, high copy number plasmids in E.coli and can therefore be easily manipulated b) their transfer to yeast and plants is very efficient in contrast to the transfer of IncQ plasmids c) derivatives with two oriT sequences in direct repeat will lead to the formation of “T-DNAs” lacking vector parts as is the case in the Ti plasmid d) characteristics of the mobilization proteins may be exploited to direct their use not only for nuclear transformation, but also for organel transformation; e) similarly they offer advantages for the use of CloDF13 as a vector for gene targeting by homologous and site specific recombination.

MATERIALS AND METHODS

Recombinant DNA Techniques
Unless specified, standard protocols were followed for plasmid DNA isolation, cloning, restriction enzyme analysis, PCR amplifications, DNA gel electrophoresis and DNA hybridization (Sambrook et al., 1989). Total DNA from yeast was isolated using the method described by Holm et al. (1986).

Plasmid Constructions
pCloLEU was constructed by insertion of: (i) a 4.6-kb BamHI-
SalI region from plasmidCloDF13 (co-ordinates 1476-6624, anti-clock sense, Nijkamp et al., 1986), containing the plasmid mobility region; (ii) a 3.4-kb HindIII-SalI fragment from plasmid pBEJ16 (Hadfield et al., 1990) containing the 2μm ori-STS region for replication and mitotic stability in S. cerevisiae plus LEU2 as a yeast auxotrophic marker, into the IncP vector pRBJ (J. Escudero, unpublished) which is a pBin19 derivative from which one of the BglII fragments is deleted and replaced by the MCS of pUC19, containing a kanamycin resistance gene for selection in bacteria (see Figure 1). pCloGUS was constructed similarly as pCloLEU but in this case a 2.5-kb fragment containing the [CaMV 35S promoter-modified GUS-CaMV 35S terminator] gene cassette from plasmid pBG5 (Shen et al., 1993) was used as marker gene for specific expression in plants (see Figure 1). Agrobacterium strains were electroporated with these plasmid constructs as described by Mozo & Hooykaas (1991).

Bacterial and Yeast Strains

The Agrobacterium tumefaciens strains used in this work are listed in the Table 4. All bacterial strains contain the original C58 chromosomal background and either an octopine type pTiB6 plasmid with a wild-type vir-gene region or derivatives of it. The Escherichia coli strain used for cloning was DH5α (Sambrook et al., 1989). Saccharomyces cerevisiae strain RSY12 (MATa leu2-3,112 his3-11,15 ura3Δ::HIS3) was used as recipient cell in conjugation experiments with bacteria.

Plasmid-DNA Transfer Assays

Conjugation assays between agrobacteria harboring pCloLeu and yeast were carried out as follows. The bacterial donor cells were grown for 2-3 days at 28°C on LC-agar (Hooykaas et al.
1979) medium plates in the presence of appropriate antibiotics (rifampicin, 10 mg/l; kanamycin, 100 mg/l; gentamycin, 80 mg/l; carbenicillin, 75 mg/l). From fresh cultures, a single colony was inoculated into 10 ml of LC liquid medium with the same antibiotic specification. Growth was allowed overnight at 28°C, shaking at 200 rpm to reach an OD$_{600}$ between 1.0-1.5. Then bacteria were collected by centrifugation and washed with a 10 mM Mg SO$_4$ solution. Thereafter, bacteria were diluted to OD$_{600}$ ≈ 0.2 in two kinds of minimal liquid media: (i) MM (Hooykaas et al., 1979), which is regularly adjusted to pH 7. (ii) IM [containing the same composition as the MM, plus 0.5 % (w/v) glycerol, 40 mM 2-(N-morpholino) ethanesulfonic acid (MES) and optional 0.2 mM AS], which is adjusted to pH 5.3. Bacteria in minimal liquid medium were further cultured for 8-10 hr at 28°C, shaking at 200 rpm, before being used for mating with the yeast cells. The yeast recipient cells were grown on YPD-agar (Sherman et al., 1983) medium plates and a single colony was cultured overnight at 30°C in YPD-liquid medium. Yeast cells were then diluted 20 times in fresh YPD liquid medium and subsequently cultured for 8-10 hr at 30°C. Yeast cells were then collected by centrifugation and washed with either MM or with IM, then concentrated 10 times in the same medium before use. Subsequently, 50 μl of both the bacterial and yeast suspensions were gently mixed in an Eppendorf tube and finally placed on 0.45 μm cellulose nitrate filters. Bacteria-yeast conjugations were carried out either on MM-agar plates or on IM-agar plates, containing 5 mM glucose and the relevant amino acids (leucine and uracil at 30 mg/l and histidine at 20 mg/l). After co-cultivation the filter with the cell mixture was immersed in 1 ml of PZ [physiological salt solution, 9 g/l (w/v) NaCl] and shaked vigorously for 10-15 min. Afterwards, 100 μl aliquots of this conjugation mixture were plated out on MY-agar medium (Zonneveld, 1986)
plates containing 0.2 mM cefotaxim, to counterselect bacterial growth, and lacking leucine. The number of Leu\(^+\) transformed RSY12 colonies obtained in this way, after incubation for one week at 30°C, was taken as an estimate of the efficiency of successful plasmid transfer from agrobacteria to yeast. The output number of bacteria (donor cells) and yeast (recipient cells) was accurately determined by plating out dilutions of the conjugation mixture in the PZ solution: for bacteria on LC-agar medium containing the relevant antibiotics and for yeast on MY-agar medium containing the full set of required aminoacids. Plasmid DNA was isolated from the Leu\(^+\) transformed yeast colonies and used to transform E. coli cells for a proper characterisation by restriction analysis.

Plasmid pCloGUS transfer assays to plants were carried out as follows. Agrobacteria were grown and treated as specified above for the conjugation assays with yeast, except that after washing with the 10 mM Mg SO\(_4\) solution the bacterial suspension was adjusted to an OD\(_{600}\) ≈1.0 in MS-liquid medium (Murashige and Skoog, 1962) before use. Tobacco seedlings (Nicotiana tabacum, cv. Petit Havana line SR1) 7- to 10-days-old, from sterile in vitro germinated seeds in MS-agar medium, were used as plant-cell recipients. Routinely, twenty seedlings were immersed in 4 ml of bacterial suspension contained in plastic tubes and subjected to soft vacuum infiltration (~ 0.5 atm with occasional gentle shaking) during fifteen min. Subsequently, the tobacco seedlings were quickly blottedted on sterile paper and transferred to MS-agar medium plates containing 0.2mM AS.

Bacteria and plant co-cultivation was then allowed for 3 days in vitro at 23°C in a growth chamber with a 16 hr light (2000 lux)/8 hr dark regime. The tobacco plantlets were then washed in sterile distilled water and subjected to a GUS histochemical assay as described (Escudero et al., 1995). The
number of tobacco cells expressing GUS was then taken as an estimation of the efficiency of pCloGUS transfer from agrobacteria to the plant cell.

5 Tumor Formation Assays
Agrobacteria were grown as described above on LC-agar medi. with appropriate antibiotics. Bacterial cells were then resuspended in 10 mM MgSO₄ and adjusted to OD₆₀₀ ≈ 1 in Eppenforf tubes before use. Two-months-old Nicotiana glauca plants were infected by puncturing first in the stem with a sterile toothpick and subsequently applying 20 µl of the bacterial suspension to be tested into the wound. Routinely 3 infections were performed per plant and every test was repeated in at least two plants. Furthermore, independent infection experiments were carried out with different batches of plants. After infection of the plants with bacteria, plant cell proliferation (the so-called tumour formation) was due to the oncogenic nature of the native T-DNA. Plants were scored for tumour formation after 6 weeks post-infection.

20 Results

Trans-kingdom mobilization of Plasmid CloDF13 from Agrobacterium to yeast Requires vir-Gene Activation, Low Temperature and Long Mating Time
The CloDF13 plasmid is a small, non-conjugative plasmid, which can be mobilized among E. coli cells by the F plasmid and several other conjugative plasmids. The mobilization genes of CloDF13 are distinctly different from those of other mobilizable plasmids, such as the broad host range IncQ plasmid RSF1010. In addition, CloDF13 seems to encode a protein, MoDB, related to the family of coupling proteins, such as pTi VirD4 and RP4 TraG. Hence, we were interested to find out whether CloDF13 could be mobilized by the pTi virulence system in interkingdom crosses and which Vir-
proteins would be required for such a DNA transfer. Initially, we chose the yeast *Saccharomyces cerevisiae* as a recipient because of experimental convenience. Therefore, we constructed the plasmid pCloLEU (Figure 1), containing the mobilization region of CloDF13, the RK2 replicator for maintenance in agrobacteria, the yeast *LEU2* selection gene and the yeast 2μ replicator. We then did mating experiments between *A. tumefaciens* and *S. cerevisiae* RSY12, which is a haploid Leu⁻ strain, to test for plasmid transfer from bacteria to yeast. In this way indeed Leu⁺ yeast colonies were obtained indicative of pCloLEU transfer to yeast from Agrobacterium (LBA1100).

To investigate the transfer mechanism of the pCloLEU plasmid from agrobacteria to yeast, we assayed different values of four important parameters for *Agrobacterium*-mediated DNA transfer during our bacteria/yeast co-cultivation. Namely: (1) acidity of the medium (pH 5.3 versus pH 7); (2) temperature (23 °C versus 33 °C); (3) mating time (20 hr, 40 hr and 60 hr) and (4) presence or absence of the *vir*-gene inducer acetosyringone (AS). The results from Table 1 show that an acidic medium, low temperature and the inducer AS during the bacteria/yeast co-cultivation were essential for the recovery of Leu⁺ transformed yeast colonies. The length of mating time is also critical because high numbers of Leu⁺ yeast colonies were only observed after long co-cultivation. The estimated pCloLEU transfer frequency from the bacterial strain LBA1100 after 60 hr was $10^{-5}$ and this value decreased one order of magnitude per 20 hr shortening in co-cultivation time. A similar duration of co-cultivation was also necessary for T-DNA transfer from agrobacteria to yeast (Bundock et al, 1995). Hence, we concluded that pCloLEU transfer to yeast had all the characteristics of transport by the *Agrobacterium Virulence* system. For that, transcriptional activation of the *vir* regulon by the presence of AS is necessary and the particular mating complex in the agrobacterial donor, responsible for DNA/protein translocation, needs to be
functionally established requiring the proper physical conditions.

**Role of Vir-proteins in the interkingdom CloDF13 mobilization**

In order to establish which of the Vir-proteins are involved in the interkingdom transfer of CloDF13, we tested several vir-mutants for their ability to mobilize this plasmid to yeast. As it is shown in Table 2, transfer of pCloLEU did occur from agrobacteria with complete vir-systems (strains LBA1010, LBA1100 and GV3101 [pPM6000]). However, lack of vir genes (strain LBA288) resulted in no plasmid transfer. As a control, we created the plasmid pLEU, which is identical to pCloLEU but devoid of CloDF13 sequences. As expected, pLEU could not be transferred from agrobacteria to yeast cells (see below). The virA (LBA1142), virB (LBA1143) and virG (LBA1145) operons were essential for transfer to occur. This was expected since the VirA and VirG proteins are regulators of the expression of the vir-regulon, and it is believed that VirB proteins likely determine the mating structure. Mutants impaired in the gene for the single stranded DNA binding protein VirE2 (strains LBA1149 and ATΔvirE2) showed a tenfold lower frequency of transfer, as this was the case for T-DNA transfer to yeast (data not shown). The host-range gene virF (LBA1561) was not necessary for CloDF13 mobilization. As the CloDF13 mobilization region determines its own oriT sequence and the cognate relaxase protein, we expected that the pTi encoded border repeat specific relaxase VirD2 would not be necessary for CloDF13 transfer. This was indeed the case; strains with a non-polar insertion in virD2 (LBA1147) or deletion of virD2 (ATΔvirD2) were equally mobilization-proficient as the wild-type strains. Similarly, CloDF13 transfer from Agrobacterium to yeast could be accomplished from bacteria with a mutation in the gene coding for the coupling factor VirD4 (strains LBA1148 and LBA1151). As VirD4 is essential for T-DNA transfer to yeast, it is probable that
the protein encoded by the CloDF13 mobilization region, which resembles VirD4, can take over its function and interacts with the VirB complex. To confirm that such a characteristic is intrinsic for CloDF13-like plasmids, we also assayed a RSP1010 (IncQ) derivative plasmid. RSP1010 could not be transferred to yeast from the mutated virD4 bacterial strain LBA1148 (data not shown).

The CloDF13 mobB and mobC genes are Essential for Trans-kingdom Transfer

We constructed the plasmid pCloLEU by inserting the SalI-BamHI fragment (≈ 4.6 kb) from CloDF13 (Nijkamp et al., 1986), encompassing the mobilization region plus oriT, into a wide host range replicon (see Figure 1). In order to analyze the CloDF13 genetic elements that were required for the observed plasmid transfer from agrobacteria to yeast, a series of derivative plasmids was constructed by mutating stepwise the four CloDF13 genes present in pCloLEU: (i) pCloΔELEU, with a deletion of the gene E encoding the immunity protein; (ii) pCloΔEHLEU, with an additional deletion of the gene H encoding the cloacin excretion protein; (iii) pCloΔEBLEU, with an additional deletion of the gene mobB; (iv) pCloΔECLEU, with an additional deletion of the gene mobC. The results, summarized in Table 3, indicated that neither gene E nor H is essential for Agrobacterium-mediated plasmid transfer to yeast. Plasmids pCloΔELEU and pCloΔEHLEU were transferred at high frequency, as their parental pCloLEU, from all transfer-proficient bacterial strains tested. Indeed the original plasmids pCloLEU, pCloΔELEU and pCloΔEHLEU, which were harbourd by the bacterial donor, could be rescued from transformed Leu' yeast cells after the mating experiments (data not shown). However, both CloDF13 mobB and mobC turned out to be essential for transfer, as in no case the plasmid pCloΔEBLEU or pCloΔECLEU was mobilized to yeast. Hence, genetic complementation of the CloDF13 mob genes did
not occur by any agrobacterial vir counterpart, suggesting a strong specificity of the respective proteins for their cognate intermediate complex during conjugal transfer (see below).

5 CloDF13 can also be Transferred to Plant Cells and does not Inhibit Agrobacterium Virulence

We were interested to find out whether CloDF13 transfer, as was described above from agrobacteria to yeast, would also occur to plant cells. Hence, the plasmid pCloGUS was constructed (Figure 1), which is similar to pCloLEU but carrying the gene for β-glucuronidase (gus) gene under plant expression signals. This GUS marker has been previously shown to be very sensitive in detecting T-DNA expressed in plants, both in tobacco as well as in maize cells (Rossi et al., 1993; Shen et al., 1993). After co-cultivation of agrobacteria with young tobacco (Nicotiana tabacum, line SR1) plants, the expression of the GUS gene encoded in the pCloGUS plasmid was assayed histochemically in the plant tissue. Blue staining, indicative of GUS activity, in the tobacco plantlets was clearly detected with the bacterial strains LBA1010 [pCloGUS, pTiB6] and LBA1100 [pCloGUS, pAL1100], both harbouring wild-type vir genes. This transient expression of the CloDF13-derivative plasmid was very abundant in the tobacco tissue. Therefore, we compared transfer of pCloGUS and transfer of T-DNA to tobacco cells. We assayed in parallel the bacterial strain LBA1100 [pCloGUS, pAL1100] with LBA1100 [pBG5, pAL1100], which carries the mentioned GUS gene as T-DNA marker in a RK2 replicon similar to the one used to construct pCloGUS. The efficiency in transfer of the pCloGUS plasmid was similar to that of T-DNA, judged from the number of plant cells showing GUS activity (data not shown). This result evidenced that transfer of pCloGUS and expression of the marker gene in the plant-cell nucleus took place at high efficiency.
As mentioned above and similarly to what was observed in the experiments with yeast as a recipient cell, the plasmid pCloGUS could be transferred to tobacco cells from strain LBA1010, which carries a wild-type T-DNA in its pTi plasmid. Importantly, the tumour formation in Nicotiana glauca plants infected with the strain LBA1010 was efficient and irrespective of the presence of plasmid pCloGUS (data not shown). Hence, contrary to what has been observed with agrobacteria containing RSF1010-derivative plasmids (Ward et al, 1991), there seems to be no interference between the transfer of the T-DNA and the CloDF13 complexes from agrobacteria to plant cells.
Figure legend

Fig. 1 Plasmids pCloGUS and pCloLEU
REFERENCES


efficient method for isolating DNA from yeast. Gene 42, 169-173.


Table 1. Transfer efficiencies of plasmid pCloLEU from wild-type agrobacterial strain LBA1100 to yeast depending on the vir-gene induction conditions, the temperature and the extent of time during mating

<table>
<thead>
<tr>
<th>Medium</th>
<th>Temperature (°C)</th>
<th>Time (hr)</th>
<th>Titre Output (x10^9)*</th>
<th>Transfer Frequency^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.3+AS</td>
<td>23</td>
<td>20</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>pH 5.3+AS</td>
<td>23</td>
<td>40</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>pH 5.3+AS</td>
<td>23</td>
<td>60</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>pH 5.3+AS</td>
<td>33</td>
<td>60</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>pH 5.3</td>
<td>23</td>
<td>60</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>23</td>
<td>60</td>
<td>3.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The strain LBA1100 [pCloLEU] was used as bacterial donor and the strain RSY12 was the yeast recipient.

*Values represent number of bacterial donors and yeast recipient colonies per millilitre.

^b Estimated as the frequency of RSY12 Leu^+ yeast colonies per output recipient.
Table 2. Transfer of the CloDF13-derivative plasmid pCloLEU from *A. tumefaciens* donor strains to the *S. cerivisiae* recipient strain RSY12

<table>
<thead>
<tr>
<th>Bacterial Strain&lt;sup&gt;a&lt;/sup&gt; (vir mutation)</th>
<th>No. of RSY12 colonies (x10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. Leu&lt;sup&gt;+&lt;/sup&gt; RSY12 colonies&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Frequency of Leu&lt;sup&gt;+&lt;/sup&gt; per recipient cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA288 (No vir)</td>
<td>2.2</td>
<td>0</td>
<td>&lt;2.2x10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>LBA1010</td>
<td>2.3</td>
<td>1175</td>
<td>0.5x10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>LBA1100</td>
<td>2.1</td>
<td>1200</td>
<td>0.5x10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>LBA2577</td>
<td>1.3</td>
<td>2700</td>
<td>2.0x10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>LBA1147 (3' virD2)</td>
<td>1.5</td>
<td>2500</td>
<td>1.6x10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>LBA1148 (virD4)</td>
<td>1.1</td>
<td>1400</td>
<td>1.4x10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>LBA1149 (virE2)</td>
<td>2.0</td>
<td>154</td>
<td>0.7x10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>LBA2576 (virE2)</td>
<td>2.3</td>
<td>160</td>
<td>0.7x10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>LBA2561 (virF)</td>
<td>1.5</td>
<td>1250</td>
<td>0.8x10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>The bacterial strains LBA1010, LBA1100 and LBA2577 all contain a wild-type vir set of genes in their respective pTi plasmid. The bacterial strain LBA288 is a LBA1010 derivative in which the pTi plasmid was cured. All bacterial strains contained the plasmid pCloLEU and matings were performed at 23 °C, in pH 5.3 medium containing 0.2mM acetosyringone as described (see Materials and Methods).

<sup>b</sup>Counting are values per millilitre of mating mixture.
### Table 3. Effect of the different CloDF13 genetic components in the transfer efficiency of CloDF13-derivative plasmids from agrobacteria to yeast

<table>
<thead>
<tr>
<th>Bacterial Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of RSY12 colonies (x10^8)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. Leu&lt;sup&gt;+&lt;/sup&gt; RSY12 colonies&lt;sup&gt;u&lt;/sup&gt;</th>
<th>Frequency of Leu&lt;sup&gt;+&lt;/sup&gt; per recipient cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA1100 [pJleu]</td>
<td>1.8</td>
<td>0</td>
<td>&lt;1.8x10^-6</td>
</tr>
<tr>
<td>LBA1100 [pCloΔEleu]</td>
<td>3.0</td>
<td>360</td>
<td>1.2x10^-6</td>
</tr>
<tr>
<td>LBA1100 [pCloΔEHleu]</td>
<td>1.8</td>
<td>3200</td>
<td>1.7x10^-5</td>
</tr>
<tr>
<td>LBA1100 [pCloΔEBleu]</td>
<td>3.0</td>
<td>0</td>
<td>&lt;3.0x10^-8</td>
</tr>
<tr>
<td>LBA1100 [pCloΔECleu]</td>
<td>3.2</td>
<td>0</td>
<td>&lt;3.2x10^-8</td>
</tr>
<tr>
<td>LBA1148 [pCloΔEleu]</td>
<td>3.0</td>
<td>400</td>
<td>1.3x10^-6</td>
</tr>
<tr>
<td>LBA1148 [pCloΔEHleu]</td>
<td>1.7</td>
<td>2400</td>
<td>1.4x10^-5</td>
</tr>
<tr>
<td>LBA1148 [pCloΔEBleu]</td>
<td>3.5</td>
<td>0</td>
<td>&lt;3.5x10^-8</td>
</tr>
<tr>
<td>LBA1148 [pCloΔECleu]</td>
<td>2.5</td>
<td>0</td>
<td>&lt;2.5x10^-8</td>
</tr>
</tbody>
</table>
Table 4. *Agrobacterium* strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chromosomal background</th>
<th>Ti Plasmid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA288</td>
<td>C58</td>
<td>Cured of Ti, no <em>vir</em></td>
<td>Hooykaas et al. 1979</td>
</tr>
<tr>
<td>LBA1010</td>
<td>C58</td>
<td>wild-type <em>vir</em> pTiB6</td>
<td>Koekman et al. 1982</td>
</tr>
<tr>
<td>LBA1100</td>
<td>C58</td>
<td>pAL1100, i.e. pTiB6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\Delta T_{r}$, $\Delta T_{r}$, $\Delta tra$, $\Delta occ$</td>
<td>Beijersbergen et al. 1992</td>
</tr>
<tr>
<td>LBA1142</td>
<td>C58</td>
<td>pAL1100 (<em>virA::Tn3Hoho1</em>)</td>
<td>idem</td>
</tr>
<tr>
<td>LBA1143</td>
<td>C58</td>
<td>pAL1100 (<em>virB4::Tn3Hoho1</em>)</td>
<td>idem</td>
</tr>
<tr>
<td>LBA1145</td>
<td>C58</td>
<td>pAL1100 (<em>virG::Tn3Hoho1</em>)</td>
<td>idem</td>
</tr>
<tr>
<td>LBA1147</td>
<td>C58</td>
<td>pAL1100 (<em>3virD2::Tn3Hoho1</em>)</td>
<td>idem</td>
</tr>
<tr>
<td>LBA1148</td>
<td>C58</td>
<td>pAL1100 (<em>virD4::Tn3Hoho1</em>)</td>
<td>idem</td>
</tr>
<tr>
<td>LBA1149</td>
<td>C58</td>
<td>pAL1100 (<em>virE2::Tn3Hoho1</em>)</td>
<td>idem</td>
</tr>
<tr>
<td>LBA1151</td>
<td>C58</td>
<td>pAL1100 (<em>5'virD2::Tn3Hoho1</em>)</td>
<td>idem</td>
</tr>
<tr>
<td>LBA1561</td>
<td>C58</td>
<td>pAL1100 ($\Delta virF$)</td>
<td>Schrammeijer et al., 1998</td>
</tr>
<tr>
<td>LBA2577</td>
<td>C58</td>
<td>pPM6000, i.e. pTiAch5 $\Delta T_{r}$, $\Delta T_{r}$</td>
<td>Bonnard et al., 1989</td>
</tr>
<tr>
<td>LBA2576</td>
<td>C58</td>
<td>pPM6000 ($\Delta virE2$)</td>
<td>Rossi et al., 1996</td>
</tr>
<tr>
<td>LBA2584</td>
<td>C58</td>
<td>pPM6000 ($\Delta virD2$)</td>
<td>Bravo-Angel et al., 1998</td>
</tr>
</tbody>
</table>
Claims

1. A method for transferring genetic material which is not a typical T-DNA surrounded by Agrobacterium T-borders from an Agrobacterium virulence system to a eukaryotic host cell, comprising providing said genetic material on a mobilisable plasmid, capable of forming a relaxosome, bringing said mobilisable plasmid in an Agrobacterium having at least the activity of the transfer genes of Agrobacterium not present on said mobilisable plasmid, whereby the necessary gene products providing the same or similar activity as a functional VirB operon are also present and cocultivating said agrobacterium with said eukaryotic host cell.

2. A method according to claim 1, whereby said mobilisable plasmid comprises a functional oriT and a sequence encoding VirD-like mobilization activity as well as a sequence encoding a VirD4-like coupling factor.

3. A method according to claim 1 or 2, whereby said mobilisable plasmid comprises a functional oriT, but whereby VirD-like mobilization functions and the VirD4-like coupling factor are provided in trans.

4. A method according to any one of claims 1-3, whereby said mobilisable plasmid is derived from a group of mobilisable plasmids present in enterobacteria, which plasmids are non-conjugative.

5. A method according to claim 4, wherein said group of mobilisable plasmids comprises small plasmids which can be maintained in high copy number in enterobacteria.

6. A method according to claim 5, wherein said group of enterobacteria comprises E.coli.
7. A method according to any one of the foregoing claims, wherein said mobilisable plasmid is derived from CloDF13.

8. A method according to any one of the foregoing claims whereby said mobilisable plasmid is produced and or multiplied in an enterobacterium.

9. A method according to claim 8, wherein said enterobacterium is E.coli.

10. A mobilisable plasmid comprising genetic material to be transferred into a eukaryotic cell by Agrobacterium transfer, said mobilisable plasmid further comprising a functional oriT, sequences encoding functional virD-like mobilisation products, a VirD4-like coupling factor, and sequences encoding functional virB-like activity.

11. A mobilisable plasmid according to claim 10, which encodes the Mob functions of CloDF13.

12. A mobilisable plasmid according to claim 10 or 11, which is derived from a group of mobilisable plasmids present in enterobacteria, which plasmids are non-self-conjugative.

13. A mobilisable plasmid according to claim 12, which is derived from CloDF13.

14. Use of a plasmid according to any one of claims 10-13 for the transfer of genetic material to eukaryotic cells.

15. Use according to claim 14, wherein said transfer is to the nucleus or an organelle of said eukaryotic cell.
16. Use according to claim 14 or 15, wherein said eukaryotic cell is a plant cell.

17. Use according to claim 14 or 15, wherein said eukaryotic cell is a yeast cell and/or a fungal cell.
FIGURE 1

pCloGUS

pCloLEU
### A. CLASSIFICATION OF SUBJECT MATTER

| IPC  | C12N15/82 | C12N15/74 | C12N15/80 | C12N15/81 | A01H5/00 |

According to International Patent Classification (IPC) or to both national classification and IPC.

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

| IPC  | C12N |

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

### Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

- EPO-Internal, WPI Data, PAJ, BIOSIS, CAB Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>BEIJERBERGEN A ET AL: &quot;CONJUGATIVE TRANSFER BY THE VIRULENCE SYSTEM OF AGROVACTERIUM TUMEFACIENS&quot; SCIENCE, US, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, vol. 256, 29 May 1992 (1992-05-29), pages 1324-1327, XP000674721 ISSN: 0036-8075 cited in the application the whole document</td>
<td>1, 2, 4-6, 8-10, 12, 14-16</td>
</tr>
</tbody>
</table>

### X Further documents are listed in the continuation of box C.  

### X Patent family members are listed in annex.

### Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance.
- "E" earlier document but published on or after the international filing date.
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified).
- "O" document referring to an oral disclosure, use, exhibition or other means.
- "P" document published prior to the international filing date but later than the priority date claimed.

### Date of the actual completion of the international search

7 June 2001

### Date of mailing of the international search report

15/06/2001

### Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rivwijk Tel: (+31-70) 340-2040, Tx: 31 651 epo nl, Fax: (+31-70) 340-3016

Authorized officer:

Oderwald, H
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>NOMURA N ET AL: &quot;Genetic organization of a DNA-processing region required for mobilization of a non-self-transmissible plasmid, pEC3, isolated from Erwinia carotovora subsp. carotovora&quot; GENE, NL, ELSEVIER BIOMEDICAL PRESS, AMSTERDAM, vol. 170, no. 1, 17 April 1996 (1996-04-17), pages 57-62, XP004042870 ISSN: 0378-1119 the whole document</td>
<td>1,3-6,8, 9</td>
</tr>
<tr>
<td>X</td>
<td>US 4 680 264 A (PUHLER ALFRED ET AL) 14 July 1987 (1987-07-14) the whole document</td>
<td>1,3-6,8, 9</td>
</tr>
<tr>
<td>X</td>
<td>WO 88 03564 A (BIOTEKNIKA INTERNATIONAL) 19 May 1988 (1988-05-19) the whole document</td>
<td>1,3-6,8, 9</td>
</tr>
<tr>
<td>X</td>
<td>BUCHANAN-WOLLASTON ET AL: &quot;The mob and oriT mobilization functions of a bacterial plasmid promote its transfer to plants&quot; NATURE, GB, MACMILLAN JOURNALS LTD, LONDON, vol. 328, 9 July 1987 (1987-07-09), pages 172-175, XP002094285 ISSN: 0028-0836 cited in the application the whole document</td>
<td>1,3-6,8, 9</td>
</tr>
<tr>
<td>A</td>
<td>Cabezón E ET AL.: &quot;Genetic evidence of a coupling role for the TraG protein family in bacterial conjugation&quot; MOLECULAR AND GENERAL GENETICS, vol. 254, 1997, pages 400-406, XP002144852 cited in the application the whole document</td>
<td>1,3-6,8, 9</td>
</tr>
</tbody>
</table>

---
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>
| A        | DE GROOT M J A ET AL.: "Agrobacterium tumefaciens-mediated transformation of filamentous fungi"  
            cited in the application  
            the whole document | ____ |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td>US 4680264 A</td>
<td>14-07-1987</td>
<td>NONE</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 8240087 A</td>
<td>01-06-1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0288547 A</td>
<td>02-11-1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 1501598 T</td>
<td>08-06-1989</td>
</tr>
</tbody>
</table>