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T-DNA Transfer, Integration, Stability and Quantification in Transgenic Plants

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La science consiste à passer d'un étonnement à un autre.
[Aristote]
La science ne sert guère qu'à nous donner une idée de l'étendue de notre ignorance.
[Félicité de Lamennais]
La vraie science est une ignorance qui se sait.
[Montaigne]

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Abbreviations

A	A	Adenine
	AACC	American Association of Cereal Chemists
	AFLP	Amplified Fragment Length Polymorphism
	ANOVA	Analysis of variance
	att	Attachment
B	bp	Base pair
C	C	Cytosine
	cDNA	Complementary DNA
	Chv	Chromosomal virulence
	CPB	Cartagena Protocol on Biosafety
	Cre	Recombinase of cre/lox recombinase system
	CRL	Community Reference Laboratory
	CRM	Certified Reference Material
	CSCE	Conformational Sensitive Capillary Electrophoresis
	C_T	Threshold cycle
	CTAB	Cethyl Trimethyl Ammonium Bromide
D	D	Dark
	DNA	Deoxyribonucleic acid
	ds	Double stranded
	DSB	Double-stranded break
	DSBR	Double-stranded break repair
	Dtr	DNA transfer and replication
E	EC	European Commission
	EFSA	European Food Safety Authority
	ELISA	Enzyme linked immunosorbent assay
	EU	European Union
F	FAO	Food and Agriculture Organization
	FRET	Fluorescence resonance energy transfer
G	G	Guanine
	GM	Genetically modified
	GMDD	GMO Detection Method Database
	GMO	Genetically Modified Organism
	Gus	β-glucuronidase gene
H	h	Hours
	HGE	Haploid Genome Equivalent
	HIV	Human Immunodeficiency Virus
	HR	homologous recombination
I	IHCP	Institute of Health and Consumer Protection
	indels	Insertions/deletions

	IRMM	Institute for Reference Materials and Measurements
J	JRC	Joint Research Center
L	L	Light
	LB	Left T-DNA border
	LMO	Living Modified Organism
N	NHEJ	Non-homologous end-joining
	NHR	Non homologous recombination
	NIRS	Near infrared spectroscopy
	NLS	Nuclear localization signal
	nos	Nopaline synthase gene
	nptII	Neomycin phosphotransferase gene
O	OSTP	Office of Science and Technology Policy
P	P35S	Cauliflower mosaic virus 35S promoter
	PCR	Polymerase Chain Reaction
	PEG	polyethylene glycol
Q	QC-PCR	Quantitative competitive polymerase chain reaction
	QRT-PCR	Quantitative real time polymerase chain reaction
R	rat	Resistant to *Agrobacterium* transformation
	RB	Right T-DNA border
	RFLP	Restriction Fragment Length Polymorphism
	RNA	Ribonucleic acid
S	SDSA	Synthesis-dependent strand annealing
	SERK1	Somatic embryogenesis receptor kinase 1 promoter
	SNP	Single nucleotide polymorphism
	ss	Single-stranded
	SSIIb	Maize starch synthase gene
T	T	Thymine
	T4SS	Type IV Secretion System
	T-DNA	Transferred DNA
	Ti plasmid	Tumor inducing plasmid
V	VIP	VirE2 interacting protein
	Vir	Virulence factors
W	WHO	World Health Organization

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Part 1: *Agrobacterium* Mediated Plant Transformation

1. *Agrobacterium*: history, nomenclature and host range

The earliest study reporting plant tumor caused by a bacterium was made more than hundred years ago. The bacterium was then called "*Bacterium tumefaciens*" and described as a "rod shaped", medium size and polar flagelled organism unable to grow well at 37ºC" (Smith and Townsend, 1907). *Agrobacterium*, a new bacterium genus, was later proposed to comprise all plant pathogens closely related to the crown gall organism with *Agrobacterium tumefaciens* as its type species (Conn, 1942). To date several other species are members of the *Agrobacterium* genus. Nomenclature and classification of these species is still subject to a debate amongst scientists. Based on pathogenic symptoms, they were grouped in different species: *tumefaciens, radiobacter, rhizogenes, rubi, vitis and larrymoorei. Agrobacterium tumefaciens* was the name given to strains capable of inducing tumorigenic reactions in a wide range of host plant species (Smith and Townsend, 1907; Conn, 1942). *Agrobacterium radiobacter*, originally proposed as *Bacillus radiobacter* by Beijerinck and Van Delden (1902), comprised non-pathogenic strains. *Agrobacterium rhizogenes* was the name given to bacteria causing the hairy root or rhizogenic symptom in a range of plant species (Riker et al., 1930; Conn, 1942). *Agrobacterium rubi* was allocated to species considered to be specific to *Rubus* (Starr and Weiss, 1943). Ophel and Kerr (1990) re-examined a subpopulation of tumorigenic strains isolated from grapes, previously described as *Agrobacterium radiobacter* and grouped them as *Agrobacterium vitis.* A last group of tumorigenic pathogens was isolated from aerial tumors in *Ficus benjamina* and was named *Agrobacterium larrymoorei* (Bouzar and Jones, 2001).

Agrobacterium strains were also classified based on their biochemical and physiological properties which led to the definition of three biotypes (Kerr and Panagopoulos, 1977). More recent Young et al., (2001) proposed to rename the *Agrobacterium* genus, member of the *Rhizobiaceae* family, as a member of the

closely related *Rhizobium* genus and also proposed renaming the species. Farrand et al., (2003), in a reply to Young‘s proposition, argued that sufficient differences exist between *Rhizobium* and *Agrobacterium* such that the genus names should not be changed. A summary of the different nomenclatures allocated to the *Agrobacterium* genus is presented in Table 1.

In 1974, the genetic element conferring pathogenicity to *Agrobacterium tumefaciens* was characterized by the group of Schell and Van Montagu as a circular DNA molecule that they called Ti (tumor inducing) plasmid or pTi (Zaenen et al., 1974). The plasmid of *Agrobacterium rhizogenes* is called Ri plasmid (root inducing tumor) (White et al., 1982). Nester et al., (1977) showed that the tumor is based on the transfer of a DNA fragment from the Ti plasmid, called T-DNA, to the plant cell chromosomes. Later studies revealed that the T-DNA is well defined within the Ti plasmid by the presence of two 25 bp flanking borders as direct repeat, characterized by a high homology in their sequences and referred to as the left and right T-DNA borders (Yadav et al., 1982; Zambryski et al., 1982). T-DNA transfer and integration in the host genome will be described in the next section of this introductory chapter. These findings were the starting point of plant genome engineering. Indeed, any foreign DNA placed between the T-DNA borders can be transferred to plant cells. Thus, the first vector systems for plant transformation were constructed (de Framond et al., 1983; Hoekema et al., 1983; Deblaere et al., 1985; Hooykaas and Schilperoort, 1992; Hamilton, 1997).

On the natural T-DNA, the onco (*onc)* genes and the opine synthase genes required for disease establishment are located. The *onc* genes code for proteins that are involved in the production of plant growth factors, such as auxins and cytokinins, resulting in an uncontrolled cell division and crown gall production after gene transfer. Via the opine synthase enzymes, specific opines are produced in the plant cells. These molecules can be used exclusively by *Agrobacterium* as carbon and nitrogen source. In this way, *Agrobacterium* creates a distinct ecological niche for itself (Schell et al., 1979; Guyon et al., 1980). Opines have been used for classifying *Agrobacterium* strains and their Ti/Ri plasmids. Octopine, nopaline, succinamopine, agropine, agropine/mannopine, mannopine, chrysopine/succinamopine, chrysopine/nopaline, cucumopine/mikimopine (Pionnat et al., 1999; Moriguchi et al.,

2001) octopine/cucumopine and vitopine (Szegedi et al., 1988) strains and plasmids have been identified. A null-type category has been proposed for cases in which no opine could be detected. Although different opines were identified, their use for *Agrobacterium* classification was rejected for three reasons. First, some Ti/Ri plasmids, initially classified as null-type, were found later to produce opines (Guyon et al., 1980; Chang et al., 1983; Chilton et al., 1984; Unger et al., 1985; Hood et al., 1986). Second, opine synthesis and/or utilization genes occupy relatively small parts of the large Ti/Ri plasmids. The third reason is the ability of one plasmid to induce several types of opines and different plasmids can specify different combinations of opines (Otten et al., 2008).

The Ti plasmid carries also the transfer (*tra*), the opine catabolism and the virulence (*vir*) genes (Zhu et al., 2000 for review). The *tra* genes are involved in the conjugative transfer of the Ti plasmid between different *Agrobacterium* cells, whereas the *vir* genes code for proteins that process and transport the T-DNA to the plant cell. The opine catabolism genes code for enzymes that metabolize the specific plant-produced opines.

Three genetic elements are necessary for transfer and integration of the T-DNA in the plant genome: the border repeats that delimit the T-DNA, the *vir* genes that code for the *in trans*-acting type IV secretion system, and various bacterial chromosomal genes that are necessary for attachment and the early stages of transformation (Yadav et al., 1982; Zambryski et al., 1982; Lee and Gelvin, 2008 for review). These chromosomal genes are generally involved in bacterial exopolysaccharide synthesis and secretion (Douglas et al., 1985; Cangelosi et al., 1987; Robertson et al., 1988; Cangelosi et al., 1989; Matthysse et al., 1995a; Matthysse et al., 1995b). However, some chromosomal genes important for virulence likely mediate the bacterial response to the environment (Xu and Pan, 2000; Saenkham et al., 2007).

As a genus, *Agrobacterium* can transfer DNA to a broad group of organisms including numerous dicotyledon and monocotyledon angiosperm species (DeCleene and DeLey, 1976; Anderson and Moore, 1979; Porter, 1991; van Wordragen and Dons, 1992) and gymnosperms (Loopstra et al., 1990; Morris and Morris, 1990; Stomp et al., 1990; McAfee et al., 1993; Yibrah et al., 1996; Levee et al., 1999;

Wenck et al., 1999). In addition, *Agrobacterium* can transform fungi, including yeast (Bundock et al., 1995; Bundock and Hooykaas, 1996; Piers et al., 1996), ascomycetes (de Groot et al., 1998; Abuodeh et al., 2000), and basidiomycetes (de Groot et al., 1998). *Agrobacterium* was also reported to transfer DNA to human cells (Kunik et al., 2001) and to grass species (Wang and Ge, 2005; Vogel and Hill, 2008; Zale et al., 2009).

The molecular and genetic basis for the host range of a given *Agrobacterium* strain remains unclear. Early work indicated that the Ti plasmid, rather than chromosomal genes, was the major genetic determinant to control host range (Loper and Kado, 1979; Thomashow et al., 1980). Several virulence (*vir*) loci on the Ti plasmid, including *virC* (Yanofsky and Nester, 1986) and *virF* (Melchers et al., 1990; Regensburg-Tuink and Hooykaas, 1993), were shown to determine the range of plant species that could be transformed into growing crown gall tumors. The *virH* locus appeared to be involved in the ability of *Agrobacterium* to transform maize, as established by an assay in which symptoms of maize streak virus infection were determined following agroinoculation of maize plants (Jarchow et al., 1991). Other *vir* genes, including *virG*, contribute to the “hypervirulence” of particular strains (Hood et al., 1986). However, it became clear that *Agrobacterium* host range is determined by both bacterial and plant factors. For example, many monocot plant species, including some cultivars of grasses such as maize (Ishida et al., 1996), rice (Chan et al., 1993; Hiei et al., 1994) and wheat (Cheng et al., 1997), can now be genetically transformed by many *Agrobacterium* strains. However, these plant species do not support the growth of crown gall tumors. Host range may further result from an interaction of particular Ti plasmids with certain bacterial chromosomal backgrounds. For example, the Ti plasmid pTiBo542, when in its natural host strain *A. tumefaciens* Bo542, directs limited tumorigenic potential when assayed on many leguminous plant species. However, when placed in the C58 chromosomal background, pTiBo542 directs strong virulence toward soybeans and other legumes (Hood et al., 1987).

Table 1: Comparison of nomenclature allocated to the genus *Agrobacterium* (adapted from Young, 2008).

Species names based on natural classification			Species names based on pathogenicity	
After Young et al., (2001)	After Holmes and Roberts, (1981); Holmes, (1988); Moore et al., (2001)	After Kaene et al., (1970); Kerr and Panagopoulos, (1977); Panagopolous et al., (1978)	After Allen and Holding, (1974); Skerman et al., (1980)	After Kersters and De Ley, (1984)
R. larrymoorei[a]	*A. larrymoorei*[b]	NR[c]	NR	NR
R. Radiobacter (Ti)	*A. tumefaciens*[d] (tumorigenic)	*A. radiobacter* biovar *tumefaciens* (biotype 1)	*A. tumefaciens*	*A. tumefaciens* (biovar 1)
R. Radiobacter (Ti)	*A. tumefaciens*[d] (tumorigenic)	*A. radiobacter* biovar *tumefaciens* (biotype 1)	*A. tumefaciens*	*A. tumefaciens* (biovar 1)
R. Radiobacter (Ri)	*A. tumefaciens*[d] (rhizogenic)	*A. radiobacter* biovar *rhizogenes* (biotype 1)	*A. rhizogenes*	*A. rhizogenes* (biovar 1)
R. Radiobacter	*A. tumefaciens* (non -pathogenic)	*A. radiobacter* biovar *radiobacter* (biotype 1)	*A. radiobacter*	*A. radiobacter* (biovar 1)
R. rhizogenes (Ti)	*A. rhizogenes* (tumorigenic)	*A. radiobacter* biovar *tumefaciens* (biotype 2)	*A. tumefaciens*	*A. tumefaciens* (biovar 2)
R. rhizogenes (Ri)	*A. rhizogenes* (rhizogenic)	*A. radiobacter* biovar *rhizogenes* (biotype 2)	*A. rhizogenes*	*A. rhizogenes* (biovar 2)
R. rhizogenes	*A. rhizogenes* (non-pathogenic)	*A. radiobacter* biovar *radiobacter* (biotype 2)	*A. radiobacter*	*A. radiobacter* (biovar 2)
R. rubi[b]	*A. rubi*[b]	*A. radiobacter* biovar *tumefaciens* (biotype 2)	*A. rubi*	*A. rubi*
R. vitis (Ti)	*A. vitis* (tumorigenic)[e]	*A. radiobacter* biovar *tumefaciens* (biotype 3)	NR	*A. tumefaciens* (biovar 3)
R. vitis	*A. vitis* (non-pathogenic)	NR	NR	NR

R: *Rhizobium*
A: *Agrobacterium*
Ri: virulence plasmid of *A*. *rhizogenes*
Ti: tumor-inducing plasmid
[a] Oncogene designations are indicated when necessary for clarity.
[b] Only tumorigenic (Ti) capability has been reported for this species
[c] NR = not recorded
[d] The correct name for this species is *A. radiobacter* (see Sawada et al., 1993; Young et al., 2006)
[e] (Moore et al., 2001)

2. *Agrobacterium* plant transformation process

The different steps underlying the plant transformation process via *Agrobacterium* were extensively studied and documented in several excellent reviews: Gelvin, 2000; Tzfira and Citovsky, 2000; Zupan et al., 2000; Tzfira and Citovsky, 2002; Gelvin, 2003; Tzfira and Citovsky, 2003; McCullen and Binns, 2006; Citovsky et al., 2007; Banta and Montenegro, 2008. Six steps were arbitrarily identified: (1) chemical recognition of the host and (2) activation of virulence gene expression followed by the physical recognition and interaction between the bacterium and the host. The third step (3) is the production of transferred substrates and transfer machinery leading to the next step (4) which is the transfer of substrates out of the bacterium and into the host cell. Once in the plant cell, the movement of substrates into the nucleus starts (5) followed by the integration of the T-DNA into the host genome (6) and expression of the T-DNA gene(s) (Figure 1).

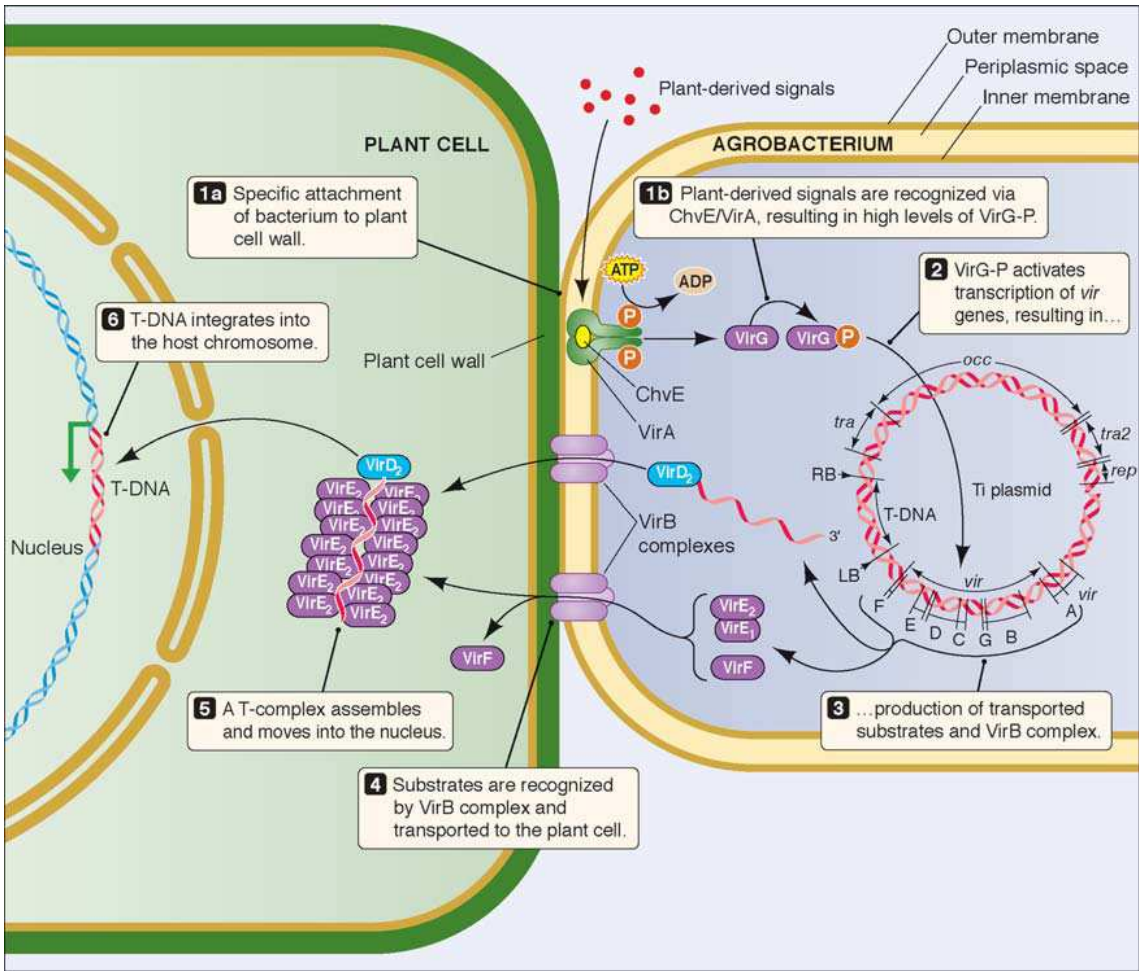

Figure 1: A model for molecular interactions during *Agrobacterium*-mediated genetic transformation of plant cells (McCullen and Binns, 2006) *(Legend continued next page)*

Figure 1 *(legend continued from previous page)* The transformation process begins with recognition of plant signals by the bacterial VirA/VirG sensory system, followed by activation of the *vir* loci and attachment of the bacterium to the host cell (1a,1b). The T-strand is excised from the T-DNA region by VirD2/VirD1 (2,3) and exported with a covalently attached VirD2 molecule and with several other *vir* proteins, into the plant cell cytoplasm via a VirB/D4 type IV secretion system (4). Inside the plant cell the intranuclear transport of the T-complex to the host chromosome takes place (5) and T-DNA integration into the host cell genome is mediated by VirD2 and/or VirE2 and by host factors (6) (see text for details).

2.1. *Agrobacterium* plant cell recognition

The first step of the transformation process is the recognition of the plant cell by the bacteria. It has been proposed that the wounded plant cell is the preferred substrate for *Agrobacterium* infection, although inefficient infection via stomata (Escudero and Hohn, 1997) and transformation of non wounded plants (Escudero and Hohn, 1997; Brencic et al., 2005) have also been reported. In nature, wounded plant tissues secrete a wide range of low molecular compounds such as phenolic and sugar compounds provoking a chemotactic response of the bacteria towards the plant cell (Shaw, 1991). Simultaneously with the chemotaxis, the two-component regulatory system VirA-VirG of *Agrobacterium*, necessary for virulence (*vir*) activation, is induced (Stachel and Zambryski, 1986; Shaw, 1991). VirA is a membrane bound sensor kinase that has a large periplasmic domain (Melchers et al., 1989; Chang and Winans, 1992) and interacts directly with plant phenolic components (Lee et al., 1995). VirA interacts also with ChvE, a chromosomally encoded and abundant periplasmic protein. ChvE is homologous to a series of periplasmic sugar-binding proteins that are involved in sugar transport and chemotaxis to sugars (Gao et al., 2006). The interaction of both proteins makes VirA supersensitive (Chang and Winans, 1992; Shimoda et al., 1993; Peng et al., 1998; Gao and Lynn, 2005). VirA functions as a homodimer and its autophosphorylation reaction is in fact an intradimer transphosphorylation reaction (Brencic et al., 2004a). The unstable phosphate bond is transferred to an aspartic acid residue of VirG, the cytoplasmic response regulator (Jin et al., 1990). The activated VirG protein binds to a specific 12bp DNA sequence of the *vir* operon promoters (*vir* boxes), leading to the transcription of the inducible *vir* genes (Pazour and Das, 1990; Scheeren-Groot et al., 1994; Brencic and Winans, 2005) (Figure 2).

Although the phenolic compounds are essential for the sensing and the movement of *Agrobacterium* towards the plant cell, they are toxic for the bacteria and thus have to be degraded. Two proteins, encoded by the *virH* operon and showing high similarity to cytochrome P450 enzymes, are ideal candidates for detoxification (Brencic et al., 2004b). Other environmental factors play a very important role in the induction of the *vir* genes such as temperature, pH and phosphate concentration (Winans, 1990; Dillen et al., 1997; Gao and Lynn, 2005). pH regulation of the VirG promoter may be mediated through a separate pH-sensing two-component system, ChvG/ChvI. This system is chromosomally encoded and required for both *vir* gene expression and virulence (Li et al., 2002).

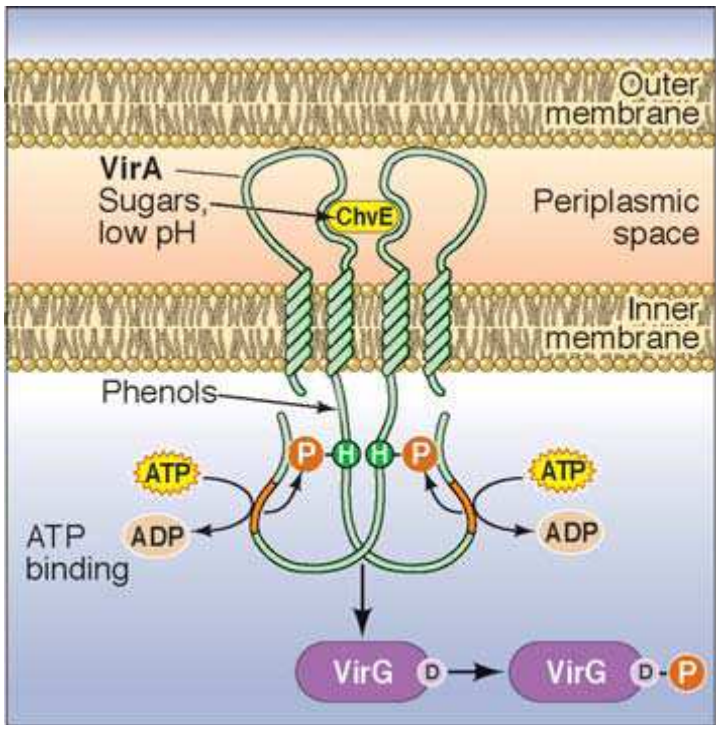

Figure 2: Plant and environmental signals recognition by *Agrobacterium* leads to the activation of the ChvE/VirA/VirG signal transduction system (McCullen and Binns, 2006).

2.2. Attachment of *Agrobacterium* to plant cells

Following recognition of the plant cell, the attachment of *Agrobacterium* to its host results in the formation of a biofilm of bacteria on the surface of the plant tissue (Danhorn and Fuqua, 2007). This attachment occurs in a polar manner and in two

steps. The first step is likely mediated by a cell-associated acetylated acidic capsular polysaccharide present on the bacterial envelope (Reuhs et al., 1997; Matthysse et al., 2000), a ricadhesin (Swart et al., 1994), the gene products from the chromosomally encoded *chvA*, *chvB*, and *pscA* genes (involved in synthesis, processing and export of cyclic beta-1, 2-glucans and other sugars) (Cangelosi et al., 1989; O'Connell and Handelsman, 1989; Uttaro et al., 1990), and the pAtC58 localized *att* region (encoding ABC transporter systems and genes required for the synthesis of surface molecules; Matthysse et al., 1996). The implications of these genes were revealed after the isolation of *Agrobacterium tumefaciens* mutants that were unable to bind to plant cells (Matthysse et al., 1996; Matthysse and McMahan, 1998). The second step in the attachment process stabilizes the previous described step and involves the elaboration of cellulose fibrils by the bacterium (Matthysse et al., 1981; Matthysse, 1987; Matthysse et al., 1995a; Matthysse et al., 1995b). It has been proposed that plant pathogen virulence proteins, such as ligninases, pectinases and xylanases might also be involved in *Agrobacterium*-infection by means of breaching the cell wall prior to T-DNA transfer (Wood et al., 2001).

The attachment of *Agrobacterium* to the plant cell wall also involves plant molecules. Plant vitronectin-like proteins were thought to be involved in the attachment as *Agrobacterium* mutants defective to attach the plant cells showed reduced binding to vitronectin (Wagner and Matthysse, 1992). A more recent study showed that there was no correlation between the occurrence of vitronectin, attachment of bacteria to the cells and susceptibility to *Agrobacterium*-mediated transformation (Clauce-Coupel et al., 2008). The latter does not support a functional role of plant vitronectin as the receptor for site-specific *Agrobacterium* attachment to the plant cells (Clauce-Coupel et al., 2008). In addition to vitronectin, a rhicadhesin−binding protein was proposed to play a role in *Agrobacterium* binding to the host cells (Swart et al., 1994).

Other plant proteins playing a role in *Agrobacterium* attachment to the plant cell were identified by the isolation of *Arabidopsis thaliana* mutants resistant to *Agrobacterium* transformation called *rat* mutants (Nam et al., 1999; Zhu et al., 2003a). These *rat* mutants were obtained after screening libraries of T-DNA insertion mutant lines of *Arabidopsis* for recalcitrance to *Agrobacterium* root transformation. In

the selected *rat* mutants, plant DNA fragments flanking the T-DNA insertion sites were identified (Feldmann, 1991; Nam et al., 1999; Zhu et al., 2003a). The *rat1* locus encodes a Lysine-rich arabinogalactan protein which might be involved in the reduction of systemic acquired resistance responses during the infection process (Gaspar et al., 2004). This observation is pointing to the fact that efficient transformation of plants is depending on the inactivity of the plant defense mechanisms or its inhibition by *Agrobacterium* (Ditt et al., 2005). Recently, it was shown that *Arabidopsis* G-protein *β*-subunit 1 is required for the defense response against *Agrobacterium tumefaciens* (Ishikawa, 2009). The *rat3* locus probably encodes an unidentified plant cell wall protein. Mutant *rat4*, blocked at early stages of root transformation, codes for the cellulose synthase-like gene *CSLA9* (Zhu et al., 2003b). This *CSLA9* gene is expressed in the elongation zone of the roots and lateral root primordia, a region which is very susceptible to *Agrobacterium*-mediated transformation (Yi et al., 2002). The *rat*4 mutant has a decreased number of lateral roots, is less able to bind *Agrobacterium*, but displays no major alterations in cell wall sugar composition or linkage structure (Zhu et al., 2003b). Interestingly, transformation by the *rat1, rat3* and *rat4* mutants via infection of female gametophytes was as efficient as the transformation of their respective wild-type parental ecotypes (Mysore et al., 2000; Zhu et al., 2003a), suggesting that different surface molecules are involved in the attachment to different plant tissues (Mysore et al., 2000).

2.3. Production of the single stranded T-DNA

As mentioned earlier, sensing of the wounded plant cell by the bacterium induces the VirA-VirG two-component regulatory system. The phosphorylated VirG actively binds the *vir* boxes and activates transcription of the *vir* operons. Amongst the induced Vir proteins are VirD1 and VirD2, coding for a site-specific endonuclease and a relaxase respectively. Those enzymes are involved in the T-strand DNA processing (Filichkin and Gelvin, 1993). VirD2 nicks the Ti plasmid between the third and the fourth base in the “bottom strand” of the right and left border repeats (Scheiffele et al., 1995; Relic et al., 1998) determining in this way the initiation and

termination sites for T-strand formation. Nicking of the double-stranded (ds) Ti plasmid by VirD2 also requires VirD1, which probably acts as topoisomerase that converts the supercoiled DNA to relaxed DNA (Jayaswal et al., 1987).

Another sequence called ‘overdrive’ flanks the T-DNA right border and, together with VirC1 and VirC2 proteins, stimulates tumorigenesis several hundredfold (Peralta et al., 1986). VirC1 binds the overdrive sequence and the VirD2 protein also interacts with this sequence (Toro et al., 1989). Although the precise role of the overdrive and the *virC* encoded proteins remain unknown, they appear to distinguish the right and left border sequences (the origin and terminus of T-DNA transfer respectively). As T-DNA transfer is unidirectional (Miranda et al., 1992) and requires the right border in its wild type orientation (Peralta and Ream, 1985), the relaxosome must distinguish between the left and the right border of the T-DNA which are functionally equivalent in their interaction with the VirD1/VirD2 nicking enzyme (Yanofsky et al., 1986; Albright et al., 1987). Thus it was proposed that the overdrive allows the transfer apparatus to recognize the right border as the origin of transfer (Fullner and Nester, 1996).

After nicking the right border sequence, a single-stranded T-strand is produced in an unidirectional way that proceeds toward the left border (Albright et al., 1987). Upon nicking, the VirD2 remains covalently attached to the 5’ end of the processed T-strand (Herrera-Estrella et al., 1988; Durrenberger et al., 1989; Howard et al., 1989; Gelvin, 2003). Thus, a single-stranded protein/nucleic acid complex called the T-complex is transferred to the plant cell (Tinland et al., 1994; Christie, 2004; Vergunst et al., 2005). Several roles were attributed to the VirD2 protein. Durrenberger et al., (1989) suggested that VirD2 is protecting the T-DNA strand against exonucleolytic degradation. Nuclear localization signals (NLS), present at the amino- and carboxy termini of the VirD2 protein, target the T-strand to the nucleus (Herrera-Estrella et al., 1990; Howard et al., 1992; Tinland et al., 1992). Recognition of VirD2 was shown to be mediated by widely conserved nuclear factors in eukaryotes (Bako et al., 2003). Furthermore, VirD2 also has a function in the precise integration and ligation of the T-DNA ends to the plant DNA (Tinland et al., 1995). This role is still controversial. Indeed, Ziemienowicz et al., (2000) showed that not VirD2 but plant enzymes are involved in ligation of the T-DNA to the plant DNA

genome. However, the study conducted by Pelczar et al., (2004) suggests that virulence proteins, mainly VirD2 and VirE2, are exported in association with VirE1 and that these proteins only bind the single-stranded (ss) T-DNA in the plant cell. Indeed, these proteins were sufficient for the integration of a synthetic T-DNA into an eukaryotic genome in the absence of other bacterial or plant factors (Pelczar et al., 2004).

2.4. Transfer of the T−strand complex to the plant

Following the activation of the virulence genes and attachment to the host, the *Agrobacterium* T-strand together with particular proteins will be transferred to the plant cells. These molecules need to cross both the bacterial and the cellular membranes and cell walls. A specialized transporter complex encoded by the VirB operon and VirD4 (Zupan et al., 2000; Christie, 2004) is employed. The VirB complex is a prototypical type IV secretion system (T4SS), a class of transporters found across a broad range of gram-negative bacteria, involved in the conjugative transfer of plasmids between bacteria (for review see Cascales and Christie, 2003; Nagai and Roy, 2003; Christie et al., 2005; Schroder and Lanka, 2005). In *Agrobacterium*, this apparatus is assembled from 11 proteins encoded by the VirB operon and VirD4 (Krall et al., 2002; Ward et al., 2002) (Figure 3). These proteins are required for virulence, associate with the cell envelope and form a multisubunit envelope-spanning structure (Christie et al., 2005). Substrates transported into host cells by the VirB complex include the VirD2-T strand, VirE2, VirE3, VirF and VirD5 (Vergunst et al., 2005).

VirD4, a member of the ATPases family (Gomis-Ruth et al., 2004) has been implicated in the coupling of transfer intermediates with the export apparatus (Hamilton et al., 2000). Indeed, a direct interaction between VirE2 (a type IV secreted protein) and VirD4 has been reported (Atmakuri et al., 2003). The different VirB proteins either act in forming a membrane channel or in the interaction with the host cell receptors (Kado, 2000).

The VirB1 protein belongs to the superfamily of the lysozyme-like glycosylases (Koraimann, 2003) and was proposed to participate in the hydrolysis of the peptidoglycan layer (Lai et al., 2000; Llosa et al., 2000). There is controversy concerning the importance of VirB1 in the transfer process. Berger and Christie, (1994) and Lai et al., (2000) mentioned that the protein only improves the transfer efficiencies while, in a more recent study, Zupan et al., (2007) provided genetic evidence that VirB1 is an essential factor for T-pilus assembly. The same authors proposed that the protein is bifunctional as the N-terminal lytic transglycosylase domain provides localized lysis of the peptidoglycan cell wall to allow insertion of the T4SS and the C-terminal VirB1 domain promotes T-pilus assembly through protein-protein interactions with T-pilus subunits.

VirB11 is a member of a large family of ATPases (Krause et al., 2000; Planet et al., 2001). Structural studies of the protein showed that the protein possesses domains that ensures a coordination of ATP utilization (Hare et al., 2006). The VirB4 protein is the largest and most evolutionarily conserved protein in T4SS (Fernandez-Lopez et al., 2006). VirB4 is also a putative ATPase that energizes DNA and protein substrate transfer as well as pilus assembly (Tato et al., 2005; Arechaga et al., 2008). Some studies showed that VirB4 protein is predominantly cytoplasmic with possible periplasmic loops (Dang and Christie, 1997; Rabel et al., 2003) located at the entrance to the VirB/D4 channel (Middleton et al., 2005). VirD4, VirB11 and VirB4 are the energetic compounds of the VirB/D4 machine.

VirB6, VirB8, VirB10 and VirB3 are the inner membrane components (Figure 3) characterized by the presence of trans-membrane segment(s). VirB6 has been shown to stabilize other VirB proteins such as VirB3, VirB5 and VirB7 homodimers and to play a role in the formation of an outer membrane-associated VirB7-VirB9 heterodimer (Hapfelmeier et al., 2000; Jakubowski et al., 2003). VirB8 assembles as a homodimer and interacts with other VirB subunits (Kumar and Das, 2001; Ward et al., 2002). VirB10 senses the use of ATP energy by the inner membrane proteins (VirD4 and VirB11) for a dynamic association with the outer membrane protein VirB9 (Cascales and Christie, 2004). VirB10 also interacts with other T4SS subunits (Beaupre et al., 1997; Das and Xie, 2000; Llosa et al., 2000; Ward et al., 2002; Atmakuri et al., 2004; Jakubowski et al., 2005; Jakubowski et al., 2009). The function

of VirB3 is still unknown. An early study localized the VirB3 protein at the outer membrane (Jones et al., 1994). This localization was not supported by the findings of Christie et al., (2005) stating that VirB3 and VirB4 are fused as a single polypeptide.

VirB2 is the major pilin subunit of the *Agrobacterium tumefaciens* VirB/D4 T-pilus and an essential component of the secretion channel (Jones et al., 1996; Lai and Kado, 1998). Several *Arabidopsis* proteins have been identified (Hwang and Gelvin, 2004) that interact with VirB2, a major component of the bacterium-host cell attachment structure termed T-pilus (Lai and Kado, 2000). These proteins include VirB2 interactors (BTIs) BTI1, BTI2 and BTI3 with unknown functions, and a membrane associated GTPase, AtRAB8. Interestingly, BTI expression was transiently increased after *Agrobacterium* infection, indicating a positive feedback communication between *Agrobacterium* and its host cell (Hwang and Gelvin, 2004).

VirB5 subunits are exported to the periplasm and they are localized extracellularly as components of the T-pilus (Schmidt-Eisenlohr et al., 1999; Aly and Baron, 2007; Backert et al., 2008). VirB2 and VirB5 might function as adhesions that mediate host-cell targeting through binding to specific host receptors (Backert et al., 2008). The outer membrane proteins VirB7 and VirB9 play an important role in the initiation and stabilization of the transporter assembly (Baron et al., 1997). The role of the T-pilus in VirB/D4 mediated translocation is poorly understood. In analogy with the bacterial conjugation systems, the T-pilus will likely initiate contact with target cells and thus with specific plant cell receptors. It is reasonable to assume that during T-DNA transfer, plant host factors are involved. Four plant proteins have been identified that can interact with the VirB2 protein (BTI1, BTI2, BTI3 and a membrane-associated GTPase, AtRAB8). Such proteins could serve as T-pilus receptors (Hwang and Gelvin, 2004).

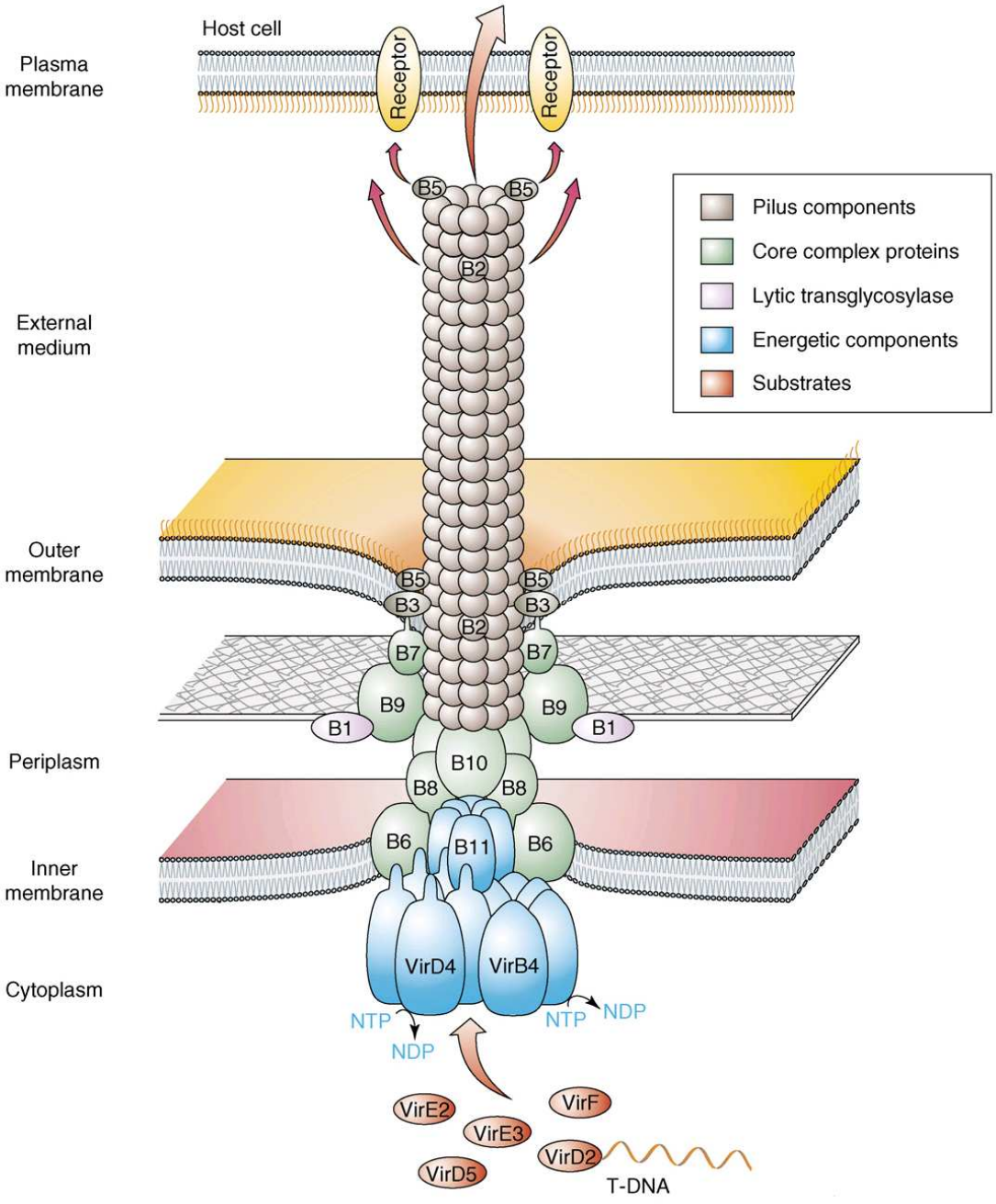

Figure 3: A proposed architecture for the VirB/D4 secretion channel (adapted from Backert et al., 2008). VirB10 senses ATP energy used by VirD4 and VirB11 and, as a consequence, interacts stably with the outer membrane-associated VirB7-VirB9 complex. The model depicts the VirB/D4 T4S as structurally dynamic complex, wherein signals including substrate binding, ATP energy and target cell contacts trigger structural transitions between a quiescent transenvelope complex and an active transport channel.

2.5. Intracellular movement and nuclear import of the T-strand

Citovsky et al., (1992) showed that VirE2 could function in a plant cell as transgenic VirE2-expressing tobacco plants could “complement” infection by a *virE2* mutant *Agrobacterium* strain. However, it has been shown that VirE2 can transfer to the plant cell in the absence of a T-strand (Otten et al., 1984; Lee et al., 1999, Vergunst et al., 2000). Hence, it is possible that VirE2 complexes with the T-strand either in the bacterial export channel or within the plant cell (see Gelvin, 2000 and 2003 for review).

The T-DNA complex must travel trough the cytoplasm to reach the host cell nucleus. The dense structure of the cytoplasm (Luby-Phelps, 2000) and the large size of the T-complex (Tzfira, 2006) suggest that the complex is actively transported. This idea was supported by the demonstration of active transport of artificial T-complexes along microtubules in a cell-free system (Salman et al., 2005). A Dynein-like *Arabidopsis* protein (dyneins are one of the three families of cytoskeleton-based molecular motors; Tzfira, 2006), DLC3, was suggested to be involved in the intracellular transport of the *Agrobacterium* T-complex through interaction with the host protein VIP1 (Tzfira et al., 2001). This interaction mediates the recognition of VirE2 by the nuclear import machinery of the plant cell (Citovsky et al., 2007). In such scenario, DCL3 may function as a molecular link between VIP1-VirE2-T-DNA complexes and the microtubule track system. *Arabidopsis* VIP1 and VIP2 proteins have been shown to interact with VirE2 (Tzfira et al., 2001). Presumably, VIP1 interacts with an importin and is imported in the nucleus (Tzfira et al., 2000), while VIP2 mediates the interaction with chromatin structures and could facilitate the integration of the T strand (Ward and Zambryski, 2001).

The diameter of the mature T-complex exceeds the diffusion size exclusion limit of the nuclear pore complex (Abu-Arish et al., 2004). This indicates that also the entry of the T-complex into the cell nucleus is an active mechanism mediated by the nuclear import machinery of the host cell. As T-complexes are polar structures, their nuclear import is thought to occur in a polar fashion initiated by the 5’ end attached VirD2 molecule (Sheng and Citovsky, 1996). *Agrobacterium* VirD2 and VirE2 proteins are involved in the nuclear T-DNA import process as they contain nuclear localization

signals (NLS). Both VirD2 and VirE2 contribute, in a different and complementary way, to the nuclear import of the T-DNA. Potentially, VirD2 is sufficient to target the T-DNA to the nucleus pore while VirE2 is required for the passage through the pore (Ballas and Citovsky, 1997). As the T-complex is longer than the channel of the nuclear pore, the single VirD2 molecule will arrive in the nucleus at early stage. VirE2 will then present the T-DNA in a continuous structure compatible with its passage through the nuclear pore (Ziemienowicz et al., 2001). This model is supported by the identification of the different plant molecules interacting with VirD2. Indeed, VirD2 interacts with three plant host cyclophilins: RocA, Roc4 and CypA probably acting as chaperones that can alter the VirD2 conformation in the plant cell (Deng et al., 1998). VirD2 was also found to interact with *Arabidopsis* α-importin proteins (AtKAPα) (Ballas and Citovsky, 1997; Gelvin, 2003). This interaction was suggested to mediate nuclear uptake of the T-complex. Also, VirD2 interacts with a type 2C serine/threonine protein phosphatase that might dephosphorylate the VirD2-NLS and negatively regulate nuclear import of the VirD2-T-DNA complex (Gelvin, 2000). Bako et al., (2003) showed that VirD2 is also bound by nuclear TATA-box binding proteins and a nuclear protein kinase. VirE2 interacts with VIP1 and VirE3 (Duckely and Hohn, 2003). VIP1 is imported into the plant cell nucleus via the karyopherin α-dependent pathway and might function as a molecular bridge between VirE2 and karyopherin α proteins (Tzfira and Citovsky, 2000; Tzfira et al., 2001; Ziemienowicz et al., 2001; Tzfira et al., 2002; Ward et al., 2002; Citovsky et al., 2004). Later findings suggested that VirE3 can partially complement the absence of VIP1 (Lacroix et al., 2005) and is transported into the nucleus. There, it may interact with the transcription factor pBrp to induce the expression of genes needed for tumor development (Garcia-Rodriguez et al., 2006). Once inside the nucleus, the T-complex is uncoated of the VirE2 proteins, a process in which VirF might be involved (Tzfira et al., 2004a). Indeed, VirF is an F-box protein that can mediate the proteolysis of the *Agrobacterium* VirE2 protein and the *Arabidopsis* VIP1 protein (Tzfira et al., 2004a).

2.6. T-DNA integration in the host genome

Once in the nucleus, the T-DNA will become double stranded (ds). Indeed, the results reported by De Neve et al., (1997) and De Buck et al., (1999) showed that the frequently observed linkages of two different T-DNAs about the right border in transformed plants can only arise when second-strand synthesis results in a double-stranded T-DNA substrate prior to linkage. T-DNA was shown to integrate into double strand breaks created in *Nicotiana tabacum* genome by transiently expressed rare cutting endonucleases (Chilton and Que, 2003; Tzfira et al., 2003). This observation is a strong evidence that the T-DNA strand is first converted to double stranded intermediates that is subsequently integrated into the plant genome (Chilton and Que, 2003; Tzfira et al., 2003). Moreover, several studies reported the expression of the T-DNA encoded genes without being integrated in the genome of the host cell corresponding to transient expression (Liu et al., 1992; Shen et al., 1993; Kapila et al., 1997; Maximova et al., 1998; De Buck et al., 2000a). These observations indicated, on one hand, that the T-DNA is rapidly converted into a dsDNA. T-DNA expression was detected within 48 hours after the start of plant cells cocultivation with *Agrobacterium* (Rossi et al., 1993; Kapila et al., 1997). This early expression of T-DNA-encoded genes showed a peak at 3-4 days after cocultivation and then declined. The extent of transient expression after 4 days was in correlation with stable integration (Janssen and Gardner, 1989). On the other hand, detection of T-DNA transient expression indicated that the frequency of gene transfer from *Agrobacterium* to plant cells is higher that the frequency of stably integrated T-DNAs suggesting that the integration step may be an inefficient process that occurs from a large pool of ds T-DNAs by a host-encoded mechanism (Janssen and Gardner, 1989).

T-DNA transient expression provides a rapid and versatile way for achieving very high levels of gene expression in plants. *Agrobacterium*-mediated transient expression systems have been useful for inducing silencing processes (Voinnet and Baulcombe, 1997; Johansen and Carrington, 2001) and for dissecting the mechanism of gene silencing. Indeed, in plants, cytoplasmic RNA silencing can be induced efficiently by agroinfiltration, a strategy for transient expression of T-DNA

vectors after delivery by *Agrobacterium tumefaciens*. The transiently expressed DNA encodes either a single- or double-stranded RNA, which is typically a hairpin (hp) RNA (Johansen and Carrington, 2001; Mlotshwa et al., 2002; Tenllado et al., 2003; Voinnet et al., 2003). T-DNA transient expression after *Agrobacterium* infiltration of *Nicotiana benthamiana* leaves was also used for protein expression (Sainsbury et al., 2009).

The mechanism of T-DNA integration is not well established and questions concerning this process still need to be answered. Amongst these questions, the following ones can be cited: how does the T-DNA “choose” its target site within the chromosomes? What is the precondition for a genomic site to be “chosen” as T-DNA integration point? Is it related to DNA breaks or to the availability of chromatin in a relaxed conformation with reduced nucleosome content? How does the T-DNA integrate in the host genome?

2.6.1. Molecules and models involved in T-DNA integration

It is logic that T-complex associated factors and/or plant host factors are involved in T-DNA integration. Experiments involving the transformation of HeLa cells with a T-DNA complex, reconstructed *in vivo*, showed that VirD2 and VirE2 are the only virulence proteins absolutely required for T-DNA integration (Pelczar et al., 2004). VirD2 protein is suggested to be involved in T-DNA integration as it is covalently attached to the 5’end of the transferred T-DNA strand. Two functions were attributed to VirD2 protein: to mediate integration (Tinland et al., 1995) and to contain ligase activity (Pansegrau et al., 1993). The finding that plant DNA ligases and not VirD2 mediated T-DNA ligation *in vitro* has weakened the direct involvement of VirD2 in T-DNA integration (Ziemienowicz et al., 2001). Nevertheless, the potential function of VirD2 in T-DNA integration cannot be denied as it interacts with plant proteins that are involved in T-DNA integration. Indeed, VirD2 has been shown to interact with nuclear TATA-box binding proteins and a nuclear protein kinase (Bako et al., 2003). VirE2 coats the T-DNA inside the plant cell and protects it from nucleolytic degradation (Rossi et al., 1996). This protection probably represents only an indirect function of VirE2 in T-DNA integration as VirE2 interacting proteins (VIP) might aid in

targeting the T-strand to suitable loci. VIP2 is known to mediate the interaction with chromatin structures in which the host DNA is exposed (Tzfira and Citovsky, 2000; Ward and Zambryski, 2001), and VIP1 was shown to interact with the H2A histone (Li et al., 2005).

That plant factors are involved in T-DNA integration was first demonstrated by the characterization of two radiation-sensitive *Arabidopsis thaliana* mutants, *uvh*1 (UV-hypersensitive) and *rad*5 (γ-radiation hypersensitive), recalcitrant to stable transformation but susceptible to transient transformation (Sonti et al., 1995). *Arabidopsis rat* mutant screening (see 2.2) allowed the identification of plant factors involved in T-DNA integration. Nam et al., (1999) used a T-DNA insertion library and identified five *rat* mutants: *rat5*, *rat17*, *rat18*, *rat20* and *rat22*, all deficient in T-DNA integration. Two of these mutants have been further characterized. The *rat17* mutant harbors a disrupted gene for a *myb*-like transcription factor (Gelvin, 2000), while the *rat5* mutant carries the T-DNA in a histone H2A gene (Mysore et al., 2000). The *rat*5 mutant is recalcitrant to root transformation, but not to flower vacuum infiltration (Mysore et al., 2000; Yi et al., 2002; Zhu et al., 2003a). After exposure of root segments to phytohormones or wounding, both histone H2A expression and the transformation frequency increased, whereas the response of a cyclin gene (*Cyc1 AT*) important for cell division (Ferreira et al., 1994) was not affected by these treatments (Yi et al., 2002). It was thus proposed that *H2A* gene expression is not strictly linked to the S-phase of the mitotic cell cycle, but expression of this gene is a marker and a predictor of plant cells most susceptible to *Agrobacterium* transformation. Recently it has been shown that *HTA* genes (*Arabidopsis thaliana* histone genes), encoding H2A histones, were able to compensate for the loss of *H2A* gene activity when overexpressed from a strong promoter (Yi et al., 2006). Disruption of histone H3 genes results in a *rat* phenotype (Zhu et al., 2003a). Also, knockout of other genes encoding chromatin-modifying proteins, such as histone deacetylases, histone acetyl transferases, and chromatin-modifying proteins, suggest the importance of chromatin structure in T-DNA integration (Zhu et al., 2003a). Plant genes involved in *Agrobacterium* transformation have also been identified using cDNA-AFLP (Ditt et al., 2001; Gelvin, 2003) and DNA microarrays (Veena et al., 2003). Analysis of the genes that are differentially expressed upon *Agrobacterium* infection indicate that, in general, plant genes necessary for T-DNA transformation

(e.g. histones and ribosomal proteins) are induced, while host defense responses were, in general, induced early after *Agrobacterium* inoculation and suppressed later during the infection process (Citovsky et al., 2007).

In addition to chromatin structure and depending on the mechanism of integration, other host factors/pathways can be involved in T-DNA integration. Double strand breaks (DSBs) may serve as a target sites for T-DNA integration. Indeed, Salomon and Puchta, (1998) showed that T-DNAs could be captured at induced DSBs in plants genome. This finding has been further substantiated by Tzfira et al., (2003) and Chilton and Que, (2003). Salomon and Puchta, (1998) observed a significantly high T-DNA integration at induced DSB which led to the suggestions that DSBs represent the preferred substrate for T-DNA integration and that the number of breaks in a cell might be rate limiting for T-DNA integration. In yeast cells, DSBs are repaired dominantly via homologous recombination (HR) by the factors Rad51 and Rad52 (Van Attikum et al., 2001). In higher eukaryotes, non homologous recombination (NHR) or non homologous end joining (NHEJ) is the dominant pathway. In plants, several factors have been shown to mediate NHEJ such as Ku70, Ku80, the Mre11/Rad50/Nbs1 complex, XRCC4 and Lig4 (reviewed in Tzfira et al., 2004b; Bray and West, 2005). Alternatively, DSBs can be also repaired via HR employing either double-strand break repair (DSBR) or synthesis-dependent single strand annealing (SDSA) involving RPA, Rad51 and likely Rad54/Rad57 proteins (reviewed in Tzfira et al., 2004b; Bray and West, 2005). A detailed description of *Agrobacterium* T-DNA integration models is provided in Tzfira et al., (2004b) and Windels et al., (2008).

2.6.2. Distribution of T-DNA inserts

Early studies suggested that T-DNA insertion within the host genome occurs in a random manner (Ambros et al., 1986; Wallroth et al., 1986; Gheysen et al., 1991). However, with the availability of the *Arabidopsis thaliana* genome sequence, the draft sequence of the rice genome (Goff et al., 2002) and large T-DNA insertion collections, preferential insertion sites were attributed to the T-DNA. At the gene level and by using promoterless marker genes, T-DNA integrates preferentially into transcription

active regions (Koncz et al., 1989; Herman et al., 1990; Kertbundit et al., 1991). Moreover, T-DNA insertions within the *Arabidopsis thaliana* genome were found more frequently at the sites of transcription initiation and termination than within genes (Schneeberger et al., 2005; Li et al., 2006). T-DNA insertions in the rice genome were also biased towards both the 5' and 3' regulatory regions of genes, outside the coding sequences (Alonso et al., 2003; Chen et al., 2003; Schneeberger et al., 2005; Zhang et al., 2007). It was speculated that a gene-rich region is usually actively transcribed and more frequently in an 'open' state, and thus more accessible to T-DNA integration (Barakat et al., 2000; Sha et al., 2004). Recently, a fascinating link between gene activity and intranuclear localization of active genes was established for *Saccharomyces cerevisiae*. This link associates T-DNA insertion to the entry of the T-DNA complex through the nuclear pores and the physical proximity of active chromatin to explain preferential integration into active genes. This hypothesis was however analyzed exclusively in yeast (Casolari et al., 2004; Rodriguez-Navarro et al., 2004; Cabal et al., 2006; Schmid et al., 2006; Taddei et al., 2006; Luthra et al., 2007) and it is not sure that it can be generalized.

At the chromosome level, T-DNA insertions were found in all five chromosomes of *Arabidopsis thaliana*, but more frequently in gene rich regions (euchromatin) then in centromeric, paracentrometric and telomeric sequences (heterochromatin) (Brunaud et al., 2002; Sessions et al., 2002; Szabados et al., 2002; Alonso et al., 2003; Rosso et al., 2003). The same observation was made for T-DNA integration in rice `(An et al., 2003; Chen et al., 2003; Sallaud et al., 2004; Zhang et al., 2007). Brunaud et al., (2002) analyzed 9000 T-DNA flanking plant genomic sequences in *Arabidopsis* to determine whether T-DNA integration exhibits a sequence specific determinant. They found that T-DNA integration is favored in A-T rich plant DNA regions. This has been suggested to enrich the flexibility of the target site thus promoting the T-DNA and the repair machineries access.

Despite the fact that the above mentioned studies indicate that T-DNA integration is not random within the *Arabidopsis* genome, care should be taken when interpreting these data. When analyzing the T-DNA distribution pattern in transgenic plants, it should be noted that the studied population is enriched for T-DNA loci that harbor an actively expressed T-DNA and also for T-DNA loci with amplifiable border

regions. Indeed, it has been shown that T-DNA insertions in heterochromatin were occurring with a higher frequency in the absence of selection than under selection conditions (Francis and Spiker, 2005; Kim et al., 2007).

2.6.3. T-DNA integration patterns

Transgene integration is more complex than a simple ligation of the T-DNA, delimited by the left and the right borders, to the host genome. Truncated T-DNAs and non T-DNA sequences could also be integrated in a simple or a complex pattern. Rearrangements in the host genome were also reported. In this section, an overview of these observations is provided.

Upon T-DNA transfer, more than one T-DNA copy can integrate into the host genome (Depicker et al., 1985; Koncz et al., 1989; Hobbs et al., 1993; Cheng et al., 1997; De Neve et al., 1997; De Buck et al., 1999). These multiple copies can be present at the same genomic locus or at different loci. T-DNAs present at one locus were reported for several plant species and are often linked in direct or inverted orientation (Cluster et al., 1996; De Block and Debrouwer, 1991; De Neve et al., 1997; Krizkova and Hrouda, 1998; Kumar and Fladung, 2000; Kim et al., 2003; Sallaud et al., 2003). It was postulated that multiple copies are the result of the replication of a single T-DNA and their repair or the ligation of T-DNAs before or during integration into the host genome (Jorgensen et al., 1987; Van Lijsebettens et al., 1986). Studying plant co-transformation using different T-DNAs showed that multiple T-DNA copies can also be the result of cotransformation of a single plant cell by different bacteria (Depicker et al., 1985; De Block and Debrouwer, 1991; De Neve et al., 1997; De Buck et al., 1998; De Buck et al., 1999; De Buck et al., 2009; Radchuk et al., 2005). In this case, ligation of the two T-DNAs occurs extrachromosomal and before integration (De Neve et al., 1997; De Buck et al., 1999). Recently, De Buck et al., (2009), suggested that the T-DNA integration pattern complexity is determined by the targeted plant cell rather than by the agrobacteria.

Integrated T-DNAs can be truncated at one or both ends of the T-DNA (Deroles and Gardner, 1988). These deletions probably originate after transfer in the plant cell, before or during integration as a consequence of attack by plant

nucleases. The T-DNA could also break during integration through annealing of internal T-DNA sequences with homologous plant DNA at the target site (Windels et al., 2003a). Both borders are equally prone to end processing; however, the deletions at the LB junction are usually larger than these at the RB junction (Brunaud et al., 2002; Meza et al., 2002; Kim et al., 2003; Windels et al., 2003a), and it is postulated that this difference is due to the presence of VirD2 at the RB (Jasper et al., 1994).

Upon more careful analysis of the transferred DNA in the transformed plant cells, it was recognized that not only T-DNA sequences but also Ti plasmid or T-DNA vector sequences (vector backbone sequences) were often found to be associated with the integrated T-DNA copies (Ramanathan and Veluthambi, 1995; van der Graaff et al., 1996; Kononov et al., 1997; Wenck et al., 1997; Jakowitsch et al., 1999; De Buck et al., 2000b; McCormac et al., 2001; Kim et al., 2003; Breitler et al., 2004; Huang et al., 2004; Vain et al., 2004; Podevin et al., 2006; Permyakova et al., 2009; Lange et al., 2006). Integration of these vector sequences is related to a wrong recognition of the T-DNA borders during its production in *Agrobacterium*. One of the reasons is that the LB repeat is recognized as the initiation site of T-DNA production (Ramanathan and Veluthambi, 1995; van der Graaff et al., 1996). A second reason is inefficient recognition and nicking at the LB repeat, resulting in a “read-through” vector backbone transfer. It was reported that two T-DNAs separated by the entire vector backbone integrated in the host genome (Wenck et al., 1997; De Buck et al., 2000b). Recently, generation of vector-backbone free transformants via plant ovaries dripping (dipping of individual flowers with the *Agrobacterium* culture) was reported (Yang et al., 2009). The key features of this method focused on the complete removal of the whole styles, and the subsequent application of a vector backbone-free and selectable marker-free linear GFP cassette directly to the ovaries (Yang et al., 2009). Petti et al., (2009) showed that *Agrobacterium* strain LBA4404 had a greater potential to integrate non-T-DNA vector sequences than the *Agrobacterium* AGL1 strain after *Solanum tuberosum* (potato) internodal explants transformation, indicating a dependency between the *Agrobacterium* genotype and vector backbone sequence integration. Finally, Podevin et al., (2006) did not find a correlation between the *Agrobacterium vir* plasmids and vector backbone sequence integration after *Arabidopsis thaliana* floral dip transformation.

Host genome rearrangements were reported in the literature as a consequence of *Agrobacterium* transformation. Illegitimate recombination underlying T-DNA integration is correlated to a high extent with the deletion of plant genomic target site sequences (Gheysen et al., 1991; Mayerhofer et al., 1991; Meza et al., 2002; Kim et al., 2003). Also insertion of filler sequences at the T-DNA/plant DNA junctions are found in nearly 50% of the studied integration events (Gheysen et al., 1991; Mayerhofer et al., 1991; Windels et al., 2003a). Filler sequences are scrambled DNA segments that originate primarily from the plant target DNA and/or the T-DNA ends (Windels et al., 2003a). These rearrangements are small as most described deletions were less than 75 bp and the size of the filler insertions is less than 100 bp (Gheysen et al., 1991; Mayerhofer et al., 1991; Meza et al., 2002; Forsbach et al., 2003; Kim et al., 2003; Windels et al., 2003a; Windels et al., 2008). More pronounced rearrangements such as duplication of the plant target in combination with a 27 bp deletion (Gheysen et al., 1991; Ohba et al., 1995), duplication/translocation of large regions (Tax and Vernon, 2001), large scale deletion/reciprocal translocation and large inversion (Nacry et al., 1998; Laufs et al., 1999; Forsbach et al., 2003) or insertion of organelle DNA (Szabados et al., 2002) were observed in *Arabidopsis thaliana*. Chromosomal DNA deletions associated with the transformation process were also reported for other species such as aspen and rice (Kumar and Fladung, 2002; Kim et al., 2003).

3. *Agrobacterium* plant transformation: two main methods

As described in section 1 of this thesis, wild-type *Agrobacterium* strains harboring a tumor-inducing (Ti) plasmid are the causative agent of crown gall tumor disease in dicotyledonous plants. Any foreign DNA can be transferred to the plant nucleus via the *vir* system provided the segment is flanked by T-DNA border repeats. Because the *vir* functions operate in *trans*, the T-DNA and the *vir* genes are not needed to be located on the same plasmid (Hoekema et al., 1983). Thus, experimentally, the *vir* functions are often provided by a non tumorigenic Ti plasmid derivative called a disarmed Ti plasmid or a *vir* helper plasmid, in which the native oncogenes and the T-DNA borders have been removed. The *Agrobacterium* strain is

then called “cured”. Such *vir* helper plasmids have been constructed from several Ti plasmid types (Hood et al., 1986, 1993; Hoekema et al., 1983; Torisky et al., 1997; Zambryski et al., 1983). Genes to be transferred are contained on a binary vector that code for two border repeats flanking a multiple cloning site, a plant-selectable marker, and other useful sequences such as replication origins allowing their stable maintenance in *Escherichia coli* and *Agrobacterium* cells (Jones et al., 1992).

Agrobacterium-mediated plant transformation can be achieved in two ways: regeneration dependent (*in vitro*) or regeneration independent (*in planta*). In the regeneration-dependent transformation methods, protoplasts, leaf-discs, root fragments, suspension-cultured cells, callus cells, organ parts or tissue explants can be incubated with an *Agrobacterium* culture and plants are regenerated from the cocultivated cells using plant tissue culture (Horsch et al., 1985; Valvekens et al., 1988; Hooykaas and Schilperoort, 1992; Christou, 1996). For *Arabidopsis* roots transformation, selection is essential to efficiently obtain transformants (De Buck et al., 1998; De Buck et al., 2000a). For many plant species, the lack of a suitable regeneration method is undoubtedly one of the main bottlenecks in developing a transformation procedure. During *Arabidopsis in vitro* transformation, cells located at the wounded sites, dedifferentiating mesophyl cells and pericycle cells are competent for transformation (Sangwan et al., 1991; Sangwan et al., 1992; De Buck et al., 2000a).

Vacuum infiltration was reported as a tissue culture-independent *Agrobacterium* transformation method by Bechtold et al., (1993). *Arabidopsis* plants at the early stages of flowering were uprooted and incubated in a solution of *Agrobacterium*. A vacuum was applied and then released, causing air trapped within the plant to bubble off and be replaced with the *Agrobacterium* solution. Plants were transplanted back to soil, grown until seeds were dry, and in the next generation stably transformed lines could be selected using the antibiotic or herbicide appropriate for the selectable marker gene present on the T-DNA. Later, the floral-dip method was described as an alternative and more simple method (Clough and Bent, 1998). The flowers are simply dipped in an *Agrobacterium* culture, the plants are allowed to mature and dry, and then the T1 seeds can be harvested and screened for the presence of any marker encoded on the T-DNA. Three research groups worked

in parallel to determine the target cells of regeneration-independent transformation methods (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000). All their results indicated that developing ovules, before fertilization, are the primary target for productive transformation in the *Arabidopsis* floral dip or vacuum infiltration transformation procedures (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000).

Until 1990, *Arabidopsis thaliana* was mainly transformed via regeneration dependent methods. *In planta* methods are however easy to conduct and not time consuming which recently resulted in their predominant use for transgenic *Arabidopsis* production. Nevertheless, more T-DNA copies are integrated in floral dip transformants than in root transformants (De Buck et al., 2004; De Buck et al., 2009; Marjanac et al., 2008; De Buck et al., 2009). The prevalent complex T-DNA integration pattern in floral dip transformants might be a disadvantage for some studies or some applications as expression variability and silencing are more related to multiple than to single-transgene copies (Hobbs et al., 1993; Meyer and Saedler, 1996; Muskens et al., 2000; Jorgensen et al., 1996; De Buck et al., 2004; Schubert et al., 2004).

4. Factors influencing *Agrobacterium*-mediated transformation

Attempts to improve transformation procedures focused on the identification of environmental, bacterial or plant factors influencing the transformation efficiency. Sangwan et al., (1991) and (1992) showed that a pre-culture of *Arabidopsis* embryo, root and leaf explants on a phytohormone containing medium was a perquisite for successful transformation as it renders the cell competent for transformation by activation of cell dedifferentiation and division. The same authors reported also that the three *Arabidopsis* ecotypes used in their study didn't react in the same way to this pre-culture treatment (Sangwan et al., 1991). This observation was confirmed later by Akama et al., (1992) as they found that different combinations of *Agrobacterium* strains and plant ecotypes had an influence on the transformation efficiency. Osmotic treatment was shown to enhance *Agrobacterium* transformation of rice (Hiei et al.,

1994), maize (Frame et al., 2002) but not of wheat (Cheng et al., 2003). Co-cultivation of Canola hypocotyls explants with *Agrobacterium tumefaciens* for 48 hours (h) resulted in higher transformation efficiency in comparison with co-cultivation for 24 and 72h (Cardoza and Stewart, 2003). Another factor that improves crop transformation is desiccation of explants prior to, or post, *Agrobacterium* infection. It is unclear which factors are affected by air-drying. Arencibia et al., (1998) reported that air-drying sugarcane suspension cells prior to inoculation slightly improved the transformation efficiency. Similarly, air-drying calluses derived from rice suspension cultures increased the transformation efficiency 10-fold or more compared to the control without air-drying (Urushibara et al., 2001). Air-drying of precultured immature embryos and embryogenic calluses of wheat prior to inoculation did not have the same effect as in sugarcane and rice (Cheng et al., 2003). Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) was reported as a method to transform meristematic tissue buried under several cell layers. SAAT increased transient transformation efficiency 100 to 1400 fold in several different plant tissues including leaf tissue, immature cotyledons, somatic and zygotic embryos, roots, stems, shoot apices, embryogenic suspension cells and whole seedlings. For soybean, SAAT treatment was necessary to obtain stable transformation (Trick and Finer, 1997). Sonication followed by vacuum infiltration was described to increase citrus *Agrobacterium* transformation efficiency (de Oliveira et al., 2009). The effect of temperature during co-culture on T-DNA delivery was also reported. Co-culture at 25ºC led to the highest number of transformed plants of tobacco, even though 19ºC was optimal for T-DNA delivery (Salas et al., 2001). In monocotyledons, co-culture temperature for most crops ranged from 24 to 25ºC, and in some cases, 28ºC was used (Rashid et al., 1996; Arencibia et al., 1998; Hashizume et al., 1999). Higher transformation frequency was observed in maize immature embryo transformation at 20ºC than at 23ºC when using a standard binary vector (Frame et al., 2002).

While these studies reported on T-DNA stable transformation, others reported on the T-DNA transfer itself by studying transient expression of genes carried by the T-strand. Genotype influence on the early expression of transferred genes was observed with different *Arabidopsis* ecotypes (Chateau et al., 2000). Dillen et al., (1997) showed that transient expression levels in *Nicotiana tabacum* leaves and *Phaseolus acutifolius* callus were influenced by the temperature. They tested

temperatures comprised between 15 and 29ºC and concluded that 22ºC was optimal for T-DNA transfer. The same optimal temperature for T-strand transfer was reported for garlic calluses (Kondo et al., 2000). Recently, Boyko et al., (2009) showed that exposure of plants to high concentration of ammonium nitrate during growth increases the frequency of *Agrobacterium*-mediated transient and stable transformation of tobacco cotyledons and leaves respectively.

Another environmental factor that was investigated for its contribution to T-DNA transfer was light which has been shown to promote gene transfer from *Agrobacterium* to *Arabidopsis thaliana* roots and *Phaseolus acutifolius* callus (De Clercq et al., 2002; Zambre et al, 2003). The effect of light conditions during co-cultivation on the gene transfer was assessed by applying two different light regimes (complete darkness or a 16 hours light/8 hours dark photoperiod) during *Phaseolus acutifolius* calli cocultivation with *Agrobacterium*. Co-cultivation in darkness conditions was deleterious for the *Phaseolus acutifolius* calli transformation as almost no GUS activity could be detected and the explant survival rate was drastically reduced (De Clercq et al., 2002). In Zambre et al., (2003), the effect of light on T-DNA transfer was investigated during coculture of *Arabidopsis* roots and *Phaseolus acutifolius* calli with *Agrobacterium*. In addition to complete darkness and 16 hours light/8 hours dark photoperiod, also the continuous light regime was applied. Under continuous light, T-DNA transfer was higher in comparison with the two other used photoperiods. This result was independent from the explants type, plant species or genotype as well as from the *Agrobacterium vir* plasmids or co-culture period (Zambre et al., 2003). In contrast, higher transient T-DNA activity occurred in dark cultured calli than in light cultured calli of the wetland monocot *Typha latifolia* (Nandakumar et al., 2004). Similarly, darkness also promoted gene transfer in rice callus during *Agrobacterium* cocultivation as the transformation efficiency under 24h period of dark (77.5%) was higher than the efficiencies obtained under 16 hours light/ 8 hours dark (62.5%) and continuous light (50%) (Ali et al., 2007).

Sangwan et al., (1992) reported that there was a difference between early expression of the reporter gene carried by the T-DNA and the number of stable transformants. The latter was much lower showing that not all transferred T-DNAs integrated in the host genome. This observation raises the question concerning the

relative extent by which T-DNA transfer and/or T-DNA integration influence the transformation efficiencies. In order to answer this question, plant co-transformation using one or more *Agrobacterium* strains and two or more distinguishable T-DNAs was employed in some studies. Depicker et al., (1985) reported that when T-DNAs are located on the same Ti plasmid vector within one bacterial strain, tobacco protoplasts cotransformation frequency (60-70%) was significantly higher than the product of the single transformation frequencies (35%). These results gave an indication that the plant cell competence to acquire, integrate and express a T-DNA is more limiting than the *Agrobacterium* competence to transform a plant cell (Depicker et al., 1985). Later reports confirmed that cell competence to integrate the transgene is a determining factor during transformation (Nam et al., 1997; De Buck et al., 1998; McCormac et al., 2001). De Buck et al., (1998) co-transformed both *Arabidopsis thaliana* root explants and tobacco protoplasts using two T-DNAs carried by different *Agrobacterium* strains. The authors reported that the co-transformation frequencies (between 21-47% for *Arabidopsis* root explants and 41-44% for tobacco protoplasts) were higher than the single transformation frequencies (below 1% for *Arabidopsis* explants and between 11 and 12% for protoplasts). These results reproduced previous ones (Petit et al., 1986; De Block and Debrouwer, 1991; Komari et al., 1996). Furthermore, De Buck et al., (1998) reported also that the number of accessible and/or competent plant cells in tissue explants was lower than the number of competent protoplasts. Both studies reported by Depicker et al., (1985) and De Buck et al., (1998) focused on stable transformation meaning that the T-DNA was integrated within the host genome while the number of T-DNAs transferred to the plant cells could be much higher. Nam et al., (1997) performed transient expression assays by monitoring the expression of a *gus*-intron gene in inoculated *Arabidopsis* root segments. They detected early (2-3 days after infection) expression of the reporter gene in two *Arabidopsis* ecotypes (UE-1 and Aa-0) but detected stable *gus* expression only in Aa-0 ecotype. They concluded that both *Arabidopsis* ecotypes were competent to take up transferred T-DNA strands and expressed those but that they had different T-DNA integration abilities (Nam et al., 1997).

De Buck et al., (2000a) provides a detailed study on the extent by which T-DNA transfer and/or T-DNA integration hampers the transformation efficiencies during *Arabidopsis* root explant transformation. They used a strategy based on the

Cre recombinase system (Dale and Ow, 1990) to discriminate between the transfer and the integration of the T-DNA. In a first experiment, root explants of transgenic *Arabidopsis* carrying a first T-DNA were co-cultivated with an *Agrobacterium* carrying a second T-DNA. The latter, harboring the Cre recombinase, is able to recognize and delete a specific sequence comprised between two *lox* sites and lying on the first resident T-DNA. After cocultivation of root explants, containing the first resident T-DNA, with an *Agrobacterium* containing the second T-DNA, shoots were regenerated in the absence of selection for the second T-DNA. Activity and integration of the Cre recombinase in the regenerated shoots was monitored by different PCR reactions. The authors reported that none of the regenerants was stably transformed with the second T-DNA, allowing them to conclude that the transformation frequency was below 0.5%. However, in four of the 84 regenerants, the T-DNA was transiently expressed as witnessed by the deletion of the floxed sequence, meaning that T-DNA transfer was 10 fold higher than the T-DNA integration frequency. In a second experiment, the authors co-transformed *Arabidopsis* root explants using two *Agrobacterium* strains harboring separately the above described T-DNAs. After co-cultivation, they selected only for the presence of the first T-DNA carrying the target sequence for the Cre recombinase. Co-transfer and activity of the second T-DNA, carrying the Cre recombinase, was monitored by different PCR reactions (De Buck et al., 2000a). The authors observed that T-DNA transfer of a second unselected T-DNA occurred in approximately 50 % of the cells which were already transformed with a first T-DNA. Furthermore, T-DNA transfer of the second unselected T-DNA was 10 fold higher than the T-DNA transfer frequencies when no selection was applied for the first T-DNA. The 10-fold difference in T-DNA transfer frequency in regenerants that were not selected (5%) compared with those that were (50%) selected for a first transformation event clearly showed that the T-DNA transfer frequency is an important limiting step during *Arabidopsis* root transformation process (De Buck et al., 2000a). The results also revealed that about half of the cotransferred T-DNAs were integrated into the host genome if another T-DNA did integrate (De Buck et al., 2000a) showing that cells able to stabilize a first selected T-DNA have a high chance to stabilize a second incoming T-DNA. Thus, besides T-DNA transfer, T-DNA integration into the host genome is also limiting during *Arabidopsis* root explant transformation. T-DNA integration but not transfer was

reported to hamper maize *Agrobacterium* transformation (Narasimhulu et al., 1996). Another study concluded that bacterial/cell interaction was limiting tobacco leaf disc transformation (McCormac et al., 2001). To date, the extent by which T-DNA transfer and/or T-DNA integration limits the transformation efficiency during *Agrobacterium in planta* transformation methods was not reported.

5. Alternatives to *Agrobacterium*-mediated transformation

Although *Agrobacterium*-based transformation is the preferred method for most plant species, direct gene transfer methods have also been widely used. These methods (described in Newell, 2000; van den Eede et al., 2004) were developed due to the early difficulties with *Agrobacterium* transformation of several agronomical important crop plants including maize, wheat, barley and rice. The transfer of 'naked' DNA to plant cells and successful transgene expression have been achieved with diverse methods: electroporation (Shigekawa and Dower, 1988), macro- and microinjection (Crossway et al., 1986; Nomura and Komamine, 1986), vortexing with silicon carbide fibers (Kaeppler et al., 1990), polyethylene glycol (PEG) (Paszkowski et al., 1984), ultrasound- (Sawahel, 1996), laser-mediated uptake (Weber et al., 1988) and particle bombardment (Christou, 1992). Some of these methods, such as polyethylene glycol (PEG) mediated transformation or electroporation, were most successful with protoplasts, which in cereals are laborious to isolate and difficult to regenerate from. Particle or microprojectile bombardment (also called biolistics) became a widely used method for cereal transformation. In addition, biolistics has also been used to deliver DNA to chloroplasts and mitochondria (Sanford et al., 1993).

6. Plant transformation applications

Genetic engineering was used to introduce several traits in plants. Applications of the obtained transgenic plants are very diverse. A brief description of

these applications is given in the following sections. For a detailed review, the reader is referred to Newell, 2000; Halford, 2006; Banta and Montenegro, 2008.

6.1. Science

Plant transformation has been widely used in gene-function studies. *Agrobacterium in planta* transformation represents a high throughput method that can be adapted for both gene loss and gene gain of function, hence for forward and reverse genetics (Alonso and Ecker, 2006). Forward genetics approaches start with a mutant phenotype from which the nature of the genetic lesion is deduced, whereas reverse genetics uses the opposite approach: it starts with a lesion in a known gene leading to a mutant organism that is tested for a specific phenotype. If the T-DNA inserts within the boundaries of a gene, it can drastically alter, and in many cases completely abolish, gene function (Krysan et al., 1999). Thus, large T-DNA mutant collections were generated that are designed exclusively to yield ‘knockouts’ (Sessions et al., 2002; Alonso et al., 2003). T-DNA can also carry promoter-less reporter genes (such as β-glucuronidase, *GFP* or luciferase), which can ‘trap’ the regulatory sequences of the ‘tagged’ gene (Springer, 2000; Nakayama et al., 2005). Consequently, the reporter gene will display the original expression patterns of the tagged gene. Promoter trap lines provide an excellent tool for studying gene-expression patterns (Springer, 2000). Systematic analysis of trap lines provides molecular markers that can be used to monitor *in vivo* developmental processes, such as flower or root morphogenesis, responses to stress and circadian rhythms (Alonso and Ecker, 2006).

6.2. Agriculture and food

Most genetically modified crop plants are designed to improve the agronomic characteristics such as pest and disease resistance to the benefit of the farmers, by improved yields. Resistance against insects, viruses and herbicide tolerance are common traits in genetically modified crops.

Herbicide tolerance is introduced for easy crop management by chemical weed control of large areas of monoculture. The resistant transgenic crop plants will survive the herbicide application whereas competing weeds will die. Herbicide tolerance plants can be produced by the introduction of the EPSPS gene coding for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the *gox* gene coding for the enzyme glyphosate oxidase (GOX), the *bar* gene encoding the phosphinothricin-N-acetyltransferase (PAT) enzyme or the *bxn* gene that encodes a nitrilase enzyme. Resistance of transgenic crops to insect pests is achieved by introducing genes coding for insect toxins. Crystalline proteins (Cry1Ab, Cry1Ac, Cry1F, Cry2Ab, Cry3A, Cry3B1, Cry9c), encoded by *cry* genes are used for this purpose (Baum et al., 2004; Li et al., 2004; Siqueira et al., 2004; Halford, 2006). Transgenic plants were also developed with resistance to pathogenic viruses infections by inserting virus-derived sequences that encode the viral coat proteins (CP) (Abel et al., 1986). For an overview of approved GM plants, details on the mechanism and pathways involved in conferring different resistance traits after introduction of these genes into plants the reader is referred to the Agbios GM database (http://www.agbios.com).

Additionally, several transgenic crops have been developed offering new product qualities which should give more directly benefit to the consumers, such as delayed fruit ripening, altered fatty acid composition, increased starch- or protein production or increased iron- and beta-carotene content (Goto et al., 1999; Ye et al., 2000). Fruits with delayed ripening have increased shelf-life, increased firmness and show a reduced deterioration of quality. This trait is a benefit also for the producer since it allows the harvesting, transport and storage with fewer losses. The alteration of the plant content, e.g. a fatty acid composition that is considered to be healthier, renders a benefit to the consumer by increasing the nutritional value. Augmented iron-, beta-carotene (Golden Rice) (Ye et al., 2000; Paine et al., 2005), starch or protein content also increases the nutritional value of the transgenic food products (Huang et al., 2002).

New genetically modified plants are developed with characteristics that should allow cultivation of crops in areas with adverse environmental conditions. These plants should have traits that confer so-called stress resistance against drought,

flooding, temperature changes, salinity, pH (acidity or alkalinity), heavy metal-polluted soils (Willekens et al., 1994; Xu et al., 1996; Frommer et al., 1999; Blumwald and Grover, 2006; Mundree et al., 2006). Other attempts are made to produce shorter (dwarfed) plants with a greater resistance to damage by wind, resulted from a reduced response to plant hormones (Peng et al., 1999).

6.3. Medical

Plant transformation has been also used to engineer plants producing clinically relevant metabolites and proteins (molecular farming). Vaccines, antibodies (Rodgers et al., 1999; Nicholson et al., 2006) and human therapeutic proteins (Staub et al., 2000) could be produced in transgenic plants. To date, most such molecules have been produced in tobacco, although potatoes, alfalfa, soybean, rice and wheat have also been used successfully (Banta and Montenegro, 2008). Oral immunization has been achieved using transgenic potatoes (Richter et al., 2000; Tacket et al., 2000; Kong et al., 2001) and transgenic alfalfa (Dus Santos et al., 2005). While green tissue has an advantage in terms of productivity, seeds or tubers are most useful for delivery of an edible product such as vaccine; they can be stored for long periods of time (Daniell et al., 2001) and shipped long distances at ambient temperatures (Streatfield et al., 2001). However, there are no transgenic plant-derived pharmaceuticals in commercial production yet (Ma et al., 2005). This may change in the near future, as a large European consortium with collaborators in South Africa is actively engaged in developing plant-based production platforms for pharmaceuticals targeted to tuberculosis, diabetes, rabies and human immunodeficiency virus (HIV). This group would be the first to carry out clinical trials of plant-derived candidate pharmaceuticals within the European Union regulatory framework (http://www.pharma-planta.org).

Part 2: Genetically Modified Organisms (GMO) legislation and detection methods

In this second part of the introductory chapter, a short overview of the international legislations guiding the introduction of agricultural transgenic crops and GM-derived food and feed will be given with emphasis on the current European legislation. In addition, the analytical methodology that can be used in order to implement the current legislation will be described.

7. GMOs: a steady production increase

When looking at the status of approved transgenic lines grown worldwide, one cannot deny that more and more transgenic lines enter the agricultural area and are used in food and feed. During the thirteen-year period from 1996 to 2008, there was a steady and continual growth resulting in a 74-fold increase of the global GM crops (also called Biotech crops) growth rate and forecasts are that this will remain. In 2008, a total estimated area of 125 million hectares of transgenic crops was grown (Figure 4). The US together with Argentina and Brazil were responsible for 79.5% of this global acreage (Figure 5). Biotech soybean continued to be the principal biotech crop in 2008, occupying 65.8 million hectares or 53% of global biotech area, followed by biotech maize (37.3 million hectares at 30%), biotech cotton (15.5 million hectares at 12%) and biotech canola (5.9 million hectares at 5% of the global biotech crop area) (James, 2008). From the first commercialization in 1996 to 2008, herbicide tolerance has consistently been the dominant trait. In 2008, herbicide tolerance deployed in soybean, maize, canola, cotton and alfalfa occupied 63% or 79 million hectares of the global biotech area of 125 million hectares. In 2008, the stacked double and triple traits occupied a larger area (26.9 million hectares, or 22% of global biotech crop area) than insect resistant varieties (19.1 million hectares) at 15% (James, 2008).

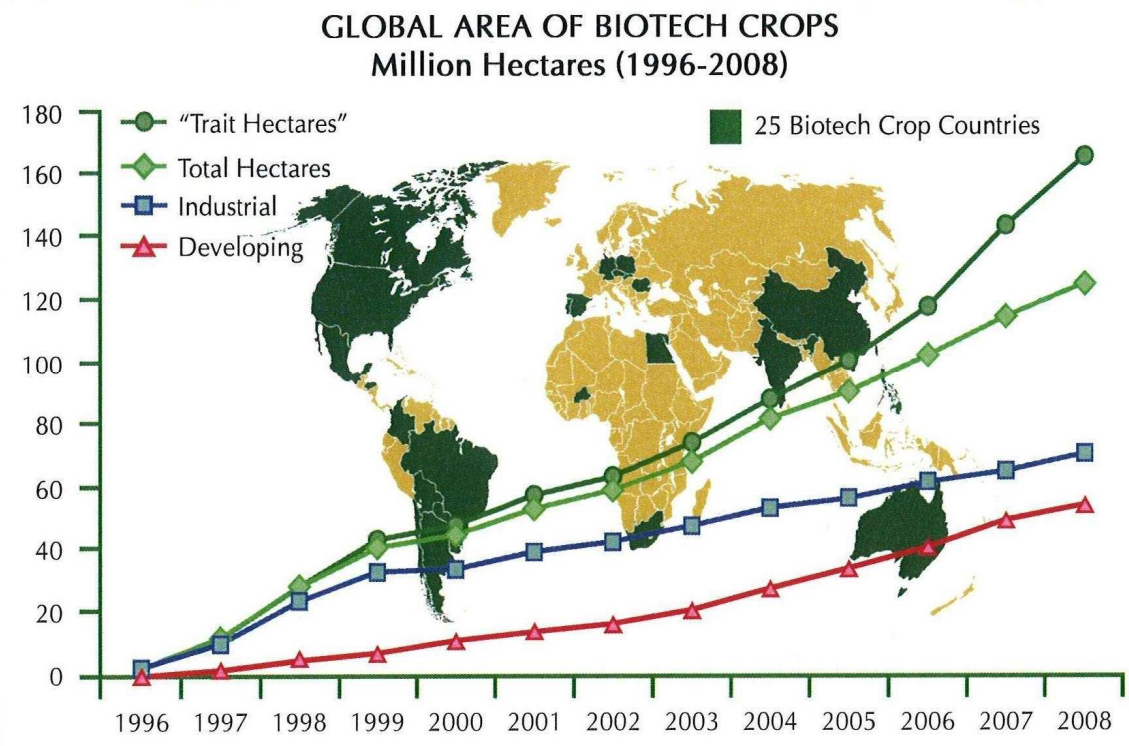

Figure 4: Increase of the worldwide genetically modified plants cultivation areas, in millions of hectares, from 1996 to 2008 (adapted from James, 2008). Trait hectares: areas, in million hectares, where GM crops with two or three “stacked traits” (containing two or three genes in a single GM crop) are cultivated. Total hectares: the total area (in million hectares) of cultivated GM crops in industrial countries (blue squares) and developing countries (red rectangles)

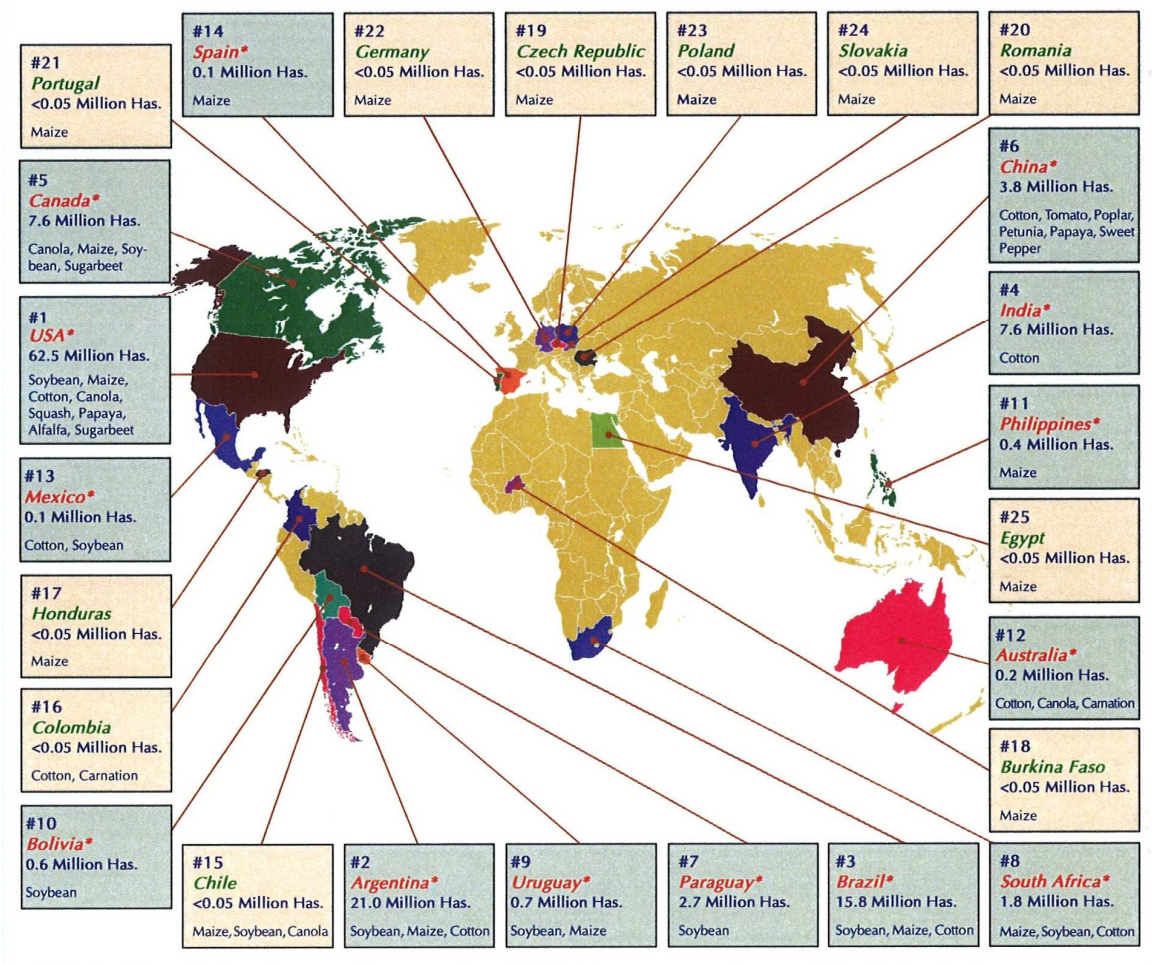

Figure 5: Global map of countries growing GM plant crops in 2008 (adapted from James, 2008). Has: hectares.

8. Global GMO legislation

Although GMOs have nowadays, besides their scientific value, very important agronomic and commercial issues, no global regulation governing their culture, production and commercialization is available (Varzakas et al., 2007). The absence of global regulation is partly compensated by the existence of some international organizations involved in developing protocols for GMOs such as the Cartagena protocol and the Codex Alimentarius Commission (Codex). The Cartagena Protocol on Biosafety (CPB) (http://www.cbd.int/), an environmental treaty legally binding for its parties, regulates trans-boundary movements of living modified organisms (LMOs). GM foods are within the scope of the protocol only if they contain LMOs that are capable of transferring or replicating genetic material. The cornerstone of the

CPB is a requirement that exporters seek consent from importers before the first shipment of LMOs intended for release into the environment. The Codex Alimentarius Commission is the joint Food and Agriculture Organization-World Health Organization (FAO/WHO) body responsible for compiling the standards, codes of practice, guidelines, and recommendations that constitute the Codex Alimentarius: the international food code (http://www.codexalimentarius.net/web/index_en.jsp). Codex is developing principles for the human health risk analysis of GM foods. The premise of these principles dictates a pre-market assessment, performed on a case-by-case basis and including an evaluation of both direct effects (from the inserted gene) and unintended effects (that may arise as a consequence of insertion of the new gene(s)). Codex principles do not have a binding effect on national legislation, but are referred to specifically in the Sanitary and Phytosanitary Agreement of the World Trade Organization (SPS Agreement), and can be used as a reference in case of trade disputes.

In term of GMO regulation, three groups of countries can be distinguished. The first group concerns countries that produce GMOs such as the United States, Canada and Argentina. These “producer” countries are in favor of GMO development and commercialization as they consider that GMOs are not distinct from their non-biotech counterparts and thus not harmful for human and animal health. GMO labeling is not applied in these countries. In the U.S., the Office of Science and Technology Policy (OSTP) proposed a plan for the regulation of crops issued from biotechnology. This plan, published by the OSTP in 1986, is still in use today. It is based on the principle that techniques of biotechnology are not inherently risky and that biotechnology should not be regulated as a process, but rather that the products of biotechnology should be regulated in the same way as products of other technologies (OSTP, February 1992). The framework outlined rules and policies of the federal agencies and contained the conclusions that: (i) existing laws were, for the most part, adequate for biotechnology products (ii) the products, not the process, would be regulated (iii) genetically engineered organisms are not fundamentally different from non-modified ones and (vi) authority should be exercised only where there is evidence that the risk posed by the introduction is unreasonable. Three U.S. agencies share responsibility for regulating agricultural biotechnology. The Animal

and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) is responsible for ensuring that the growth of genetically engineered plants does not harm the agricultural environment. The Environmental Protection Agency (EPA) is responsible for assuring the human and environmental safety of pesticidal substances engineered into plants, and the Food and Drug Administration (FDA) is responsible for assuring that foods derived through genetic engineering are as safe as their traditional counterparts.

In a second group of countries, mainly European Union countries, GMOs are subject of specific regulations and directives, due to the intentional and directed change(s) introduced in their genome (Davison and Bertheau, 2007). An overview of the European legislation governing the use and commercialization of GM crops will be given in the next section.

In a third group of countries, GMOs are not popular due to a lack of information, scientific knowledge and capacities to control the development and production of GM crops. These countries are mainly third world countries. However, progress towards genetic modification technologies is taking place. An example is South Africa where GMO production is already in place (1% of the world production). Other countries started some field trials such as Burkina Faso, Zimbabwe, Egypt, Kenya and Tanzania (http://www.africabio.com).

9. GMOs in Europe

The continuous global acreage growth of transgenic crops does not reflect the consumer acceptance. The European Union is one of the world's regions where opposition against the deliberate release of GM crops and the subsequent use of these crops and derived products in food and feed is irrefutably intense. A Eurobarometer report published in March 2008 indicated that the majority of Europeans (58%) declared that they are opposed to the use of GMOs while around a fifth (21%) supports their use. A further 9% say they have never heard of GMOs (Eurobarometer, 2008).

An integrated risk assessment analysis prior to consent for deliberate release, labeling provisions and a reliable traceability system are the major topics in the current European legislation on genetically modified crops. First, there is Community legislation on the authorization for deliberate release of these crops. This envisages specific legislation to protect the European citizen and the environment; hence an environmental risk assessment is required. The main legislation is Directive 2001/18/EC (EU, 2001) on the deliberate release into the environment of genetically modified organisms (including GM-seeds), applicable since 17 October 2002 and repealing Directive 90/220/EEC (EU, 1990). Directive 2001/18/EC covers both the release of GM crops for experimental purposes, such as field experiments, as well as the introduction of GM crops on the market. Second, there is a Regulation on GM food and feed (Regulation (EC) N°1829/2003), which is in force since 7 November 2003 (EC, 2003a). It replaces the GM part of Regulation (EC) N° 258/97 (EU, 1997) on novel foods and novel food ingredients. This regulation sets out rules for the authorization of GMO-derived foods and GMO-derived food ingredients. Under this regulation, labeling of these novel foods is mandatory. Third, there is a Regulation on traceability and labeling of GMOs and traceability of food and feed produced from GMOs (Regulation (EC) N° 1830/2003) that entered in to force 7 November 2003 (EC, 2003b). This regulation lays down comprehensive traceability requirements for GMOs as well as for food and feed produced from GMOs. A summary of the EU Directives/Regulation related to GMOs (Varzakas et al., 2007) is provided in the annex.

9.1. Directive 2001/18/EC

Directive 2001/18/EC entered into force in 2002 as replacement of Directive 90/220/EEC. Under this Directive, no GMO should be released into the environment, either for field experiments or for release on the market, without the written consent of the involved Member State or the European Union respectively. A producer intending to release a GMO for experimental purposes or for marketing should, prior to the release of a GMO, submit a notification to the competent authority of the Member State within whose territory the deliberate release or initial marketing will

take place. Notifications for the deliberate release of a GM-crop for any other purpose than for placing on the market are submitted under part B of Directive 2001/18/EC, while notifications for placing on the market are submitted under Part C of the former Directive. The consent for the deliberate release for placing onto the market of a GMO, under Directive 2001/18/EC, is given for a maximum period of 10 years. This in order to evaluate new information that might become available due to the development of new scientific and technological methods. In contrast to its predecessor, Directive 2001/18/EC also includes some additional provisions. Phasing out of the use of genes conferring resistance to certain antibiotics is a key action. It also includes a provision on the post-release monitoring of GM crops and a provision on the traceability and labeling of GM products. Due to the traceability provisions, each Member State should ensure that, once a GMO is released onto the European market, at all stages of the introduction, the GMO is labeled as such. A label should clearly contain the words “This product contains genetically modified organisms”. A Regulation on the traceability and labeling of GMOs has been adopted by the European Council that amends Directive 2001/18/EC (Windels, 2004). A weekly update on the status of transgenic crops notified for marketing in the European Union can be accessed at the website of the Institute of Health and Consumer Protection (IHCP) of the European Commission’s Joint Research Centre (JRC): http://gmoinfo.jrc.ec.europa.eu/ At this website, a database containing all Summary Notification Information Formats (SNIFs) for new transgenic crops is maintained.

9.2. Regulation (EC) 1829/2003

Public and political debates, questioning the impact of GMOs on human health and the environment, led the European Union to implement some new Regulations. Regulation (EC) 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed, applied since January 2005, aims at the harmonization of procedures for the scientific assessment and the authorization of genetically modified food and feed. This general Regulation is accompanied by two Regulations for the traceability and the labeling of GM food and

feed. The first is Regulation (EC) 1830/2003 of the European Parliament and of the Council concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms. Traceability, as formulated by the European Commission, entails the ability to trace products through the production and distribution chains. The second is Regulation (EC) 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for GMOs. These identifiers are simple numeric or alphanumeric codes that allow distinguishing between different transgenic events.

9.3. Regulation (EC) 1830/2003

GMO-labeling (Regulation (EC) 1830/2003) covers all food and feed products derived from GMOs. Essentially, if a food or a feed product is produced using genetic engineering, this must be indicated on its label. This revised legislation on labeling rules is currently applied to all GM-products, even if the genetic modification itself is not detectable in the final product anymore (example: some highly processed products like sugars and oil). Thus, GM products or the ingredients of a GM product must be labeled either as "genetically modified...", or as "produced from genetically modified...". Adventitious (unintentional and technically unavoidable) contaminations with GMOs authorized in the EU only need to be labeled if the GM content exceeds a fixed threshold of 0.9% of the original ingredient. The producer has to prove that any contamination was not intended and could not have been avoided by good practice. This threshold is different from country to another. In Russia the threshold was in 2004, lowered from 5 to 0.9% (similar to the E.U). It is equal to 5% in Japan and Taiwan and 1% in Brazil. When the product contains a mixture of GMOs, if each ingredient (analytically translated as content per taxon) does not exceed 0.9%, no labeling is required, even if the sum is higher than the fixed threshold. Example, a product containing 0.9% GM maize and 0.3% GM soya will be labeled while a product containing 0.5% GM maize and 0.8% GM soya will not be labeled. A typical decision tree under EC regulations is shown in Figure 6.

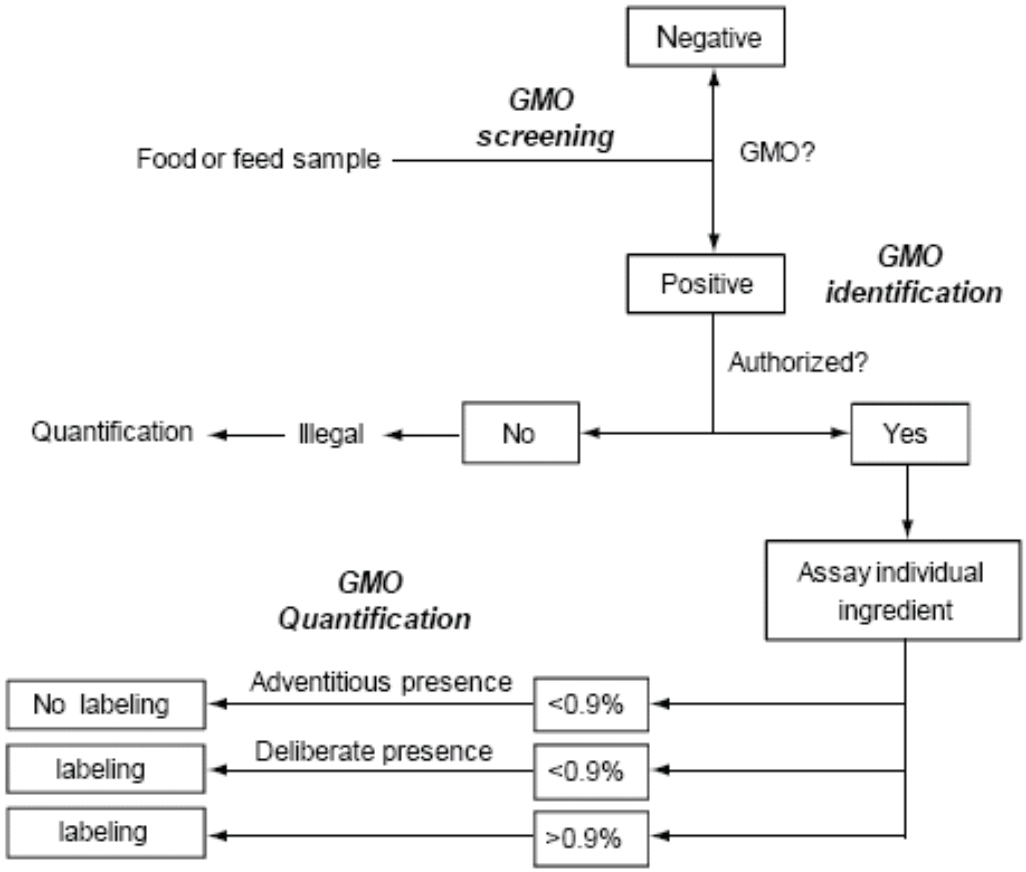

Figure 6: A decision tree for labeling of GMO food and feed (adapted from Davison and Bertheau, 2007).

10. DNA based GMO detection and quantification methods

According to the Regulation (EC) No 1829/2003, an analytical method for GMO analysis (sampling, identification and detection) has to be provided as part of the authorization dossier of any new GMO (EC, 2003a). Several GMO detection and quantification methods were described in the literature (Bonfini et al., 2001; Ahmed, 2002; Holst-Jensen et al., 2003; Garcia-Canas et al., 2004; Miraglia et al., 2004; Elenis et al., 2008; Jasbeer et al., 2008 for review). Recently, a GMO Detection Method Database (GMDD) was developed where almost all previously developed and reported GMO detection methods were collected (Dong et al., 2008). GMO detection methods could be distinguished based on their target molecules. Within the European regulation, proteins and nucleic acids are accepted as target molecules.

When targeting nucleic acids, polymerase chain reaction (PCR) and/or quantitative real-time PCR (QRT-PCR) are used as main methods (Luthy, 1999). DNA and RNA are the two existing nucleic acids. To date, RNA is not used in routine analysis for GMO detection and quantification. This is explained by the difficult RNA extraction process, the variable levels of RNA synthesis in different tissues and their high instability (Jasbeer et al., 2008). DNA based methods are very sensitive and small amounts of these molecules can be easily detected; thus DNA is the most reliable target used for GMOs detection and quantification. In addition, it has been shown that treatments such as high temperature treatment, high pression and sonication did not influence the quantitative real-time PCR quantification (Debode et al., 2007). Two DNA-based methods could be used for DNA detection: Southern blotting and PCR (Bertheau et al., 2002; Griffiths et al., 2002). In Southern blotting, the isolated DNA is transferred to a nitrocellulose or nylon membrane. Highly specific, labeled oligonucleotide probes are then allowed to hybridize to the fixed DNA. Free probes are washed away while bound probes are visualized radiographically, fluorescently or by chemiluminescence. A color reaction indicates the presence of GMO-specific DNA (Ahmed, 2002). As Southern blotting is not widely used for GMO analysis, this method will not further be described.

Targeting DNA implies that the molecule needs to be first isolated from the sample, meaning that sampling, sample grinding, homogenization and DNA extraction precede the proper DNA detection and quantification. Recommendation 2004/787/EC gives a technical guidance for sampling procedures (EC, 2004). Sampling procedures should result in a sample representative in characteristics and composition of the lot from which it was taken (Davison and Bertheau, 2007). In some cases, several steps of sampling have to be made (Lipp et al., 2005). Several sampling procedures were described by international organizations such as ISO (ISO 542, ISO 6644) or associations such as the American Association of Cereal Chemists (AACC). To date no universal method is available for sample homogenization. Considerations to be observed in designing a sampling plan have been summarized by Gilbert, (1999). These considerations are the type of material (raw material, derived ingredients or finished processed foods), the threshold limit for acceptance of the presence of GM material, cost of sampling analysis and the speed required to release the results. For covering the European legislation needs, the JRC

launched, several years ago, a research program called Kernel Lot Distribution Assessment "*KeLDA*" (Miraglia et al., 2004; Paoletti et al., 2006) and a software package, called Kernel Sampling Technique Evaluation "*KeSTE*". The objective of KeLDA was to investigate if GM material is randomly distributed within each given lot, or if it shows any deviation from randomness. The results of the KeLDA research program gave evidence that the distribution of GM material is heterogeneous within a lot (Paoletti et al., 2006). Both programs were taken into consideration in a European recommendation. However, this recommendation was not effectively implemented following an attempt by the European member states at determining the costs of such a sampling plan. In general, the cost inhibits the use of adequate sampling plans, as long as the safety issues are not foreseen (Davison and Bertheau, 2007).

After sampling, the final sample will be grinded and homogenized. DNA extraction is the final step preceding GMOs detection and quantification in a given sample. This step is crucial as the quality of the extracted DNA is determining the final results of detection and quantification. The extraction can be performed using Cethyl Trimethyl Ammonium Bromide (CTAB) (Meyer and Jaccoud, 1997; Lipton et al., 2000) or commercialized kits such as silica-based DNA extraction kits or phenol-based extraction procedures.

10.1. PCR as GMO detection method

DNA-based detection methods that make use of PCR, exponentially amplify the target of interest (a DNA sequence) with high specificity. The PCR technique allows amplification of a certain genomic segment under specific conditions *in vitro*. Therefore, short DNA oligomers, referred to as PCR primers, which are complementary with the target to be detected (the template DNA), are used to prime a DNA polymerization reaction (Kleppe et al., 1971). By using a thermostable DNA polymerase, in combination with a thermal cycling program, the template DNA is amplified exponentially (Saiki et al., 1988). In this way, one can obtain a high number of DNA copies starting from a very low initial template copy number. Upon PCR amplification, the amplified fragment is analyzed using gel electrophoresis and can be visualized. A positive PCR signal indicates that the template of interest was

present during the PCR reaction and as a result, the testing sample is positive for the presence of the DNA sequence to be detected. A negative PCR signal indicates that during PCR amplification the template of interest was absent.

Whereas the first PCR methods for GMO detection and quantification were simplex, amplifying a single target in a tube, later also duplex (amplifying two targets simultaneously) and multiplex PCR (amplifying more than two targets simultaneously) methods have been developed (Matsuoka et al., 2001; Terry and Harris, 2001; James et al., 2003; Germini et al., 2004; Huang and Pan, 2004; Onishi et al., 2005; Peano et al., 2005; Leimanis et al., 2006; Nadal et al., 2006; Yamaguchi et al., 2006; Xu et al., 2007; Chaouachi et al., 2008). Duplex and multiplex PCR would be useful methods for GMO analysis, due to the continuous increase of GM crops. However, the sensitivity of multiplex PCR might be affected when one GMO event is more concentrated than the others as the amplification from a more concentrated event might inhibit the amplification of other less abundant targets (Jasbeer et al., 2008). Moreover, optimization of these methods is time-consuming due to the different optimal conditions required to obtain each amplicon (Garcia-Canas et al., 2004).

Different specificity levels can be reached when using PCR for GMO detection (Garcia-Canas et al., 2004; Miraglia et al., 2004; Jasbeer et al., 2008). Classification of PCR types according to their specificity is illustrated in Figure 7. Detection methods target regulatory elements that are widely used in GM-crop technology such as promoter sequences, terminator sequences and genes coding for antibiotics resistance. A positive screening result is an indication that GM-derived DNA might be present. However, using this approach, there is no indication as to the identity of the transgenic event present in the tested sample. In the literature, several PCR screening methods have been reported. For instance the 35S promoter (Pietsch et al., 1997) and 35S terminator sequence (Matsuoka et al., 2002) from Cauliflower mosaic virus, the terminator region from the nopaline synthase gene (*nos* gene) from *Agrobacterium tumefaciens* (Pietsch et al., 1997) and the coding sequence of the neomycin phosphotransferase gene (*nptII* gene) (Matsuoka et al., 2002) are regularly used.

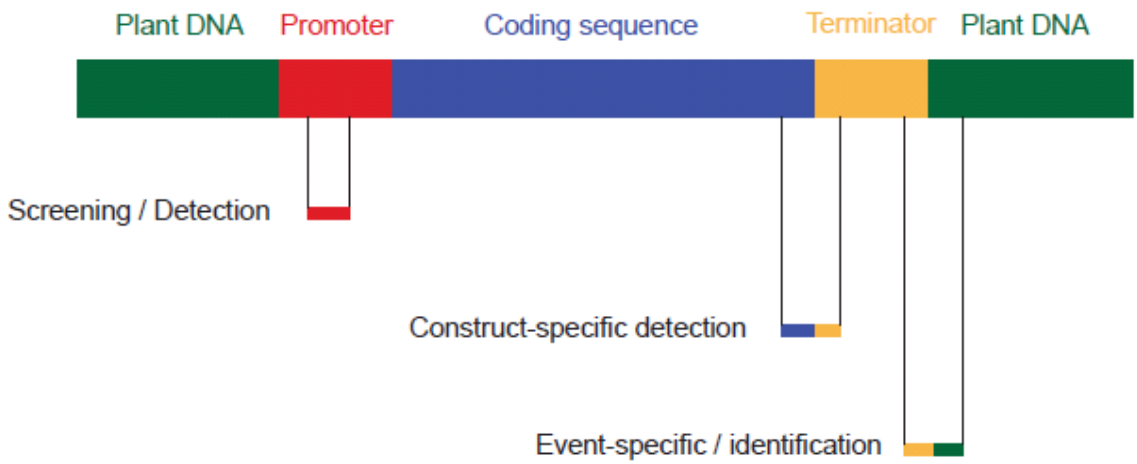

Figure 7: Detection and identification of transgenic crops. Horizontal levels of DNA-based PCR analysis (adapted from Windels, 2004).
Depending on the target, a different level of specificity is obtained. The lowest level of specificity involves PCR primers that target a particular region of the transgene insert. These techniques only allow to determine whether a particular transgene element is present. A second level of specificity involves the use of cross-border fragments within the transgene. More information is thus generated with regard to which transgenic crop is present in the analysis sample. Although, still different transgene events might be present. The highest level of specificity is obtained when the border between the transgene insert and the adjacent plant DNA is targeted. Indeed, the border region is specific and unique for each transgene event.

A higher level of specificity is obtained by targeting construct-specific or cross-border regions. Here, a PCR primer pair is developed that targets the junction between two adjacent genetic elements present in one transgene construct (Figure 7). This assay offers a higher level of specificity, since a positive PCR result identifies the transgene construct that has been used. Construct specific PCR analytical methods have been described that target transgene constructs used in several commercialized transgenic maize varieties (Matsuoka et al., 2001; Matsuoka et al., 2002) that target the Roundup Ready soybean construct (Wurz and Willmund, 1997) and the Zeneca tomato (Busch et al., 1999). However, even construct-specific methods do not allow identification of the transgene event present in the sample since the same transgene construct may have been used for the transformation of a number of different transgenic events.

The highest level of specificity is obtained when the transgenic event is identified. Transgenic event refers to the unique DNA integration that took place in

one plant cell, which was then used to generate entire transgenic plant. For identification purposes, DNA sequences unique for each transgenic event must be targeted. Junction regions, also known as edge fragments, are the DNA sequences at the transition between the plant genomic DNA and the newly introduced exogenous transgene. Since transgene integration is a random process, the exact position of the transgene locus within the host genome is unique for a transgene event. Therefore, a PCR primer pair that spans the junction region between the plant genomic DNA and the inserted DNA, is the basis for the development of a highly specific DNA based identification method that is unique for a specific transgene event (Figure 7). A prerequisite for the development of identification methods for transgenic crops is the availability of detailed information on their molecular structure. A wide range of molecular techniques is available to amplify and characterize these regions. Inverse PCR and a series of techniques such as anchored-PCR (Theuns et al., 2002), adapter ligation PCR and ligation mediated PCR, which have in common a ligation step of a known adapter sequences to a pool of generated restriction fragments, can be used (Spertini et al., 1999). Once the DNA sequence of a junction of a transgenic event is known, primers specific for the junction region are developed. The obtained primer pairs are event-specific and are used in an analytical PCR methodology to identify GMOs in food and feed. Event- or line-specific PCR methods have been developed for several events such as Roundup Ready soybean (Taverniers et al., 2001; Terry and Harris, 2001), MON810 maize (Holck et al., 2002; Hernandez et al., 2003; Hernandez et al., 2004a), CBH-351 or StarLink maize (Windels et al., 2003b), Bt11 maize (Zimmermann et al., 2000; Ronning et al., 2003; Hernandez et al., 2004a), NK 603 maize (Nielsen et al., 2004), GA21 maize (Hernandez et al., 2004a; Hernandez et al., 2004b), T25 maize (Hernandez et al., 2004a) and Oxy-235 Canola (Yang et al., 2008). GMO detection methods are listed on the CRL website: http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm. When the presence of GM-derived DNA material is shown, quantification is the next step. This is necessary because, under EU legislation, a 0.9% threshold is set for the adventitious presence of GMOs.

PCR-ELISA, which consists of a qualitative PCR followed by the enzyme-linked immunosorbent assay (ELISA) technique, was also used for GMO detection. A

PCR-ELISA resembles the classical ELISA as a typical ‘sandwich’ structure is formed. The antibodies are replaced by target-specific probes. The target PCR product is hybridized to this probe and subsequently bound to a second, ‘detector probe’, which is labeled with biotin or digoxigenin. After adding a streptavidin pyruvate kinase or a streptavidin-HRP conjugate, detection occurs through luminescence or colorization respectively (Ahmed, 2002; Griffiths et al., 2002). Several PCR-ELISAs have been developed for GMOs targeting the CaMV-35S promoter or *Agrobacterium tumefaciens* 3’nos terminator (Brunnert et al., 2001; Liu et al., 2004).

10.2. PCR as GMO quantification method

The purpose of quantification is to determine the amount of one or more GMOs in a product or seed lot which will enable the laboratory to assess compliance with the threshold regulation. PCR was adapted for GMO quantification purposes which led to the creation of the quantitative-competitive PCR (QC-PCR) and the quantitative real-time PCR (QRT-PCR).

10.2.1. Quantitative competitive PCR (QC-PCR)

The principle of QC-PCR is the co-amplification of two similar targets: the specific gene target and a synthetic internal control target also called competitor. Co-amplification is performed using the same primer pair, in a single reaction tube. Because the two targets compete for available nucleotides, primers and DNA polymerase, the relative quantity of end product is assumed to correspond to the relative quantity at the beginning of the first PCR cycle (Wiseman, 2002). Usually, competitor DNA fragments are constructed by recombinant DNA technology using the cloned target sequence as a background in order to introduce *in vitro* small insertions, deletions, or single nucleotide changes that will modify the size or the restriction pattern in relation to the original target sequence (Garcia-Canas et al., 2004). The competitor is added to the sample in a series of known concentrations. After amplification, length difference of both amplicons can be visualized after separation on agarose gel. Quantification is then done at the equivalence point

where both amplicons give the same intensity (Figure 8) (Hubner et al., 1999; Ahmed, 2002; Anklam et al., 2002; Elenis et al., 2008; Jasbeer et al., 2008). During QC-PCR, any variation in amplification efficiency caused by changes in reaction conditions or the presence of inhibitors affects the amplification of the target and the control target equally, so that the ratio of their PCR products is constant through the PCR phases: exponential and plateau phase (Elenis et al., 2008). Therefore, the target sequence can be compared to the known internal standard for what concerns concentration (Ahmed, 2002; Wiseman, 2002; Holst-Jensen et al., 2003; Garcia-Canas et al., 2004; Elenis et al., 2008).

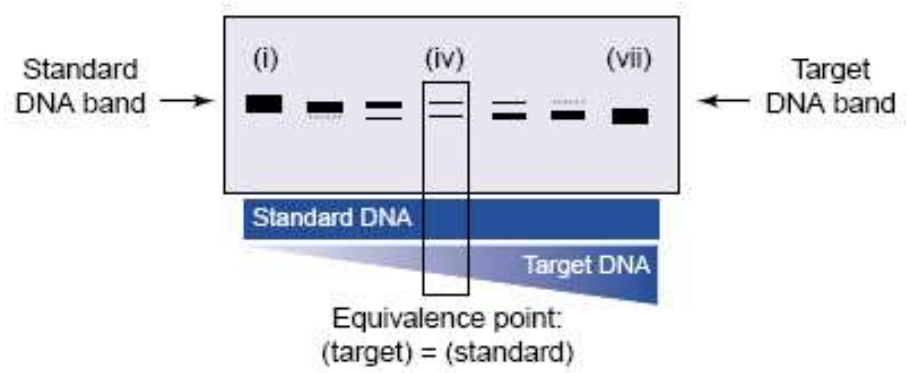

Figure 8: Principle of QC-PCR: standard DNA and target DNA are co-amplified in the same reaction tube. After PCR, the products are separated by agarose gel electrophoresis. Difference in amplicons length allows to distinguish the amplified standard DNA from the amplified target DNA. At the equivalence point the starting concentrations of internal standard and of target are equal (adapted from Ahmed, 2002).

A “good” quantification can only be obtained when the target and the competitor sequences are amplified with the same efficiency throughout the reaction time. This makes the design of the competitor DNA crucial for the development of any QC-PCR method. Care should be taken when manipulating the size of the competitors because it could greatly influence the amplification efficiency (McCulloch et al., 1995; Zimmermann and Mannhalter, 1996). Moreover, a difference in amplification efficiencies between the competitor and the template DNA can take place due to the presence of inhibitors in the extracted DNA in contrast with the designed competitor. Multi-specific competitors, containing targets for several pairs of

primers corresponding to several GMOs, have also been designed (Bouaboula et al., 1992; Cottrez et al., 1994).

The first QC-PCR methods only quantified the GM gene but did not allow to normalize it to the amount of the sample DNA (Gilliland et al., 1990). Hence double QC-PCR methods, which involve two competitive PCRs for the determination of the GMO-specific gene and a reference gene, were described (Hubner et al., 1999; Wurz et al., 1999; Hupfer et al., 2000; Dinelli et al., 2006). However, these methods never became routinely applied for quantitative GMO analysis due to the fact that their development is time consuming and to the influence of the technology used for quantification of the amplified sequences after PCR, discussed in Garcia-Canas et al., (2004).

PCR-ELISA, previously described, can be quantitative when the PCR is stopped before a significant decrease in amplification efficiency occurs (Anklam et al., 2002). ELISA has been used to quantify relatively low amounts of PCR products (Landgraf et al., 1991; Gonzalez et al., 1999). Despite the fact that a GMO detection kit using PCR-ELISA has been commercialized (D-Genos, Angers, France), this technique has not been widely adopted for accurate GMO quantification purposes.

All PCR techniques described earlier for GMO detection and quantification are called end-point measurements as they detect the amplified DNA after the PCR has completely finished. Griffiths et al., (2002) also mentioned the term ‘heterogeneous’ for end-point determinations, as detection is performed separately from the PCR.

10.2.2. Quantitative real-time PCR (QRT-PCR)

QRT-PCR was originally developed in 1992 by Higuchi and co-workers (Higuchi et al., 1992) and it is a ‘homogeneous’ method. A unique feature of this PCR technique is that the amplification of the target DNA sequence can be followed during the whole reaction by indirect monitoring of the product formation. The monitoring is achieved by the use of special chemistries, generally fluorescently labeled probes in the PCR. Several types of probes exist, including DNA-binding dyes like SYBR greenI, hydrolysis probes (5’→3’ nuclease probes), hybridization probes, Molecular

Beacons™ and Scorpions™ (reviewed in Giulietti et al., 2001; Wilhelm and Pingoud, 2003; Kubista et al., 2006). Each type of probe has its own unique characteristics, but the strategy for each is simple. They must link a change in fluorescence to DNA amplification.

SYBR Green I binds to the minor groove of dsDNA, emitting 1000 fold greater fluorescence than when it is free in solution (Wittwer et al., 1997). Therefore, the higher the amount of dsDNA present in the reaction tube, the higher the amount of DNA binding and fluorescent signal from SYBR green I. Thus any amplification of DNA in the reaction tube is measured. The primary concern with the usage of sequence independent dsDNA-binding probes is specificity. To help ensure specificity, the dissociation curve of the amplified product can be analyzed to determine the melting point. If there are two or more peaks, it suggests that more than one amplified sequence was obtained, and the amplification was not specific for a single DNA target (Valasek and Repa, 2005; Kubista et al., 2006).

Hydrolysis probes offer an alternative approach to the problem of specificity. These are likely the most widely used fluorogenic probe format (Mackay, 2004). Three oligonucleotides are used: a forward primer, a reverse primer and a probe. All of them are specific for the target and are able to bind to it. The TaqMan assay uses a probe technology that exploits the 5'→ 3' nuclease activity of an enzyme, the most commonly used being *Taq* polymerase. The probe is an oligonucleotide with a reporter dye at the 5' end and a quencher at the 3' end. The fluorescent reporter dye is attached covalently to the 5' end and can be FAM (6-carboxyfluorescein), TET (tetrachloro-6-carboxyfluorescein), JOE (2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein), HEX (hexacholoro-6-carboxyfluo-rescein), or VIC. The reporter is quenched by TAMRA (6-carboxytetramethylrhodamine), bound to the 3' end by a linker arm (Giulietti et al., 2001). When the probe is intact the quencher dye absorbs the fluorescence of the reporter dye due to the proximity between both. This proximity permits fluorescence resonance energy transfer (FRET). By the 5'→3' exonuclease activity of the *Taq* polymerase during amplification, the probe is hydrolyzed and the reporter dye is separated from the quencher (Figure 9) resulting in an increase in the fluorescence emission. The increase of the reporter signal

corresponds to the specific amplification of the DNA (Giulietti et al., 2001; Wilhelm and Pingoud, 2003; Mackay, 2004; Kubista et al., 2006).

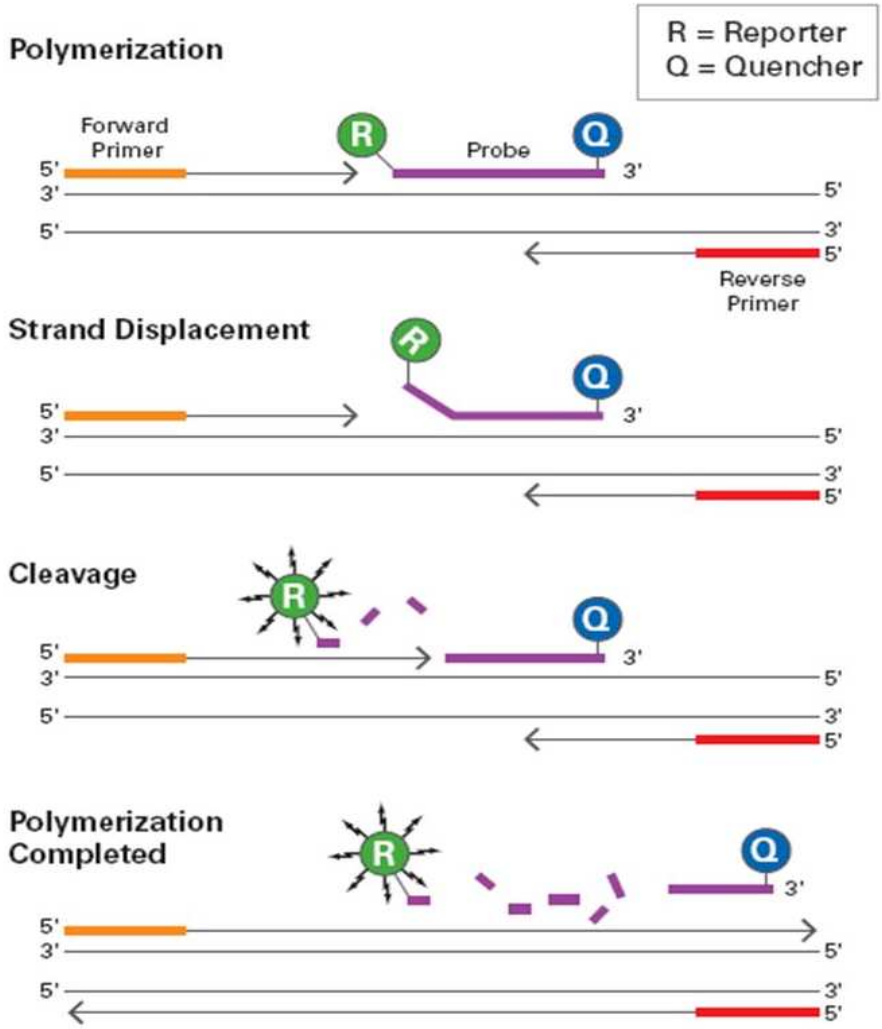

Figure 9: Quantitative real-time PCR using TaqMan probes: Probes and primers anneal to target sequence. TaqMan probes have two covalently linked fluorescent dyes: a reporter (R) and a quencher (Q). On the probe, the reporter dye emission is quenched. During each extension cycle, the 5'→3' exonuclease activity of Taq DNA polymerase cleaves the reporter dye from the probe. Once separated from the quencher, the reporter dye emits its characteristic fluorescence, which is measured in every cycle by the sequence detector.

Using any of the developed chemistries, the increase in fluorescence emission can be read by a sequence detector in “real time” during the amplification reaction. Several instrumentations for QRT-PCR were developed (Giulietti et al., 2001). A

computer software program calculates a ΔRn using the equation $\Delta Rn = Rn^{+} - Rn^{-}$, where Rn^{+} is the fluorescence emission of the product at each time point and Rn^{-} is the fluorescence emission of the baseline. Thus, this value expresses the probe degradation during the PCR reaction. The computer software constructs amplification plots or curves using the fluorescence emission data that is collected during the amplification. The ΔRn values are plotted versus the cycle number resulting in an amplification curve with three segments corresponding to three phases (Figure 10) (Giulietti et al., 2001). An early background phase or baseline (below the detection level of the instrument) is followed by an exponential growth phase (or log phase). The exponential phase begins when sufficient product has accumulated to be detected above the background and ends when the reaction efficiency falls. During this exponential phase, the reaction efficiency is maximal and the PCR is not limited due to depletion of nucleotides, primers or probes (for TaqMan applications). The last phase of the amplification curve is the plateau (end-point) phase where no more significant specific product is generated, as a consequence of reaction exhaustion (Tichopad et al., 2003). An arbitrary threshold is chosen in the early phase of signal detection, based on the variability of the baseline, usually determined as 10 times the standard deviation of the baseline, set from cycles 3 to 15. Threshold cycle (C_T) values are then calculated by determining the point at which fluorescence exceeds the chosen threshold limit. C_T is reported as the cycle number at this point. For TaqMan applications, a horizontal threshold line is defined that crosses the amplification curve. The intersection point then defines the C_T value on the x-axis. C_T values decrease linearly with increasing the input target quantity (Figure 10). This can be used as a quantitative measurement of the input target (Giulietti et al., 2001).

All GMO detection methods, considered by the European Network of GMO Laboratories (ENGL) as complying with EC regulations and with its performance criteria, use the QRT-PCR. All validated methods are listed on the Community Reference Laboratory (CRL) web site http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm and mostly use TaqMan chemistry. To determine the GMO percentage in a given sample after QRT-PCR, two different methods are commonly used: the standard curve method and the comparative threshold method.

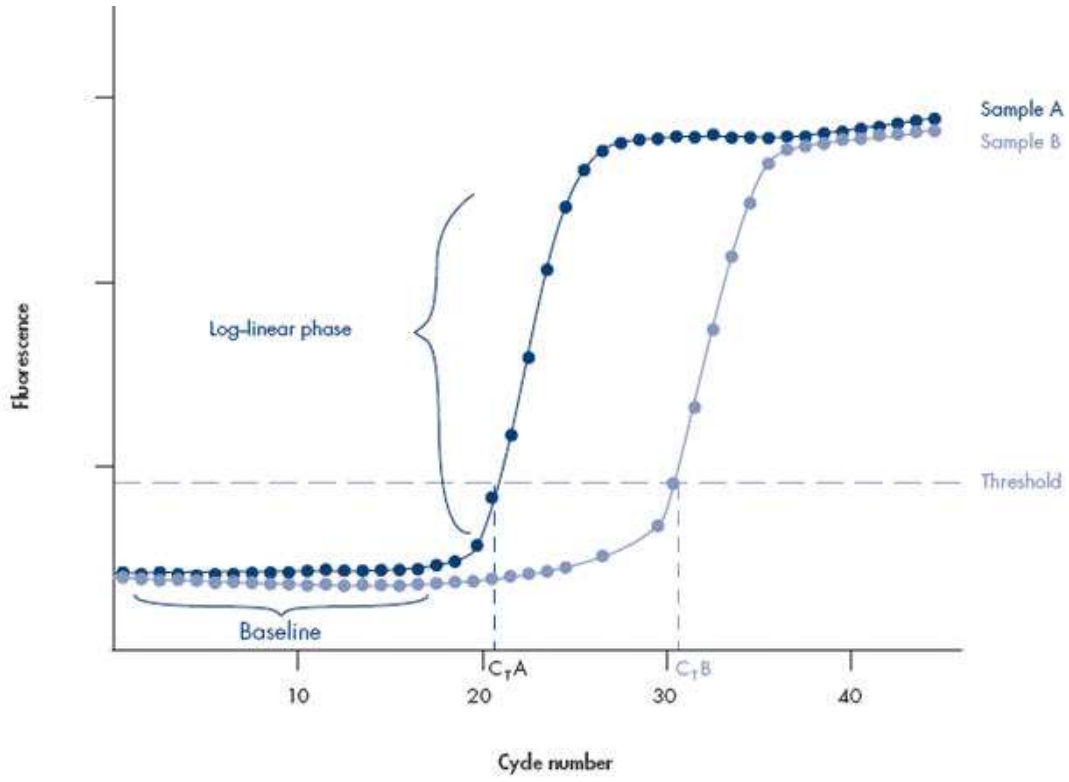

Figure 10: Typical QRT-PCR amplification plots showing increases in fluorescence from two samples A and B. Three phases are observed: a baseline phase, an exponential or log-linear phase and a plateau phase. The threshold is set at an early stage of signal detection. Sample A contains a higher amount of starting template than sample B as its C_T value is lower than the C_T value of sample B.

Standard curve method for GMO quantification

In this quantification approach, standard curves (also called calibration curves) of reference material are used to quantify the unknown sample. A reference material contains a known amount of the analyte of interest and is called certified reference material (CRM) if it was validated by a recognized institution; for example the Institute for Reference Materials and Measurements (IRMM; http://irmm.jrc.ec.europa.eu/html/homepage.htm); CRM database: http://www.erm-crm.org/ermcrmdb/. Besides IRMM, examples of other sources of reference material are Bayer, Crop Science and American Oil Chemists Society (AOCS; http://www.aocs.org/tech/crm/). CRMs consist of mixtures with defined mass fractions of conventional and GMO-specific material, which were produced applying a dry-mixing technique of homogenized seed powder to prevent DNA degradation. Since

these CRMs are made by mixing GM seeds with non-GM seeds in certain concentrations, a relative percentage of GMO in this case represents a weight/weight percentage. CRMs are available for soybean (line Roundup Ready), several maize lines, potato (line EH92-527-1), sugar beet (line H7-1) and cotton seed (Jasbeer et al., 2008) in 0.1, 0.5, 1, 2 and 5 weight-percentages, a range that is of relevance for the actual European labeling threshold limits. Standards with 0% or 100% pure GMO material such as seeds, leaves or grains of transgenic material are also available. Solutions of DNA isolated from powder CRMs are used as calibrators.

Alternative to genomic DNA, isolated from a matrix material, plasmid DNA material has been used as calibrants for real-time PCR quantification of GMOs. Since 2001, different types of plasmid calibrants have been introduced: single target plasmid (STP) DNA molecules (Taverniers et al., 2001) dual target, “tandem marker” or so-called “pJANUS^{TM} plasmids” (Weighardt et al., 2004; Mattarucchi et al., 2005) and multiple target plasmid (MTP) DNA materials (Kuribara et al., 2002; Shindo et al., 2002; Taverniers et al., 2004; Burns et al., 2006) report on the compared performances in real-time PCR of different types of DNA calibrants. Plasmid DNA provides a suitable alternative to genomic DNA for use as a calibrant in GMO quantification. These studies reported that plasmid calibrants gave equal or better performance characteristics in terms of precision and closeness to the expected value, than their genomic equivalents. With regard to the ease of production, storage, distribution, high stability and its performance, plasmid DNA calibrators may be preferred over genomic DNA calibrators in the future. One drawback of plasmid DNA calibrators is that they, as such, only contain the pure analyte and are not similar to real samples of interest (Taverniers, 2005). This may be overcome by ‘matrix matching’, which is mixing the plasmid DNA with a background of genomic DNA as done in a study by Taverniers et al., (2004).

A defined amount of DNA extracted from reference materials with different GM percentages, is amplified using the GMO specific QRT-PCR detection system. Using the resulting C_T values a standard curve for the GM target is generated by plotting C_T values versus the logarithm of the known GMO amount (or copy number) of the reference sample. The linear regression line through the data points allows the quantification of the unknown samples of which the GMO amount is determined by

amplifying an equal amount of DNA and interpolating the resulting C_T value to the generated standard curve.

For reliable quantification, the GMO amount should be normalized to the total DNA amount of the plant species (taxon-specific sequence or endogene). Hence, two reactions of the same concentration of the template DNA must be performed, one targeting the endogene and one targeting a GM-specific sequence. The GM content is calculated from the ratio of the target transgenic specific DNA sequence copy number versus the DNA sequence copy number of the respective target plant species (taxon gene sequence) (Ahmed, 2002; Holst-Jensen et al., 2003; Elenis et al., 2008; Jasbeer et al., 2008). If more than one GMO event (mixed samples) are present in the unknown sample, each of them has to be considered separately with an appropriate event-specific detection system. For mixed samples consisting of diverse ingredients the amount of a distinct ingredient has to be determined first to be able to normalize the amount of the corresponding genetically modified counterpart. No CRMs are available for mixed composites therefore serial dilutions of genomic DNA extracts are used to generate the needed standard curve for the endogene quantification, similar as for the transgene quantification. Relative GMO contents are then derived by dividing the calculated amount of transgene target (in copy numbers, corresponding to the haploid genome equivalents (HGE) unit recommended for EU laboratories) by the calculated amount of endogene target, and multiplying with 100% (Ahmed, 2002; EC, 2004; Elenis et al., 2008; Jasbeer et al., 2008).

Comparative threshold method for GMO quantification

The comparative threshold or $\Delta\Delta C_T$ method, which relies on the direct comparison of C_T values, is an alternative approach to the absolute standard curve method for GMO quantification. While still involving an endogenous target amplification and a transgene target amplification, it differs from the standard curve method by relying on equal PCR efficiencies of the transgene and the endogenous control genes (Livak and Schmittgen, 2001). The ΔC_T value for each sample is determined by calculating the difference between the GMO target gene C_T value and the endogenous reference gene C_T value. This is determined for each unknown sample as well as for a calibrator sample of known GMO amount.

$$\Delta C_T \text{ (sample)} = C_T \text{ GMO target gene} - C_T \text{ endogenous reference gene}$$

$$\Delta C_T \text{ (calibrator)} = C_T \text{ GMO target gene} - C_T \text{ endogenous reference gene}$$

Then, the $\Delta\Delta C_T$ value for each sample is determined by subtracting the ΔC_T value of the calibrator from the ΔC_T value of the sample:

$$\Delta\Delta C_T = \Delta C_T \text{ (sample)} - \Delta C_T \text{ (calibrator)}$$

The normalized amount of GMO target gene is calculated by using the formula:

$$\text{Normalized amount of GMO target gene of sample} = 2^{-\Delta\Delta C_T}$$

The efficiency of PCR amplification of the target gene and the endogene is a very important issue for the application of this method. Indeed, both efficiencies should be approximately equal. If the efficiencies are not the same, which is often the case, the comparative threshold method cannot be used. While using the standard curve method for quantification, the final quantitative estimate is based on comparing estimated quantity of GM to estimated quantity of reference (Holst-Jensen et al., 2003; Jasbeer et al., 2008). This feature is the advantage that the standard curve method has over the comparative threshold method.

10.3. Factors influencing the quantitative real-time PCR

The efficiency of the PCR reaction depends on the quality, the integrity and the purity of the extracted template DNA. These factors vary according to the material, the degree of processing of the sample and the DNA extraction method. Genomic DNA extracted from food samples can be strongly fragmented due to food processing, heating or pH conditions (Monma et al., 2005). If the targeted sequence is fragmented, amplification will not occur. In a recent study, it has been shown that the quality and the integrity of the template DNA is dependent on the combination of the starting material (from which the DNA will be extracted) and the used DNA extraction method (Mafra et al., 2008). DNA extraction method was shown to have an influence on the QRT-PCR reactions used for GMO analysis (Cankar et al., 2006; Charels et al., 2007; Corbisier et al., 2007).

Other factors that can influence the performance of the PCR are contaminants inhibiting compounds that might be present in the DNA extracts. Presence of contaminants in the analytical sample is a well known phenomenon typical for food matrices, but also resulting from certain extraction buffers or reagents. Inhibitors may be polysaccharides, proteins, lipids, fats, polyphenols, and caramelized sugar (Zimmermann et al., 1998; Ahmed, 2002) or chemicals such as CTAB or alkali (Anklam et al., 2002).

In addition to the purity and the integrity of the DNA templates, the amount of DNA subjected to the PCR is of crucial importance. A minimum number of target sequences is required for efficient amplification. The use of increased amounts of DNA will usually not enhance PCR, due to the high inhibition risk. The genome size should also be considered as the number of target will inversely vary with the genome size.

10.4. DNA based methods for GMO analysis: advantages and disadvantages

DNA detection methods offer certain advantages. These methods are suitable for a range of applications from screening methods to event-specific methods. Any part of a plant can be used for GMO detection as the DNA composition is the same in all cells of an organism. GMO quantification is possible using DNA-based assays thus they allow the implementation of the legislations governing GMO introduction on the market. DNA-based assays are sensitive and primer design and amplification optimization are rather fast processes. However, DNA based methods present also some disadvantages as they are expensive, require rather sophisticated and expensive equipment as well as highly trained personnel, and may be unsuitable for on-site testing. The template DNA can ‘loose’ its integrity after extraction, especially from processed food and amplification might be inhibited due to the presence of contaminants. Moreover, these methods present a contamination risk, hence a series of positive and negative controls should be included within the run (Griffiths et al., 2002; Jasbeer et al., 2008). Indeed, ‘false positive’ result is defined as an analysis

result which is positive, while the sample should be negative. The most probable origin of false positive PCR results is cross-contamination of the sample to be analyzed, due to the presence of the target sequence(s) in other samples, in apparatuses, on working surfaces and/or in the air. 'False negative' PCR results occur when no signal is detected whereas a positive result is expected for the sample. Here, probable reasons are matrix- or extraction-based effects such as DNA degradation due to processing and PCR inhibition due to the presence of specific inhibiting components in the DNA solution (see above), or low specificity and/or sensitivity of the PCR (Taverniers, 2005).

11. Alternative techniques for GMOs analysis

Alternative methods for GMO analysis have been developed and described in the literature. These methods are not commonly used for routine GMO detection and quantification analysis. A brief description of these alternative methods is provided in the following sections. More details concerning these methods can be found in Ahmed, 2002; Anklam et al., 2002; Elenis et al., 2008; Jasbeer et al., 2008.

11.1. Protein based methods

Insertion of one or several new genes into a GM plant will usually lead to the synthesis of one or more novel protein(s). Characterization of GMO is thus possible through fractionation, separation and profiling of proteins and peptides. Protein based approaches, mainly immunoassays (using antibodies as test reagents), have been used for GMO analysis. These methods include ELISA, western blot and lateral flow strip (Taverniers, 2005).

ELISA involves testing for the presence of specific proteins by exploiting the specificity of binding between expressed antigen (transgene encoded protein) and target antibody. Antibodies are immobilized on a solid support. The newly synthesized proteins are captured by the antibodies, after which a second antibody is

bound to the antibody-protein complex. This structure is called a ‘double antibody sandwich’. In ELISA, the intensity of color indicates the amount of the protein present. Several ELISA methods have been developed that are specific for gene products widely expressed in GM plants such as the neomycin phosphotransferase II (*nptII*) gene product, the enzyme 5-pyruvylshikimate-3-phosphate synthase (EPSPS), the *Bacillus thuringiensis* (*Bt*) insecticide Cry1Ab, and herbicide-tolerant phosphinotricin acetyltransferase (*PAT*) protein. ELISA tests are not event-specific (Bonfini et al., 2001). Commercial immunoassay methods are currently available for detection and quantification of GM crops expressing Cry1Ab, Cry1Ac, Cry3A, Cry2A, Cry9C, CP4 EPSPS and PAT protein.

Western blots are based on electrophoretic separation of proteins in a denaturing gel, transfer of the gel to a solid support such as a nitrocellulose membrane and binding of antibodies to specific sites of the protein on the membrane (Ahmed, 2002).

Lateral flow strip technology is a variation of ELISA with antibodies that are immobilized onto a test strip in specific zones. Flow strips provide a rapid test for GMO detection. The test can be performed with a kit and does not require any major equipment. These strips are suitable for on-site use, with minimal training required. Sample preparation simply involves crushing the sample and mixing it with protein extraction solutions provided in the kit (Griffiths et al., 2002).

Protein detection methods are more economic than DNA based methods. They are fast as a minimum of sample preparation is needed. Another advantage of immunoassays is their high target-specificity and can be used on-site for GM grains verification (Bonfini et al., 2001; Griffiths et al., 2002; Jasbeer et al., 2008). However, some disadvantages also exist. These methods are generally less sensitive than DNA detection methods and antibodies production is a slow and difficult task that requires a great deal of skill and experience. Moreover, the target protein must be correctly folded in order to be recognized by the antibody. Protein levels are not the same in all cells, and can also vary at different stages of the cell’s life cycle. The protein encoded by the transgene is not expressed in some cases due to transgene post-transcriptional silencing for instance (Kubista et al., 2006; Jasbeer et al., 2008).

11.2. Biological and chemical methods

Bioassays, used to detect GMOs, are screening methods that are based on different plant behavior in the presence of a specific herbicide. Prerequisite for this type of tests is the inclusion of negative and positive trait seeds as controls. Further investigations are necessary to confirm the presence of the transgene in the tested seeds. These methods are only applicable to seedlings harboring newly introduced genes for a specific herbicide tolerance (e.g. RoundupReady™ or LibertyLink™ GM traits), they are time consuming, inexpensive and very useful for seed companies (Bertheau et al., 2002).

Chromatography is used when the composition of GMO ingredient (fatty acids, triglycerides…) is altered. This method allows to distinguish the GMO from its conventional counterpart. Chromatography is only qualitative and can be applied only if significant changes occur in the composition of GMO plants or derived products (Anklam et al., 2002).

Near infrared spectroscopy (NIRS) may be used if the transgenic plant presents altered fibre structure whereas no significant differences are observed in the content of protein and oil. NIRS is only applied on raw products such as grains or beans. The capacity of NIRS to resolve small quantities of GMO varieties in non-GMO products is assumed to be low (Anklam et al., 2002).

References

Abel PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science **232:** 738-743

Abu-Arish A, Frenkiel-Krispin D, Fricke T, Tzfira T, Citovsky V, Wolf SG, Elbaum M (2004) Three-dimensional reconstruction of *Agrobacterium* VirE2 protein with single-stranded DNA. The Journal of biological chemistry **279:** 25359-25363

Abuodeh RO, Orbach MJ, Mandel MA, Das A, Galgiani JN (2000) Genetic transformation of Coccidioides immitis facilitated by *Agrobacterium tumefaciens*. J Infect Dis **181:** 2106-2110

Ahmed FE (2002) Detection of genetically modified organisms in foods. Trends Biotechnol **20:** 215-223

Akama K, Shiraishi H, Ohta S, Nakamura K, Okada K, Shimura Y (1992) Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. Plant cell reports **12:** 7-11

Albright LM, Yanofsky MF, Leroux B, Ma DQ, Nester EW (1987) Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. Journal of bacteriology **169:** 1046-1055

Ali S, Xianyin Z, Xue Q, Hassan MJ, Qian H (2007) Investigations for Improved Genetic Transformation Mediated by *Agrobacterium tumefaciens* in Two Rice Cultivars. Biotechnology **6:** 138-147

Allen ON, Holding AJ (1974) Genus II. *Agrobacterium* Conn. 1942. 359. *In* RE Buchanan, NE Gibbons, eds, Bergey's Manual of Determinative Bacteriology, 8th ed. The williams and Wilkins Co., Baltimore, pp 264-267

Alonso JM, Ecker JR (2006) Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in *Arabidopsis*. Nat Rev Genet **7:** 524-536

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science New York, N.Y **301:** 653-657

Aly KA, Baron C (2007) The VirB5 protein localizes to the T-pilus tips in *Agrobacterium tumefaciens*. Microbiology **153:** 3766-3775

Ambros PF, Matzke AJ, Matzke MA (1986) Localization of *Agrobacterium rhizogenes* T-DNA in plant chromosomes by in situ hybridization. The EMBO journal **5:** 2073-2077

An S, Park S, Jeong DH, Lee DY, Kang HG, Yu JH, Hur J, Kim SR, Kim YH, Lee M, Han S, Kim SJ, Yang J, Kim E, Wi SJ, Chung HS, Hong JP, Choe V, Lee HK, Choi JH, Nam J, Park PB, Park KY, Kim WT, Choe S, Lee CB, An G (2003) Generation and analysis of end sequence database for T-DNA tagging lines in rice. Plant Physiol **133:** 2040-2047

Anderson A, Moore LW (1979) Host specificity in the genus *Agrobacterium.* Phytopathology **69:** 320-323

Anklam E, Gadani F, Heinze P, Pijnenburg H, Van Den Eede G (2002) Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products Eur Food Res Technol **214:** 3-26

Arechaga I, Pena A, Zunzunegui S, del Carmen Fernandez-Alonso M, Rivas G, de la Cruz F (2008) ATPase activity and oligomeric state of TrwK, the VirB4 homologue of the plasmid R388 type IV secretion system. Journal of bacteriology **190:** 5472-5479

Arencibia AD, Carmona ERC, Tellez P, Chan MT, Yu SM, Trujillo LE, Oramas P (1998) An efficient protocol for sugarcane (Saccharum spp. L) transformation mediated by *Agrobacterium tumefaciens*. Transgenic Res **7:** 213-222

Atmakuri K, Cascales E, Christie PJ (2004) Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. Molecular microbiology **54:** 1199-1211

Atmakuri K, Ding Z, Christie PJ (2003) VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of Agrobacterium tumefaciens. Molecular microbiology **49:** 1699-1713

Backert S, Fronzes R, Waksman G (2008) VirB2 and VirB5 proteins: specialized adhesins in bacterial type-IV secretion systems? Trends in microbiology **16:** 409-413

Bako L, Umeda M, Tiburcio AF, Schell J, Koncz C (2003) The VirD2 pilot protein of *Agrobacterium*-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. Proceedings of the National Academy of Sciences of the United States of America **100:** 10108-10113

Ballas N, Citovsky V (1997) Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. Proceedings of the National Academy of Sciences of the United States of America **94:** 10723-10728

Banta LM, Montenegro M (2008) *Agrobacterium* and plant biotechnology. *In* T Tzfira, V Citovsky, eds, *Agrobacterium*: From Biology to Biotechnology. Springer, New York, pp 73-148

Barakat A, Gallois P, Raynal M, Mestre-Ortega D, Sallaud C, Guiderdoni E, Delseny M, Bernardi G (2000) The distribution of T-DNA in the genomes of transgenic *Arabidopsis* and rice. FEBS letters **471:** 161-164

Baron C, Thorstenson YR, Zambryski PC (1997) The lipoprotein VirB7 interacts with VirB9 in the membranes of *Agrobacterium tumefaciens*. Journal of bacteriology **179:** 1211-1218

Baum JA, Chu CR, Rupar M, Brown GR, Donovan WP, Huesing JE, Ilagan O, Malvar TM, Pleau M, Walters M, Vaughn T (2004) Binary toxins from Bacillus thuringiensis active against the western corn rootworm, Diabrotica virgifera virgifera LeConte. Appl.Environ.Microbiol. **70:** 4889-4898

Beaupre CE, Bohne J, Dale EM, Binns AN (1997) Interactions between VirB9 and VirB10 membrane proteins involved in movement of DNA from *Agrobacterium tumefaciens* into plant cells. Journal of bacteriology **179:** 78-89

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Paris, Life Sciences **316:** 1194-1199

Bechtold N, Jaudeau B, Jolivet S, Maba B, Vezon D, Voisin R, Pelletier G (2000) The maternal chromosome set is the target of the T-DNA in the in planta transformation of *Arabidopsis thaliana*. Genetics **155:** 1875-1887

Beijerinck MW, Van Delden A (1902) Ueber die Assimilation des freien Stickstoffs durch Bakterien. *Zentbl Bakt Parasitenk Infekt Abt II* **9:** 3-43

Berger BR, Christie PJ (1994) Genetic complementation analysis of the *Agrobacterium tumefaciens* virB operon: virB2 through virB11 are essential virulence genes. Journal of bacteriology **176:** 3646-3660

Bertheau Y, Diolez A, Kobilinsky A, Magin K (2002) Detection methods and performance criteria for genetically modified organisms. J AOAC Int **85:** 801-808

Blumwald E, Grover A (2006) Salt tolerance. *In* NG Halford, ed, Plant Biotechnology: current and future applications of genetically modified crops. Wiley, U.K., pp 206-224

Bonfini L, Petra H, Simon K, Van den Eede G (2001) Review of GMO dtection and quantification techniques. *In*. Institute for Health and Consumer Protection

Bouaboula M, Legoux P, Pessegue B, Delpech B, Dumont X, Piechaczyk M, Casellas P, Shire D (1992) Standardization of mRNA titration using a polymerase chain reaction method involving co-amplification with a multispecific internal control. J Biol Chem **267:** 21830-21838

Bouzar H, Jones JB (2001) *Agrobacterium larrymoorei* sp. nov., a pathogen isolated from aerial tumours of Ficus benjamina. Int J Syst Evol Microbiol **51:** 1023-1026

Boyko A, Matsuoka A, Kovalchuk I (2009) High frequency *Agrobacterium tumefaciens*-mediated plant transformation induced by ammonium nitrate. Plant cell reports

Bray CM, West CE (2005) DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. The New phytologist **168:** 511-528

Breitler JC, Meynard D, Van Boxtel J, Royer M, Bonnot F, Cambillau L, Guiderdoni E (2004) A novel two T-DNA binary vector allows efficient generation of marker-free transgenic plants in three elite cultivars of rice (Oryza sativa L.). Transgenic research **13:** 271-287

Brencic A, Angert ER, Winans SC (2005) Unwounded plants elicit *Agrobacterium* vir gene induction and T-DNA transfer: transformed plant cells produce opines yet are tumour free. Molecular microbiology **57:** 1522-1531

Brencic A, Eberhard A, Winans SC (2004b) Signal quenching, detoxification and mineralization of vir gene-inducing phenolics by the VirH2 protein of *Agrobacterium tumefaciens*. Molecular microbiology **51:** 1103-1115

Brencic A, Winans SC (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. Microbiology and molecular biology reviews **69:** 155-194

Brencic A, Xia Q, Winans SC (2004a) VirA of *Agrobacterium tumefaciens* is an intradimer transphosphorylase and can actively block vir gene expression in the absence of phenolic signals. Molecular microbiology **52:** 1349-1362

Brunaud V, Balzergue S, Dubreucq B, Aubourg S, Samson F, Chauvin S, Bechtold N, Cruaud C, DeRose R, Pelletier G, Lepiniec L, Caboche M, Lecharny A (2002) T-DNA integration into the *Arabidopsis* genome depends on sequences of pre-insertion sites. EMBO reports **3:** 1152-1157

Brunnert HJ, Spener F, Borchers T (2001) PCR-ELISA for the CaMV-35S promoter as a screening method for genetically modified Roundup Ready Soybeans. Eur Food Res Technol **213:** 366-371

Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJ (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. The EMBO journal **14:** 3206-3214

Bundock P, Hooykaas PJ (1996) Integration of *Agrobacterium tumefaciens* T-DNA in the *Saccharomyces cerevisiae* genome by illegitimate recombination. Proceedings of the National Academy of Sciences of the United States of America **93:** 15272-15275

Burns M, Corbisier P, Wiseman G, Valdivia H, McDonald P, Bowler P, Ohara K, Schimmel H, Charels D, Damant A, Harris N (2006) Comparison of plasmid and genomic DNA calibrants for the quantification of genetically modified ingredients. Eur Food Res Technol **224:** 249-258

Busch U, Mühlbauer B, Schulze M, Zagon J (1999) Screening- und spezifische Nachweismethoden für transgene Tomaten (Zeneca) mit der Polymerasekettenreaktion. Deutsche Lebensmittelrundschau Heft **2:** 52-56

Cabal GG, Genovesio A, Rodriguez-Navarro S, Zimmer C, Gadal O, Lesne A, Buc H, Feuerbach-Fournier F, Olivo-Marin JC, Hurt EC, Nehrbass U (2006) SAGA interacting factors confine sub-diffusion of transcribed genes to the nuclear envelope. Nature **441:** 770-773

Cangelosi GA, Hung L, Puvanesarajah V, Stacey G, Ozga DA, Leigh JA, Nester EW (1987) Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. Journal of bacteriology **169:** 2086-2091

Cangelosi GA, Martinetti G, Leigh JA, Lee CC, Theines C, Nester EW (1989) Role for [corrected] *Agrobacterium tumefaciens* ChvA protein in export of beta-1,2-glucan. Journal of bacteriology **171:** 1609-1615

Cankar K, Stebih D, Dreo T, Zel J, Gruden K (2006) Critical points of DNA quantification by real-time PCR--effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. BMC Biotechnol **6:** 37

Cardoza V, Stewart CN (2003) Increased *Agrobacterium*-mediated transformation and rooting efficiencies in canola (Brassica napus L.) from hypocotyl segment explants. Plant cell reports **21:** 599-604

Cascales E, Christie PJ (2003) The versatile bacterial type IV secretion systems. Nat Rev Microbiol **1:** 137-149

Cascales E, Christie PJ (2004) *Agrobacterium* VirB10, an ATP energy sensor required for type IV secretion. Proceedings of the National Academy of Sciences of the United States of America **101:** 17228-17233

Casolari JM, Brown CR, Komili S, West J, Hieronymus H, Silver PA (2004) Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. Cell **117:** 427-439

Chan MT, Chang HH, Ho SL, Tong WF, Yu SM (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric alpha-amylase promoter/beta-glucuronidase gene. Plant molecular biology **22:** 491-506

Chang CC, Chen CM, Adams BR, Trost BM (1983) Leucinopine, a characteristic compound of some crown-gall tumors. Proceedings of the National Academy of Sciences of the United States of America **80:** 3573-3576

Chang CH, Winans SC (1992) Functional roles assigned to the periplasmic, linker, and receiver domains of the *Agrobacterium tumefaciens* VirA protein. Journal of bacteriology **174:** 7033-7039

Chaouachi M, Chupeau G, Berard A, McKhann H, Romaniuk M, Giancola S, Laval V, Bertheau Y, Brunel D (2008) A high-throughput multiplex method adapted for GMO detection. J Agric Food Chem **56:** 11596-11606

Charels D, Broeders S, Corbisier P, Trapmann S, Schimmel H, Linsinger T, Emons H (2007) Toward metrological traceability for DNA fragment ratios in GM quantification. 2. Systematic study of parameters influencing the quantitative determination of MON 810 corn by real-time PCR. J Agric Food Chem **55:** 3258-3267

Chateau S, Sangwan RS, Sangwan-Norreel BS (2000) Competence of *Arabidopsis thaliana* genotypes and mutants for *Agrobacterium tumefaciens*-mediated gene transfer: role of phytohormones. J Exp Bot **51:** 1961-1968

Chen S, Jin W, Wang M, Zhang F, Zhou J, Jia Q, Wu Y, Liu F, Wu P (2003) Distribution and characterization of over 1000 T-DNA tags in rice genome. The Plant journal **36:** 105-113

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic Transformation of Wheat Mediated by *Agrobacterium tumefaciens*. Plant Physiol **115:** 971-980

Cheng M, Hu T, Layton JI, Liu CN, Fry JE (2003) Desiccation of plant tissues post-*Agrobacterium* infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. In *Vitro* Cell. Dev. Biol. Plant **39:** 595-604

Chilton MD, Que Q (2003) Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. Plant Physiol **133:** 956-965

Chilton WS, Tempe J, Matzke M, Chilton MD (1984) Succinamopine: a new crown gall opine. Journal of bacteriology **157:** 357-362

Christie PJ (2004) Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. Biochimica et biophysica acta **1694:** 219-234

Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E (2005) Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu Rev Microbiol **59:** 451-485

Christou P (1992) Genetic transformation of crop plants using microprojectiles bombardment. `The plant journal **2:** 275-281

Christou P (1996) Transformation technology. Trends Plant Sci **1:** 423-431

Citovsky V, Kapelnikov A, Oliel S, Zakai N, Rojas MR, Gilbertson RL, Tzfira T, Loyter A (2004) Protein interactions involved in nuclear import of the *Agrobacterium* VirE2 protein in vivo and in vitro. The Journal of biological chemistry **279:** 29528-29533

Citovsky V, Kozlovsky SV, Lacroix B, Zaltsman A, Dafny-Yelin M, Vyas S, Tovkach A, Tzfira T (2007) Biological systems of the host cell involved in *Agrobacterium* infection. Cellular microbiology **9:** 9-20

Citovsky V, Zupan J, Warnick D, Zambryski P (1992) Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. Science **256:** 1802–1805

Clauce-Coupel H, Chateau S, Ducrocq C, Niot V, Kaveri S, Dubois F, Sangwan-Norreel B, Sangwan RS (2008) Role of vitronectin-like protein in *Agrobacterium* attachment and transformation of *Arabidopsis* cells. Protoplasma **234:** 65-75

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant journal **16:** 735-743

Cluster PD, O'Dell M, Metzlaff M, Flavell RB (1996) Details of T-DNA structural organization from a transgenic Petunia population exhibiting co-suppression. Plant molecular biology **32:** 1197-1203

Conn HJ (1942) Validity of the Genus Alcaligenes. Journal of bacteriology **44:** 353-360

Corbisier P, Broothaerts W, Gioria S, Schimmel H, Burns M, Baoutina A, Emslie KR, Furui S, Kurosawa Y, Holden MJ, Kim HH, Lee YM, Kawaharasaki M, Sin D, Wang J (2007) Toward metrological traceability for DNA fragment ratios in GM quantification. 1. Effect of DNA extraction methods on the quantitative determination of Bt176 corn by real-time PCR. J Agric Food Chem **55:** 3249-3257

Cottrez F, Auriault C, Capron A, Groux H (1994) Quantitative PCR: validation of the use of a multispecific internal control. Nucleic Acids Res **22:** 2712-2713

Crossway A, Oakes JV, Irvine JM, Ward B, Knauf VC, Shewmaker CK (1986) Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. Molecular and General Genetics **202:** 179-185

Dale EC, Ow DW (1990) Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase. Gene **91:** 79-85

Dang TA, Christie PJ (1997) The VirB4 ATPase of *Agrobacterium tumefaciens* is a cytoplasmic membrane protein exposed at the periplasmic surface. Journal of bacteriology **179:** 453-462
Danhorn T, Fuqua C (2007) Biofilm formation by plant-associated bacteria. Annu Rev Microbiol **61:** 401-422
Daniell H, Streatfield SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci **6:** 219-226
Das A, Xie YH (2000) The *Agrobacterium* T-DNA transport pore proteins VirB8, VirB9, and VirB10 interact with one another. Journal of bacteriology **182:** 758-763
Davison J, Bertheau Y (2007) EU regulations on the traceability and detction of GMOs: difficulties in interpretation, implementation and complinace. Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources **77:** 1-14
De Block M, Debrouwer D (1991) Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor. Appl. Genet. **82:** 257-263
De Buck S, De Wilde C, Van Montagu M, Depicker A (2000a) Determination of the T-DNA transfer and the T-DNA integration frequencies upon cocultivation of *Arabidopsis thaliana* root explants. Molecular plant-microbe interactions **13:** 658-665
De Buck S, De Wilde C, Van Montagu M, Depicker A (2000b) T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. Molecular Breeding **6:** 459-468
De Buck S, Jacobs A, Van Montagu M, Depicker A (1998) *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Molecular plant-microbe interactions **11:** 449-457
De Buck S, Jacobs A, Van Montagu M, Depicker A (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. The Plant journal **20:** 295-304
De Buck S, Windels P, De Loose M, Depicker A (2004) Single-copy T-DNAs integrated at different positions in the *Arabidopsis* genome display uniform and comparable beta-glucuronidase accumulation levels. Cellular and molecular life sciences **61:** 2632-2645
De Buck S, Podevin N, Nolf J, Jacobs A, Depicker A (2009) The T-DNA integration pattern in Arabidopsis transformants is highly determined by the transformed target cell. The Plant journal **60:** 134-145
De Clercq J, Zambre M, Van Montagu M, Dillen W, Angenon G (2002) An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius A. Gray* (tepary bean). Plant Cell Rep. **21:** 333-340
de Framond AJ, Barton KA, Chilton MD (1983) Mini-Ti: a new vector strategy for plant genetic engineering. Biotechnology **1:** 262–269
de Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nature biotechnology **16:** 839-842
De Neve M, De Buck S, Jacobs A, Van Montagu M, Depicker A (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. The Plant journal **11:** 15-29
de Oliveira ML, Febres VJ, Costa MG, Moore GA, Otoni WC (2009) High-efficiency *Agrobacterium*-mediated transformation of citrus via sonication and vacuum infiltration. Plant cell reports **28:** 387-395
Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucleic acids research **13:** 4777-4788
Debode F, Janssen E, Berben G (2007) Physical degradation of genomic DNA of soybean flours does not impair relative quantification of its transgenic content. Eur Food Res Technol **226:** 273-280
DeCleene M, DeLey J (1976) The host range of crown gall. Bot. Rev. **42:** 389-466
Deng W, Chen L, Wood DW, Metcalfe T, Liang X, Gordon MP, Comai L, Nester EW (1998) *Agrobacterium* VirD2 protein interacts with plant host cyclophilins. Proceedings of the National Academy of Sciences of the United States of America **95:** 7040-7045
Depicker A, Herman L, Jacobs A, Schell J, Van Montagu M (1985) Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. Mol Gen Genet **201:** 477-484

Deroles SC, Gardner RC (1988) Analysis of the T-DNA structure in a large number of transgenic *Petunias* generated by *Agrobacterium*-mediated transformation. Plant Mol. Biol. **11:** 365-377

Desfeux C, Clough SJ, Bent AF (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. Plant physiology **123:** 895-904

Dillen W, De Clerq J, Kapila J, Zambre M, Van Montagu M, Angenon G (1997) The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant J **12:** 1459-1463.

Dinelli G, Bonetti A, Marotti I, Minelli M, Navarrete-Casas M, Segura-Carretero A, Fernandez-Gutierrez A (2006) Quantitative-competitive polymerase chain reaction coupled with slab gel and capillary electrophoresis for the detection of roundup ready soybean and maize. Electrophoresis **27:** 4029-4038

Ditt RF, Nester E, Comai L (2005) The plant cell defense and *Agrobacterium tumefaciens*. FEMS Microbiol Lett **247:** 207-213

Ditt RF, Nester EW, Comai L (2001) Plant gene expression response to *Agrobacterium tumefaciens*. Proceedings of the National Academy of Sciences of the United States of America **98:** 10954-10959

Dong W, Yang L, Shen K, Kim B, Kleter GA, Marvin HJ, Guo R, Liang W, Zhang D (2008) GMDD: a database of GMO detection methods. BMC Bioinformatics **9:** 260

Douglas CJ, Staneloni RJ, Rubin RA, Nester EW (1985) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. Journal of bacteriology **161:** 850-860

Duckely M, Hohn B (2003) The VirE2 protein of Agrobacterium tumefaciens: the Yin and Yang of T-DNA transfer. FEMS Microbiol Lett **223:** 1-6

Durrenberger F, Crameri A, Hohn B, Koukolikova-Nicola Z (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proceedings of the National Academy of Sciences of the United States of America **86:** 9154-9158

Dus Santos MJ, Carrillo C, Ardila F, Rios RD, Franzone P, Piccone ME, Wigdorovitz A, Borca MV (2005) Development of transgenic alfalfa plants containing the foot and mouth disease virus structural polyprotein gene P1 and its utilization as an experimental immunogen. Vaccine **23:** 1838-1843

EC (2003a) Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed 2001/18/EC. *In*. Off. J. Eur. Commun., pp 24-28

EC (2003b) Regulation (EC) No. 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending directive 2001/18/EC. . *In*, Vol *Legis.* 2003, L268. Off. J. Eur. Commun., pp 24-28

EC (2004) EC/787/2004 of 04.10.2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisns as or in products in the context of Regulation (EC) No 1830/2003. . *In*, Vol L348. Off. J. Eur. Union, pp 18-26

Elenis DS, Kalogianni DP, Glynou K, Ioannou PC, Christopoulos TK (2008) Advances in molecular techniques for the detection and quantification of genetically modified organisms. Anal Bioanal Chem **392:** 347-354

Escudero J, Hohn B (1997) Transfer and integration of T-DNA without cell injury in the host plant. Plant cell **9:** 2135-2142

EU (1990) Council Directive 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms. *In*, Vol L117. Off J Eur Commun, pp 15-27

EU (1997) Regulation (EC) N°258/97 of the European P arliament and of the Council of 27 January 1997 concerning Novel Foods and novel food ingredients. *In*, Vol L43. Off J Eur Commun, pp 1-7

EU (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *In*, Vol L106. Off J Eur Commun, pp 1-39

EU (2004) Recommendation 2004/787/EC. Commission Recommendation of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material

produced from genetically modified organisms as or in products in the context of Regulation (EV) No 1830/2003.
Eurobarometer (2008) Attitudes of European citizens towards the environment. *In*,
Farrand SK, Van Berkum PB, Oger P (2003) *Agrobacterium* is a definable genus of the family *Rhizobiaceae*. Int J Syst Evol Microbiol **53:** 1681-1687
Feldmann KA (1991) T-DNA insertion mutagenesis in Arabidopsis: Mutational spectrum. Plant J. **1:** 71-82.
Fernandez-Lopez R, Garcillan-Barcia MP, Revilla C, Lazaro M, Vielva L, de la Cruz F (2006) Dynamics of the IncW genetic backbone imply general trends in conjugative plasmid evolution. FEMS Microbiol Rev **30:** 942-966
Ferreira PC, Hemerly AS, Engler JD, van Montagu M, Engler G, Inze D (1994) Developmental expression of the *Arabidopsis* cyclin gene cyc1At. The Plant cell **6:** 1763-1774
Filichkin SA, Gelvin SB (1993) Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1,D2 endonuclease. Molecular microbiology **8:** 915-926
Forsbach A, Schubert D, Lechtenberg B, Gils M, Schmidt R (2003) A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. Plant molecular biology **52:** 161-176
Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SE, Li B, Nettleton DS, Pei D, Wang K (2002) *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol **129:** 13-22
Francis KE, Spiker S (2005) Identification of *Arabidopsis thaliana* transformants without selection reveals a high occurrence of silenced T-DNA integrations. The Plant journal **41:** 464-477
Frommer WB, Ludewig U, Rentsch D (1999) Taking transgenic plants with a pinch of salt. Science **285:** 1222-1223
Fullner KJ, Nester EW (1996) Environmental and genetic factors affecting RSF1010 mobilization between strains of *Agrobacterium tumefaciens*. *In* W Ream, SB Gelvin, eds, Crown gall: advances in understanding interkingdom gene transfer. American Phytopathological society, pp 15-29
Gao R, Lynn DG (2005) Environmental pH sensing: resolving the VirA/VirG two-component system inputs for *Agrobacterium* pathogenesis. Journal of bacteriology **187:** 2182-2189
Gao R, Mukhopadhyay A, Fang F, Lynn DG (2006) Constitutive activation of two-component response regulators: characterization of VirG activation in *Agrobacterium tumefaciens*. Journal of bacteriology **188:** 5204-5211
Garcia-Canas V, Cifuentes A, Gonzalez R (2004) Detection of genetically modified organisms in foods by DNA amplification techniques. Crit Rev Food Sci Nutr **44:** 425-436
Garcia-Rodriguez FM, Schrammeijer B, Hooykaas PJ (2006) The *Agrobacterium* VirE3 effector protein: a potential plant transcriptional activator. Nucleic acids research **34:** 6496-6504
Gaspar YM, Nam J, Schultz CJ, Lee LY, Gilson PR, Gelvin SB, Bacic A (2004) Characterization of the *Arabidopsis* lysine-rich arabinogalactan-protein AtAGP17 mutant (rat1) that results in a decreased efficiency of *Agrobacterium* transformation. Plant Physiol **135:** 2162-2171
Gelvin SB (2000) *Agrobacterium* and Plant Genes Involved in T-DNA Transfer and Integration. Annu Rev Plant Physiol Plant Mol Biol **51:** 223-256
Gelvin SB (2003) Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiology and molecular biology reviews **67:** 16-37, table of contents
Germini A, Zanetti A, Salati C, Rossi S, Forre C, Schmid S, Marchelli R, Fogher C (2004) Development of a seven-target multiplex PCR for the simultaneous detection of transgenic soybean and maize in feeds and foods. J Agric Food Chem **52:** 3275-3280
Gheysen G, Villarroel R, Van Montagu M (1991) Illegitimate recombination in plants: a model for T-DNA integration. Genes Dev **5:** 287-297
Gilbert A (1999) Sampling of raw materials and processed foods for the presence of GMOs. Food Control **10:** 363-365
Gilliland G, Perrin S, Blanchard K, Bunn HF (1990) Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc Natl Acad Sci U S A **87:** 2725-2729
Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods **25:** 386-401

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchison D, Martin C, Katagiri F, Lange BM, Moughamer T, Xia Y, Budworth P, Zhong J, Miguel T, Paszkowski U, Zhang S, Colbert M, Sun WL, Chen L, Cooper B, Park S, Wood TC, Mao L, Quail P, Wing R, Dean R, Yu Y, Zharkikh A, Shen R, Sahasrabudhe S, Thomas A, Cannings R, Gutin A, Pruss D, Reid J, Tavtigian S, Mitchell J, Eldredge G, Scholl T, Miller RM, Bhatnagar S, Adey N, Rubano T, Tusneem N, Robinson R, Feldhaus J, Macalma T, Oliphant A, Briggs S (2002) A draft sequence of the rice genome (Oryza sativa L. ssp. japonica). Science (New York, N.Y **296:** 92-100

Gomis-Ruth FX, Sola M, de la Cruz F, Coll M (2004) Coupling factors in macromolecular type-IV secretion machineries. Curr Pharm Des **10:** 1551-1565

Gonzalez I, Garcia T, Fernandez A, Sanz B, Hernandez PE, Martin R (1999) Rapid enumeration of Escherichia coli in oysters by a quantitative PCR-ELISA. J Appl Microbiol **86:** 231-236

Goto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F (1999) Iron fortification of rice seed by the soybean ferritin gene. Nat.Biotechnol. **17:** 282-286

Griffiths K, Partis L, Croan D, Wang N, Emslie KR (2002) Review of technologies for detecting genetically modified materials in commodities and food. Australian Government Analytical Laboratories, Prepared for: Department of Agroculture, Fisheries and Forestry - Australia**:** p118

Guyon P, Chilton MD, Petit A, Tempe J (1980) Agropine in "null-type" crown gall tumors: Evidence for generality of the opine concept. Proceedings of the National Academy of Sciences of the United States of America **77:** 2693-2697

Halford NG (2006) Plant Biotechnology: current and future applications of genetically modified crops. Wiley, U.K.

Hamilton CM (1997) A binary-BAC system for plant transformation with high-molecular-weight DNA. Gene **200:** 107-116

Hamilton CM, Lee H, Li PL, Cook DM, Piper KR, von Bodman SB, Lanka E, Ream W, Farrand SK (2000) TraG from RP4 and TraG and VirD4 from Ti plasmids confer relaxosome specificity to the conjugal transfer system of pTiC58. Journal of bacteriology **182:** 1541-1548

Hapfelmeier S, Domke N, Zambryski PC, Baron C (2000) VirB6 is required for stabilization of VirB5 and VirB3 and formation of VirB7 homodimers in *Agrobacterium tumefaciens*. Journal of bacteriology **182:** 4505-4511

Hare S, Bayliss R, Baron C, Waksman G (2006) A large domain swap in the VirB11 ATPase of Brucella suis leaves the hexameric assembly intact. Journal of molecular biology **360:** 56-66

Hashizume F, Tsuchiya T, Ugaki M, Niwa Y, Tachibana N, Kowyama Y (1999) Efficient *Agrobacterium*-mediated transformation and the usefulness of a synthetic GFP reporter gene in leading varieties of japonical rice. Plant Biotechnol. **16:** 397-401

Herman L, Jacobs A, Van Montagu M, Depicker A (1990) Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. Molecular & general genetics **224:** 248-256

Hernandez M, Duplan MN, Berthier G, Vaitilingom M, Hauser W, Freyer R, Pla M, Bertheau Y (2004a) Development and comparison of four real-time polymerase chain reaction systems for specific detection and quantification of Zea mays L. J Agric Food Chem **52:** 4632-4637

Hernandez M, Esteve T, Prat S, Pla M (2004b) Development of real-time PCR systems based on SYBR Green I, amplifluorTM and TaqManR technologies for specific quantitative detection of the transgenic maize event GA21. J Cereal Sci **39:** 99-107

Hernandez M, Pla M, Esteve T, Prat S, Puigdomenech P, Ferrando A (2003) A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard based on the 3'-transgene integration sequence. Transgenic Res **12:** 179-189

Herrera-Estrella A, Chen ZM, Van Montagu M, Wang K (1988) VirD proteins of *Agrobacterium tumefaciens* are required for the formation of a covalent DNA--protein complex at the 5' terminus of T-strand molecules. The EMBO journal **7:** 4055-4062

Herrera-Estrella A, Van Montagu M, Wang K (1990) A bacterial peptide acting as a plant nuclear targeting signal: the amino-terminal portion of *Agrobacterium* VirD2 protein directs a beta-galactosidase fusion protein into tobacco nuclei. Proceedings of the National Academy of Sciences of the United States of America **87:** 9534-9537

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant journal **6:** 271-282

Higuchi R, Dollinger G, Walsh PS, Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y) **10:** 413-417

Hobbs SL, Warkentin TD, DeLong CM (1993) Transgene copy number can be positively or negatively associated with transgene expression. Plant molecular biology **21:** 17-26

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of the *vir* and T-region of the *Agrobacterium tumefaciens* Ti plasmid. Nature **303:**179-180

Holck A, Vaïtilingom M, Didierjean L, Rudi K (2002) 5'-Nuclease PCR for quantitative event-specific detection of the genetically modified Mon810 MaisGard maize. Eur Food Res Technol **214:** 449-454

Holmes B (1988) The taxonomy of *Agrobacterium*. Acta Hort **225:** 47-52

Holmes B, Roberts P (1981) The classification, identification and nomenclature of agrobacteria. J Appl Bacteriol **50:** 443-467

Holst-Jensen A, Ronning SB, Lovseth A, Berdal KG (2003) PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal Bioanal Chem **375:** 985-993

Hood EE, Fraley RT, Chilton MD (1987) Virulence of *Agrobacterium tumefaciens* Strain A281 on Legumes. Plant Physiol **83:** 529-534

Hood EE, Gelvin SB, MelchersLS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgen. Res. **2:**208-218

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. Journal of bacteriology **168:** 1291-1301

Hooykaas PJ, Schilperoort RA (1992) *Agrobacterium* and plant genetic engineering. Plant molecular biology **19:** 15-38

Howard EA, Winsor BA, De Vos G, Zambryski P (1989) Activation of the T-DNA transfer process in *Agrobacterium* results in the generation of a T-strand-protein complex: Tight association of VirD2 with the 5' ends of T-strands. Proceedings of the National Academy of Sciences of the United States of America **86:** 4017-4021

Howard EA, Zupan JR, Citovsky V, Zambryski PC (1992) The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. Cell **68:** 109-118

Huang HY, Pan TM (2004) Detection of genetically modified maize MON810 and NK603 by multiplex and real-time polymerase chain reaction methods. J Agric Food Chem **52:** 3264-3268

Huang J, Pray C, Rozelle S (2002) Enhancing the crops to feed the poor. Nature **418:** 678-684

Huang S, Gilbertson LA, Adams TH, Malloy KP, Reisenbigler EK, Birr DH, Snyder MW, Zhang Q, Luethy MH (2004) Generation of marker-free transgenic maize by regular two-border *Agrobacterium transformation* vectors. Transgenic research **13:** 451-461

Hubner P, Studer E, Luthy J (1999) Quantitative competitive PCR for the detection of genetically modified organisms in food Food Control **10:** 353-358

Hupfer C, Hotzel H, Sachse K, Moreano F, Engen KH (2000) PCR-based quantification of genetically modified Bt maize: single-competitive versus dual-competitive approach. Eur Food Res Technol **212:** 95-99

Hwang HH, Gelvin SB (2004) Plant proteins that interact with VirB2, the *Agrobacterium tumefaciens* pilin protein, mediate plant transformation. The Plant cell **16:** 3148-3167

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (Zea mays L.) mediated by *Agrobacterium tumefaciens*. Nature biotechnology **14:** 745-750

Ishikawa A (2009) The *Arabidopsis* G-protein beta-subunit is required for defense response against *Agrobacterium tumefaciens*. Biosci Biotechnol Biochem **73:** 47-52

Jakowitsch J, Papp I, Moscone EA, van der Winden J, Matzke M, Matzke AJ (1999) Molecular and cytogenetic characterization of a transgene locus that induces silencing and methylation of homologous promoters in trans. The Plant journal **17:** 131-140

Jakubowski SJ, Cascales E, Krishnamoorthy V, Christie PJ (2005) *Agrobacterium tumefaciens* VirB9, an outer-membrane-associated component of a type IV secretion system, regulates substrate selection and T-pilus biogenesis. Journal of bacteriology **187:** 3486-3495

Jakubowski SJ, Kerr JE, Garza I, Krishnamoorthy V, Bayliss R, Waksman G, Christie PJ (2009) *Agrobacterium* VirB10 domain requirements for type IV secretion and T pilus biogenesis. Molecular microbiology **71:** 779-794

Jakubowski SJ, Krishnamoorthy V, Christie PJ (2003) *Agrobacterium tumefaciens* VirB6 protein participates in formation of VirB7 and VirB9 complexes required for type IV secretion. Journal of bacteriology **185:** 2867-2878

James C (2008) Global Status of Commercialized Biotech/GM Crops: 2008. *In*, Vol Brief No. 39. ISAAA, Ithaca, NY.

James D, Schmidt AM, Wall E, Green M, Masri S (2003) Reliable detection and identification of genetically modified maize, soybean, and canola by multiplex PCR analysis. J Agric Food Chem **51:** 5829-5834

Janssen BC, Gardner RC (1989) Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Mol Biol **14**:61- 72

Jarchow E, Grimsley NH, Hohn B (1991) virF, the host-range-determining virulence gene of *Agrobacterium tumefaciens*, affects T-DNA transfer to Zea mays. Proceedings of the National Academy of Sciences of the United States of America **88:** 10426-10430

Jasbeer K, Ghazali FM, Cheah YK, Son R (2008) Application of DNA immunoassay analytical methods for GMO testing in Agricultural crops and plant-derived products. ASEAN Food Journal **15:** 1-25

Jasper F, Koncz C, Schell J, Steinbiss HH (1994) *Agrobacterium* T-strand production in vitro: sequence-specific cleavage and 5' protection of single-stranded DNA templates by purified VirD2 protein. Proceedings of the National Academy of Sciences of the United States of America **91:** 694-698

Jayaswal RK, Veluthambi K, Gelvin SB, Slightom JL (1987) Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in Escherichia coli by a virD-encoded border-specific endonuclease from *Agrobacterium tumefaciens*. Journal of bacteriology **169:** 5035-5045

Jin SG, Prusti RK, Roitsch T, Ankenbauer RG, Nester EW (1990) Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of VirG. Journal of bacteriology **172:** 4945-4950

Johansen LK, Carrington JC (2001) Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. Plant Physiol. **126:** 930–938

Jones AL, Lai EM, Shirasu K, Kado CI (1996) VirB2 is a processed pilin-like protein encoded by the *Agrobacterium tumefaciens* Ti plasmid. Journal of bacteriology **178:** 5706-5711

Jones AL, Shirasu K, Kado CI (1994) The product of the virB4 gene of *Agrobacterium tumefaciens* promotes accumulation of VirB3 protein. Journal of bacteriology **176:** 5255-5261

Jones JDG, Shlumukov L, Carland F, English J, Scofield SR, Bishop GJ, Harrison K (1992) Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgen. Res. **1:**285-297

Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA (1996) Chalcone synthase co suppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Mol Biol. **31:** 957-973

Jorgensen R, Snyder C, Jones JDC (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol Gen Genet **207:** 471-477

Kado CI (2000) The role of the T-pilus in horizontal gene transfer and tumorigenesis. Current opinion in microbiology **3:** 643-648

Kaene PJ, Kerr A, New PB (1970) Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aus J Biol Sci **23:** 585-595

Kaeppler H, Gu W, Somers DA, Rines HW, Cockburn AF (1990) Silicon carbide fiber-mediated DNA delivery into plant cells. Plant cell reports **9:** 415-418

Kapila J, Rycke RD, Montagu MV, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. Plant science **122:** 101-108

Kerr A, Panagopoulos CG (1977) Biotypes of *Agrobacterium radiobacter* var. *tumfaciens* and their biological control. Phytopath Z **90:** 172-179

Kersters K, De Ley J (1984) Genus III. *Agrobacterium* Conn. *In* NR Krieg, JG Holt, eds, Bergey's Manual of Determinative Bacteriology, Vol. 1, 8th ed. The Williams and Wilkins Co., Baltimore, London

Kertbundit S, De Greve H, Deboeck F, Van Montagu M, Hernalsteens JP (1991) *In vivo* random P-glucuronidase gene fusions in *Arabidopsis rhaliana.* Proc Natl Acad Sci USA **88:** 5212-5216

Kim SI, Veena, Gelvin SB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. The Plant journal **51:** 779-791

Kim SR, Lee J, Jun SH, Park S, Kang HG, Kwon S, An G (2003) Transgene structures in T-DNA-inserted rice plants. Plant molecular biology **52:** 761-773

Kleppe K, Ohtsuka E, Kleppe R, Molineux I, Khorana HG (1971) Studies on polynucleotides. XCVI. Repair replications of short synthetic DNA's as catalyzed by DNA polymerases. J Mol Biol **56:** 341-361

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. The Plant journal **10:** 165-174

Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Korber H, Redei GP, Schell J (1989) High-frequency T-DNA-mediated gene tagging in plants. Proceedings of the National Academy of Sciences of the United States of America **86:** 8467-8471

Kondo T, Hasegawa H, Suzuki M (2000) Transformation and regeneration of garlic (*Allium sativum L.*) by *Agrobacterium*-mediated gene transfer. Plant cell reports **19:** 989-993

Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proceedings of the National Academy of Sciences of the United States of America **98:** 11539-11544

Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. The Plant journal **11:** 945-957

Koraimann G (2003) Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria. Cellular and molecular life sciences **60:** 2371-2388

Krall L, Wiedemann U, Unsin G, Weiss S, Domke N, Baron C (2002) Detergent extraction identifies different VirB protein subassemblies of the type IV secretion machinery in the membranes of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Sciences of the United States of America **99:** 11405-11410

Krause S, Barcena M, Pansegrau W, Lurz R, Carazo JM, Lanka E (2000) Sequence-related protein export NTPases encoded by the conjugative transfer region of RP4 and by the cag pathogenicity island of Helicobacter pylori share similar hexameric ring structures. Proceedings of the National Academy of Sciences of the United States of America **97:** 3067-3072

Krizkova L, Hrouda M (1998) Direct repeats of T-DNA integrated in tobacco chromosome: characterization of junction regions. The Plant journal **16:** 673-680

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in *Arabidopsis*. The Plant cell **11:** 2283-2290

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, Sindelka R, Sjoback R, Sjogreen B, Strombom L, Stahlberg A, Zoric N (2006) The real-time polymerase chain reaction. Mol Aspects Med **27:** 95-125

Kumar RB, Das A (2001) Functional analysis of the *Agrobacterium tumefaciens* T-DNA transport pore protein VirB8. Journal of bacteriology **183:** 3636-3641

Kumar S, Fladung M (2000) Transgene repeats in aspen: molecular characterisation suggests simultaneous integration of independent T-DNAs into receptive hotspots in the host genome. Molecular & general genetics **264:** 20-28

Kumar S, Fladung M (2002) Transgene integration in aspen: structures of integration sites and mechanism of T-DNA integration. The Plant journal **31:** 543-551

Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C, Citovsky V (2001) Genetic transformation of HeLa cells by *Agrobacterium*. Proceedings of the National Academy of Sciences of the United States of America **98:** 1871-1876

Kuribara H, Shindo Y, Matsuoka T, Takubo K, Futo S, Aoki N, Hirao T, Akiyama H, Goda Y, Toyoda M, Hino A (2002) Novel reference molecules for quantitation of genetically modified maize and soybean. J AOAC Int **85:** 1077-1089

Lacroix B, Vaidya M, Tzfira T, Citovsky V (2005) The VirE3 protein of *Agrobacterium* mimics a host cell function required for plant genetic transformation. The EMBO journal **24:** 428-437

Lai EM, Chesnokova O, Banta LM, Kado CI (2000) Genetic and environmental factors affecting T-pilin export and T-pilus biogenesis in relation to flagellation of *Agrobacterium tumefaciens*. Journal of bacteriology **182:** 3705-3716

Lai EM, Kado CI (1998) Processed VirB2 is the major subunit of the promiscuous pilus of *Agrobacterium tumefaciens*. Journal of bacteriology **180:** 2711-2717
Lai EM, Kado CI (2000) The T-pilus of *Agrobacterium tumefaciens*. Trends in microbiology **8:** 361-369
Landgraf A, Reckmann B, Pingoud A (1991) Direct analysis of polymerase chain reaction products using enzyme-linked immunosorbent assay techniques. Anal Biochem **198:** 86-91
Lange M, Vincze E, Moller MG, Holm PB (2006) Molecular analysis of transgene and vector backbone integration into the barley genome following *Agrobacterium*-mediated transformation. Plant Cell Rep **8:** 815-820
Laufs P, Autran D, Traas J (1999) A chromosomal paracentric inversion associated with T-DNA integration in *Arabidopsis*. The Plant journal **18:** 131-139
Lee LY, Gelvin SB (2008) T-DNA binary vectors and systems. Plant Physiol **146:** 325-332
Lee LY, Gelvin SB, Kado CI (1999) pSa causes oncogenic suppression of Agrobacterium by inhibiting VirE2 protein export. J. Bacteriol. **181:**186–196
Lee YW, Jin S, Sim WS, Nester EW (1995) Genetic evidence for direct sensing of phenolic compounds by the VirA protein of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Sciences of the United States of America **92:** 12245-12249
Leimanis S, Hernandez M, Fernandez S, Boyer F, Burns M, Bruderer S, Glouden T, Harris N, Kaeppeli O, Philipp P, Pla M, Puigdomenech P, Vaitilingom M, Bertheau Y, Remacle J (2006) A microarray-based detection system for genetically modified (GM) food ingredients. Plant Mol Biol **61:** 123-139
Levee V, Garin E, Klimaszewska K, Seguin A (1999) Stable genetic transformation of white pine (*Pinus strobus* L.) after cocultivation of embryogenic tisues with *Agrobacterium tumefaciens.* . Mol. Breeding **5:** 429-440
Li H, Gonzalez-Cabrera J, Oppert B, Ferre J, Higgins RA, Buschman LL, Radke GA, Zhu KY, Huang F (2004) Binding analyses of Cry1Ab and Cry1Ac with membrane vesicles from Bacillus thuringiensis-resistant and -susceptible Ostrinia nubilalis. Biochem Biophys Res Commun **323:** 52-57
Li J, Krichevsky A, Vaidya M, Tzfira T, Citovsky V (2005) Uncoupling of the functions of the *Arabidopsis* VIP1 protein in transient and stable plant genetic transformation by *Agrobacterium*. Proceedings of the National Academy of Sciences of the United States of America **102:** 5733-5738
Li L, Jia Y, Hou Q, Charles TC, Nester EW, Pan SQ (2002) A global pH sensor: *Agrobacterium* sensor protein ChvG regulates acid-inducible genes on its two chromosomes and Ti plasmid. Proceedings of the National Academy of Sciences of the United States of America **99:** 12369-12374
Li Y, Rosso MG, Ulker B, Weisshaar B (2006) Analysis of T-DNA insertion site distribution patterns in *Arabidopsis thaliana* reveals special features of genes without insertions. Genomics **87:** 645-652
Lipp M, Shillito R, Giroux R, Spiegelhalter F, Charlton S, Pinero D, Song P (2005) Polymerase chain reaction technology as analytical tool in agricultural biotechnology. J AOAC Int **88:** 136-155
Lipton CR, Dautlick JX, Grothaus GD, Hunst PL, Magin KM, Mihaliak CA, Rubio FM, Stave JW (2000) Guidelines for the validation and use of immunoassays for determination of introduced proteins in biotechnology anhanced crops and derived food ingredients. Food Agric Immunol **12:** 153-164
Liu CN, Li XQ, Gelvin SB (1992) Multiple copies of virG enhance the transient transformation of celery, carrot and rice tissues by *Agrobacterium tumefaciens*. Plant molecular biology **20:** 1071-1087
Liu G, Su W, Xu Q, Long M, Zhou J, Song S (2004) Liquid-phase hybridization based PCR-ELISA for detection of genetically modified organisms in food. Food Control **15:** 303-306
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:** 402-408
Llosa M, Zupan J, Baron C, Zambryski P (2000) The N- and C-terminal portions of the *Agrobacterium* VirB1 protein independently enhance tumorigenesis. Journal of bacteriology **182:** 3437-3445
Loopstra CA, Stomp AM, Sederoff RR (1990) *Agrobacterium*-mediated DNA transfer in sugar pine. Plant molecular biology **15:** 1-9
Loper JE, Kado CI (1979) Host range conferred by the virulence-specifying plasmid of *Agrobacterium tumefaciens*. Journal of bacteriology **139:** 591-596

Luby-Phelps K (2000) Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. Int Rev Cytol **192:** 189-221

Luthra R, Kerr SC, Harreman MT, Apponi LH, Fasken MB, Ramineni S, Chaurasia S, Valentini SR, Corbett AH (2007) Actively transcribed GAL genes can be physically linked to the nuclear pore by the SAGA chromatin modifying complex. The Journal of biological chemistry **282:** 3042-3049

Luthy J (1999) Detection strategies for food authenticity and gentically modified food. Food Control **10:** 359-361

Ma JK, Chikwamba R, Sparrow P, Fischer R, Mahoney R, Twyman RM (2005) Plant-derived pharmaceuticals--the road forward. Trends Plant Sci **10:** 580-585

Mackay IM (2004) Real-time PCR in the microbiology laboratory. Clin Microbiol Infect **10:** 190-212

Mafra I, Silva SA, Moreira EJMO, Ferreira da Silva CS, Beatriz M, Oliveira PP (2008) Comparative study of DNA extraction methods for soybean derived food products Food Control **19:** 1183-1190

Marjanac G, De Paepe A, Peck I, Jacobs A, De Buck S, Depicker A (2008) Evaluation of CRE-mediated excision approaches in *Arabidopsis thaliana*. Transgenic research **17:** 239-250

Matsuoka T, Kuribara H, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, Hino A (2001) A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. Shokuhin Eiseigaku Zasshi **42:** 24-32

Matsuoka T, Kuribara H, Takubo K, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, Hino A (2002) Detection of recombinant DNA segments introduced to genetically modified maize (Zea mays). J Agric Food Chem **50:** 2100-2109

Mattarucchi E, Weighardt F, Barbati C, Querci M, Van den Eede G (2005) Development and applications of real-time PCR standards for GMO quantification based on tandem-marker plasmids Eur Food Res Technol **221:** 511-519

Matthysse AG (1987) Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. Journal of bacteriology **169:** 313-323

Matthysse AG, Holmes KV, Gurlitz RH (1981) Elaboration of cellulose fibrils by *Agrobacterium tumefaciens* during attachment to carrot cells. Journal of bacteriology **145:** 583-595

Matthysse AG, McMahan S (1998) Root colonization by *Agrobacterium tumefaciens* is reduced in cel, attB, attD, and attR mutants. Appl Environ Microbiol **64:** 2341-2345

Matthysse AG, Thomas DL, White AR (1995a) Mechanism of cellulose synthesis in *Agrobacterium tumefaciens*. Journal of bacteriology **177:** 1076-1081

Matthysse AG, White S, Lightfoot R (1995b) Genes required for cellulose synthesis in *Agrobacterium tumefaciens*. Journal of bacteriology **177:** 1069-1075

Matthysse AG, Yarnall H, Boles SB, McMahan S (2000) A region of the *Agrobacterium tumefaciens* chromosome containing genes required for virulence and attachment to host cells. Biochimica et biophysica acta **1490:** 208-212

Matthysse AG, Yarnall HA, Young N (1996) Requirement for genes with homology to ABC transport systems for attachment and virulence of *Agrobacterium tumefaciens*. Journal of bacteriology **178:** 5302-5308

Maximova SN, Dandekar AM, Guiltinan MJ (1998) Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. Plant molecular biology **37:** 549-559

Mayerhofer R, Koncz-Kalman Z, Nawrath C, Bakkeren G, Crameri A, Angelis K, Redei GP, Schell J, Hohn B, Koncz C (1991) T-DNA integration: a mode of illegitimate recombination in plants. The EMBO journal **10:** 697-704

McAfee BJ, White EE, Pelcher LE, M.S. L (1993) Root induction in pine (*Pinus*) and larch (*Larix*) spp. using *Agrobacterium rhizogenes.* Plant Cell Tissue Organ Cult **34:** 53-62

McCormac AC, Fowler MR, Chen DF, Elliott MC (2001) Efficient co-transformation of Nicotiana tabacum by two independent T-DNAs, the effect of T-DNA size and implications for genetic separation. Transgenic research **10:** 143-155

McCullen CA, Binns AN (2006) *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annual review of cell and developmental biology **22:** 101-127

McCulloch RK, Choong CS, Hurley DM (1995) An evaluation of competitor type and size for use in the determination of mRNA by competitive PCR. PCR Methods Appl **4:** 219-226

Melchers LS, Maroney MJ, den Dulk-Ras A, Thompson DV, van Vuuren HA, Schilperoort RA, Hooykaas PJ (1990) Octopine and nopaline strains of *Agrobacterium tumefaciens* differ in virulence; molecular characterization of the virF locus. Plant molecular biology **14:** 249-259

Melchers LS, Regensburg-Tuink TJ, Bourret RB, Sedee NJ, Schilperoort RA, Hooykaas PJ (1989) Membrane topology and functional analysis of the sensory protein VirA of *Agrobacterium tumefaciens*. The EMBO journal **8:** 1919-1925

Meyer P, Saedler H (1996) Homology-Dependent Gene Silencing in Plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 23-48

Meyer R, Jaccoud E (1997) Detection of genetically modified soya in processed food products: Developement and validation of PCR assay for the specific detection of glyphosate tolerant soybean. Eur Food Chem **1:** 23-28

Meza TJ, Stangeland B, Mercy IS, Skarn M, Nymoen DA, Berg A, Butenko MA, Hakelien AM, Haslekas C, Meza-Zepeda LA, Aalen RB (2002) Analyses of single-copy *Arabidopsis* T-DNA-transformed lines show that the presence of vector backbone sequences, short inverted repeats and DNA methylation is not sufficient or necessary for the induction of transgene silencing. Nucleic acids research **30:** 4556-4566

Middleton R, Sjolander K, Krishnamurthy N, Foley J, Zambryski P (2005) Predicted hexameric structure of the *Agrobacterium* VirB4 C terminus suggests VirB4 acts as a docking site during type IV secretion. Proceedings of the National Academy of Sciences of the United States of America **102:** 1685-1690

Miraglia M, Berdal KG, Brera C, Corbisier P, Holst-Jensen A, Kok EJ, Marvin HJ, Schimmel H, Rentsch J, van Rie JP, Zagon J (2004) Detection and traceability of genetically modified organisms in the food production chain. Food Chem Toxicol **42:** 1157-1180

Miranda A, Janssen G, Hodges L, Peralta EG, Ream W (1992) *Agrobacterium tumefaciens* transfers extremely long T-DNAs by a unidirectional mechanism. Journal of bacteriology **174:** 2288-2297

Mlotshwa S, Voinnet O, Mette MF, Matzke M, Vaucheret H, Ding SW, Pruss G, Vance VB (2002) RNA silencing and the mobile silencing signal. Plant Cell **14:** S289–301

Monma K, Araki R, Sagi N, Satoh M, Ichikawa H, Satoh K, Tobe T, Kamata K, Hino A, Saito K (2005) Detection of genetically modified organisms in foreign-made processed foods containing corn and potato. Shokuhin Eiseigaku Zasshi **46:** 79-85

Moore LW, Bouzar H, Burr TJ (2001) *Agrobacterium*. *In* NW Schaad, JB Jones, W Chun, eds, Laboratoy guide for identification of plant pathogenic bacteria. American Phytopatological Society Press, St. Paul, Minnesota, pp 17-33

Moriguchi K, Maeda Y, Satou M, Hardayani NS, Kataoka M, Tanaka N, Yoshida K (2001) The complete nucleotide sequence of a plant root-inducing (Ri) plasmid indicates its chimeric structure and evolutionary relationship between tumor-inducing (Ti) and symbiotic (Sym) plasmids in *Rhizobiaceae*. Journal of molecular biology **307:** 771-784

Morris JW, Morris RO (1990) Identification of an *Agrobacterium tumefaciens* virulence gene inducer from the pinaceous gymnosperm *Pseudotsuga menziesii*. Proceedings of the National Academy of Sciences of the United States of America **87:** 3614-3618

Mundree SG, Iyer R, Baker B, Conrad N, Davis EJ, Govender K, Maredza AT, Thomson JA (2006) Prospects for using gentic modificationto engineer drought tolerance in crops. *In* NG Halford, ed, Plant Biotechnology: current and future applications of genetically modified crops. Wiley, U.K., pp 193-205

Muskens, MW, Vissers, AP, Mol JN, Kooter JM (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Mol. Biol. **43:** 243-260

Mysore KS, Nam J, Gelvin SB (2000) An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. Proceedings of the National Academy of Sciences of the United States of America **97:** 948-953

Nacry P, Camilleri C, Courtial B, Caboche M, Bouchez D (1998) Major chromosomal rearrangements induced by T-DNA transformation in *Arabidopsis*. Genetics **149:** 641-650

Nadal A, Coll A, La Paz JL, Esteve T, Pla M (2006) A new PCR-CGE (size and color) method for simultaneous detection of genetically modified maize events. Electrophoresis **27:** 3879-3888

Nagai H, Roy CR (2003) Show me the substrates: modulation of host cell function by type IV secretion systems. Cellular microbiology **5:** 373-383

Nakayama N, Arroyo JM, Simorowski J, May B, Martienssen R, Irish VF (2005) Gene trap lines define domains of gene regulation in *Arabidopsis* petals and stamens. The Plant cell **17:** 2486-2506

Nam J, Matthysse AG, Gelvin SB (1997) Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. Plant Cell **9:** 317-333

Nam J, Mysore KS, Zheng C, Knue MK, Matthysse AG, Gelvin SB (1999) Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. Molecular & general genetics **261:** 429-438

Nandakumar R, Chen L, Rogers SMD (2004) Factors affecting the Agrobacterium-mediated transient transformation of the wetland monocot, *Typha latifolia*. Plant Cell, Tissue, Organ Culture **79:** 31-38

Narasimhulu SB, Deng XB, Sarria R, Gelvin SB (1996) Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. The Plant cell **8:** 873-886

Nester EW, Merlo DJ, Drummond MH, Sciaky D, Montoya AL, Chilton MD (1977) The incorporation and expression of *Agrobacterium* plasmid genes in crown gall tumors. Basic Life Sci **9:** 181-196

Newell CA (2000) Plant transformation technology. Developments and applications. Molecular biotechnology **16:** 53-65

Nicholson L, Canizares MC, G.P. L (2006) Production of vaccines in GM plants. *In* NG Halford, ed, Plant Biotechnology: current and future applications of genetically modified crops. Wiley, U.K., pp 164-192

Nielsen CR, Berdal KG, Holst-Jensen A (2004) Characterization of the 5' integration site and development of an event-specific real-time PCR assay for NK603 maize from a low starting copy number. Eur Food Res Technol **219:** 421-427

Nomura K, Komamine A (1986) Embryogenesis from microinjected single cells in a carrot suspension culture. Plant Science **44:** 53-58

O'Connell KP, Handelsman J (1989) chvA locus may be involved in export of neutral cyclic beta-1,2-linked D-glucan from *Agrobacterium tumefaciens*. Molecular plant-microbe interactions **2:** 11-16

Ohba T, Yoshioka Y, Machida C, Machida Y (1995) DNA rearrangement associated with the integration of T-DNA in tobacco: an example for multiple duplications of DNA around the integration target. The Plant journal **7:** 157-164

Onishi M, Matsuoka T, Kodama T, Kashiwaba K, Futo S, Akiyama H, Maitani T, Furui S, Oguchi T, Hino A (2005) Development of a multiplex polymerase chain reaction method for simultaneous detection of eight events of genetically modified maize. J Agric Food Chem **53:** 9713-9721

Ophel K, Kerr A (1990) *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. Int J Syst Bacteriol **40:** 236-241

OSTP (February 1992) Exercise of Federal oversight within scope of statutory authority: Planned introductions of biotechnology products into the environment *In*, Vol 57 FR 6753, *Federal Register* 27

Otten L, Burr T, Szegedi E (2008) *Agrobacterium*: a disease-causing bacterium. *In* T Tzfira, V Citovsky, eds, *Agrobacterium* from biology to biotechnology. Springer, pp 1-46

Otten L, DeGreve H, Leemans J, Hain R, Hooykaas P, Schell J (1984) Restoration of virulence of *vir* region mutants of *Agrobacterium tumefaciens* strain B6S3 by coinfection with normal and mutant *Agrobacterium* strains. Mol. Gen. Genet. **195:** 159–163

Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchliffe E, Adams JL, Silverstone AL, Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat Biotechnol **23:** 482-487

Panagopolous CG, Psallidas PG, Alivizatos AS (1978) Studies on biotype 3 of *Agrobacterium radiobacter* var. *tumefaciens*. *In* SdPve phytobactériologie, ed, Plant pathogenic bacteria Proc 4th Internat Conf Plant Path Bact, Angers, France

Pansegrau W, Schoumacher F, Hohn B, Lanka E (1993) Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. Proceedings of the National Academy of Sciences of the United States of America **90:** 11538-11542

Paoletti C, Heissenberger A, Mazzara M, Larcher S, Grazioli E, Corbisier P, Hess Gilbert Berben N, Lubeck PS, De Loose M, Moran G, Henry C, Imma Floch CB, Ovesna J, Van Den Eede G (2006) Kernel lot distribution assessment (KeLDA): a study on the distribution of GMO in large soybean shipments. Eur Food Res Technol **224:** 129-139

Paszkowski J, Shillito RD, Saul M, Mandak V, Hohn T, Hohn B, Potrykus I (1984) Direct gene transfer to plants. **3:** 2717-2722

Pazour GJ, Das A (1990) Characterization of the VirG binding site of *Agrobacterium tumefaciens*. Nucleic acids research **18:** 6909-6913

Peano C, Bordoni R, Gulli M, Mezzelani A, Samson MC, Bellis GD, Marmiroli N (2005) Multiplex polymerase chain reaction and ligation detection reaction/universal array technology for the traceability of genetically modified organisms in foods. Anal Biochem **346:** 90-100

Pelczar P, Kalck V, Gomez D, Hohn B (2004) *Agrobacterium* proteins VirD2 and VirE2 mediate precise integration of synthetic T-DNA complexes in mammalian cells. EMBO reports **5:** 632-637

Peng WT, Lee YW, Nester EW (1998) The phenolic recognition profiles of the *Agrobacterium tumefaciens* VirA protein are broadened by a high level of the sugar binding protein ChvE. Journal of bacteriology **180:** 5632-5638

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, Sudhakar D, Christou P, Snape JW, Gale MD, Harberd NP (1999) 'Green revolution' genes encode mutant gibberellin response modulators. Nature **400:** 256-261

Peralta EG, Hellmiss R, Ream W (1986) Overdrive, a T-DNA transmission enhancer on the *A. tumefaciens* tumour-inducing plasmid. The EMBO journal **5:** 1137-1142

Peralta EG, Ream LW (1985) T-DNA border sequences required for crown gall tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America **82:** 5112-5116

Permyakova N, Shumnyi V, Deineko E (2009) Agrobacterium -mediated transformation of plants: Transfer of vector DNA fragments in the plant genome**.** Russian Journal of Genetics 45: 266-275

Petit A, Berkaloff A, Tempé J (1986) Multiple transformation of plant cells by *Agrobacterium* may be responsible for the complex organization of T-DNA in crown gall and hairy root. Mol. Gen. Genet. **202:** 388-393

Petti C, Wendt T, Meade C, Mullins E (2009) Evidence of genotype dependency within *Agrobacterium tumefaciens* in relation to the integration of vector backbone sequence in transgenic Phytophthora infestans-tolerant potato. J Biosci Bioeng **107:** 301-306

Piers KL, Heath JD, Liang X, Stephens KM, Nester EW (1996) *Agrobacterium tumefaciens*-mediated transformation of yeast. Proceedings of the National Academy of Sciences of the United States of America **93:** 1613-1618

Pietsch K, Waiblinger HU, Brodmann P, Wurz A (1997) Screeningverfahren zur Identifizierung gentechnischen veränderter Lebensmittel. . Deutsche Lebensm Rundsch **93:** 35-38

Pionnat S, Keller H, Hericher D, Bettachini A, Dessaux Y, Nesme X, Poncet C (1999) Ti plasmids from *Agrobacterium* characterize rootstock clones that initiated a spread of crown gall disease in Mediterranean countries. Appl Environ Microbiol **65:** 4197-4206

Planet PJ, Kachlany SC, DeSalle R, Figurski DH (2001) Phylogeny of genes for secretion NTPases: identification of the widespread tadA subfamily and development of a diagnostic key for gene classification. Proceedings of the National Academy of Sciences of the United States of America **98:** 2503-2508

Podevin N, De Buck S, De Wilde C, Depicker A (2006) Insights into recognition of the T-DNA border repeats as termination sites for T-strand synthesis by Agrobacterium tumefaciens. Transgenic research **15:** 557-571

Porter JR (1991) Host range and implications of plant infection by *Agrobacterium rhizogenes.* Crit. Rev. Plant Sci. **10:** 387-421

Rabel C, Grahn AM, Lurz R, Lanka E (2003) The VirB4 family of proposed traffic nucleoside triphosphatases: common motifs in plasmid RP4 TrbE are essential for conjugation and phage adsorption. Journal of bacteriology **185:** 1045-1058

Radchuk VV, Thi Van D, Klocke E (2005) Multiple gene co-integration in Arabidopsis thaliana predominantly occurs in the same genetic locus after simultaneous in planta transformation with distinct Agrobacterium tumefaciens strains. Plant Sci **168:** 1515-1523

Ramanathan V, Veluthambi K (1995) Transfer of non-T-DNA portions of the *Agrobacterium tumefaciens* Ti plasmid pTiA6 from the left terminus of TL-DNA. Plant molecular biology **28:** 1149-1154

Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in indica rice. Plant cell reports **15:** 727-730

Regensburg-Tuink AJ, Hooykaas PJ (1993) Transgenic N. glauca plants expressing bacterial virulence gene virF are converted into hosts for nopaline strains of *A. tumefaciens*. Nature **363:** 69-71

Relic B, Andjelkovic M, Rossi L, Nagamine Y, Hohn B (1998) Interaction of the DNA modifying proteins VirD1 and VirD2 of *Agrobacterium tumefaciens*: analysis by subcellular localization in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America **95:** 9105-9110

Reuhs BL, Kim JS, Matthysse AG (1997) Attachment of *Agrobacterium tumefaciens* to carrot cells and Arabidopsis wound sites is correlated with the presence of a cell-associated, acidic polysaccharide. Journal of bacteriology **179:** 5372-5379

Richter LJ, Thanavala Y, Arntzen CJ, Mason HS (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nature biotechnology **18:** 1167-1171

Riker AJ, Banfield WM, Wright WH, Keitt GW, Sagen HE (1930) Studies on infectious hairy roots of nursery trees of apples. J Agr Res **41**

Robertson JL, Holliday T, Matthysse AG (1988) Mapping of *Agrobacterium tumefaciens* chromosomal genes affecting cellulose synthesis and bacterial attachment to host cells. Journal of bacteriology **170:** 1408-1411

Rodgers PB, Hamilton WD, Adair JR (1999) The therapeutic potential of plant-derived vaccines and antibodies. Expert Opin Investig Drugs **8:** 211-227

Rodriguez-Navarro S, Fischer T, Luo MJ, Antunez O, Brettschneider S, Lechner J, Perez-Ortin JE, Reed R, Hurt E (2004) Sus1, a functional component of the SAGA histone acetylase complex and the nuclear pore-associated mRNA export machinery. Cell **116:** 75-86

Ronning SB, Berdal K, Holst-Jensen A (2003) Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). Eur Food Res Technol **216:** 347-354

Rossi L., Escudero J., Hohn B., Tinland B. (1993) Efficient and sensitive assay for T-DNA dependent transient gene expression. Plant Mol Biol Rep **12:** 220-229

Rossi L, Hohn B, Tinland B (1996) Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Sciences of the United States of America **93:** 126-130

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant molecular biology **53:** 247-259

Saenkham P, Eiamphungporn W, Farrand SK, Vattanaviboon P, Mongkolsuk S (2007) Multiple superoxide dismutases in *Agrobacterium tumefaciens*: functional analysis, gene regulation, and influence on tumorigenesis. Journal of bacteriology **189:** 8807-8817

Sainsbury F, Thuenemann EC, Lomonossoff GP (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnology Journal **7:** 682 - 693

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:** 487-491

Salas MC, Park SH, Srivatanakul M, Smith RH (2001) Temperature influence on stable T-DNA integration in plant cells. Plant cell reports **20:** 701-705

Sallaud C, Gay C, Larmande P, Bes M, Piffanelli P, Piegu B, Droc G, Regad F, Bourgeois E, Meynard D, Perin C, Sabau X, Ghesquiere A, Glaszmann JC, Delseny M, Guiderdoni E (2004) High throughput T-DNA insertion mutagenesis in rice: a first step towards in silico reverse genetics. The Plant journal **39:** 450-464

Sallaud C, Meynard D, van Boxtel J, Gay C, Bes M, Brizard JP, Larmande P, Ortega D, Raynal M, Portefaix M, Ouwerkerk PB, Rueb S, Delseny M, Guiderdoni E (2003) Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa L.*) functional genomics. Theor Appl Genet **106:** 1396-1408

Salman H, Abu-Arish A, Oliel S, Loyter A, Klafter J, Granek R, Elbaum M (2005) Nuclear localization signal peptides induce molecular delivery along microtubules. Biophys J **89:** 2134-2145

Salomon S, Puchta H (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. The EMBO journal **17:** 6086-6095

Sanford JC, Smith FD, Russell JA (1993) Optimizing the biolistic process for different biological applications. Methods in enzymology **217:** 483-509

Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan BS (1992) Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. Planta **188:** 439-456

Sangwan RS, Bourgeois Y, Sangwan-Norreel BS (1991) Genetic transformation of *Arabidopsis thaliana* zygotic embryos and identification of critical parameters influencing transformation efficiency. Molecular & general genetics **230:** 475-485

Sawada H, Ieki H, Oyaizu H, Matsumoto S (1993) Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. Int J Syst Bacteriol **43:** 694-702

Sawahel WA (1996) Ultrasound-mediated stable transformation of potato tuber discs. Biotechnology Techniques **10:** 821-824

Scheeren-Groot EP, Rodenburg KW, den Dulk-Ras A, Turk SC, Hooykaas PJ (1994) Mutational analysis of the transcriptional activator VirG of *Agrobacterium tumefaciens*. Journal of bacteriology **176:** 6418-6426

Scheiffele P, Pansegrau W, Lanka E (1995) Initiation of *Agrobacterium tumefaciens* T-DNA processing. Purified proteins VirD1 and VirD2 catalyze site- and strand-specific cleavage of superhelical T-border DNA in vitro. The Journal of biological chemistry **270:** 1269-1276

Schell J, Van Montagu M, De Beuckeleer M, De Block M, Depicker A, De Wilde M, Engler G, Genetello C, Hernalsteens JP, Holsters M, Seurinck J, Silva B, Van Vliet F, Villarroel R (1979) Interactions and DNA transfer between *Agrobacterium tumefaciens*, the Ti-plasmid and the plant host. Proceedings of the Royal Society of London. Series B, Containing papers of a Biological character **204:** 251-266

Schmid M, Arib G, Laemmli C, Nishikawa J, Durussel T, Laemmli UK (2006) Nup-PI: the nucleopore-promoter interaction of genes in yeast. Mol Cell **21:** 379-391

Schmidt-Eisenlohr H, Domke N, Angerer C, Wanner G, Zambryski PC, Baron C (1999) Vir proteins stabilize VirB5 and mediate its association with the T pilus of *Agrobacterium tumefaciens*. Journal of bacteriology **181:** 7485-7492

Schneeberger RG, Zhang K, Tatarinova T, Troukhan M, Kwok SF, Drais J, Klinger K, Orejudos F, Macy K, Bhakta A, Burns J, Subramanian G, Donson J, Flavell R, Feldmann KA (2005) *Agrobacterium* T-DNA integration in *Arabidopsis* is correlated with DNA sequence compositions that occur frequently in gene promoter regions. Functional & integrative genomics **5:** 240-253

Schroder G, Lanka E (2005) The mating pair formation system of conjugative plasmids-A versatile secretion machinery for transfer of proteins and DNA. Plasmid **54:** 1-25

Schubert D, Lechtenberg B, Forsbach A, Gils M, Bahadur S, Schmidt R (2004) Silencing in *Arabidopsis* T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. The Plant cell **16:** 2561-2572

Sessions A, Burke E, Presting G, Aux G, McElver J, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C, Clarke JD, Cotton D, Bullis D, Snell J, Miguel T, Hutchison D, Kimmerly B, Mitzel T, Katagiri F, Glazebrook J, Law M, Goff SA (2002) A high-throughput *Arabidopsis* reverse genetics system. The Plant cell **14:** 2985-2994

Sha Y, Li S, Pei Z, Luo L, Tian Y, He C (2004) Generation and flanking sequence analysis of a rice T-DNA tagged population. Theor Appl Genet **108:** 306-314

Shaw CH (1991) Swimming against the tide: chemotaxis in *Agrobacterium*. Bioessays **13:** 25-29

Shen WH, Escudero J, Schlappi M, Ramos C, Hohn B, Koukolikova-Nicola Z (1993) T-DNA transfer to maize cells: histochemical investigation of beta-glucuronidase activity in maize tissues. Proceedings of the National Academy of Sciences of the United States of America **90:** 1488-1492

Sheng J, Citovsky V (1996) *Agrobacterium*-plant cell DNA transport: have virulence proteins, will travel. The Plant cell **8:** 1699-1710

Shigekawa K, Dower WJ (1988) Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. BioTechniques **6:** 742-751

Shimoda N, Toyoda-Yamamoto A, Aoki S, Machida Y (1993) Genetic evidence for an interaction between the VirA sensor protein and the ChvE sugar-binding protein of *Agrobacterium*. The Journal of biological chemistry **268:** 26552-26558

Shindo Y, Kuribara H, Matsuoka T, Futo S, Sawada C, Shono J, Akiyama H, Goda Y, Toyoda M, Hino A (2002) Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. J AOAC Int **85:** 1119-1126

Siqueira HA, Moellenbeck D, Spencer T, Siegfried BD (2004) Cross-resistance of Cry1Ab-selected

Ostrinia nubilalis (Lepidoptera: Crambidae) to Bacillus thuringiensis delta-endotoxins. J Econ.Entomol. **97:** 1049-1057

Skerman VBD, McGowan V, Sneath PHA (1980) Approved lists of Bacterial names. Int J Syst Bacteriol **30:** 225-420

Smith EF, Townsend CO (1907) A Plant-Tumor of Bacterial Origin. Science (New York, N.Y **25:** 671-673

Sonti RV, Chiurazzi M, Wong D, Davies CS, Harlow GR, Mount DW, Signer ER (1995) *Arabidopsis* mutants deficient in T-DNA integration. Proceedings of the National Academy of Sciences of the United States of America **92:** 11786-11790

Spertini D, Beliveau C, Bellemare G (1999) Screening of transgenic plants by amplification of unknown genomic DNA flanking T-DNA. Biotechniques **27:** 308-314

Springer PS (2000) Gene traps: tools for plant development and genomics. The Plant cell **12:** 1007-1020

Stachel SE, Zambryski PC (1986) virA and virG control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46:** 325-333

Starr MP, Weiss JE (1943) Growth of phytopathogenic bacteria in a synthetic asparagine medium. Phytopathology **33:** 314-318

Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. Nature biotechnology **18:** 333-338

Stomp AM, Loopstra C, Chilton WS, Sederoff RR, Moore LW (1990) Extended Host Range of *Agrobacterium tumefaciens* in the Genus Pinus. Plant Physiol **92:** 1226-1232

Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA (2001) Plant-based vaccines: unique advantages. Vaccine **19:** 2742-2748

Swart S, Logman TJ, Smit G, Lugtenberg BJ, Kijne JW (1994) Purification and partial characterization of a glycoprotein from pea (Pisum sativum) with receptor activity for rhicadhesin, an attachment protein of *Rhizobiaceae*. Plant molecular biology **24:** 171-183

Szabados L, Kovacs I, Oberschall A, Abraham E, Kerekes I, Zsigmond L, Nagy R, Alvarado M, Krasovskaja I, Gal M, Berente A, Redei GP, Haim AB, Koncz C (2002) Distribution of 1000 sequenced T-DNA tags in the *Arabidopsis* genome. The Plant journal **32:** 233-242

Szegedi E, Czako M, Otten L, Koncz C (1988) Opines in crown gall tumors induced by biotype 3 isolates of *Agrobacterium tumefaciens.* Physiol Mol Plant Pathol **32:** 237-247

Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ (2000) Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. J Infect Dis **182:** 302-305

Taddei A, Van Houwe G, Hediger F, Kalck V, Cubizolles F, Schober H, Gasser SM (2006) Nuclear pore association confers optimal expression levels for an inducible yeast gene. Nature **441:** 774-778

Tato I, Zunzunegui S, de la Cruz F, Cabezon E (2005) TrwB, the coupling protein involved in DNA transport during bacterial conjugation, is a DNA-dependent ATPase. Proceedings of the National Academy of Sciences of the United States of America **102:** 8156-8161

Taverniers I (2005) Development and implementation of strategies for GMO quantification in an evolving European context. Ghent University, Ghent

Taverniers I, Van Bockstaele E, De Loose M (2001) Use of cloned DNA fragments as reference materials for event specific quantification of genetically modified organisms (GMOs). Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet **66:** 469-472

Taverniers I, Van Bockstaele E, De Loose M (2004) Cloned plasmid DNA fragments as calibrators for controlling GMOs: different real-time duplex quantitative PCR methods. Anal Bioanal Chem **378:** 1198-1207

Tax FE, Vernon DM (2001) T-DNA-associated duplication/translocations in *Arabidopsis*. Implications for mutant analysis and functional genomics. Plant Physiol **126:** 1527-1538

Tenllado F, Barajas D, Vargas M, Atencio FA, Gonzalez-Jara P, Diaz-Ruiz JR (2003) Transient expression of homologous hairpin RNA causes interference with plant virus infection and is overcome by a virus encoded suppressor of gene silencing. Mol. Plant Microbe Interact. **16:** 149–158

Terry CF, Harris N (2001) Event-specific detection of Roudup Ready soya using two different real time PCR detection chemistries. Eur Food Res Technol **213:** 425-431

Theuns I, Windels P, De Buck S, Depicker A, Van Bockstaele E, De Loose M (2002) Identification and characterization of T-DNA inserts via T-DNA fingerprinting. Euphytica **123:** 75-84
Thomashow MF, Panagopoulos CG, Gordon MP, Nester EW (1980) Host range of *Agrobacterium tumefaciens* is determined by the Ti plasmid. Nature **283:** 794-796
Tichopad A, Dilger M, Schwarz G, Pfaffl MW (2003) Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Res **31:** e122
Tinland B, Hohn B, Puchta H (1994) *Agrobacterium tumefaciens* transfers single-stranded transferred DNA (T-DNA) into the plant cell nucleus. Proceedings of the National Academy of Sciences of the United States of America **91:** 8000-8004
Tinland B, Koukolikova-Nicola Z, Hall MN, Hohn B (1992) The T-DNA-linked VirD2 protein contains two distinct functional nuclear localization signals. Proceedings of the National Academy of Sciences of the United States of America **89:** 7442-7446
Tinland B, Schoumacher F, Gloeckler V, Bravo-Angel AM, Hohn B (1995) The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. The EMBO journal **14:** 3585-3595
Torisky RS, Kovacs L, Avdiushko S, Newman JD, Hunt AG, Collins GB (1997) Development of a binary vector system for plant transformation based on the supervirulent *Agrobacterium tumefaciens* strain Chry5. Plant Cell Rep. **17:**102-108
Toro N, Datta A, Carmi OA, Young C, Prusti RK, Nester EW (1989) The *Agrobacterium tumefaciens* virC1 gene product binds to overdrive, a T-DNA transfer enhancer. Journal of bacteriology **171:** 6845-6849
Trick HN, Finer JJ (1997) SAAT: sonication-assisted *Agrobacterium* mediated transformation. Transgenic Research, **6:** 329-336.
Tzfira T (2006) On tracks and locomotives: the long route of DNA to the nucleus. Trends in microbiology **14:** 61-63
Tzfira T, Citovsky V (2000) From host recognition to T-DNA integration: the function of bacterial and plant genes in the *agrobacterium*-plant cell interaction. Mol. Plant Pathol **1:** 201-212
Tzfira T, Citovsky V (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol **12:** 121-129
Tzfira T, Citovsky V (2003) The *Agrobacterium*-plant cell interaction. Taking biology lessons from a bug. Plant physiology **133:** 943-947
Tzfira T, Frankman LR, Vaidya M, Citovsky V (2003) Site-specific integration of *Agrobacterium tumefaciens* T-DNA via double-stranded intermediates. Plant physiology **133:** 1011-1023
Tzfira T, Li J, Lacroix B, Citovsky V (2004b) *Agrobacterium* T-DNA integration: molecules and models. Trends Genet **20:** 375-383
Tzfira T, Rhee Y, Chen MH, Kunik T, Citovsky V (2000) Nucleic acid transport in plant-microbe interactions: the molecules that walk through the walls. Annu Rev Microbiol **54:** 187-219
Tzfira T, Vaidya M, Citovsky V (2001) VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. The EMBO journal **20:** 3596-3607
Tzfira T, Vaidya M, Citovsky V (2002) Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. Proceedings of the National Academy of Sciences of the United States of America **99:** 10435-10440
Tzfira T, Vaidya M, Citovsky V (2004a) Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature **431:** 87-92
Unger L, Ziegler SF, Huffman GA, Knauf VC, Peet R, Moore LW, Gordon MP, Nester EW (1985) New class of limited-host-range *Agrobacterium* mega-tumor-inducing plasmids lacking homology to the transferred DNA of a wide-host-range, tumor-inducing plasmid. Journal of bacteriology **164:** 723-730
Urushibara S, Tozawa Y, Kawagishi-Kobayashi M, Wakasa K (2001) Efficient transformation of suspension-cultured rice cells mediated by *Agrobacterium tumefaciens*. Breed. Sci. **5:** 33-38
Uttaro AD, Cangelosi GA, Geremia RA, Nester EW, Ugalde RA (1990) Biochemical characterization of avirulent exoC mutants of *Agrobacterium tumefaciens*. Journal of bacteriology **172:** 1640-1646
Vain P, Harvey A, Worland B, Ross S, Snape JW, Lonsdale D (2004) The effect of additional virulence genes on transformation efficiency, transgene integration and expression in rice plants using the pGreen/pSoup dual binary vector system. Transgenic research **13:** 593-603
Valasek MA, Repa JJ (2005) The power of real-time PCR. Adv Physiol Educ **29:** 151-159

Valvekens D, Montagu MV, Lijsebettens MV (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci **85:** 5536-5540

van Attikum H, Bundock P, Hooykaas PJJ (2001) Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration. EMBO J **20:** 6550- 6558

van den Eede G, Aarts H, Buhk HJ, Corthier G, Flint HJ, Hammes W, Jacobsen B, Midtvedt T, van der Vossen J, von Wright A, Wackernagel W, Wilcks A (2004) The relevance of gene transfer to the safety of food and feed derived from genetically modified (GM) plants. Food and chemical toxicology **42:** 1127-1156

van der Graaff E, den Dulk-Ras A, Hooykaas PJ (1996) Deviating T-DNA transfer from *Agrobacterium tumefaciens* to plants. Plant molecular biology **31:** 677-681

Van Lijsebettens M, Inze D, Schell J, Van Montagu M (1986) Transformed cell clones as a tool to study T-DNA integration mediated by *Agrobacterium tumefaciens*. *J. Mol. Biol.* **188:** 129−145

van Wordragen MF, Dons HJM (1992) *Agrobacterium tumefaciens* mediated transformation of recalcitrant crops. Plant Mol. Biol. Rep. **10:** 12-36

Varzakas TH, Arvanitoyannis IS, Baltas H (2007) The politics and science behind GMO acceptance. Crit Rev Food Sci Nutr **47:** 335-361

Veena, Jiang H, Doerge RW, Gelvin SB (2003) Transfer of T-DNA and Vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. The Plant journal **35:** 219-236

Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CMT, Regensburg-Tuink TJG, Hooykaas PJJ (2000) VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. Science **290:** 979–982

Vergunst AC, van Lier MC, den Dulk-Ras A, Stuve TA, Ouwehand A, Hooykaas PJ (2005) Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. Proceedings of the National Academy of Sciences of the United States of America **102:** 832-837

Vogel J, Hill T (2008) High-efficiency *Agrobacterium*-mediated transformation of Brachypodium distachyon inbred line Bd21-3. Plant cell reports **27:** 471-478

Voinnet O, Baulcombe DC (1997) Systemic signalling in gene silencing. Nature **389:** 553

Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. **33:** 949–956

Wagner VT, Matthysse AG (1992) Involvement of a vitronectin-like protein in attachment of *Agrobacterium tumefaciens* to carrot suspension culture cells. Journal of bacteriology **174:** 5999-6003

Wallroth M, Gerats AGM, Rogers SG, R.T. F, Horsch RB (1986) Chromosomal localization of foreign genes in *Petunia Hybrida*. Mol Gen Genet **202:** 6-15

Wang ZY, Ge Y (2005) *Agrobacterium*-mediated high efficiency transformation of tall fescue (Festuca arundinacea). J Plant Physiol **162:** 103-113

Ward DV, Draper O, Zupan JR, Zambryski PC (2002) Peptide linkage mapping of the *Agrobacterium tumefaciens* vir-encoded type IV secretion system reveals protein subassemblies. Proceedings of the National Academy of Sciences of the United States of America **99:** 11493-11500

Ward DV, Zambryski PC (2001) The six functions of *Agrobacterium* VirE2. Proceedings of the National Academy of Sciences of the United States of America **98:** 385-386

Ward DV, Zupan JR, Zambryski PC (2002) *Agrobacterium* VirE2 gets the VIP1 treatment in plant nuclear import. Trends Plant Sci **7:** 1-3

Weber G, Monajembashi S, Greulich KO, Wolfrum J (1988) Genetic manipulation of plant cells and organelles with a laser microbeam. Plant Cell, Tissue and Organ Culture **12:** 219-222

Weighardt F, Barbati C, Paoletti C, Querci M, Kay S, De Beuckeleer M, Van den Eede G (2004) Real-time polymerase chain reaction-based approach for quantification of the pat gene in the T25 Zea mays event. J AOAC Int **87:** 1342-1355

Wenck A, Czako M, Kanevski I, Marton L (1997) Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. Plant molecular biology **34:** 913-922

Wenck AR, Quinn M, Whetten RW, Pullman G, Sederoff R (1999) High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (Picea abies) and loblolly pine (Pinus taeda). Plant molecular biology **39:** 407-416

White FF, Ghidossi G, Gordon MP, Nester EW (1982) Tumor induction by *Agrobacterium rhizogenes* involves the transfer of plasmid DNA to the plant genome. Proc Natl Acad Sci USA **79:** 3193-3197

Wilhelm J, Pingoud A (2003) Real-time polymerase chain reaction. Chembiochem **4:** 1120-1128

Willekens H, Van Camp W, Van Montagu M, Inze D, Langebartels C, Sandermann H J (1994) Ozone, Sulfur Dioxide, and Ultraviolet B Have Similar Effects on mRNA Accumulation of Antioxidant Genes in Nicotiana plumbaginifolia L. Plant Physiol **106:** 1007-1014

Winans SC (1990) Transcriptional induction of an *Agrobacterium* regulatory gene at tandem promoters by plant-released phenolic compounds, phosphate starvation, and acidic growth media. Journal of bacteriology **172:** 2433-2438

Windels P (2004) Integration of exogenous DNA into plant chromosomes: Applicability of and theoretical considerations on plant DNA/T-DNA junctions. Ghent University, Ghent

Windels P, Bertrand S, Moens W, Depicker A, Van Bockstaele E, De Loose M (2003b) Qualitative and event-specific PCR real-time detection methods for StarLink maize. Eur Food Res Technol **216:** 259-263

Windels P, De Buck S, Depicker A (2008) *Agrobacterium tumefaciens*-mediated transformation: patterns of T-DNA integration into the host genome. *In* T Tzfira, V Citovsky, eds, Agrobacterium: From Biology to Biotechnology. Springer, New York, pp 441-481

Windels P, De Buck S, Van Bockstaele E, De Loose M, Depicker A (2003a) T-DNA integration in Arabidopsis chromosomes. Presence and origin of filler DNA sequences. Plant Physiol **133:** 2061-2068

Wiseman G (2002) State of the art and limitations of quantitative polymerase chain reaction. J AOAC Int **85:** 792-796

Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques **22:** 130-131, 134-138

Wood DW, Setubal JC, Kaul R, Monks DE, Kitajima JP, Okura VK, Zhou Y, Chen L, Wood GE, Almeida NF, Jr., Woo L, Chen Y, Paulsen IT, Eisen JA, Karp PD, Bovee D, Sr., Chapman P, Clendenning J, Deatherage G, Gillet W, Grant C, Kutyavin T, Levy R, Li MJ, McClelland E, Palmieri A, Raymond C, Rouse G, Saenphimmachak C, Wu Z, Romero P, Gordon D, Zhang S, Yoo H, Tao Y, Biddle P, Jung M, Krespan W, Perry M, Gordon-Kamm B, Liao L, Kim S, Hendrick C, Zhao ZY, Dolan M, Chumley F, Tingey SV, Tomb JF, Gordon MP, Olson MV, Nester EW (2001) The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science (New York, N.Y **294:** 2317-2323

Wurz A, Bluth A, Zeltz P, Pfeifer C, Willmund R (1999) Quantitative analysis of genetically modified organisms (GMO) in processed food by PCR-based methods. Food Control **10:** 385-389

Wurz A, Willmund R (1997) Identification of transgenic glyphosate-resistant soybeans. *In* GA Schreiber, KW Bögl, eds, Foods produced by means of genetic engineering. 2nd status report. Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany pp 115-117

Xu D, Duan X, Wang B, Hong B, Ho T, Wu R (1996) Expression of a Late Embryogenesis Abundant Protein Gene, HVA1, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice. Plant Physiol **110:** 249-257

Xu J, Zhu S, Miao H, Huang W, Qiu M, Huang Y, Fu X, Li Y (2007) Event-specific detection of seven genetically modified soybean and maizes using multiplex-PCR coupled with oligonucleotide microarray. J Agric Food Chem **55:** 5575-5579

Xu XQ, Pan SQ (2000) An *Agrobacterium* catalase is a virulence factor involved in tumorigenesis. Molecular microbiology **35:** 407-414

Yadav NS, Vanderleyden J, Bennett DR, Barnes WM, Chilton MD (1982) Short direct repeats flank the T-DNA on a nopaline Ti plasmid. Proceedings of the National Academy of Sciences of the United States of America **79:** 6322-6326

Yamaguchi A, Shimizu K, Mishima T, Aoki N, Hattori H, Sato H, Ueda N, Watanabe T, Hino A, Akiyama H, Maitani T (2006) Detection method for genetically modified papaya using duplex PCR. Shokuhin Eiseigaku Zasshi **47:** 146-150

Yang AF, Su Q, An LJ (2009) Generation of vector backbone-free and selectable marker-free transgenic maize (Zea mays L.) via ovary-drip method. Yi Chuan **31:** 95-100

Yang L, Guo J, Zhang H, Liu J, Zhang D (2008) Qualitative and quantitative event-specific PCR detection methods for oxy-235 canola based on the 3' integration flanking sequence. J Agric Food Chem **56:** 1804-1809

Yanofsky MF, Nester EW (1986) Molecular characterization of a host-range-determining locus from *Agrobacterium tumefaciens*. Journal of bacteriology **168:** 244-250

Yanofsky MF, Porter SG, Young C, Albright LM, Gordon MP, Nester EW (1986) The virD operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. Cell **47:** 471-477

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. The Plant journal **19:** 249-257

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science **287:** 303-305

Yi H, Mysore KS, Gelvin SB (2002) Expression of the *Arabidopsis* histone H2A-1 gene correlates with susceptibility to *Agrobacterium* transformation. The Plant journal **32:** 285-298

Yi H, Sardesai N, Fujinuma T, Chan CW, Veena, Gelvin SB (2006) Constitutive expression exposes functional redundancy between the *Arabidopsis* histone H2A gene HTA1 and other H2A gene family members. The Plant cell **18:** 1575-1589

Yibrah HS, Gronroos R, Lindroth A, Franzen H, Clapham D, von Arnold S (1996) *Agrobacterium rhizogenes*-mediated induction of adventitious rooting from *Pinus contorta* hypocotyls and the effect of 5-azacytidine on transgene activity. Transgenic research **5:** 75-85

Young JM (2008) *Agrobacterium*-Taxonomy of plant-pathogenic *Rhizobium* species. *In* T Tzfira, V Citovsky, eds, Agrobacterium: From Biology to Biotechnology. Springer, New York, pp 183-220

Young JM, Kuykendall LD, Martinez-Romero E, Kerr A, Sawada H (2001) A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium* undicola de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. Int J Syst Evol Microbiol **51:** 89-103

Young JM, Pennycook SR, Watson DR (2006) Proposal that *Agrobacterium radiobacter* has priority over *Agrobacterium tumefaciens*. Request for an opinion. Int J Syst Evol Microbiol **56:** 491-493

Zaenen I, Van Larebeke N, Van Montagu M, Schell J (1974) Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. Journal of molecular biology **86:** 109-127

Zale JM, Agarwal S, Loar S, Steber CM (2009) Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens*. Plant cell reports

Zambre M, Terryn N, De Clercq J, De Buck S, Dillen W, Van Montagu M, Van Der Straeten D, Angenon G (2003) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta **216:** 580-586

Zambryski P, Depicker A, Kruger K, Goodman HM (1982) Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. J Mol Appl Genet **1:** 361-370

Zambryski P, Joos H, Genetello C, Leemans J, van Montagu M, Schell J (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J. **4:**3069-3076

Zhang J, Guo D, Chang Y, You C, Li X, Dai X, Weng Q, Chen G, Liu H, Han B, Zhang Q, Wu C (2007) Non-random distribution of T-DNA insertions at various levels of the genome hierarchy as revealed by analyzing 13 804 T-DNA flanking sequences from an enhancer-trap mutant library. The Plant journal **49:** 947-959

Zhu Y, Nam J, Carpita NC, Matthysse AG, Gelvin SB (2003b) *Agrobacterium*-mediated root transformation is inhibited by mutation of an *Arabidopsis* cellulose synthase-like gene. Plant Physiol **133:** 1000-1010

Zhu Y, Nam J, Humara JM, Mysore KS, Lee LY, Cao H, Valentine L, Li J, Kaiser AD, Kopecky AL, Hwang HH, Bhattacharjee S, Rao PK, Tzfira T, Rajagopal J, Yi H, Veena, Yadav BS, Crane YM, Lin K, Larcher Y, Gelvin MJ, Knue M, Ramos C, Zhao X, Davis SJ, Kim SI, Ranjith-Kumar CT, Choi YJ, Hallan VK, Chattopadhyay S, Sui X, Ziemienowicz A, Matthysse AG, Citovsky V, Hohn B, Gelvin SB (2003a) Identification of *Arabidopsis* rat mutants. Plant Physiol **132:** 494-505

Zhu J, Oger P, Schrammeijer B, Hooyakaas PJJ, Farrand SK, Winans SC (2000) The bases of crown gall tumorigenesis. J. Bacteriol. **182:** 3885-3895

Ziemienowicz A, Merkle T, Schoumacher F, Hohn B, Rossi L (2001) Import of *Agrobacterium* T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins. The Plant cell **13:** 369-383

Ziemienowicz A, Tinland B, Bryant J, Gloeckler V, Hohn B (2000) Plant enzymes but not *Agrobacterium* VirD2 mediate T-DNA ligation in vitro. Mol Cell Biol **20:** 6317-6322

Zimmermann A, Lüthy J, Pauli U (1998) Quantitative and qualitative evaluation of nine different extraction methods for nucleic acids on soya bean food samples Z Lebensm Unters Forsch A **207:** 81-90

Zimmermann A, Lüthy J, Pauli U (2000) Event specific transgene detection in Bt11 corn by quantitative PCR at the integration site. Lebensm Wiss u Technol **33:** 210-216

Zimmermann K, Mannhalter JW (1996) Technical aspects of quantitative competitive PCR. Biotechniques **21:** 268-272, 274-269

Zupan J, Hackworth CA, Aguilar J, Ward D, Zambryski P (2007) VirB1* promotes T-pilus formation in the vir-Type IV secretion system of *Agrobacterium tumefaciens*. Journal of bacteriology **189:** 6551-6563

Zupan J, Muth TR, Draper O, Zambryski P (2000) The transfer of DNA from *agrobacterium tumefaciens* into plants: a feast of fundamental insights. The Plant journal **23:** 11-28

Chapter 2: Objectives

Agrobacterium-mediated transformation is a widely used method to introduce foreign genes into the plant genome. For the model plant *Arabidopsis thaliana*, two *Agrobacterium* mediated transformation methods can be distinguished: regeneration-dependent (*in vitro*) methods such as root and leaf disc tissue transformation (Horsch et al., 1985; Valvekens et al., 1988) or regeneration-independent methods (*in planta*) transformation methods such as vacuum infiltration and floral dip (Bechtold et al., 1993; Clough and Bent, 1998). Although *Agrobacterium* has been used to produce transgenic plants since the beginning of the eighties, several aspects of the transformation process are still poorly understood and the transformation procedure can still be improved.

The objectives of this thesis were plural. In order to improve *Agrobacterium* transformation frequencies and to obtain more insights on the molecular process underlying this transformation process, the model plant *Arabidopsis thaliana* was used. We first evaluated the influence of the *Agrobacterium* chromosomal background on the floral dip transformation frequency. 12 different bacterial strains were tested including the commonly used strains C58C1 Rif^R (pMP90) and LBA4404. Transformation efficiencies, obtained after floral dipping the model plant *Arabidopsis thaliana* Col0 and C24 ecotypes with different bacterial strains, were compared . The results are summarized in chapter 3. Second, we wanted to assess the influence of two different light regimes on T-DNA stable integration after *Arabidopsis in vitro* root explants transformation, and to investigate the extent to which T-DNA transfer and/or T-DNA integration into the host genome is limiting the transformation during *Arabidopsis thaliana* floral dip. As this method is frequently used, more insights into the molecular mechanisms underlying the transformation process could help to improve its transformation efficiencies and/or to apply this method to other crops. The results are presented in chapter 4.

Since the development of the first transformed plant cells in 1983, a continual growth in the global area of genetically engineered crops was registered. In 2008, the global cultivation area of biotech crops continued to grow reaching 125 million

hectares denoting a 74 fold hectare increase between 1996, the first year of commercialization, and 2008 (James, 2008). Within the European Union, specific legislations have been developed to guide the introduction of commercialized transgenic crops on the European market. These legislations involve traceability of transgenic crops, risk evaluation studies prior to environmental release and labeling.

The integration locus of each transgenic event is unique and the junctions between the plant-DNA and the T-DNA are used for the unequivocal identification of the transgenic event and the quantification of genetically modified content. Therefore, we wanted to evaluate the stability of the transgene flanking sequences and the integration locus under cultivation practices and high light as an abiotic stress condition (chapter 5). Further, we evaluated the reliability of the quantitative real-time PCR (QRT-PCR) which is the method used for genetically modified organisms (GMOs) quantification. As single point mutations (SNP) can be present in the genome of plants (Koorneef et al., 2004), we assessed the impact of a primer/template mismatch which can be created by the presence of a genomic SNP in the primer attachment site, on the real-time PCR quantification (chapter 6).

In summary, the goal of the work in this thesis was to contribute to the knowledge on plant transformation and on the methods used to trace and quantify transgenic events.

References

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Paris, Life Sciences **316:** 1194-1199

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant journal **16:** 735-743

Horsch RB, Fry GE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A Simple and General Method for Transferring Genes into Plants. Science (New York, N.Y **227:** 1229-1231

James C (2008) Global Status of Commercialized Biotech/GM Crops: 2008. ISAAA Briefs **No. 39**

Koorneef M, Alonso-Blanco C, Vreugdenhil D (2004) Naturally Occurring Genetic Variation in *Arabidopsis thaliana*. Annu Rev Plant Biol **55**

Valvekens D, Montagu MV, Lijsebettens MV (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci **85:** 5536-5540

Chapter 3

Comparative study of *Arabidopsis thaliana* floral dip transformation mediated by 12 different *Agrobacterium* strains

Rim Ghedira, Sylvie De Buck, Anni Jacobs and Ann Depicker

Contribution to this work: Rim Ghedira performed all practical work described in this chapter except for the introduction of *vir* plasmids and binary vector into the different *Agrobacterium* strains which was performed by Anni Jacobs. The body of this chapter was entirely written by Rim Ghedira

Abstract

Agrobacterium is widely employed for plant transformation using tissue culture dependent (*in vitro*) or tissue culture independent (*in planta*) methods. In order to evaluate the chromosomal background influence of different *Agrobacterium* strains on the floral dip transformation frequency, 12 *Agrobacterium* strains, harboring a T-DNA with the glucuronidase reporter gene, were tested in the *Arabidopsis thaliana* Columbia (Col0) and C24 ecotypes. *Arabidopsis* Col0 floral dip transformation showed high variability within and across the performed biological repeats while the *Arabidopsis* C24 ecotype showed over all very low floral dip transformation frequencies. After *Arabidopsis* Col0 floral dip, the widely used C58C1 Rif^R chromosomal background in combination with the pMP90 virulence plasmid showed high transformation frequencies while the LBA4044 reference strain resorted in the group with low floral dip transformation frequencies. Floral dip transformation frequencies obtained with the chromosomal background of LMG201, which is genetically close to C58, were higher than those obtained with C58 derived strains. All the other tested *Agrobacterium* chromosomal backgrounds had transformation frequencies intermediate to the LBA4404 and C58C1 Rif^R (pMP90) reference strains. Our results indicate that especially the plant characteristics but also the *Agrobacterium* strain influence *Arabidopsis thaliana* Col0 floral dip transformation frequencies.

1. Introduction

Agrobacterium is a genus of gram-negative, aerobic, rod-shaped bacteria found in soil. Most members of this genus can invade the roots and stems of plants, via wounds, causing the transformation of the plant cells into proliferating tumor cells (plant tumors called crown gall) (Tzfira and Citovsky, 2000; Tzfira et al., 2004; Yelin et al., 2008). On the molecular level, *Agrobacterium* plant infection results from the transfer and integration of a bacterial DNA fragment, the so called T-DNA (transferred DNA), into the plant cell genome. The T-DNA is transferred to the host cell nucleus, integrated into plant chromosomal DNA and expressed. The multistep transformation process was described in detail in many review articles (Gelvin, 2000; Tzfira and Citovsky, 2000, 2002; Gelvin, 2003; McCullen and Binns, 2006; Citovsky et al., 2007; Yelin et al., 2008) and starts with the chemical recognition of the host and activation of virulence gene expression. Afterwards, the physical recognition and interaction between the bacterium and the host takes place.

Three genetic elements are necessary for the transfer and the integration of the T-DNA in the plant genome: the border repeats that delimit the T-DNA, the *vir* genes that code for the *in trans*-acting type IV secretion system, and various bacterial chromosomal genes that are necessary for attachment and the early stages of transformation (Lee and Gelvin, 2008). Genes involved in attachment are located on the *Agrobacterium* chromosome and mutants in these loci (*chvA, chvB* and *pcsA or exoC)* are avirulent on many plant species (Thomashow et al., 1987; Zorreguieta et al., 1988; Leigh and Coplin, 1992). The *chvA, chvB* proteins are involved in β-1,2 glucan synthesis and mutants in the encoding genes are deficient in their ability to attach to and infect plants (Douglas et al., 1985; Cangelosi et al., 1987; Zorreguieta et al., 1988). The *pcsA or exoC* gene is required for the synthesis of exopolysaccharides (Cangelosi et al., 1987; Thomashow et al., 1987; Uttaro et al., 1990). Mutations in other *Agrobacterium* chromosomal genes resulted in strains deficient in attachment to the host cell and were called *att* genes (Matthysse, 1987; Matthysse et al., 2000). From these genes, a*ttR* was reported to be involved in the synthesis of surface molecules (Reuhs et al., 1997; Matthysse and McMahan, 1998; Matthysse et al., 2000). The published genome sequence of *Agrobacterium*

tumefaciens C58 revealed that the *att* genes are located on the pAtC58 plasmid of the bacteria (Goodner et al., 2001). However, the study reported by Nair et al., (2003) showed that pAtC58 is not essential for virulence if the strains contain the pTiC58 plasmid but pAtC58 is required along with pTiC58 for maximal virulence. Furthermore, a disruption in *attR* does not affect the capacity of pAtC58 to influence tumorigenesis. The genome of the frequently used *A. tumefaciens* strain C58 has four distinct replicons: a circular chromosome, a linear chromosome, and the plasmids pAtC58 and pTiC58 (Goodner et al., 2001; Wood et al., 2001). The chromosomal background of the other commonly used *Agrobacterium* strain LBA4404 is that of Ach5 (Hoekema et al., 1983).

Using *Agrobacterium*, two main methods can be employed for plant transformation: *in planta* methods such as vacuum infiltration and floral dip transformation (Bechtold et al., 1993; Clough and Bent, 1998) and tissue culture dependent methods such as root and leaf explants transformation (Horsch et al., 1985; Valvekens et al., 1988). *In Arabidopsis,* both transformation methods are possible but currently the floral dip transformation method is most often used because it is an easy, cheap and quick protocol.

The *Agrobacterium*-mediated plant transformation frequency is influenced by several plant, bacterial and environmental factors (Gelvin, 2000; Tzfira and Citovsky, 2000; Tzfira et al., 2002). On the plant level, it has been reported that *Arabidopsis* ecotypes C24 and Wassilewskija (*Ws*) are more efficiently transformed than Landsberg *erecta* (*Ler*) and Col0 with root transformation (Valvekens et al., 1988). On the bacterial side, the density of the bacterial culture (Cheng et al., 2004; Opabode, 2006) and the strain ability to attach and transfer its T-DNA to the host cells (Shen et al., 1993; Nam et al., 1997; Cheng et al., 2004) were described to influence the transformation frequency. Different *Agrobacterium* strains were also reported to influence the transformation frequency depending on the transformed plant or crop. For the *Arabidopsis* Ws ecotype, shoot regeneration was most efficient when using the EHA101 compared to a C58C1 rifampicin resistant (Rif^R) (pTiR225) and the LBA4404 strains (Akama et al., 1992). Leaf disc tissue transformation frequency of apple with *Agrobacterium* EHA101 (pIG121Hm) was also higher in comparison with C58C1 Rif^R (pGV2260) (De Bondt et al., 1994). However, *in vitro*

transformation of *Arabidopsis Ler* ecotype was more effective with the C58C1 Rif^R (pTiR225) strain than with the EHA101 strain (Akama et al., 1992). Tumor induction experiments revealed that strains with nopaline Ti plasmids (such as pTiC58) were weakly virulent on *Nicotiana glauca* in contrast to strains with wild type octopine or leucinopine Ti plasmids (Melchers et al., 1990). Dillen et al., (1997) reported that *Agrobacterium* C58C1 Rif^R harboring either the nopaline pMP90 or the octopine pGV2260 *vir* plasmid was more efficient than *Agrobacterium* C58C1 Rif^R harboring the agropine/succinamopine pEHA101 *vir* plasmid for the transformation of *Nicotiana tabacum* and *Phaseolus acutifolius* via tissue culture dependent methods. Also, one transformation method can be more efficient than another. This is illustrated by the fact that *Arabidopsis Ler* ecotype could be transformed using the root explant regeneration method, while no transformants could be obtained after *in planta* transformation (Valvekens et al., 1988; Katavic et al., 1994). All these studies demonstrate that the combination of the bacterial strain, the plant to be transformed and the transformation method is important to achieve high transformation frequencies.

The goal of this study was to identify an *Agrobacterium* strain with higher transformation frequencies after *Arabidopsis thaliana* floral dip transformation than the currently used *Agrobacterium* strains. Therefore, 8 different wild type *Agrobacterium* strains were selected from 5 different genetic species out of the “Belgian Co-ordinated Collections of Micro-organisms” in order to screen for better performing chromosomal backgrounds. The wild type strains were first made rifampicin resistant and subsequently the virulence plasmid pMP90 (Koncz et al., 1986) and binary vector pTJK136 (Kapila et al., 1997) were introduced. Floral dip transformation of *Arabidopsis thaliana ecotype* Col0 and C24 was then performed using the 8 newly obtained bacterial strains. In parallel, the widely used strain LBA4404, with the chromosomal background of Ach5 and the pTiAch5 *vir* plasmid (Hoekema et al., 1983), and 3 variant strains based on the C58C1 chromosomal background (cured strain derived from C58; Van Larebeke et al., 1974) were used in the same transformation experiments. This allowed us to compare the floral dip transformation frequencies of the 8 newly constructed strains with 4 reference strains.

2. Results

2.1. *Agrobacterium* strains

The wild type LMG *Agrobacterium* strains used in this study were purchased from the Belgian Co-ordinated Collections of Micro-organisms (BCCM: http://bccm.belspo.be/index.php) (Table 1).

De Ley, (1972, 1974) grouped *Agrobacterium* strains on the basis of DNA-DNA renaturation kinetics in the following groups: LMG148 and LMG62 strains belong to the B6 group; LMG26 to the 0362 group; LBA4404, LMG232 and LMG142 to the TT111 group; C58 and LMG201 are derived from the TT9 group and LMG146 and LMG147 belong to M2/1 group. Later, Portier et al., (2006) classified different *Agrobacterium* strains into genomic species and provided their phylogenomic relatedness using the amplification fragment length polymorphism (AFLP) method. These authors reported that LMG201 and C58 strains belong to the same genomic species G8. LMG62 and LMG147 belong to the genomic species G4 and G2 respectively while LMG26 belongs to the genomic species G9 (Portier et al., 2006). Later, Costechareyre et al., (2009) reported that C58 and TT9 strains belong to genomic group species G8, whereas the 0362 strain belongs to group G9. The G4 and G2 groups contain B6 and M2/1 strains respectively and TT111 strains are part of G1 group species (Figure 1).

To be able to introduce a *vir* plasmid into these strains, rifampicin resistant (Rif^R) mutants were isolated by selection on YEB medium supplemented with rifampicin. Subsequently, the *vir* helper plasmid pMP90 was introduced into all these Rif resistant strains by bi-parental conjugation with the donor strain C58C1 Ecm^R (pMP90) strain as donor (material and methods).

Table 1: Classification of the wild type *Agrobacterium* species used in this study.

LMG number	Group according to De Ley, (1972, 1974)	Group according to Portier et al., (2006) and Costechareyre et al., (2009)	Name	Pathogenic
LMG26	362	G9	*Agrobacterium sp.*	+
LMG148	B6	G4	*A. radiobacter*	-
LMG62	B6	G4	*A. radiobacter*	-
LMG146	M2/1	G2	*A. radiobacter*	-
LMG147	M2/1	G2	*A. radiobacter*	-
LMG142	TT111	G1	*A. radiobacter*	-
LMG232	TT111	G1	*A. tumefaciens*	-
LMG201	TT9	G8	*A. tumefaciens*	+

+ indicates that the wild type strain is pathogenic; - indicates that the wild type strain is not pathogenic. LMG: Laboratorium voor Microbiologie Gent, A: *Agrobacterium. sp.*: species.

Figure 1: Neighbor-joining tree of *Agrobacterium* based on *ChvA* sequences (adapted from Costechareyre et al., 2009). Bootstrap percentage values (≥ 70%) are presented. Genomic species used in this study are underlined. According to De Ley (1972, 1974), C58 and LMG201 are TT9 strains, LMG26 is a 0362 strain; LMG148 and LMG62 are B6 strains; LMG146 and LMG147 are M2/1 strains; LBA4404, LMG232 and LMG142 are TTT111 strains.

The binary vector pTJK136, with a T-DNA carrying the intron containing β glucuronidase (*gus*) reporter gene under the control of the 35S promoter and the kanamycin resistance gene (Kapila et al., 1997), was introduced in all obtained bacterial strains via tri-parental conjugation (Lee, 2006). The 12 obtained *Agrobacterium* strains are listed in Table 2.

Floral dip transformation (Clough and Bent, 1998; material and methods) of the *Arabidopsis* Col0 ecotype was performed in three biological repeats using all bacterial strains except for C58C1 Rif^R (pMP90,pTJK136) and C58C1 Rif^R (pGV2260,pTJK136) where four and two biological repeats were performed, respectively. Floral dip transformation of C24 ecotype plants was carried out in two biological repeats (Table 2). In biological repeat c, the same bacterial cultures were used for floral dip of both the *Arabidopsis* Col0 and C24 ecotypes. In each biological repeat, 5 individual plants were dipped using the same *Agrobacterium* culture (material and methods). The optical density (OD) of the different bacterial cultures was measured at 600 nm and the *Agrobacterium* titer of each culture could be determined (material and methods).

To determine whether both the *vir* plasmid and the binary T-DNA vector were stably maintained in the different *Agrobacterium* strains, their presence was assayed in fully grown cultures without selection. As the pMP90 plasmid carries a gentamycin resistance gene (Koncz and Schell, 1986), we plated a 10^{-6} dilution of all overnight grown bacterial strains carrying the pMP90 helper plasmid on YEB medium for the number of bacteria and YEB supplemented with gentamycin (40 µg/ml) for the number of bacteria with the pMP90 plasmid. We obtained, for all strains, comparable number of colonies on the selective and non-selective medium except for LMG146 Rif^R (pMP90,pTJK136) (results not shown). Indeed, 100 µl of 10^{-6} dilution of LMG146 culture gave only one colony on gentamycin containing YEB medium while 297 colonies were obtained on non selective medium. This result showed that the pMP90 plasmid was stably maintained in all strains but was lost in LMG146 Rif^R (pMP90,pTJK136) cultures. This explains the very low transformation frequencies obtained using this strain (see further).

Table 2: Overview of the *Agrobacterium* strains used for floral dip transformation and the number of floral dip repeats performed for *Arabidopsis thaliana* Col0 and C24 ecotypes

Agrobacterium strains	Col0 biological repeats*	C24 biological repeats*
LMG26 Rif^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
LMG148 Rif^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
LMG62 Rif^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
LMG146 Rif^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
LMG147 Rif^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
LMG142 Rif^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
LMG232 Rif^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
LBA4404 Rif^R (pTJK136)	3 (a, b and c)	2 (c and d)
LMG201 Rif^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
C58C1 Ecm^R (pMP90,pTJK136)	3 (a, b and c)	2 (c and d)
C58C1 Rif^R (pMP90,pTJK136)	4 (a, b, c and d)	2 (c and d)
C58C1 Rif^R (pGV2260,pTJK136)	2 (c and d)	2 (c and d)

For biological repeats c and d, the same *Agrobacterium* suspension was used for the floral dip of both ecotypes. * Each repeat included 5 dipped plants, yielding 5 different T1 seedstocks per experiment and per *Agrobacterium* culture.

After dipping 5 plants per *Agrobacterium* culture, the plants were allowed to set seeds, and the seeds were collected per individual plant. Knowing that 25 mg seed is equal to approximately 1000 *Arabidopsis* seeds, 2000 seeds, harvested from each dipped plant, were sown on kanamycin selective medium. Three weeks later, the number of kanamycin resistant *Arabidopsis* plants was scored for all dipped plants.

2.2. *Arabidopsis thaliana* Col0 floral dip transformation frequencies are very variable

The number of transformants obtained after *Arabidopsis thaliana* Col0 ecotype floral dip within and across the different performed repeats is summarized in Table 3. It is clear from the numbers as well as from the boxplots (Figure 2) that there is a high variability in the number of transformants obtained using the different *Agrobacterium* strains. First of all, we observed a lot of variation in the number of

transformants in different T1 seedstocks obtained after dipping with the same bacterial strain. Nevertheless, the plants to be dipped were grown at the same time in the same room and although the dipping of 5 *Arabidopsis* plants was performed with the same *Agrobacterium* culture at the same time, the transformation frequencies were highly variable. This variability was observed for all used strains and within the different performed repeats. For instance, in floral dip c, using *Agrobacterium* LMG147 Rif^R (pMP90,pTJK136), 4 transformants were derived from 2000 seeds of plant 3 (P3) and 82/2000 seeds from plant 5 (P5). In floral dip c with *Agrobacterium* strain LMG62 Rif^R (pMP90,pTJK136), 20 transformants out of 2000 seeds were obtained from P1 while 101 transformants were obtained from 2000 P5 seeds. Also the LMG232 Rif^R (pMP90,pTJK136) strain gave 2 transformants per 2000 seeds from P2 and 32 transformants per 2000 seeds from P3 in repeat b. After floral dip a with C58C1 Ecm^R (pMP90,pTJK136), 36 kanamycin resistant plants were regenerated from 2000 seeds of P5 versus 2 regenerated from P3 (Table 3, figure 1). The high variability in the number of transformants obtained using the same bacterial culture indicates that plant characteristics are highly determining the transformation frequencies during floral dip transformation method.

Not surprisingly, there was also considerable variability between the different biological repeats of plants floral dipped with an independently grown culture of the analyzed strains. For instance, floral dip transformation using LMG62 Rif^R (pMP90,pTJK136) resulted in 2 to 10 transgenic plants per 2000 seeds in repeat a while the same strain gave 20 to 101 transformants per 2000 seeds in repeat c (Table 3; strain 3 in Figure 2). The transformation frequency (determined from the transformants average of the 5 dipped plants within each repeat) with strain LMG148 Rif^R (pMP90,pTJK136) (Table 3; strain 2 in Figure 2) varied between 0% and 1.06% for 5 different dipped plants. This variability was observed with almost all used strains. C58C1 Rif^R (pGV2260,pTJK136) transformation frequencies were comparable for both performed biological repeats: 0.68% for floral dip repeat d and 0.69% for floral dip repeat c (Table 3; strain 13 Figure 2). For the other strains, transformation frequencies showed a 2-fold ((LMG26 Rif^R (pMP90,pTJK136) repeat a and b)) to 15-fold ((LMG62 Rif^R (pMP90,pTJK136) repeat a and c)) difference across the performed repeats (Table 3). Floral dip repeats were carried out in the same conditions (temperature, surfactant concentration, inoculation time…). The only

differences were the *Agrobacterium* inoculums and the individual *Arabidopsis* Col0 plants that were dipped. The concentration of *Agrobacterium* in the different used cultures ranged from $5.2x10^7$ to $7.7x10^8$ (Table 3) and no correlation between the bacterial densities and the transformation frequencies could be found. For some strains, such as LMG62 Rif^R (pMP90,pTJK136) and LMG148 Rif^R (pMP90,pTJK136) in floral dip a and c respectively, the *Agrobacterium* inoculum concentrations were similar ($2.4x10^8$ and $2.1x10^8$ respectively). However, the transformation frequencies were 0.16% in floral dip repeat a using LMG62 Rif^R (pMP90,pTJK136) and 1.06% in floral dip repeat c using LMG148 Rif^R (pMP90,pTJK136) (Table 3). Hence, lower transformation frequencies cannot be solely attributed to lower *Agrobacterium* concentrations in the culture medium.

The data suggest that the *vir* helper plasmid influences Col0 floral dip transformation frequencies. The *Agrobacterium* strain C58C1 Rif^R harboring the pMP90 *vir* plasmid gave transformation frequencies ranging from 1.32% to 1.57% per plant and per repeat while when harboring the pGV2260 *vir* helper plasmid the transformation frequencies were as low as 0.68% and 0.69% (repeats c and d in Table 3; strain 12 and 13 Figure 2). Also the transformation frequencies obtained with the C58C1 Ecm^R mutant chromosomal background (0.1 to 0.52%) were consistently lower than those obtained with C58C1 Rif^R chromosomal background (0.76 to 1.57%) showing that mutations in the bacterial chromosome may also strongly influence the floral dip transformation frequencies (Table 3).

Table 3: Number of transformants among 2000 seeds obtained after floral dip transformation of 5 *Arabidopsis thaliana* plants ecotype Col0 using different *Agrobacterium* strains.

Agrobacterium strain	repeat	Bacteria per ml	Col0 P1	P2	P3	P4	P5	fcy/repeat
LMG26 Rif^R (pMP90,pTJK136)	*a*	$3.7\ 10^8$	6	2	0	0	2	0.1
	b	$3.9\ 10^8$	2	14	2	8	0	0.26
	c	$2.8\ 10^8$	1	0	9	2	3	0.15
LMG148 Rif^R (pMP90,pTJK136)	*a*	$8.5\ 10^7$	0	0	0	0	0	0
	b	$7\ 10^7$	2	40	0	0	10	0.52
	c	$2.1\ 10^8$	5	0	2	32	67	1.06
LMG62 Rif^R (pMP90,pTJK136)	*a*	$2.4\ 10^8$	2	4	0	0	10	0.16
	b	$2.5\ 10^8$	38	2	8	36	26	1.1
	c	$2.04\ 10^8$	20	64	28	27	101	2.4
LMG146 Rif^R (pMP90,pTJK136)	*a*	$3.6\ 10^8$	0	0	2	0	0	0.02
	b	$4.4\ 10^8$	0	0	0	0	0	0
	c	$2.7\ 10^8$	0	0	0	0	0	0
LMG147 Rif^R (pMP90,pTJK136)	*a*	$2.4\ 10^8$	2	2	0	6	4	0.14
	b	$1.8\ 10^8$	14	44	10	6	8	0.82
	c	$2.6\ 10^8$	16	29	4	26	82	1.57
LMG142 Rif^R (pMP90,pTJK136)	*a*	$3.3\ 10^8$	6	0	4	4	0	0.14
	b	$5.1\ 10^8$	26	0	12	4	0	0.42
	c	$2.4\ 10^8$	3	28	10	14	10	0.65
LMG232 Rif^R (pMP90,pTJK136)	*a*	$1.8\ 10^8$	28	16	10	20	0	0.74
	b	$1.7\ 10^8$	8	2	32	6	46	0.94
	c	$2.4\ 10^8$	8	2	25	3	1	0.39
LBA4404 Rif^R (pTJK136)	*a*	$4.4\ 10^8$	8	0	12	0	0	0.2
	b	$6.3\ 10^8$	2	8	2	2	4	0.18
	c	$2.5\ 10^8$	2	0	4	0	0	0.06
LMG201 Rif^R (pMP90,pTJK136)	*a*	$4.2\ 10^8$	22	50	20	22	8	1.22
	b	$3.2\ 10^8$	38	98	62	18	12	2.28
	c	$2.08\ 10^8$	56	18	2	44	2	1.22
C58C1 Ecm^R (pMP90,pTJK136)	*a*	$2.1\ 10^8$	2	6	2	0	0	0.1
	b	$1.2\ 10^8$	10	4	16	16	6	0.52
	c	$5.2\ 10^7$	1	5	3	5	11	0.25
C58C1 Rif^R (pMP90,pTJK136)	*a*	$3.1\ 10^8$	14	18	2	6	36	0.76
	b	$7.7\ 10^8$	66	40	12	22	0	1.4
	c	$2.9\ 10^8$	14	73	8	21	16	1.32
	d	$7.07\ 10^8$	17	8	36	92	4	1.57
C58C1 Rif^R (pGV2260,pTJK136)	*c*	$2.8\ 10^8$	8	11	12	3	35	0.69
	d	$5.28\ 10^8$	10	18	8	27	5	0.68

Table 3 (*legend continued from previous page*) All strains carried the pTJK136 binary vector and pMP90 or pGV2260 as *vir* helper plasmid except for LBA4404 which has its own *vir* plasmid (see text). Each *Agrobacterium* culture was used to floral dip 5 different Col0 plants (P1, P2, P3, P4 and P5) and this in 2, 3 or 4 independent experiments (a-b-c-d). 2000 seeds from the T1 seedstock obtained from each dipped plant were sown on kanamycin selective medium and the number of resistant plants was scored. Bacteria per ml: titer of the *Agrobacterium* cultures in the dipping medium extrapolated from the measured OD_{600} values (material and methods). Fcy/repeat is the transformation frequency for each repeat calculated as follows: ((sum of transformants within the repeat)/(5*2000))*100.

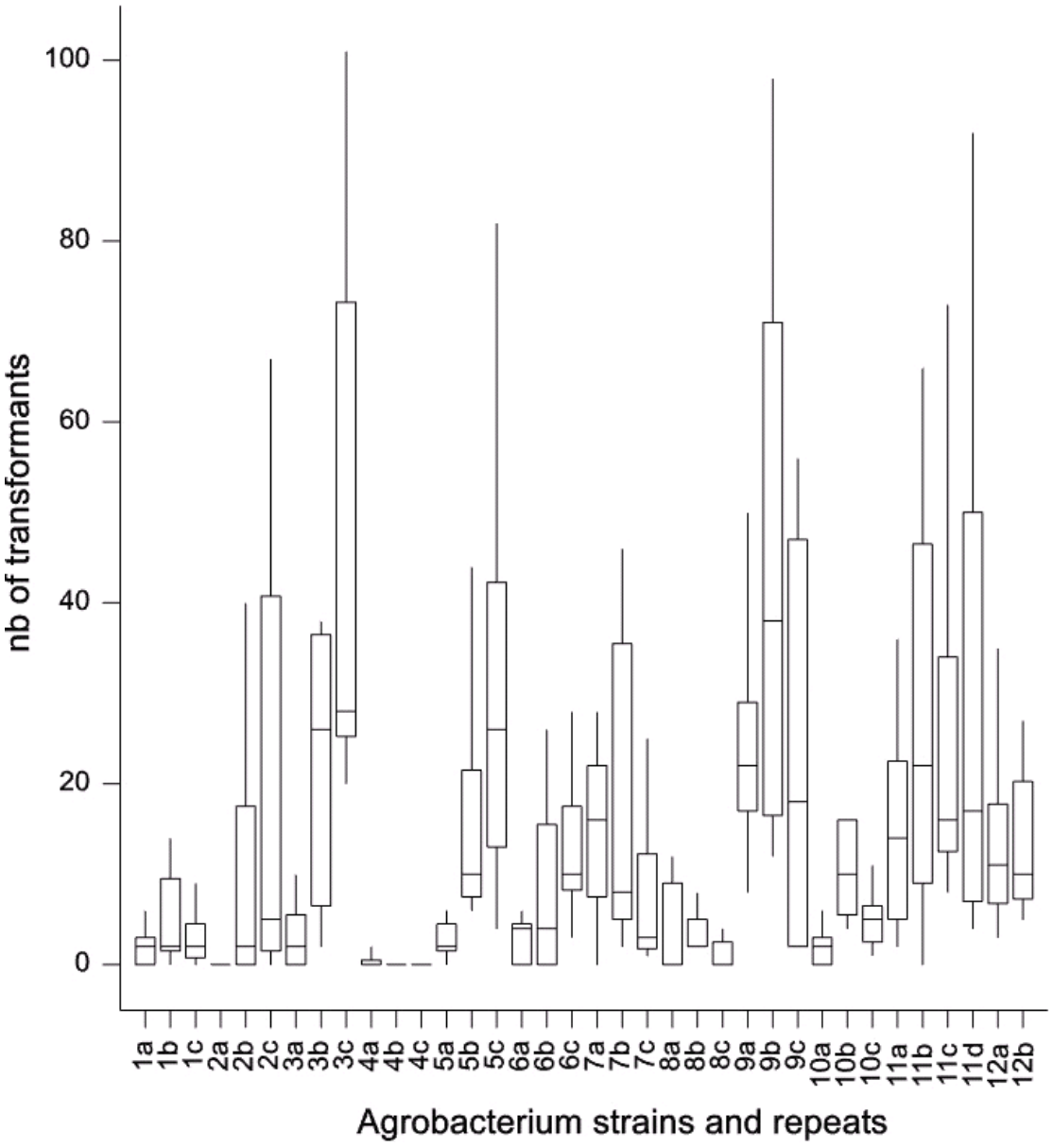

Figure 2: Boxplots showing the intra- and intervariability of the number of transformants per 2000 seeds in 5 different T1 seedstocks in 2, 3, 4 biological floral dip experiments with 12 different *Agrobacterium* strains. From the dipped plants, 2000 seeds were sown on selective medium (*legend continued next page*).

Figure 2: (*legend continued from previous page*) 1: LMG26 Rif^R (pMP90,pTJK136); 2: LMG148 Rif^R (pMP90,pTJK136); 3: LMG62 Rif^R (pMP90,pTJK136); 4: LMG146 Rif^R (pMP90,pTJK136); 5: LMG147 Rif^R (pMP90,pTJK136) 6: LMG142 Rif^R (pMP90,pTJK136); 7: LMG232 Rif^R (pMP90,pTJK136); 8: LBA4404 Rif^R (pTJK136); 9: LMG201 Rif^R (pMP90,pTJK136); 10: C58C1 Ecm^R (pMP90,pTJK136); 11: C58C1 Rif^R (pMP90,pTJK136); 12: C58C1 Rif^R (pGV2260,pTJK136); a, b, c and d: performed biological repeats.

After transformation with the *Agrobacterium* strain LMG146 Rif^R (pMP90,pTJK136), only two transgenic plants were obtained on a total of 30000 screened seeds (Table 3; strain 4 Figure 2). However as was found that the pMP90 *vir* plasmid was lost during culturing (see higher) this is not surprising. Therefore, we cannot conclude whether or not the transformation frequency of *Agrobacterium* LMG146 Rif^R (pMP90,pTJK136) is comparable to the one of the reference strains.

2.3. *Agrobacterium* LMG201 Rif^R (pMP90,pTJK136) shows higher transformation frequencies than the commonly used C58C1 Rif^R (pMP90,pTJK136) after *Arabidopsis thaliana* Col0 floral dip transformation

To compare the transformation performance of the 12 studied *Agrobacterium* strains, the degree of the transformation variability of each strain between different plants and different experiments was determined. Therefore, the number of floral dipped plants with no transformants per 2000 seeds, versus a low, medium or high number of transformants per 2000 seeds were plotted for each strain (Figure 3). The “low transformation frequency” class was defined as the class containing dipped plants in which 1 to 10 transformants per 2000 seeds were obtained, while the “medium transformation frequency” class contains the dipped plants from which 11 to 40 transformants per 2000 seeds were obtained (Figure 3A and B). The “high transformation frequency” class groups the dipped plants from which more than 40 transformants per 2000 seeds were obtained (Figure 3A and B). Figure 3A shows the transformation performance for all *Agrobacterium* strains except for C58C1 Rif^R (pGV2260,pTJK136). Figure 3B (generated from biological repeats c and d, Table 3),

shows the floral dip transformation performance of C58C1 Rif^R (pGV2260,pTJK136) and C58C1 Rif^R (pMP90,pTJK136) strains.

In the “null transformation frequency” class, LMG146 Rif^R (pMP90,pTJK136) is predominant with 14 out of 15 dipped plants that gave no transformants. However, as described earlier in this chapter, in *Agrobacterium* LMG146 Rif^R (pMP90,pTJK136) the pMP90 helper plasmid was no longer present. In the same class, LMG148 Rif^R (pMP90,pTJK136), LBA4404 Rif^R (pMP90) and LMG26 Rif^R (pMP90,pTJK136) (Figure 3A) are present with 8/15, 6/15 and 4/15 dipped plants respectively. In the “low transformation frequency” class, LMG26 Rif^R (pMP90,pTJK136) and C58C1 Ecm^R (pMP90,pTJK136) are present with 10/15 *Arabidopsis* Col0 plants. Using LMG147 Rif^R (pMP90,pTJK136), LMG232 Rif^R (pMP90,pTJK136) and LBA4404 Rif^R (pMP90), also 1-10 transformants were obtained from 8 out of 15 dipped plants (Figure 3, A). In the “medium transformation frequency” class, the highest number of dipped plants (9/15) is belonging to *Agrobacterium* C58C1 Rif^R (pMP90,pTJK136), followed by LMG210 Rif^R (pMP90,pTJK136) and LMG62 Rif^R (pMP90,pTJK136) (7/15 and 6/15 dipped plants respectively). In the “high transformation frequency class”, the predominant presence of LMG201 Rif^R (pMP90,pTJK136) is very striking. Indeed, using LMG201 Rif^R (pMP90,pTJK136) 5/15 dipped plants gave a number of transformants higher than 40 on 2000 seeds (Figure 3A). In the same class, 2 dipped plants that yielded more than 40 transformants were found for the strains LMG62 Rif^R (pMP90,pTJK136) and C58C1 Rif^R (pMP90,pTJK136), whereas the strains LMG232 Rif^R (pMP90,pTJK136) and LMG148 Rif^R (pMP90,pTJK136) showed one dipped plant that yielded more than 40 transformants (Figure 3, A).

The same classification as described above was used to compare the *Arabidopsis* Col0 transformation performance of *Agrobacterium* C58C1 Rif^R (pGV2260,pTJK136) and *Agrobacterium* C58C1 Rif^R (pPM90, pTJK136) in repeats c and d (Table 3). All dipped plants gave transformants and equal number of dipped plants (5 out of 10) using both strains were belonging to the “medium transformation frequency” class. 5 /10 plants transformed with *Agrobacterium* C58C1 Rif^R (pGV2260,pTJK136) belonged to the “low transformation frequency” class compared to 3/10 plants transformed using *Agrobacterium* C58C1 Rif^R (pMP90,pTJK136). In

the “high transformation frequency” class, only 2/10 dipped plants using *Agrobacterium* C58C1 Rif^R (pMP90,pTJK136) are present (Figure 3, B).

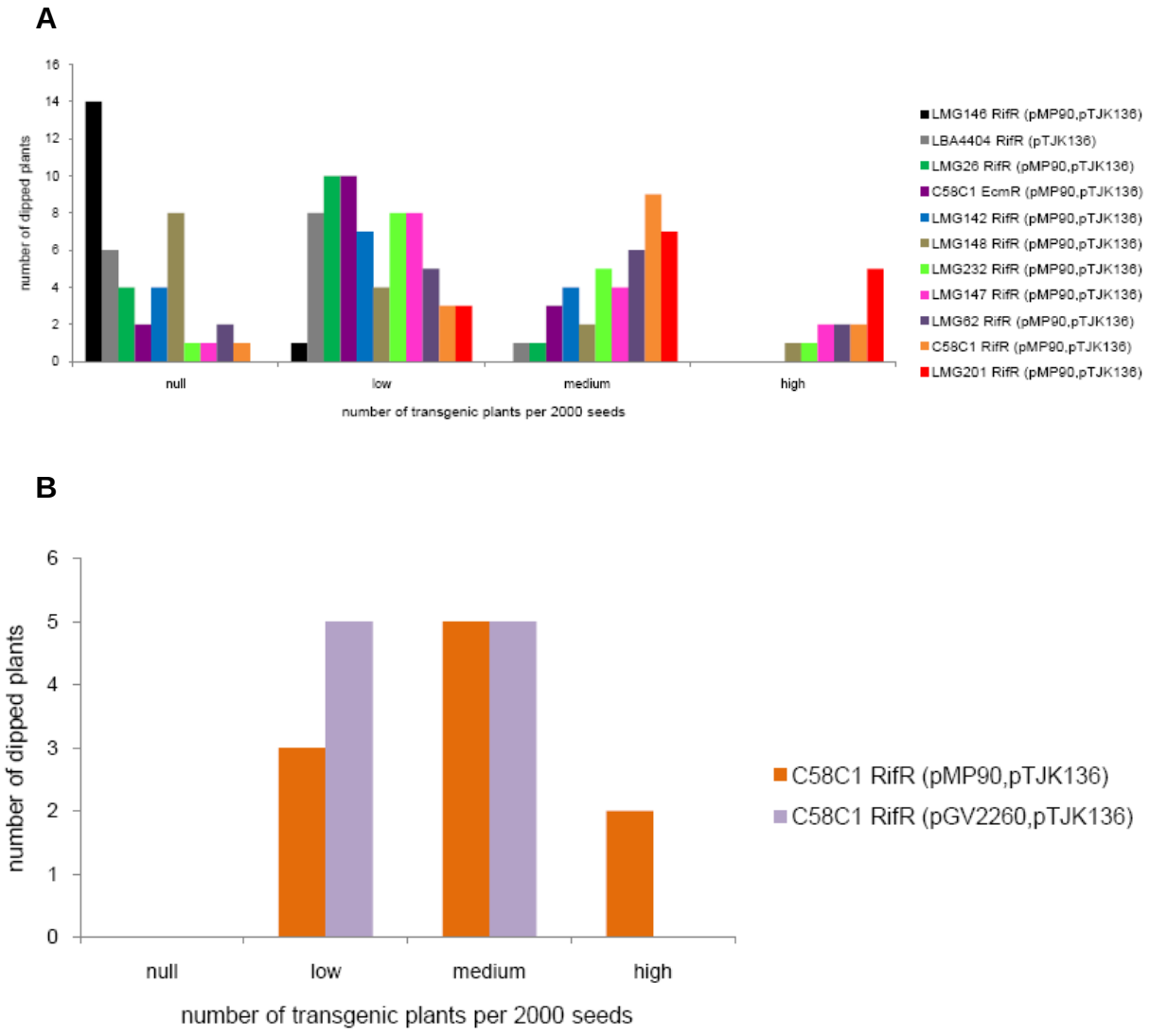

Figure 3: Transformation performance of 12 different *Agrobacterium* strains according to the different obtained transformation frequency classes (x axis) and number of *Arabidopsis thaliana* Col0 plants (y axis). A: All *Agrobacterium* strains used in this study in repeats a, b and c are presented except C58C1 Rif^R (pGV2260,pTJK136). B: *Agrobacterium* C58C1 Rif^R (pGV2260,pTJK136) and *Agrobacterium* C58C1 Rif^R (pMP90,pTJK136) repeats c and d are presented. Null represents the “null transformation frequency” class. 1-10: the “low transformation frequency” class, 11-40: “medium transformation frequency” class and >40: “high transformation frequency” class (see text).

From Figure 3, we can in general conclude that *Agrobacterium* strains LMG201 Rif^R (pMP90,pTJK136), C58C1 Rif^R (pMP90,pTJK136) and LMG62 Rif^R (pMP90,pTJK136) have the highest number of floral dipped plants with high transformation frequencies in contrast to the commonly used *Agrobacterium* LBA4404 Rif^R (pTJK136) strain.

The data presented in Table 3 were statistically analyzed (material and methods) and *Agrobacterium* C58C1 Rif^R (pMP90,pTJK136) was considered as a reference strain to which the other strains were compared. The result of the statistical analyses is presented in Table 4.

Table 4: *Arabidopsis* Col0 transformation frequencies using the different bacterial strains.

***Agrobacterium* strain**	**Transformation frequency in %[a]**	**Significance of difference in transformation frequency in comparison with C58C1 Rif^R (pMP90,pTJK136) (line 1)**
C58C1 Rif^R (pMP90,pTJK136)	1.219	-
LMG201 Rif^R (pMP90,pTJK136)	1.596	***
LMG62 Rif^R (pMP90,pTJK136)	1.212	nsd
LMG147 Rif^R (pMP90,pTJK136)	0.838	nsd
LMG232 Rif^R (pMP90,pTJK136)	0.703	nsd
C58C1 Rif^R (pGV2260, pTJK136)	0.568	*
LMG148 Rif^R (pMP90,pTJK136)	0.519	*
LMG142 Rif^R (pMP90,pTJK136)	0.401	**
C58C1 Ecm^R (pMP90,pTJK136)	0.288	**
LMG26 Rif^R (pMP90,pTJK136)	0.169	***
LBA4404 Rif^R (pTJK136)	0.149	***
LMG146 Rif^R (pMP90,pTJK136)	0.006	***

[a]: Mean values of transformation frequencies assessed as described in material and methods. *Agrobacterium* strains listed according to their mean transformation frequencies values. nsd: not significantly different. *: $p < 0.05\%$. **: $p < 0.01\%$. ***: $p < 0.001\%$.

The statistical analysis showed that the transformation performance of *Agrobacterium* LMG147 Rif^R (pMP90,pTJK136), LMG232 Rif^R (pMP90,pTJK136) and LMG62 Rif^R (pMP90,pTJK136) is not significantly different compared to the transformation performance of the commonly used C58C1 Rif^R chromosomal background harboring both pMP90 and pTJK136 (Table 4). The transformation performance of *Agrobacterium* strains LMG26 Rif^R (pMP90,pTJK136), LMG148 Rif^R

(pMP90,pTJK136), LMG142 Rif^R (pMP90,pTJK136), LBA4404 Rif^R (pTJK136), C58C1 Rif^R (pGV2260,pTJK136) and C58C1 Ecm^R (pMP90,pTJK136) is significantly different from the one of C58C1 Rif^R (pMP90,pTJK136) as the respective *p* values were lower than 0.05 and/or 0.01 and/or 0.001 (Table 4). For these strains, the assessed mean values of transformation frequencies (0.149 to 0.568) are lower than the mean value of C58C1 Rif^R (pMP90,pTJK136) (1.219; Table 4) showing that their floral dip transformation performance is significantly lower than the floral dip performance of the C58C1 Rif^R (pMP90,pTJK136) reference strain. Similarly, LMG146 Rif^R (pMP90,pTJK136) had a lower transformation performance than the reference strain (Table 4), however no conclusions can be made as we showed that the pMP90 plasmid was lost in this strain. Finally, the assessed mean transformation frequency value of LMG201 Rif^R (pMP90,pTJK136) was higher than the assessed mean value of C58C1 Rif^R (pMP90,pTJK136) (1.596 and 1.219 respectively; Table 4), showing that the *Arabidopsis* Col0 floral dip transformation performance of *Agrobacterium* LMG201 Rif^R (pMP90,pTJK136) is significantly higher than the transformation performance of C58C1 Rif^R (pMP90,pTJK136) ($p < 0.001$) under the used experimental conditions.

2.4. *Arabidopsis thaliana* C24 ecotype floral dip transformation is very inefficient

The same *Agrobacterium* strains used for *Arabidopsis* Col0 floral dip transformation (Table 2) were also used for floral dip transformation of *Arabidopsis thaliana* ecotype C24. The transformation procedure was, similarly for Col0 plants, carried out as described in material and methods. Two biological repeats were performed (c and d). Within each repeat, five plants were transformed using the same *Agrobacterium* culture. Optical density at wavelength λ=600 (OD_{600}) of each bacterial culture was measured before dipping. The obtained transformation frequencies with all used bacterial strains were rather low (Table 5). Indeed, using strains LMG26 Rif^R (pMP90,pTJK136), LMG142 Rif^R (pMP90,pTJK136), LMG146 Rif^R (pMP90,pTJK136), LMG232 Rif^R (pMP90,pTJK136), LBA4404 Rif^R (pTJK136)

and C58C1 Rif^R (pGV2260,pTJK136), transformation frequencies ranged from 0% to 0.07% in both performed biological repeats (Table 5).

Table 5: Number of transformants obtained from 2000 seeds of 5 T1 seedstocks obtained after floral dip transformation of 5 *Arabidopsis thaliana* plants ecotype C24 using 12 different *Agrobacterium* strains.

Agrobacterium strain	repeat	Bacteria per ml	C24					fcy/repeat
			P1	P2	P3	P4	P5	
LMG26 Rif^R (pMP90,pTJK136)	*c*	2.8 10^8	0	0	0	0	0	0
	d	5.28 10^8	0	0	0	0	0	0
LMG148 Rif^R (pMP90,pTJK136)	*c*	2.1 10^8	0	3	1	0	18	0.22
	d	7.04 10^7	0	0	3	1	0	0.04
LMG62 Rif^R (pMP90,pTJK136)	*c*	2.04 10^8	0	8	0	0	0	0.08
	d	3.16 10^8	3	0	-	4	5	0.12
LMG146 Rif^R (pMP90,pTJK136)	*c*	2.7 10^8	0	0	0	0	0	0
	d	4.09 10^8	0	0	0	0	0	0
LMG147 Rif^R (pMP90,pTJK136)	*c*	2.6 10^8	2	1	1	11	0	0.15
	d	2.05 10^9	0	0	0	1	1	0.02
LMG142 Rif^R (pMP90,pTJK136)	*c*	2.4 10^8	0	0	1	0	0	0.01
	d	9.4 10^8	0	0	0	0	0	0
LMG232 Rif^R (pMP90,pTJK136)	*c*	2.4 10^8	0	1	1	0	0	0.02
	d	4.14 10^8	0	0	-	6	0	0.06
LBA4404 Rif^R (pTJK136)	*c*	2.5 10^8	0	1	0	1	0	0.02
	d	5.28 10^8	0	0	0	0	0	0
LMG201 Rif^R (pMP90,pTJK136)	*c*	2.08 10^8	3	2	2	3	0	0.1
	d	3.63 10^8	1	1	1	0	0	0.03
C58C1 Ecm^R (pMP90,pTJK136)	*c*	5.2 10^7	0	8	2	1	0	0.11
	d	1.22 10^9	0	0	0	1	0	0.01
C58C1 Rif^R (pMP90,pTJK136)	*c*	2.9 10^8*	22	0	1	9	1	0.33
	d	7.07 10^8	0	0	1	3	5	0.09
C58C1 Rif^R (pGV2260,pTJK136)	*c*	2.8 10^8*	0	0	1	1	0	0.02
	d	5.28 10^8	0	-	1	0	0	0.01

P1, P2, P3, P4 and P5 designate the different *Arabidopsis* C24 dipped plants. 2000 seeds from each dipped plant were sown on kanamycin selective medium and the number of resistant plants was scored. *c* and *d* represent the performed dipping repeats. Bacteria per ml: titer of *Agrobacterium* in the dipping medium calculated from the measured OD_{600} values (material and methods). Fcy/repeat is the transformation frequency for each repeat calculated as follows: ((sum of transformants within the repeat)/(5*2000))*100.

Floral dip transformation using *Agrobacterium* strains LMG148 Rif^R (pMP90,pTJK136), LMG147 Rif^R (pMP90,pTJK136), LMG201 Rif^R (pMP90,pTJK136), C58C1 Ecm^R (pMP90,pTJK136) and C58C1 Rif^R (pMP90,pTJK136) resulted in higher transformation frequencies (0.1% to 0.33%), but only in biological repeat c (Table 5). This shows that restrictions located at the bacterial and/or the plant level are limiting *Agrobacterium*-mediated floral dip transformation of *Arabidopsis thaliana* ecotype C24. The fact that all *Agrobacterium* strains used for *Arabidopsis* C24 floral dip gave lower transformation frequencies compared to *Arabidopsis* Col0 floral dip (Table 3 and Table 5) indicates that the restrictions are mainly located at the plant level. Moreover, in repeat c the same bacterial cultures were used for both *Arabidopsis* Col0 and C24 ecotypes floral dip transformation. The transformation frequencies obtained with C24 ecotype ranged from 0% to 0.33% while it was comprised between 0% to 2.4% after Col0 transformation. Thus, *Arabidopsis* C24 ecotype is to some extent recalcitrant to *Agrobacterium* floral dip transformation.

2.5. The *Agrobacterium* strain influences the *Arabidopsis thaliana* Col0 floral dip transformation frequency

In order to asses the *Agrobacterium* strain influence on *Arabidopsis thaliana* transformation frequency in more detail and to overcome the inter- plant variability within one experiment, we performed a floral dip cotransformation of *Arabidopsis* Col0 plants using a mixture of two different *Agrobacterium* strains. Strain C58C1 Rif^R (pMP90, pAAVS1) was used as reference strain in all cotransformation experiments. The pAAVS1 T-DNA harbors the hygromycin phosphotransferase gene, conferring resistance to hygromycin (not shown). The second strain was either LMG62 Rif^R (pMP90,pTJK136), LMG201 Rif^R (pMP90,pTJK136), LBA4404 Rif^R (pTJK136) or C58C1 Rif^R (pMP90,pTJK136). The pTJK136 T-DNA plasmid carries the *nptII* gene conferring resistance to kanamycin. Separately grown *Agrobacterium* cultures were mixed at equal densities before the five plants were dipped. After floral dip, the same amount of seeds from the dipped plant were sown on K1 medium supplemented either with kanamycin or hygromycin (material and methods). If both bacterial strains present in the dipping mixture can transform *Arabidopsis* with equal frequencies, the

number of kanamycin resistant (Kan^R) plants should be comparable to the number of hygromycin resistant (Hyg^R) plants. This was indeed observed for the mixture of both C58C1 Rif^R strains (Table 6, mixture 4). After cotransformation using a mixture of LBA4404 Rif^R (pTJK136) and C58C1 Rif^R (pMP90,pAAVS1), only one Kan^R plant with the T-DNA of LBA4404 Rif^R (pTJK136) was obtained from 1000 seeds of each seedstock (5000 seeds in total) while 14, 101, 6, 12 and 3 Hyg^R plants were obtained from 1000 seeds of the five Col0 dipped plant seedstocks (Table 6, mixture 3). This result confirmed that strain LBA4404 Rif^R (pTJK136) is about 10 times less efficient than strain C58C1 Rif^R (pMP90,pTJK136) for *Arabidopsis* Col0 floral dip transformation. From the 5 dipped plants using a mixture of LMG201 Rif^R (pMP90,pTJK136) and C58C1 Rif^R (pMP90,pAAVS1), the number of transgenic plants derived from LMG201 Rif^R (pMP90,pTJK136) was higher than the number of transgenic plants derived from C58C1 Rif^R (pMP90,pAAVS1) (Table 6, mixture 2). This result clearly shows that the performance of *Agrobacterium* LMG201 Rif^R (pMP90,pAAVS1) for *Arabidopsis* Col0 floral dip transformation is higher than the performance of C58C1 Rif^R (pMP90,pAAVS1) which is in accordance with the results of the statistical analysis described in section 2.3 of this chapter.

We also observed a high inter- plant variability within one experiment using one bacterial mixture. The mixture of LMG62 Rif^R (pMP90,pTJK136) and C58C1 Rif^R (pMP90,pAAVS1) strains led to 3 transformants harboring the T-DNA of LMG62 Rif^R (pMP90,pTJK136) from P4 and 107 transformants with the same T-DNA from P1. Using the same mixture, 5 Hyg^R plants were derived from P4 and 45 from P1. These results are confirming our previous conclusions that plant characteristics are highly influencing the transformation frequencies during floral dip method. The results obtained from the mixture of C58C1 Rif^R (pMP90,pTJK136) and C58C1 Rif^R (pMP90,pAAVS1) reinforce our conclusion that the variability is not due to the bacterial culture but rather to the plant as these two similar strains gave similar number of transformants in the different seedstocks (Table 6). In general, the results obtained from the cotransformation experiment show that both the *Agrobacterium* strain and the plant characteristics are determining *Arabidopsis* Col0 transformation frequencies.

Table 6: Number of kanamycin resistant (Kan^R) and hygromycin resistant (Hyg^R) transformants in 2000 seeds obtained after *Arabidopsis* Col0 floral dip cotransformation using different *Agrobacterium* mixtures.

	Agrobacterium mixtures	Number of transgenic plants				
		P1	P2	P3	P4	P5
1	LMG62 Rif^R (pMP90,pTJK136)	107	30	20	3	82
	C58C1 Rif^R (pMP90,pAAVS1)	45	19	22	5	20
2	LMG201 Rif^R (pMP90,pTJK136)	20	27	27	43	37
	C58C1 Rif^R (pMP90,pAAVS1)	13	18	26	36	28
3	LBA4404 Rif^R (pTJK136)	0	1	0	0	0
	C58C1 Rif^R (pMP90,pAAVS1)	14	101	6	12	3
4	C58C1 Rif^R (pMP90,pTJK136)	23	20	18	9	3
	C58C1 Rif^R (pMP90,pAAVS1)	24	24	12	8	2

Within one mixture, one strain is carrying the pTJK136 T-DNA with the kanamycin resistance gene, while the other strain, C58C1 Rif^R, is carrying the pAAVS1 T-DNA with the hygromycin resistance gene. Mixtures were prepared from bacterial cultures with equal concentrations. P1-P5 designate 5 different co-transformed *Arabidopsis thaliana* Col0 plants.

2.6. *Agrobacterium* LMG201 is no longer pathogenic after introduction of the pMP90 *vir* plasmid

As *Agrobacterium* LMG201 Rif^R (pMP90,pTJK136) showed higher transformation frequencies than C58C1 Rif^R (pMP90,pTJK136) after floral dip transformation of *Arabidopsis* Col0 ecotype, we decided to further explore its properties. To check the oncogenicity of strain LMG201 after making this strain rifampicin resistant and after the introduction of both pMP90 and pTJK136 plasmids, we carried out a pathogenicity test using wounded tobacco plants (material and methods). The experiment was performed using 4 tobacco plants per strain, in two biological repeats. The wild type *Agrobacterium* strain C58 and the derived cured strain C58C1 Rif^R (Holsters et al., 1980) were used as control for crown gall induction and no tumor induction respectively. On the 8 used tobacco plants, tumor development was observed when the wild type *Agrobacterium* LMG201 and the rifampicin resistant mutant LMG201 Rif^R were applied at the wounding site. However, no tumor could be observed when LMG201 Rif^R (pMP90) and LMG201 Rif^R

(pMP90,pTJK136) (Figure 4) were applied on the 8 wounded tobacco stems showing that *Agrobacterium* LMG201 Rif^R (pMP90) was no longer oncogenic.

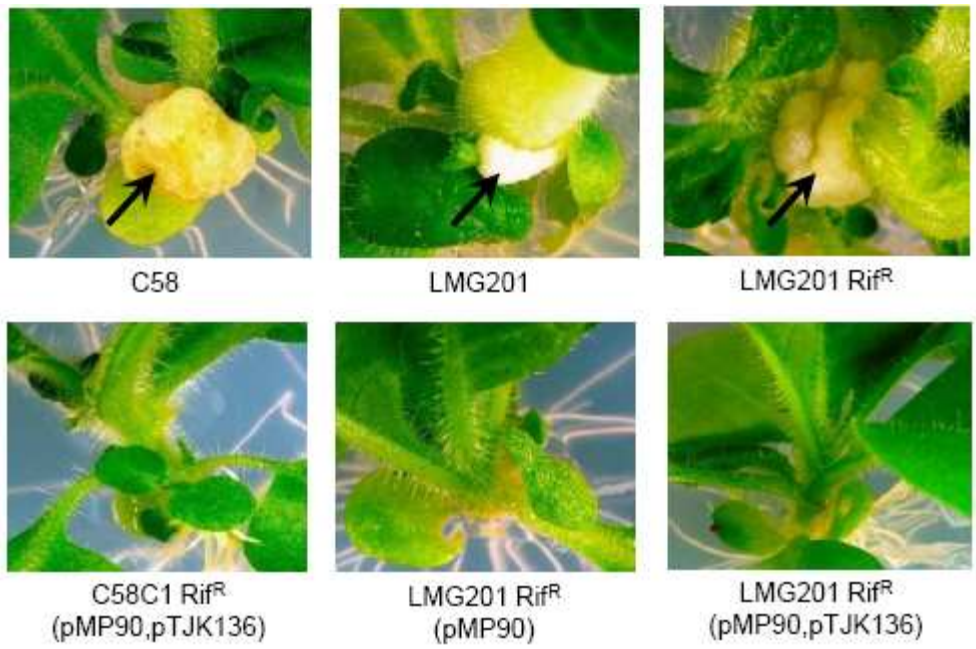

Figure 4: Pathogenicity test applied on tobacco plants. *Agrobacterium* C58 and C58C1 Rif^R (pMP90,pTJK136) were used as control for tumor development and no tumor development respectively. LMG201 and LMG201 Rif^R infection lead to tumor growth on the wounded plants (indicated by the black arrow). LMG201 Rif^R (pMP90) and LMG201 Rif^R (pMP90,pTJK136) are not oncogenic as no tumor could be observed.

2.7. *Agrobacterium* LMG201 Rif^R (pMP90,pTJK136) has a similar T-DNA integration pattern as C58C1 Rif^R (pMP90,pTJK136)

In order to determine the locus number and the T-DNA integration pattern in transformants obtained after floral dip transformation of Col0 using LMG201 Rif^R (pMP90,pTJK136), a segregation analysis and a DNA gel blot analysis were performed, respectively (material and methods). Transformants derived from the commonly used C58C1 Rif^R (pMP90,pTJK136) and LBA4404 Rif^R (pTJK136) strains were included in the analysis to allow comparison. After segregation analysis, the percentage of plants where the T-DNAs integrated in one locus was more or less similar for the three strains: 25/31 tested progeny (80%) for LMG201 Rif^R

(pMP90,pTJK136), 23/31 (74%) for C58C1 Rif^R (pMP90,pTJK136) and 7/10 (70%) for LBA4404 Rif^R (pTJK136) (data not shown). DNA gel blots were performed on DNA prepared from 31 transformants obtained using either *Agrobacterium* strain LMG201 Rif^R (pMP90,pTJK136) or *Agrobacterium* strain C58C1 Rif^R (pMP90,pTJK136). Extracted DNA was digested using the *EcoR*V restriction enzyme and two probes, the *NPTII* and *GUS* probe, were used (Figure 5A; material and methods). Inverted repeats about the right border (IRRB) are revealed by a band of approximately 2700bp with the *GUS* probe and bands longer than 3908bp with the *NPTII* probe (Figure 5A). The presence of a 5253bp band with both probes and bands more than 1345bp (*GUS* probe) and 3908bp (*NPTII* probe) is indicative of the presence of tandem repeats (TR) (Figure 5A). Inverted repeats about the left border (IRLB) can diagnosed by the presence of bands longer than 1345bp using the *GUS* probe and a band of approximately 7800bp with the *NPTII* probe (Figure 5A). The blots showed no major differences between the T-DNA integration patterns of the transformants obtained with the LMG201 Rif^R (pMP90,pTJK136) or the C58C1 Rif^R (pMP90,pTJK136) strain. A complex T-DNA integration pattern, characteristic for floral dip transformation, was observed. Indeed using the *GUS* probe, bands of 2700bp and 5300bp, revealing the presence of IRRB and tandem repeats respectively, were visualized for the different transgenic plants (Figure 5A and B). Also similar integration patterns were obtained when using the *NPTII* probe (data not shown).

This similarity in T-DNA integration pattern for both LMG201 Rif^R (pMP90,pTJK136) and C58C1 Rif^R (pMP90,pTJK136) strains also suggests similar T-DNA expression profiles. Therefore, GUS activity measurements were performed on 39 transformants obtained using LMG201 Rif^R (pMP90,pTJK136) and 26 transformants obtained using C58C1 Rif^R (pMP90,pTJK136). The obtained results revealed that in ≈90% of the LMG201 Rif^R (pMP90,pTJK136) transformants, less than 150 U GUS/mg protein was expressed while in about 10% the expected 200-300 units GUS/mg of total soluble protein was found. The same distribution was found for the GUS accumulation levels in the C58C1 Rif^R (pMP90,pTJK136) transformants (data not shown).

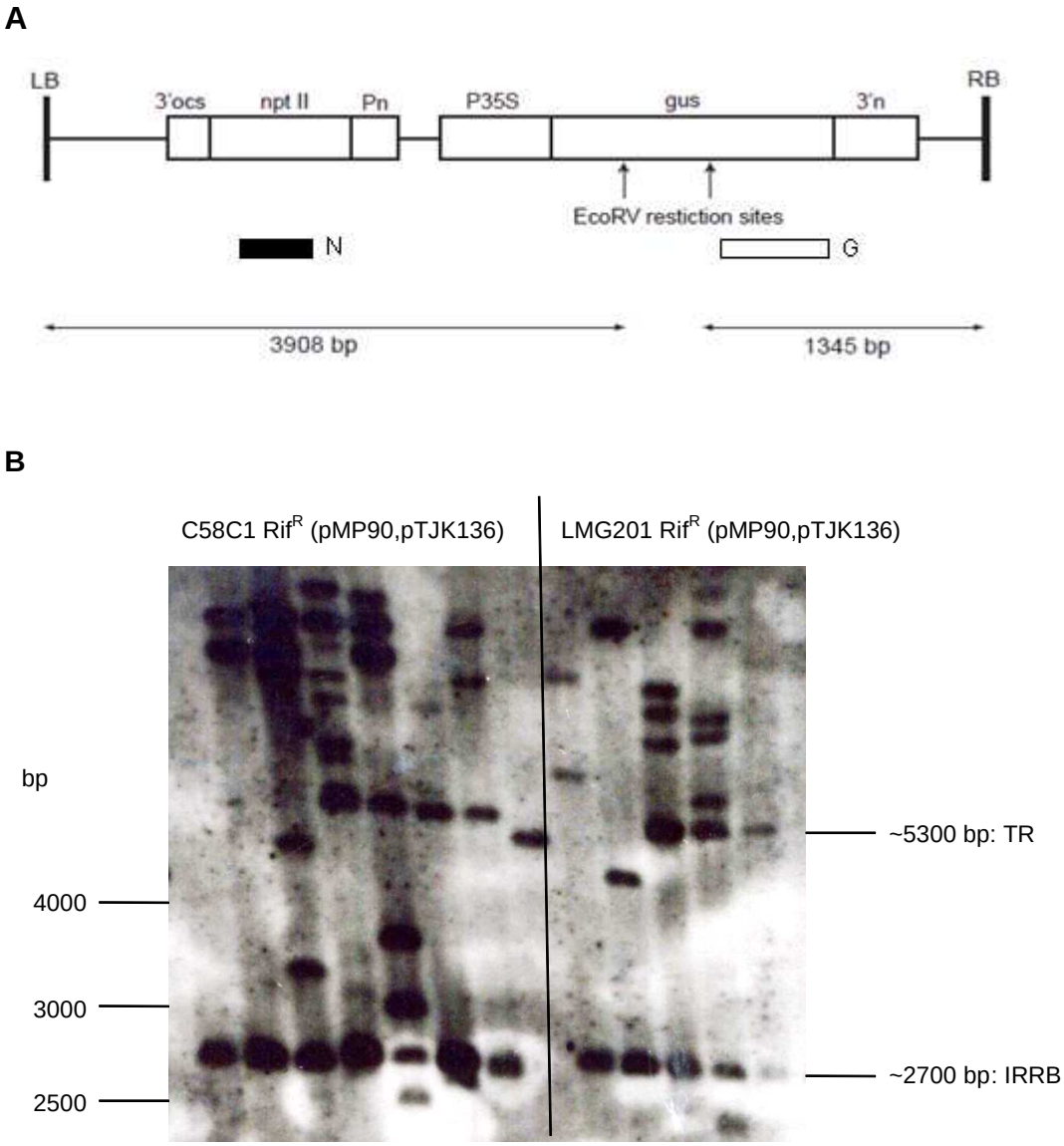

Figure 5: DNA gel blot analysis to determine the T-DNA integration pattern in transformants obtained after cocultivation with *Agrobacterium* C58C1 Rif^R (pMP90,pTJK136) and LMG201 Rif^R (pMP90,pTJK136). Two probes were used: *GUS* (G) and *NPTII* (N). A: Schematic representation of the T-DNA harbored by the pTJK136 plasmid, the *Eco*RV restriction sites (not on scale) and the expected fragment length. B: T-DNA integration pattern obtained using the *GUS* probe on DNA digested with *Eco*RV.
bp: base pair; TR: tandem repeat; IRRB: inverted repeated over the right border; 3'ocs, 3' end of the octopine synthase gene; NPTII, neomycin phosphotransferase II gene; Pn, promoter of the nopaline synthase gene; P35S, cauliflower mosaic virus 35S promoter; *GUS*, β-glucuronidase gene; 3'n, 3' end of the nopaline synthase gene.

3. Discussion

In this work we present a comparative study of 12 *Agrobacterium* strains regarding their ability to produce transformants after *Arabidopsis thaliana* floral dip transformation. Two *Arabidopsis* ecotypes were used: Col0 and C24. The commonly used strains in literature, C58C1 Rif^R (pMP90) and LBA4404 (with it's *vir* plasmid), were included in the analysis.

We observed a high variability within the same experiment using the same bacterial culture. Indeed, the 5 *Arabidopsis thaliana* Col0 plants transformed with the same *Agrobacterium* culture did not always lead to a comparable number of transgenic plants. Because this inter-plant variability was common to all strains within each repeat, the variability is located at the plant level. Indeed, performing a cotransformation with two similar strains showed correlated transformation frequencies per seedstock for the 2 strains but variable frequencies in different T1 seedstocks. It has been shown that the female tissue of *Arabidopsis thaliana,* the ovule, is the main target during *Agrobacterium* floral dip transformation. In particular transformation of the egg cell, which develops inside the ovule, is of relevance as this gives rise to stable transformants. Experimental evidence shows that T-DNA transfer occurs at the end of the female haploid phase and before fertilization (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000; Bechtold et al., 2003). It has also been reported that, using floral dip, immersion of flowers in the bacterial culture should take place a few days before anthesis: 4 to 5 days for *Arabidopsis* and 4 to 7 days for wheat (Clough and Bent, 1998; Desfeux et al., 2000; Zale et al., 2009). It is presumed that during this "time", *Agrobacterium* grows inside the developing flower where the ovary develops as a ring of cells extending to form a vase-shaped structure which is open at the top. Only late in floral development, about 3 days prior to anthesis, the locules become sealed by formation of the stigma (Wiktorek-Smagur et al., 2009). Hence for a successful transformation, it is imperative that the agrobacteria reach the immediate vicinity of the ovule and encounter the environmental conditions where virulence genes are induced. Thus it is tempting to speculate that the variability of floral dip frequency is mainly due to the variable number of developing flowers per plant where the ovule and in particular the female

gametophyte is accessible to the bacteria and where the conditions are favorable to attach to the developing female gametophyte after which the T-DNA transfer process can start.

We also observed a variability in the obtained transformation frequencies across the performed biological repeats of *Arabidopsis* Col0 floral dip. The conditions applied during the different repeats were similar (temperature, surfactant and sucrose concentration, inoculation time…) and the differences originated from the culture mediums and the dipped plants. In the conditions used in this study, there seems to be no correlation between the concentration of *Agrobacterium* in the co-culture medium and the number of transformants. Indeed the inoculum with the lowest *Agrobacterium* concentration did not lead to the lowest transformation frequency. Although our conclusion is not derived from a deep investigation of the influence of *Agrobacterium* inoculums density on transformation rate (use of different inoculums densities of the same strain for transformation), it is in agreement with the study reported by Clough and Bent, (1998) stating that the use of *Agrobacterium* at particular culture densities is not essential to successful high rate floral dip transformation.

The helper *vir* plasmid had an influence on the transformation frequency. When using strain C58C1 Rif^R with the *vir* plasmid pMP90, the transformation frequency was higher than the frequency obtained using the *vir* helper plasmid pGV2260. The small difference in transformation frequency is most probably due to the different dosage of gene products provided by the pMP90 nopaline *vir* plasmid and the pGV2260 octopine *vir* plasmid. Hood and coworkers, (1993) found a higher transformation frequency with the pMOG101 octopine *vir* plasmid in comparison with the pMOG301 nopaline *vir* plasmid after tobacco leaf discs transformation. Also in *Arabidopsis* C24, root explants cocultivation with the octopine pGV2260 *vir* plasmid was slightly better than the nopaline pMP90 plasmid in promoting transformation (unpublished data). These observations further support the statement made by Akama et al., (1992) that the combination of the bacterial strain and the target plant tissue to be transformed is very important to achieve high transformation frequencies. Moreover, Hood and coworkers, (1993) used an *in vitro* transformation method while we used an *in planta* method.

We also found that mutations in chromosomal genes can strongly influence the floral dip transformation frequency. The C58C1 Rif^R (pMP90,pTJK136) transformation frequency was at least 2 fold higher than the C58C1 Ecm^R (pMP90,pTJK136) transformation frequency in both performed biological repeats. This might be explained by the fact that the erythromycin and chloramphenicol resistance mutations most likely occurred in the genes encoding for ribosomal RNA and ribosomal proteins, thereby lowering the fitness of the mutated bacteria and interfering with their growth, movement and penetration in the ovules. This result shows that one should be careful with the choice of a chromosomal selection marker.

To compare the transformation performance of the used bacterial strains, we plotted for each strain how many of the 15 floral dipped plants yielded no transformants in 2000 seeds, 1 to 10 transformants, 11-40 transformants or more then 40 transformants per 2000 seeds. From this classification we concluded that *Agrobacterium* strains LMG201 Rif^R (pMP90,pTJK136), C58C1 Rif^R (pMP90,pTJK136) and LMG62 Rif^R (pMP90,pTJK136) have a higher transformation performance after *Arabidopsis* Col0 floral dip compared to the commonly used *Agrobacterium* strain LBA4404 Rif^R (pTJK136).

The statistical analysis showed that the transformation performance of *Agrobacterium* LMG62 Rif^R (pMP90,pTJK136) is not significantly different compared to the transformation performance of the commonly used C58C1 Rif^R (pMP90,pTJK136). The transformation performance of *Agrobacterium* LBA4404 Rif^R (pTJK136) is significantly lower from the one of C58C1 Rif^R (pMP90,pTJK136) ($p < 0.001$) while *Arabidopsis* Col0 floral dip transformation performance of *Agrobacterium* LMG201 Rif^R (pMP90,pTJK136) is significantly higher than the transformation frequency of C58C1 Rif^R (pMP90,pTJK136) ($p < 0.001$). The high transformation frequency of LMG201 Rif^R (pMP90,pTJK136) was observed in all performed biological repeats. Thus, the best performing chromosomal background for floral dip transformation is from LMG201, which belongs to the same genomic group G8 as C58 (Portier et al., 2006; Costechareyre et al., 2009) and no other *Agrobacterium* genomic groups were found to perform better then this G8 group.

Arabidopsis thaliana ecotype C24 floral dip transformation using the same bacterial strains showed low transformation frequencies (maximum 0.33%) after two performed biological repeats. This can be mainly attributed to the ecotype as some strains such as LMG201 Rif^R (pMP90,pTJK136) and C58C1 Rif^R (pMP90,pTJK136) were very efficient for *Arabidopsis* Col0 floral dip as their transformation frequencies ranged from 0.76% to 2.28%. Moreover, in biological repeat c, the same bacterial cultures were used for floral dip transformation of both Col0 and C24 *Arabidopsis* plants. The number of obtained transgenic Col0 *Arabidopsis* plants (transformation frequency: 0% - 2.4%) was higher than the number of transgenic C24 *Arabidopsis* plants (transformation frequency: 0% - 0.33%) indicating that the restrictions are mainly located at the plant level. In some literature reports, transgenic plants were obtained after *Arabidopsis* C24 floral dip (Tadege et al., 2003; Broothaerts et al., 2005; Zheng et al., 2005; Truernit and Haseloff, 2008). In these reports no indication concerning the transformation frequency is available as the method was used to express a gene of interest. However, other studies also described that different *Arabidopsis* ecotypes are transformed with variable frequencies. Clough and Bent (1998) reported that using floral dip, *Arabidopsis thaliana* ecotypes Ws-0, Nd-0 and No-0 were transformed at rates similar to Col0; in contrast Ler-0, Dijon-G and Bla-t which were transformed at 10- to 100-fold lower rates. Similarly transformants were obtained after *Arabidopsis* Col0 and RLD *in planta* transformation while no transformants could be generated from *Ler* ecotype (Katavic et al., 1994). Recalcitrance of some *Arabidopsis* ecotypes to floral dip transformation could be located at the flower level (morphology, accessibility of the ovule...). Another explanation is that the low transformation frequencies of these recalcitrant ecotypes are due to a different pathogen-response. It is known that plant defense systems are activated during *Agrobacterium* infection (Citovsky et al., 2007 and references therein) and that different *Arabidopsis thaliana* accessions have differential responses to pathogens. For instance, Adam et al., (1999) reported that C24 ecotype is resistant to two fungal powdery mildew diseases while the Col0 ecotype is susceptible for these same pathogens.

Floral dip cotransformation of *Arabidopsis* Col0 plants using different mixtures of two bacterial strains harboring either a hygromycin or kanamycin resistance gene confirmed our grouping of the tested strains. After transformation of 5 Col0 plants

with a mixture of C58C1 Rif^R (pMP90,pTJK136), carrying a kanamycin resistance gene, and C58C1 Rif^R (pMP90,pAAVS1), carrying a hygromycin resistant gene, comparable numbers of Kan^R and Hyg^R transformants were obtained. Using a mixture of LBA4404 Rif^R (pTJK136) and C58C1 Rif^R (pMP90,pAAVS1), the number of transformants harboring the T-DNA carried by LBA4404 Rif^R (pTJK136) was, for the five dipped plants, much lower than the number of transformants harboring the T-DNA carried by C58C1 Rif^R (pMP90,pAAVS1). Using a mixture of LMG201 Rif^R (pMP90,pTJK136) and C58C1 Rif^R (pMP90,pAAVS1), the number of transformants harboring the T-DNA derived from LMG201 Rif^R (pMP90,pTJK136) was higher than the number of transformants harboring the T-DNA derived from C58C1 Rif^R (pMP90,pAAVS1). In this experiment, plant variability was circumvented as the same plant was transformed using a mixture of two *Agrobacterium* strains and the difference in transformation yield is due to the bacterial strain. Thus we can conclude that for *Arabidopsis thaliana* Col0 floral dip transformation, the C58C1 Rif^R (pMP90,pTJK136) and LMG201 Rif^R (pMP90,pTJK136) strains are significantly more efficient than the LBA4404 Rif^R (pTJK136) strain.

As LMG201 Rif^R (pMP90,pTJK136) gave higher transformation frequencies in comparison with C58C1 Rif^R (pMP90,pTJK136), we were interested in further characterizing this strain. LMG201 was no longer pathogenic after introduction of the pMP90 *vir* plasmid. The loss of pathogenicity could be due to an incompatibility between the introduced plasmid and the resident Ti plasmid of LMG201. Incompatibility was previously described in the literature (Hynes et al., 1985; O'Connell et al., 1987; Uraji et al., 2002). We also compared the T-DNA integration pattern and expression profile in transformants obtained with LMG201 Rif^R (pMP90,pTJK136) to those obtained with C58C1 Rif^R (pMP90,pTJK136). No major differences were observed. This is in line with the findings that the T-DNA copy number and integration pattern is largely determined by the target plant cell (De Buck et al., 2009).

In this study, we first showed that floral dip transformation frequencies of *Arabidopsis* Col0 are characterized by a high inter- plant variability when using the same bacterial culture. This might be correlated with the number of flowers at the right stage for the T-DNA uptake. Second, we found that the chromosomal

background of *Agrobacterium* strain LMG201, not frequently used for plant transformation, was very efficient in our experimental conditions for *Arabidopsis thaliana* Col0 ecotype floral dip transformation. Transformation frequencies of LMG201 should be tested for other crops as we concluded that the combination of the plant to be transformed and the bacterial strain is very important for a successful *in planta* transformation. Third, recalcitrance of *Arabidopsis* ecotype C24 to *in planta* transformation remains unclear. A better understanding of the restricting parameters might help applying the floral dip transformation method to recalcitrant *Arabidopsis* accessions and other crops.

4. Material and methods

4.1. *Agrobacterium* strains

From all 8 wild type *Agrobacterium* strains (Table 1), except for C58C1, rifampicin resistant (Rif^R) mutants were obtained by plating them on YEB medium (Wise et al., 2006) supplemented with 100 µg/ml rifampicin. *Agrobacterium* C58C1 Rif^R (pMP90), used in this study, was described in Koncz and Schell (1986).

C58C1 (Van Larebeke et al., 1974) was made resistant to erythromycin and chloramphenicol (Ecm^R) by plating the bacteria on YEB medium supplemented with 50 µg/ml erythromycin and 50 µg/ml chloramphenicol. C58C1 Ecm^R (pMP90) was obtained from a biparental conjugation where C58C1 Rif^R (pMP90) (Koncz and Schell, 1986) was the donor strain and C58C1 Ecm^R the acceptor strain. The resulting C58C1 Ecm^R (pMP90) was afterwards used, in a second biparental conjugation, as a donor strain to introduce the pMP90 *vir* plasmid in the LMG bacterial strains (acceptor strains) described in Table 1. *Vir* plasmid pGV2260 was introduced into C58C1 Rif^R using the same method. No helper plasmid was introduced in *Agrobacterium* LBA4404 as this strain contains its own *vir* plasmid. Triparental conjugation, with *E.coli* HB101 (pRK2013) as helper, was used to transfer the

pTJK136 binary vector to the different mutant *Agrobacterium* strains listed in Table 2 (Ditta et al., 1980).

The binary vector pTJK136 (Kapila et al., 1997) contains the neomycin phosphotransferase II (*NPT*II) gene driven by the nopaline synthase gene (*nos*) promoter conferring resistance to kanamycin and a *β*-glucuronidase (*GUS*) reporter gene under the control of the P35S promoter (Odell et al., 1985).

Binary vector pAAVS1, with a T-DNA harboring the hygromycin phosphotransferase gene surrounded by the promoter and the 3' end of the nopaline synthase gene, was used for floral dip cotransformation of *Arabidopsis thaliana* ecotype Col0. The vector was introduced into C58C1 Rif^R (pMP90) by electroporation.

4.2. *Arabidopsis* ecotypes Col0 and C24 floral dip transformation

Arabidopsis thaliana seeds ecotype Colombia-0 (Col0) were first grown on 0.9% agar K1 medium containing Murashige and Skoog (MS) salts (4.308 g/L), 2.5 mM MES buffer (2-[N-Morpholino]ethane sulfonic acid) and sucrose (10 g/L); pH: 5.7. After two weeks, the seedlings were transferred to soil, kept in growth chambers under a photoperiod of 12 hours light and at 22 ºC/day and 18 ºC/night. Floral dip was performed as described by Clough and Bent, (1998). An *Agrobacterium* starter culture was prepared by inoculating 1ml LB medium with a single *Agrobacterium* colony. After incubation during 8-9 hours at 28ºC, 10ml LB medium (without antibiotic) was added to the inoculum. Another incubation at 28ºC was carried out overnight after which 1ml from the inoculums was used for OD_{600} measurement. Afterwards, 40ml of water solution containing sucrose (10%) and Silwet (0.05%) were added and the final 50 ml suspension was immediately used for dipping of six weeks old bolting, healthy *Arabidopsis* plants. The density of *Agrobacterium* in the different dipping solutions was determined from the OD_{600} values as 1OD is corresponding approximately to $8x10^8$ bacteria/ml and taking into account the final volume of the dipping solution. Dipping was performed by inverting the plant inflorescence in the *Agrobacterium* culture and gentle agitation for 5 seconds. Immediately after dipping, plants were covered with a transparent plastic bag for 24h. Five weeks later, the

dipped plants were no longer watered and were transferred to a 25ºC, 20 hours photoperiod greenhouse. When the plants were dry, the T1 seeds were separately harvested from each plant. For each *Agrobacterium* culture, five *Arabidopsis* plants were dipped. The same procedure was applied for the *Arabidopsis* plants ecotype C24 and for the cotransformation experiment. The different bacterial strains and the number of biological repeats performed for each transformation are listed in Table 2.

Cotransformation was performed using a mixture of two bacterial strains. C58C1 Rif^R (pMP90,pAASV1) was always present and was mixed with a second strain that was either LMG62 Rif^R, LMG201 Rif^R, LBA4404 Rif^R or C58C1 Rif^R. The second strain harbored plasmids pMP90 and pTJK136, except LBA4404 Rif^R where the resident *vir* helper plasmid together with pTJK136 were present. Bacteria were grown separately without selection overnight in 5 ml liquid LB medium at 28℃ under continuous agitation. Mixtures were prepared from individual bacterial cultures with equal OD values of about 3.5.

Approximately 2000 seeds (50mg of seeds) from each dipped plant were sown on K1 medium supplemented with kanamycin (50mg/l) for selection of primary transformants. The number of transformants was scored for each bacterial strain. After cotransformation experiments, 1000 seeds were sown on growth medium supplemented with either hygromycin (20mg/l) or kanamycin for selection of transformants.

4.3. Statistical analysis

The data obtained after *Arabidopsis thaliana* Col0 floral dip transformation using the different bacterial strains was statistically analyzed as follows: a General Linear Mixed Model (GLMM) of the form (random terms underlined): y = constant + strain + repeat + ε was fitted to the data using Genstat's menu facilities (Payne et al., 2009) for GLMM, specifying a binomial distribution with a probit link function. Fitting this GLMM to the data allowed estimating the mean differences between strains and their standard error. Post-hoc comparison of all strains with the reference strain was

done by means of the Fisher's LSD (Least Significant Difference) and significance was assigned.

4.4 Pathogenicity test

Pathogenicity tests were performed on *Nicotiana tabacum* plants (SR1). Tobacco seeds were sterilized by soaking them in 80% ethanol for 2 min and later in NaOCl 6% for 8 min. Afterwards washing was made using sterile water till the pH of the wash water was equal to 7. Then, seeds were kept in sterile water for at least 1 hour. Sterilized seeds were germinated on B5 medium supplemented with 1% sucrose. Three to four weeks later, one cotyledon was removed and cut at the basal end of the leaf. *Agrobacterium* strains, grown on YEB medium supplemented with the appropriate selection for 48 hours, were then applied at the wound site. The *in vitro* pathogenicity test was performed in two biological repeats. In each repeat, four tobacco plants per bacteria were infected.

4.5. Segregation analysis

To determine the T-DNA loci number, segregation analyses were performed in 31 transformants obtained from LMG201 Rif^R (pMP90,pTJK136), 31 transformants obtained from C58C1 Rif^R (pMP90,pTJK136) and 10 transformants obtained from LBA4404 Rif^R (pTJK136). At least 60 T2 seeds per transformant were sown on K1 medium supplemented with kanamycin (50 mg/l). The number of resistant (Kan^R) and sensitive (Kan^S) seedlings were calculated, allowing to deduce the most likely segregation pattern. A χ^2 analysis (Griffiths et al., 1993) was performed on the segregation ratios 3 Km^R:1 Kan^S (one T-DNA locus); 15 Kan^R:1 Kan^S (two T-DNA loci) and 63 Kan^R:1 Kan^S (three T-DNA loci). P=0.05 was chosen as a critical limit, so that the predicted segregation ratio was not rejected for P values >0.05.

4.6. Plant DNA preparation and DNA gel blot analysis

DNA of transgenic *Arabidopsis* leaf material was prepared from 10 to 50 mg of frozen plant leaves using the DNeasy Plant Mini Kit (Westburg, the Netherlands) according to the manufacturer's protocol. *NPTII* and *GUS* probes were prepared according to De Buck et al., (1999). After *EcoR*V digestion, *Arabidopsis* DNA (1µg) was loaded in each lane of a 1% agarose gel. The DNA was labelled and detected with the nonradioactive Roche labelling kit and Roche CDP star detection module (http://www.roche-applied-science.com/index.jsp)

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4.7. Protein extract preparation and GUS activity measurement

Leaf material, collected from 6 weeks old transgenic Col0 *Arabidopsis thaliana* plants, were grinded and resuspended in 100µl buffer containing 50mM phosphate buffer (pH7), 10mM β-mercaptoethanol, 100mM Na_2-EDTA and 0.1% triton X-100. Samples were then centrifuged twice at 4ºC for 10 min to eliminate insoluble material. The total amount of soluble protein in the protein extracts was determined with the Bio-Rad Protein Assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard. The GUS activity was determined as described by Breyne et al., (1993). GUS activity levels were expressed as units GUS protein relative to the total amount of soluble extracted protein (U GUS/mg protein).

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References

Adam L, Ellwood S, Wilson I, Saenz G, Xiao S, Oliver RP, Turner JG, Somerville S (1999) Comparison of Erysiphe cichoracearum and E. cruciferarum and a survey of 360 Arabidopsis thaliana accessions for resistance to these two powdery mildew pathogens. Molecular plant-microbe interactions **12:** 1031-1043

Akama K, Shiraishi H, Ohta S, Nakamura K, Okada K, Shimura Y (1992) Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. Plant cell reports **12:** 7-11

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Paris, Life Sciences **316:** 1194-1199

Bechtold N, Jaudeau B, Jolivet S, Maba B, Vezon D, Voisin R, Pelletier G (2000) The maternal chromosome set is the target of the T-DNA in the in planta transformation of *Arabidopsis thaliana*. Genetics **155:** 1875-1887

Bechtold N, Jolivet S, Voisin R, Pelletier G (2003) The endosperm and the embryo of *Arabidopsis thaliana* are independently transformed through infiltration by *Agrobacterium tumefaciens*. Transgenic research **12:** 509-517

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254

Breyne P, De Loose M, Dedonder A, Van Montagu M, Depicker A (1993) Quantitative kinetic analysis of ß-glucuronidase activities using a computer-directed microtiterplate reader. Plant Mol. Biol. Rep. **11:** 21-31

Broothaerts W, Mitchell HJ, Weir B, Kaines S, Smith LM, Yang W, Mayer JE, Roa-Rodriguez C, Jefferson RA (2005) Gene transfer to plants by diverse species of bacteria. Nature **433:** 629-633

Cangelosi GA, Hung L, Puvanesarajah V, Stacey G, Ozga DA, Leigh JA, Nester EW (1987) Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. Journal of bacteriology **169:** 2086-2091

Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. In Vitro Cell. Dev. Biol. **40:** 31-45

Citovsky V, Kozlovsky SV, Lacroix B, Zaltsman A, Dafny-Yelin M, Vyas S, Tovkach A, Tzfira T (2007) Biological systems of the host cell involved in *Agrobacterium* infection. Cellular microbiology **9:** 9-20

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant journal **16:** 735-743

Costechareyre D, Bertolla F, Nesme X (2009) Homologous recombination in *Agrobacterium*: potential implications for the genomic species concept in bacteria. Molecular biology and evolution **26:** 167-176

De Bondt A, Eggermont K, Druart P, De Vil M, Goderis I, Vanderleyden J, Broekaert WF (1994) *Agrobacterium*-mediated transformation of apple (Malus × domestica Borkh.) : an assessment of factors affecting gene transfer efficiency during early transformation steps. Plant cell reports **13:** 587-593

De Buck S, Jacobs A, Van Montagu M, Depicker A (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. The Plant journal **20:** 295-304

De Buck S, Podevin N, Nolf J, Jacobs A, Depicker A (2009) The T-DNA integration pattern in Arabidopsis transformants is highly determined by the transformed target cell. The Plant journal **60:** 134-145

De Ley J (1972) Agrobacterium: intragenic relationships and evolution. *In* HM Geesteranus, ed, Plant Pathogenic Bacteria, Wageningen, pp 251-259

De Ley J (1974) Phylogeny of the prokaryotes. Taxon **23:** 291-300

Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucleic acids research **13:** 4777-4788

Desfeux C, Clough SJ, Bent AF (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. Plant physiology **123:** 895-904

Dillen W, De Clerq J, Kapila J, Zambre M, Van Montagu M, Angenon G (1997) The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant J **12:** 1459-1463.

Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**: 7347-7351

Douglas CJ, Staneloni RJ, Rubin RA, Nester EW (1985) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. Journal of bacteriology **161:** 850-860

Gelvin SB (2000) *Agrobacterium* and Plant Genes Involved in T-DNA Transfer and Integration. Annu Rev Plant Physiol Plant Mol Biol **51:** 223-256

Gelvin SB (2003) Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiology and molecular biology reviews **67:** 16-37, table of contents

Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao Y, Askenazi M, Halling C, Mullin L, Houmiel K, Gordon J, Vaudin M, Iartchouk O, Epp A, Liu F, Wollam C, Allinger M, Doughty D, Scott C, Lappas C, Markelz B, Flanagan C, Crowell C, Gurson J, Lomo C, Sear C, Strub G, Cielo C, Slater S (2001) Genome sequence of the plant pathogen and biotechnology agent Agrobacterium tumefaciens C58. Science (New York, N.Y **294:** 2323-2328

Griffiths AJF, Miller JH, Suzuki DT, Lewontin RC, Gelbart WM (1993) An Introduction to Genetic Analysis Ed 5. W.H. Freeman and company, New York

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303:** 179-180

Holsters M, Silva B, Van Vliet F, Genetello C, De Block M, Dhaese P, Depicker A, Inze D, Engler G, Villarroel R, Van Montagu M, Schell J (1980) The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid **3:** 212–230

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic research **2:** 208-218

Horsch RB, Fry GE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A Simple and General Method for Transferring Genes into Plants. Science (New York, N.Y **227:** 1229-1231

Hynes MF, Simon R, Puhler A (1985) The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium* meliloti plasmid to eliminate pAtC58. Plasmid **13:** 99-105

Kapila J, Rycke RD, Montagu MV, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. Plant science **122:** 101-108

Katavic V, Haughn GW, Reed D, Martin M, Kunst L (1994) In planta transformation of Arabidopsis thaliana. Molecular & general genetics **245:** 363-370

Koncz C, Schell J (1986) The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet **204:** 383-396

Lee LY (2006) Integration of genes into the chromosome of *Agrobacterium tumefaciens* C58. *In* K Wang, ed, Methods in Molecular Biology: *Agrobacterium* Protocols, Vol 1. Humana Press, Iowa, pp 55-66

Lee LY, Gelvin SB (2008) T-DNA binary vectors and systems. Plant Physiol **146:** 325-332

Leigh JA, Coplin DL (1992) Exopolysaccharides in plant-bacterial interactions. Annu Rev Microbiol **46:** 307-346

Matthysse AG (1987) Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. Journal of bacteriology **169:** 313-323

Matthysse AG, McMahan S (1998) Root colonization by *Agrobacterium tumefaciens* is reduced in cel, attB, attD, and attR mutants. Appl Environ Microbiol **64:** 2341-2345

Matthysse AG, Yarnall H, Boles SB, McMahan S (2000) A region of the *Agrobacterium tumefaciens* chromosome containing genes required for virulence and attachment to host cells. Biochimica et biophysica acta **1490:** 208-212

McCullen CA, Binns AN (2006) *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annual review of cell and developmental biology **22:** 101-127

Melchers LS, Maroney MJ, den Dulk-Ras A, Thompson DV, van Vuuren HA, Schilperoort RA, Hooykaas PJ (1990) Octopine and nopaline strains of *Agrobacterium tumefaciens* differ in virulence; molecular characterization of the virF locus. Plant molecular biology **14:** 249-259

Nair GR, Liu Z, Binns AN (2003) Reexamining the role of the accessory plasmid pAtC58 in the virulence of Agrobacterium tumefaciens strain C58. Plant Physiol **133:** 989-999

Nam J, Matthysse AG, Gelvin SB (1997) Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. Plant Cell **9:** 317-333

O'Connell MP, Hynes MF, Puehler A (1987) Incompatibility between a *Rhizobium* Sym plasmid and a Ri plasmid of *Agrobacterium*. Plasmid **18:** 156-163

Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313:** 810-812

Opabode J (2006) *Agrobacterium*-mediated transformation of plants: emerging factors that influence efficiency. Biottechnology and Molecular Biology Review **1:** 12-20

Payne RW, Murray DA, Harding SA, Baird DB, Soutar DM (2009) *GenStat for Windows 12th Edition) Introduction*. VSN International, Hemel Hempstead.

Portier P, Fischer-Le Saux M, Mougel C, Lerondelle C, Chapulliot D, Thioulouse J, Nesme X (2006) Identification of genomic species in *Agrobacterium* biovar 1 by AFLP genomic markers. Appl Environ Microbiol **72:** 7123-7131

Reuhs BL, Kim JS, Matthysse AG (1997) Attachment of *Agrobacterium tumefaciens* to carrot cells and Arabidopsis wound sites is correlated with the presence of a cell-associated, acidic polysaccharide. Journal of bacteriology **179:** 5372-5379

Shen WH, Escudero J, Schlappi M, Ramos C, Hohn B, Koukolikova-Nicola Z (1993) T-DNA transfer to maize cells: histochemical investigation of beta-glucuronidase activity in maize tissues. Proceedings of the National Academy of Sciences of the United States of America **90:** 1488-1492

Tadege M, Sheldon CC, Helliwell CA, Upadhyaya NM, Dennis ES, Peacock WJ (2003) Reciprocal control of flowering time by OsSOC1 in transgenic *Arabidopsis* and by FLC in transgenic rice. Plant Biotechnol J **1:** 361-369

Thomashow MF, Karlinsey JE, Marks JR, Hurlbert RE (1987) Identification of a new virulence locus in Agrobacterium tumefaciens that affects polysaccharide composition and plant cell attachment. Journal of bacteriology **169:** 3209-3216

Truernit E, Haseloff J (2008) *Arabidopsis thaliana* outer ovule integument morphogenesis: ectopic expression of KNAT1 reveals a compensation mechanism. BMC plant biology **8:** 35

Tzfira T, Citovsky V (2000) From host recognition to T-DNA integration: the function of bacterial and plant genes in the *agrobacterium*-plant cell interaction. Mol. Plant Pathol **1:** 201-212

Tzfira T, Citovsky V (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol **12:** 121-129

Tzfira T, Li J, Lacroix B, Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. Trends Genet **20:** 375-383

Tzfira T, Vaidya M, Citovsky V (2002) Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. Proceedings of the National Academy of Sciences of the United States of America **99:** 10435-10440

Uraji M, Suzuki K, Yoshida K (2002) A novel plasmid curing method using incompatibility of plant pathogenic Ti plasmids in *Agrobacterium tumefaciens*. Genes Genet Syst **77:** 1-9

Uttaro AD, Cangelosi GA, Geremia RA, Nester EW, Ugalde RA (1990) Biochemical characterization of avirulent exoC mutants of *Agrobacterium tumefaciens*. Journal of bacteriology **172:** 1640-1646

Valvekens D, Montagu MV, Lijsebettens MV (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci **85:** 5536-5540

Van Larebeke N, Engler G, Holsters M, Van den Elsacker S, Zaenen J, Schilperoort RA, Schell J (1974) Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability Nature **252:** 169-170

Wiktorek-Smagur A, Hnatuszko-Konka K, Kononowicz AK (2009) Flower Bud Dipping or Vacuum Infiltration—Two Methods of *Arabidopsis thaliana* Transformation Russian Journal of Plant Physiology **56:** 560-568

Wise AA, Liu Z, Binns AN (2006) Culture and maintenance of *Agrobacterium* strains. *In* K Wang, ed, Methods in Molecular Biology: *Agrobacterium* Protocols, Vol 1. Humana Press, Iowa, pp 3-14

Wood DW, Setubal JC, Kaul R, Monks DE, Kitajima JP, Okura VK, Zhou Y, Chen L, Wood GE, Almeida NF, Jr., Woo L, Chen Y, Paulsen IT, Eisen JA, Karp PD, Bovee D, Sr., Chapman P, Clendenning J, Deatherage G, Gillet W, Grant C, Kutyavin T, Levy R, Li MJ, McClelland E, Palmieri A, Raymond C, Rouse G, Saenphimmachak C, Wu Z, Romero P, Gordon D, Zhang S, Yoo H, Tao Y, Biddle P, Jung M, Krespan W, Perry M, Gordon-Kamm B, Liao L, Kim S, Hendrick C, Zhao ZY, Dolan M, Chumley F, Tingey SV, Tomb JF, Gordon MP, Olson MV, Nester EW (2001) The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science (New York, N.Y **294:** 2317-2323

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. The Plant journal **19:** 249-257

Yelin M, Levy A, Tzfira T (2008) The ongoing saga of *Agrobacterium*-host interactions. Trends Plant Sci **13:** 102-105

Zale JM, Agarwal S, Loar S, Steber CM (2009) Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens*. Plant cell reports

Zheng MS, Takahashi H, Miyazakia A, Yamaguchia K, Kusanoa T (2005) Identification of the cis-acting elements in *Arabidopsis thaliana* NHL10 promoter responsible for leaf senescence, the hypersensitive response against Cucumber mosaic virus infection, and spermine treatment. Plant science **168:** 415-422

Zorreguieta A, Geremia RA, Cavaignac S, Cangelosi GA, Nester EW, Ugalde RA (1988) Identification of the product of an *Agrobacterium tumefaciens* chromosomal virulence gene. Molecular plant-microbe interactions **1:** 121-127

Chapter 4

T-DNA transfer and T-DNA integration frequencies after *Arabidopsis thaliana* floral dip and root explant cocultivation

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Contribution to this work: Rim Ghedira performed all practical work related to floral dip transformation, GUS staining and writing of the manuscript. The root explants transformation experiments and regenerants analysis were performed by Sylvie De Buck and Anni Jacobs.

Abstract

We analyzed the T-DNA transfer and integration frequencies during *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* via root and floral dip transformation with and without selection for transformation competent cells. T-DNA transfer was differentiated from T-DNA integration on the basis of the presence or absence of transient CRE recombinase activity. First, we assessed after root explant transformation the influence of the applied light photoperiod during cocultivation on transient expression and stable T-DNA integration. In the absence of selection for transformation competent cells, T-DNA transfer after cocultivation in continuous light conditions was higher compared to cocultivation under the standard 16h light/8h dark photoperiod. However, no effect on T-DNA integration frequencies was observed. When we selected for transformation competent cells, continuous light did not enhance T-DNA cotransfer and T-DNA cointegration. Second, we determined after floral dip transformation to which extent the T-DNA transfer from the bacteria to the plant cell and/or subsequent T-DNA integration into the host genome are limiting transformation frequencies. By PCR screening of 344 T1 seedlings, grown in the absence of selection, T-DNA transient expression without integration could not be observed while the T-DNA integrated in 3 of these T1 seedlings corresponding to a transformation frequency of 0.8%. of the floral dipped progeny. After floral dip cotransformation with two *Agrobacterium* strains and selection for one of the T-DNAs, 8-34% of these transformants showed cotransfer of the other unselected T-DNA. Remarkably, in all but 1% of these transformants, the unselected T-DNA was also integrated. Our data therefore suggest that especially a productive interaction between the agrobacteria and the female gametophyte rather than the T-DNA integration process limits floral dip transformation and cotransformation frequencies.

1. Introduction

Plant transformation mediated by *Agrobacterium* has become the most commonly used method for the introduction of foreign genes into plant cells. *Arabidopsis thaliana* can be transformed using *in vitro* methods such as root and leaf disc tissue transformation (Horsch et al., 1985; Valvekens et al., 1988) or *in planta* transformation methods such as vacuum infiltration and floral dip (Bechtold et al., 1993; Clough and Bent, 1998). The molecular mechanism underlying *Agrobacterium*-mediated plant transformation comprises different steps (Tzfira and Citovsky, 2002; Gelvin, 2003; McCullen and Binns, 2006; Banta and Montenegro, 2008; Gelvin, 2008, 2009). After attachment of the *Agrobacterium* to its host, the T-complex, composed of the T-strand and several VIR proteins, is transferred to the plant cell. Subsequently, the T-DNA migrates towards the nucleus, where it becomes double-stranded and transcribed soon after. Several data suggest that only a small percentage of the transferred and transiently expressed T-DNAs after tissue explant transformation are integrated (Liu et al., 1992; Shen et al., 1993; Kapila et al., 1997; Maximova et al., 1998; De Buck et al., 2000). Maximova et al., (1998) reported that the first marker signal was detected 48 h after infection of apple leaf explants with an agrobacteria harboring the green fluorescence protein (GFP) gene. For root explants transformation, *GUS* staining did not reveal any signal 24 hours after cocultivation, but from 48 hours on, clear signals were observed in the roots (De Buck et al., 2000).

The efficiency of *Agrobacterium*-mediated *in vitro* plant transformation is influenced by bacterial, plant and environmental factors. At the bacterial level, both the density of the bacterial culture and the ability of the used strain to attach to the host and to transfer its T-DNA into the plant cell were reported to influence the *Agrobacterium* transformation efficiency (Shen et al., 1993; Nam et al., 1997; Cheng et al., 2004; Opabode, 2006). At the plant level, the secretion of plant factors inducing the agrobacteria T-DNA transfer system (Gelvin, 2000; Tzfira and Citovsky, 2000; Tzfira et al., 2002), plant cell competence for transformation and regeneration (Potrykus, 1991; Sangwan et al., 1991; Sangwan et al., 1992) and cell cycle stage (Villemont et al., 1997) all contribute to the transformation efficiency. Several environmental factors, such as low temperatures (between 20ºC and 25ºC according to the transformed plant species), low pH, several sugars and continuous light, were

reported to have a positive effect on transient or stable transformation efficiencies (Hiei et al., 1994; Dillen et al., 1997; Arencibia et al., 1998; Hashizume et al., 1999; Salas et al., 2001; Frame et al., 2002; Zambre et al., 2003; Boyko et al., 2009).

After *in vitro* transformation of *Arabidopsis* roots, cotransformation frequencies varied from 10% to 47% when the T-DNAs originated from different bacteria and from 24% to 31% when the T-DNAs originated from the same bacteria (De Buck et al., 1998; De Buck et al., 2000; De Buck et al., 2009). These cotransformation frequencies were always much higher than expected on the basis of the single transformation frequencies, indicating that the cotransformation events rely on the plant cell competence to acquire, integrate and express the incoming T-DNAs rather than the *Agrobacterium* transformation competence. Additionally, De Buck et al., (2000) showed that both T-DNA transfer and T-DNA integration were restricting the transformation yield after root transformation. Indeed, upon regeneration of cocultivated root cells, 4 out of 88 shoots witnessed expression of the T-DNA, but did not integrate it. From this experiment, it can be concluded that the formation of a productive *Agrobacterium*-plant cell interaction is limiting and that the transferred T-DNA copies were not integrated in the 4 shoots. Another informative experiment was to measure the frequency of cotransfer of a second T-DNA in cells selected for a first T-DNA. Approximately 50% of the transformants showed transient expression of the second nonselected T-DNA, demonstrating that especially the number of plant cells that can engage in a productive *Agrobacterium* interaction with the plant cell is limiting the transformation frequency. Moreover, half of the cotransferred T-DNAs were also integrated into the plant genome, resulting in a stable cotransformation frequency of 25% (De Buck et al., 2000).

In planta transformation methods, such as vacuum infiltration and floral dip, are extensively used for *Arabidopsis thaliana* transformation due to their simple, cheap and not time consuming protocol (Clough and Bent, 1998). However, limited information regarding the underlying mechanisms of this transformation process is available. It is known that addition of the surfactant Silwet 77 to the culture medium may improve the floral dip transformation yield (Cheng et al., 2004; Opabode, 2006). Several studies demonstrated that female tissues of the flowers, more specifically the ovules, are the target cells during *Arabidopsis* floral dip transformation and that T-

DNA transfer occurs late in the development of the gametophytes (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000). *Arabidopsis* floral dip transformation frequencies are in general varying between 1% and 3% (Bent, 2006). Cotransformation of different T-DNAs using the floral dip method was reported with frequencies ranging from 16% to 39% for T-DNAs from different bacterial strains and from 20 to 46% for T-DNAs originating from the same *Agrobacterium* (Poirier et al., 2000; Stuitje et al., 2003; Radchuk et al., 2005; De Buck et al., 2009). Hence, it was supposed that the bacterium-plant cell interaction, rather than DNA incorporation in the plant genome, is a limiting factor during transformation (Radchuk et al., 2005). However, it is not known whether a higher percentage of the ovules are transiently transformed but do not integrate the T-DNA. Analysis of floral dip transformants showed that most of them contained multiple T-DNA copies in a single locus. Thus, it was suggested that apart from cotransformation, T-DNA replication before or during integration results in plants with a complex T-DNA integration pattern (De Buck et al., 2009). Therefore, single-copy transformants are obtained with a low frequency after floral dip (De Buck et al., 2004; Marjanac et al., 2008).

A first objective of this study was to assess the influence of light on stable T-DNA integration frequencies during *Arabidopsis* root transformation. Indeed, a previous study demonstrated that continuous light strongly promoted T-DNA transfer from the bacteria to the plant cells (De Clercq et al., 2002; Zambre et al., 2003). If also integration would be promoted, it could lead to higher transformation frequencies especially in recalcitrant species. The second objective of this study is to investigate which parameter is limiting the floral dip transformation frequency. Therefore, we identified the transient expression frequency in ovules versus the transformation frequency after floral dip. The CRE recombinase was used as a reporter for T-DNA transfer and transient expression, while PCR screening was used to assay stable integration of that T-DNA (De Buck et al., 2000). In this way, it could be assessed whether the floral dip transformation efficiency in ovules is affected by both or rather the T-DNA transfer or T-DNA integration.

2. Results

2.1. Experimental strategy to determine the T-DNA transfer and integration frequencies upon root and floral dip transformation

In order to investigate the frequency of T-DNA transfer and/or T-DNA integration upon root and floral dip transformation, a strategy based on *Cre*/lox recombination and described by De Buck et al., (2000) was applied. In this strategy, two different T-DNAs types were used. The K T-DNA harbors the P35S-*GUS* expression cassette surrounded by two tandemly repeated lox sequences, and the neomycin phosphotransferase gene (*NPTII)* expression cassette conferring resistance to kanamycin (Kan) (Figure 1; De Buck et al., 1998). The other T-DNAs carry the *CRE* expression cassette, driven by either the P35S promoter in the C T-DNA or the female germline specific somatic embryogenesis receptor kinase 1 (SERK1) promoter (Hecht et al., 2001) in the HSC-SERK1 and HSLC-SERK1 T-DNAs (Figure 1). The HSC-SERK1 T-DNA carries 2 out of frame ATGs upstream of the *CRE* coding sequence leading to a different CRE expression level compared to the HSLC-SERK1 T-DNA (Van Ex et al., 2009). Additionally, the C T-DNA carries the phosphinotricine acetyl transferase gene (*BAR*) giving rise to phosphinotricin (PPT) resistance, while the HSLC-SERK1 and HSC-SERK1 T-DNAs harbor the hygromyin phosphotransferase (*HPT*) gene conferring hygromycin (Hpt) resistance. Activity of the *CRE* recombinase will lead to the deletion of the P35S-*GUS* cassette, resulting in the formation of the Kd T-DNA (Figure 1).

The activities of both 35S and SERK1 promoters in *Arabidopsis* ovules were tested via a histochemical *GUS* staining of plants transformed with the promoter-*GUS* construct (Figure 3).

The transfer of the *CRE* containing T-DNA resulting in *CRE* activity could be monitored by two different PCR reactions with primer pairs P3+4 and P3+5 (Figures 1 and 2). When no deletion of the target DNA occurred, a fragment of 4431 bp and 1200 bp with P3+4 and P3+5, respectively, could be amplified (Figure 2, lanes 1-4). However, when the *CRE* gene was transiently expressed, deletion of the lox flanked cassette results in the formation of the Kd T-DNA and this can be diagnosed via

amplification of a 834 bp fragment with P3+4 and no PCR product with P25+26 (Figure 2, lane 10). A 734 bp fragment with P25+26 is specific for the integration of the *CRE* containing T-DNA (Figure 1, Figure 2, lanes 5-8). In some transformants of all experiments, both a 834 bp fragment with P3+4 and a 1200 bp fragment using P3+5 could be amplified (sample 9, Figure 2) indicating that deletion of the *GUS* cassette occurred in only one of the K T-DNA alleles. These plants were counted in the category of the C T-DNA being present with *CRE* activity. It should be noted that we measure in this way the minimum frequency of plant cells transiently expressing the T-DNA marker as the assay depends on the successful deletion of the P35S-*GUS* cassette and this may not be always the case if CRE levels are limiting. Indeed, no transfer of the CRE containing T-DNA is concluded when no detection of *CRE* activity and thus no PCR fragment with P25+P26 are observed.

In order to determine the T-DNA transfer and T-DNA integration frequencies after root and floral dip transformation, two approaches were followed. In a first approach, a transgenic line, harboring a single K T-DNA in homozygous condition, was used during cocultivation with an *Agrobacterium* strain harboring the Cre containing T-DNA. Root explants and seeds, obtained after root and floral dip transformation respectively, were grown on non-selective medium and the recovered seedlings were analyzed with the above described PCR reactions. Thus in this approach no selection is applied and the number of plants with transient expression and with a stably integrated T-DNA can be determined versus the total number of plants recovered from the experiment. In a second transformation experiment, the T-DNA transfer and T-DNA integration frequencies were analyzed in transformation-competent cells selected after their transformation by a first T-DNA. Therefore, roots or flowers were cocultivated with a mixture of two *Agrobacterium* strains containing the K and the *CRE* T-DNA. After selection on kanamycin and thus selecting for an accessible plant cell competent to interact with *Agrobacterium* and to integrate the K T-DNA, the seedlings were analyzed via the above described PCR assays for the transfer and the integration of the unselected CRE containing T-DNA.

To assess the influence of light on T-DNA integration frequencies, the coculvation step in both transformation experiments on *Arabidopsis thaliana* C24 root

explants was performed in continuous light and in standard 16h light (L)/8h dark (D) conditions.

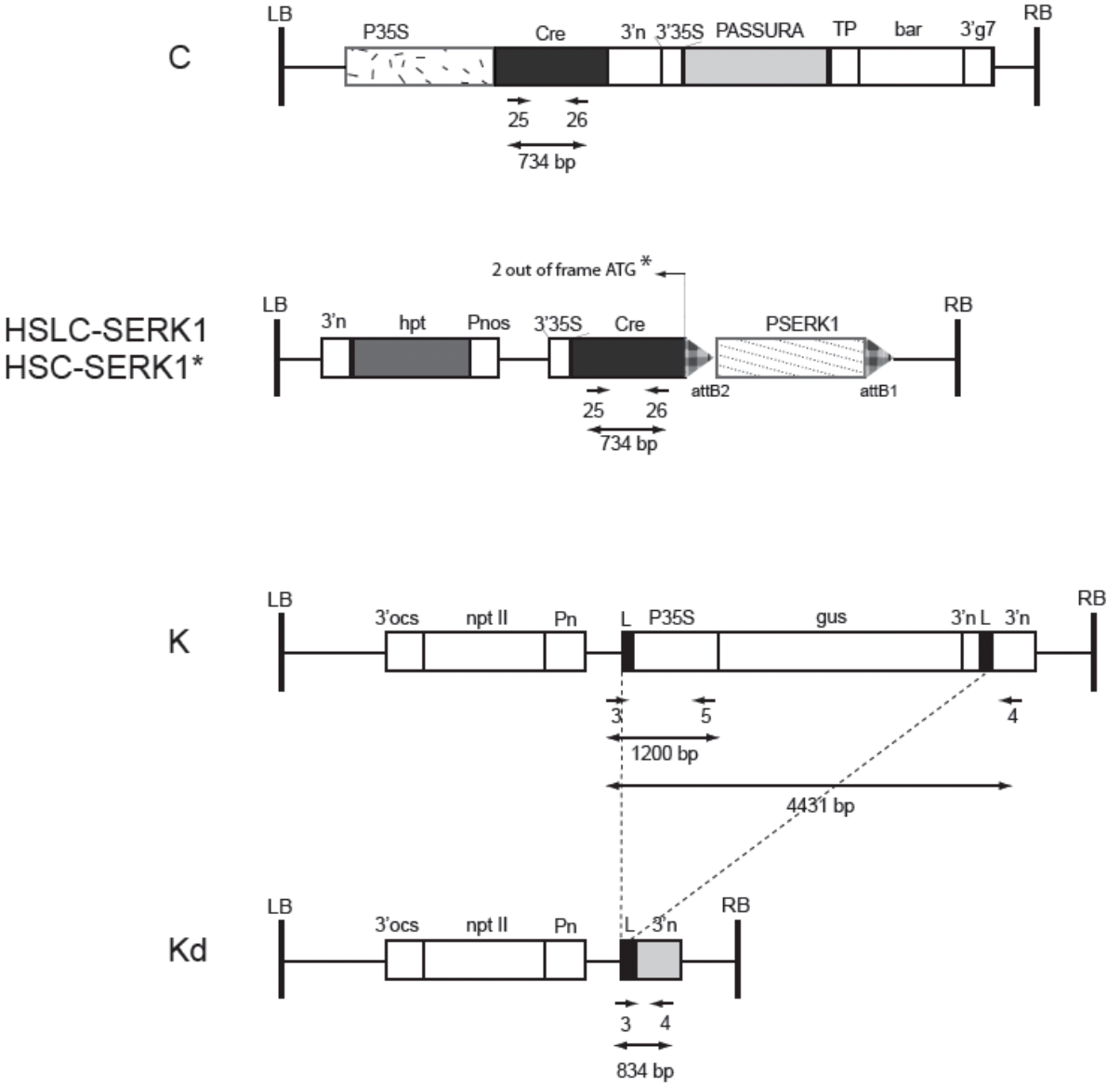

Figure 1: Schematic outline of the HSLC-SERK1, HSC-SERK1, C, K, and Kd T-DNAs (not on scale). *: 2 out of frame ATG upstream the *CRE* gene are present in HSC-SERK1 T-DNA and differentiating it from HSLC-SERK1 T-DNA. Transfer and/or integration of the C T-DNA was monitored by PCR using different primer combinations. Obtained amplicon length using P3+4 and the presence or absence of an amplicon using P3+5 gave an indication concerning the occurrence of the *GUS* cassette deletion in between the tandemly repeated *loxP* sequences. A fragment of 4431 bp and 1200 bp with P3+4 and P3+5 respectively are diagnostic for the presence of the *GUS* expression cassette, while a fragment of 834 bp with P3+4 and no PCR fragment with P3+5 are indicative for a Cre-induced deletion of the P35S-*GUS* cassette. P25+26 was employed to characterize transformants in which the *CRE* gene was integrated.

2.2. Influence of two light regimes during *Arabidopsis* root cocultivation on T-DNA transfer and T-DNA integration frequencies in the regenerated shoots

In order to assess the influence of continuous light on T-DNA integration frequencies, two different *Arabidopsis* root transformations were conducted. Root explants of KH15 (De Buck et al., 1998), a transgenic *Arabidopsis* C24 transformant harboring a resident K T-DNA (Figure 1), were cocultivated with an *Agrobacterium* carrying the C T-DNA (Figure 1). During cocultivation, two light conditions, the standard 16h L/8h D regime and continuous light, were applied and 45 and 42 shoots, respectively, were regenerated on non-selective medium (Table 1). On these shoots, the 3 PCR screening reactions (P3+4, P3+5, P25+26, Figure 2) were carried out to determine the C T-DNA transient expression and C T-DNA integration frequencies. Under continuous light, the C T-DNA was transferred in 7 out of 42 regenerated plants (Table 1). Of these 7 regenerants one was stably transformed and harbored the C T-DNA in its genome (Table 1) while in the other 6 regenerants, transient expression of the C T-DNA was witnessed by the deletion in the resident K T-DNA but the C T-DNA was not integrated. Thus, in these latter 6 shoots the C T-DNA was transferred to the plant cell, transiently expressed, but not integrated (Table 1). After cocultivation in standard light conditions (16h L/8h D), no stably transformed shoots were identified and in only in 1/45 shoots, the *CRE* gene was transiently expressed (Table 1). These results are comparable with the ones previously described by De Buck et al., (2000) (Table 1). We can conclude that under continuous light the T-DNA transfer but not the T-DNA integration is enhanced. This conclusion is supported by the results of the Fisher's exact test. Indeed the *p* value was 0.023 (<0.05) for the influence of continuous light on T-DNA transfer while the *p* value was 0.875 (>0.05) for T-DNA integration under continuous light.

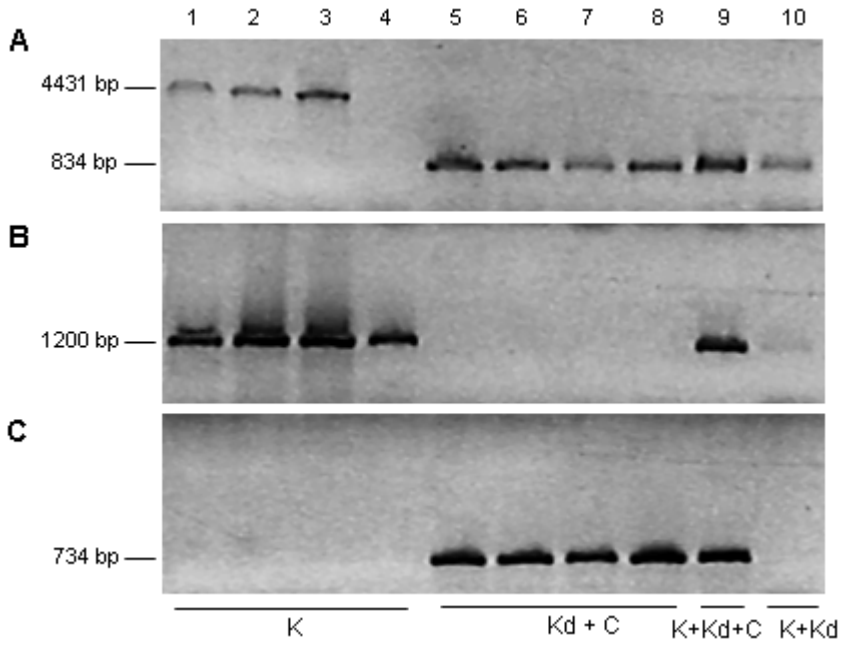

Figure 2: Example of PCR screening reactions for the *CRE*-mediated deletion in the K T-DNA and the integration of the *CRE* containing T-DNA. (a) PCR reaction using P3+4. A fragment of 834 bp is indicative for the deletion of the P35S-*GUS* cassette, while a fragment of 4431 bp is representative for the K T-DNA (b) PCR reaction using P3+5. When the P35S-*GUS* cassette is present in the plant genome, a fragment of 1200 bp will be amplified, while the presence of the Kd T-DNA does not result in an amplicon. (c) PCR reaction using P25+26 result in a fragment of 734 bp when the CRE T-DNA is integrated.

Table 1: Frequencies of regenerants with transiently expressed and/or stably integrated C T-DNAs, obtained after root cocultivation and grown on non-selective medium. Continuous light and 16h L/8h D regimes were applied during root cocultivation

	Continuous light	16h L/8h D	16h L/8h D (De Buck et al. 2000)
Total number of regenerants	42	45	84
No C T-DNA transfer	35[a] (84%)	44[a] (98%)	80 (95%)
C T-DNA transfer (+)	7[a] (16%)	1[a] (2%)	4 (5%)
C T-DNA transfer and no integration	6[b] (14%)	1[b] (2%)	4 (5%)
C T-DNA transfer and integration	1[b] (2%)	0[b] (0%)	0

Percentages (%) are calculated based on the total number of regenerants.
+: total number of regenerants with transferred C T-DNA. [a] values used during Fisher's exact test for the influence of light on T-DNA transfer frequencies. [b] values used during Fisher's exact test for the influence of light on T-DNA integration frequencies

2.3. T-DNA cotransfer and T-DNA cointegration frequencies of a second T-DNA in regenerated transformants selected for the presence of a first T-DNA upon *Arabidopsis* root cocultivation

Root explants of *Arabidopsis thaliana* C24 were cocultivated with a mixture of two *Agrobacterium* strains, harboring either the K or the C T-DNA (Figure 1). The standard and continuous light regimes were applied during cocultivation and regenerants were selected for the presence of the K T-DNA (thus we selected for transformation competent cells). After cocultivation under continuous light, 5/39 (13%) of the K transformants were cotransformed with the C T-DNA (Table 2). However, 22/39 (56%) K transformants did not contain the C T-DNA but harbored the Kd allele, implying that CRE activity had been transiently present. Thus, in 69% (5+22/39) of the K transformants, the C T-DNA was cotransferred but in only 13%, the cotransferred T-DNA was also integrated. After cocultivation under a 16h L and 8h D photoperiod, C T-DNA transfer occurred in 20/35 (57%) K transformants. 4/35 (11%) of the regenerated plants were cotransformed and had both T-DNAs integrated in their genome (Table 2). The C T-DNA was transiently expressed and lost in 46% (16/35) of the K transformants. Fisher's exact test showed that the *p* values obtained for the influence of continuous light on T-DNA transfer (p = 0.201) and T-DNA integration (p = 0.593) were higher than 0.05. Hence we can conclude that after selection for integration of a first T-DNA, the light regime does not increase the cotransfer and cointegration frequencies of the second T-DNA.

Table 2: Frequencies of root transformants, containing a first selected K T-DNA, where a second unselected C T-DNA was cotransferred and/or cointegrated. Continuous light and 16h L/8h D regimes were applied during root cocultivation

	Continuous light	16h L/8h D	16 h L/8 h D (De Buck et al. 2000)
Total number of K transformants	39	35	56
No C T-DNA transfer	12[a] (31%)	15[a] (43%)	32 (57%)
C T-DNA transfer (+)	27[a] (69%)	20[a] (57%)	24 (43%)
C T-DNA transfer and no integration	22[b] (56%)	16[b] (46%)	13 (23%)
C T-DNA transfer and integration (=cotransformation)	(4+1[c])[b] (13%)	4[b] (11%)	11 (20%)

Percentages (%) are calculated based on the total number of K transformants
+: total number of transformants with transferred C T-DNA. [a] values used during Fisher's exact test for the influence of light on T-DNA transfer frequencies. [b] values used during Fisher's exact test for the influence of light on T-DNA integration frequencies. [c] This transformant contain the CRE encoding T-DNA, but the CRE recombinase did not induce a deletion

2.4. 35S promoter activity in the ovules of *Arabidopsis* flowers is lower than the SERK1 promoter activity

To compare the activity of the 35S and the SERK1 promoter in *Arabidopsis* ovules, a histochemical *GUS* staining of *Arabidopsis* flowers, collected before anthesis stage, was performed (Materials and Methods). No GUS activity could be detected in the ovules of transformant FK24, harboring one K T-DNA copy in homozygous condition (Figure 1; De Buck et al., 2004), indicating that the P35S is not active or induces only a low, undetectable GUS activity in *Arabidopsis* female tissues (Figure 3A). The histochemical GUS staining on *Arabidopsis* flowers, harboring the PSERK1-*GUS* cassette, showed a clear *GUS* staining in the ovules, demonstrating the activity of the SERK1 promoter in *Arabidopsis* female tissues (Figure 3B). This result is in accordance with the previously described SERK1 promoter activity in *Arabidopsis* ovules (Hecht et al., 2001). It should be noted however, that GUS staining in the haploid gametophyte, the cell of interest for this study, cannot be identified by this pistil staining experiment.

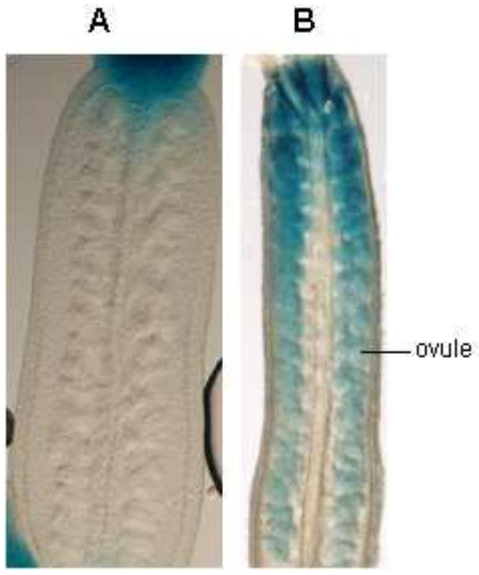

Figure 3. Histochemical *GUS* staining of *Arabidopsis* flower pistils. The 35S promoter is inactive or has a very low activity in *Arabidopsis* ovules (A), whereas the SERK1 promoter is active (B). For Col0, no blue staining was observed (data not shown).
The flowers were collected before anthesis stage. Histochemical *GUS* staining of (a) a flower pistil derived from FK24, a transformant with one K T-DNA copy in homozygous condition, and (b) a plant transformed with the pKGWFS7-SERK1 T-DNA.

2.5. Transient expression versus stable transformation frequencies in the T1 generation after floral dip transformation

To analyze the T-DNA transient expression and integration frequencies into the target ovules upon floral dip transformation, two transgenic *Arabidopsis* FK24 plants (FK24-1 and FK24-2), harboring a resident K T-DNA, were supertransformed using an *Agrobacterium* strain carrying the C T-DNA (Figure 1), and the T1 generation of seeds was harvested. By sowing approximately 1000 seeds on plant growth medium containing PPT, the transformation efficiencies of the C T-DNA were determined to be 0.50% and 0.90% for FK24-1 and FK24-2 respectively (Table 3). In parallel, 200 T1 seeds from each dipped plant were sown on non selective medium. 191 FK24-1 and 153 FK24-2 seedlings were screened via PCR reactions for the transfer and/or the integration of the C T-DNA (Figure 1). In 1/191 (0.5%) and 2/153 (1.3%) seedlings for FK24-1 and FK24-2, respectively, a 834 PCR fragment could be amplified using P3+4 indicating that the deletion of the *GUS* cassette occurred due to the presence of the C T-DNA (Table 3). In the same seedlings, the C T-DNA was integrated because a 734 bp PCR fragment using P25+26 could be amplified (Table 3). Therefore, in none of the FK24-1 and FK24-2 seedlings, transient C T-DNA expression could be detected (Table 3).

These results further demonstrated that the same stable transformation frequency is obtained whether the transformants are selected via their selectable marker or screened on the basis of a PCR diagnostic fragment.

Table 3: Frequencies of T1 seedlings with transiently expressed and/or stably integrated C T-DNAs after floral dip

	FK24-1	FK24-2
Transformation frequency Cre T-DNA[a]	0.5%	0.9%
Total number of T1 seedlings	191	153
No C T-DNA transfer[b]	190 (99.5%)	151 (98.88%)
C T-DNA transfer (+)[b]	1 (0.5%)	2 (1.12%)
C T-DNA transfer and no integration[b]	0 (0%)	0 (0%)
C T-DNA transfer and integration[b]	1 (0.5%)	2 (1.12%)

[a] Frequencies calculated after sowing 1000 T1 seeds on PPT selective medium
[b] Percentages (%) are calculated based on the total number of T1 seedlings

2.6. T-DNA transfer is a limiting step during floral dip transformation

To further determine whether T-DNA integration is not limiting the transformation frequency in floral dip transformation, selected T1 seedlings, transformed with a first T-DNA, were screened for the presence of a second non-selected T-DNA. Therefore, plants were floral dipped with a mixture of agrobacteria containing either the K or C T-DNA (Figure 1) in three biological repeats (FD1 to FD3; Table 4). Transformation frequencies of both T-DNAs, monitored by sowing approximately 1000 seeds on either Kan or PPT selective medium, ranged from 0.38% to 1.05% for the K T-DNA and from 0.03% to 0.72% for the C T-DNA (Table 4). After selection for the presence of the K T-DNA, 11% to 18% of the K seedlings were cotransformed with the unselected C T-DNA, as PCR screening with P25+26 resulted in a fragment of 734 bp in these transformants (Table 4). Not all these K transformants with an integrated C T-DNA showed the deletion of the *GUS* sequence. Indeed, 3% FD1 and 1% FD2 transformants did not show a PCR fragment of 834 bp with P3+4 (Table 4). Finally, in maximum 1% of the K transformants, a PCR fragment of 834 bp with P3+4 and no fragment with P25+26 could be amplified, indicating that the C T-DNA was not integrated but that transient expression of the *CRE* recombinase was sufficient to mediate the deletion. These results therefore indicate that a productive *Agrobacterium*-plant cell contact and/or T-DNA transfer from the bacteria to the plant cell are the limiting steps during floral dip transformation, because less than 1% of the plant cells is transiently expressed with a second unselected T-DNA. Moreover T-DNA integration is very efficient as nearly all transformants (99%) with CRE activity contained the C T-DNA.

Table 4: T-DNA transfer and integration frequencies of an unselected T-DNA upon floral dip transformation in transformants grown on kanamycin-containing medium

	PCre			HSC-Cre		HSLC-Cre	
	FD1	FD2	FD3	FD4	FD5	FD6	FD7
Transformation frequency of Cre T-DNA[a]	0.16%	0.03%	0.72%	1.37%	0.74%	1%	0.57%
Transformation frequency of K T-DNA[a]	1.05%	0.38%	1.02%	0.90%	0.50%	1%	0.57%
Total number of K transformants[b]	98	214	158	161	86	178	97
No C cotransfer[b]	87 (89%)	187 (87%)	128 (81%)	114 (71%)	79 (92%)	117 (66%)	71 (73%)
C transfer (+)[b]	11 (11%)	27 (13%)	30 (19%)	47 (29%)	7 (8%)	61 (34%)	26 (27%)
C cotransfer but no C cointegration[b]	0 (0%)	2 (1%)	1 (1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
C cotransfer and cointegration (=cotransformation)[b]	8+3[c] (8%+3%)	23+2[c] (11%+1%)	29 (18%)	21+26[c] (13%+16%)	3+4[c] (3%+5%)	44+17[c] (25%+9%)	19+7[c] (20%+7%)

[a] Frequencies calculated after sowing 1000 T1 seeds on appropriate selective medium
[b] Percentages calculated based on the total number of K transformants
[c] These transformants contain the CRE encoding T-DNA, but the CRE recombinase did not induce a deletion

Because we could not exclude that a lot of transformation events with a transiently expressed *CRE* recombinase were missed in this experiment due to the low activity of the 35S promoter in the ovules of the *Arabidopsis* flowers, two new cocultivation experiments were performed. Mixtures of agrobacteria containing the K and HSC-SERK1 or the K and the HSLC-SERK1 T-DNA (Figure 1) were used during floral dip cocultivation. Both HSC-SERK1 and HSLC-SERK1 T-DNAs carry an identical PSERK1-*CRE* expression cassette, except that the HSC-SERK1 contains two out-of-frame ATGs upstream of the *CRE* start codon (Van Ex et al., 2009). Two biological repeats per cocultivation were performed (FD4 and FD5 for K + HSC-SERK1 and FD6 and FD7 for K + HSLC-SERK1, Table 4).

The transformation frequencies of the K and the *CRE* containing T-DNAs separately were determined as described earlier using either Kan, PPT or Hyg selective medium. K T-DNA transformation frequencies ranged from 0.50% in FD5 to 1% in FD7, whereas the HSC-SERK1 and HSLC-SERK1 transformation frequencies were comprised between 0.74% and 1.37% and between 0.57% and 1% respectively (Table 4). After selection for the presence of the K T-DNA, cotransformation

frequencies of HSC-SERK1 T-DNA were 8% (FD5) and 29% (FD4), and cotransformation frequencies of HSLC-SERK1 T-DNA were 27% (FD7) and 34% (FD6) (Table 4). These cotransformation frequencies were thus at least 10-times higher than the transformation frequencies of the T-DNAs separately. In 16% (FD4), 5% (FD5), 10% (FD6) and 7% (FD7) transformants, no CRE recombinase activity was monitored, although the *CRE* containing T-DNA was integrated (Table 4).

In none of the K transformants generated in experiments FD4 to FD7, transient expression of the *CRE* recombinase could be detected. Therefore, we can conclude that during floral dip, T-DNA integration is not limiting the transformation frequency implying that besides the ovule accessibility, also the formation of an *Agrobacterium*-plant cell interaction resulting in T-DNA transfer is an important limiting step during floral dip transformation.

3. Discussion

In this study, we analyzed to which extent T-DNA transfer and/or T-DNA integration determine the transformation and cotransformation frequencies upon root and floral dip transformation. For root transformation, the influence of light on both T-DNA transfer and T-DNA integration was analyzed.

Previously, it was shown that continuous light, applied during cocultivation, promoted T-DNA transfer from *Agrobacterium* to *Arabidopsis* root cells (De Clercq et al., 2002; Zambre et al., 2003). Also in the present study, after cocultivation of roots with *Agrobacterium* and regenerating shoots from those roots in the absence of selection, C T-DNA transfer occurred in 7 out of 42 (16%) regenerants after cocultivation performed under continuous light while C T-DNA transfer occurred in only 1/45 regenerants after cocultivation in a 16h L/8h D photoperiod. Of the 7 regenerated shoots with C T-DNA transfer during continuous light cocultivation, one showed stable C T-DNA integration, whereas under 16h L/8h D photoperiod cocultivation, no stable transformant was obtained. These results therefore demonstrate that the application of continuous light during cocultivation is enhancing

T-DNA transfer (p = 0.023; Fisher's exact test) while T-DNA integration after transfer is not affected by the light regime (p = 0.875; Fisher's exact test).

Our results are in accordance with those of De Clercq et al., (2002) and Zambre et al., (2003) showing that application of continuous light during cocultivation of *Arabidopsis* root explants with *Agrobacterium* is promoting T-DNA transfer in the absence of transformation competent cells selection. The promotive effect of light on gene transfer remains unclear, however, as previously discussed in Zambre et al., (2003), light is probably enhancing plant cell competence for *Agrobacterium* attachment and T-DNA uptake rather than increasing *vir* gene inducers production. When selection for transformation competent cells was applied by selecting for the presence of a first T-DNA, T-DNA cotransfer and T-DNA cointegration of the second T-DNA were not further enhanced under continuous light. Indeed, the frequency of shoots in which we detected C T-DNA cotransfer with the K T-DNA after cocultivation under continuous light regime (27/39 representing 69% of the analyzed regenerants) was only slightly higher than the frequency after cocultivation under 16h L/8h D light regime (20/35 representing 57% of the analyzed regenerants). C T-DNA integration under continuous light was detected in 5/39 (13%) regenerants while under 16h L/8 h D, 4/35 (11%) of the regenerants had the C T-DNA integrated in their genome.

T-DNA transfer and T-DNA integration frequencies were determined after floral dip transformation in randomly picked T1 seedlings grown on non selective medium. Via PCR screening, it was found that 3 out of 344 (0.9%) plants had the C T-DNA integrated in their genome showing that selection for a T-DNA upon floral dip transformation is not needed to identify transformants. Additionally, the transformation frequencies determined by selection (0.5% and 0.9%) or by screening (0.5% and 1.12%) were identical, suggesting that the selection marker is only rarely silenced. This conclusion is contradictory to the results reported after analyzing T-DNA/plant DNA junctions generated under non-selective conditions upon *Arabidopsis* cell culture showing that a lot of T-DNAs inserted in heterochromatic regions (Kim et al., 2007). These regions are however disfavored under selective conditions. Similarly, Francis and Spiker, (2005) demonstrated that after floral dip transformation, in 30% of the transformation events identified by PCR the transgene was not expressed. The difference between our observation and the one reported by Francis

and Spiker, (2005) might be due to the fact that these authors analyzed approximately 3000 T1 seedlings while in our experiment only 191 and 153 T1 seedlings were tested.

Subsequently, floral dip T-DNA transfer and T-DNA integration frequencies were determined in competent cells by selecting for a first T-DNA after cocultivation with a mixture of two *Agrobacterium* strains carrying either the K T-DNA or a *CRE* containing T-DNA. The *CRE* gene was driven either by the 35S promoter or by the female germline specific SERK promoter (Hecht et al., 2001 and this study). We showed that P35S has no or very low activity in the ovules which is consistent with the results described by Desfeux et al., (2000) and Jenik and Irish, (2000). In the 7 biological repeats (FD1 to FD7), the cotransformation frequency of a second unselected T-DNA ranged from 8% to 34%, indicating that the *CRE* containing T-DNA was stably integrated in on average 21% of the K transformants. The C T-DNA cotransformation frequencies were in general higher after root transformation (43% to 69%, De Buck et al., 2000 and this study) then after to floral dip transformation (8% to 34%, this study) indicating that for floral dip transformation, the chance for formation of a productive interaction between the *Agrobacterium* strain and the plant cell is lower compared to root transformation. The need for a productive *Agrobacterium*-plant cell interaction to obtain transformants is also illustrated by the results obtained in our laboratory showing that *Arabidopsis thaliana* ecotype C24 floral dipping gives very low transformation frequencies compared to Col0 ecotype although dipping of both ecotypes was performed in the same conditions and using the same bacterial cultures (data not shown). The inefficient floral dip transformation of C24 ecotype compared to Col0 ecotype is probably due to a different morphology and/or physiology of the flower, containing the female gametophyte which is the *Agrobacterium* target during floral dip (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000; Bechtold et al., 2003).

In only 3 of the 470 analyzed K transformants, the 35S-*CRE* containing T-DNA was transiently expressed but not integrated, while this T-DNA was integrated in 65 of those 470 K transformants. These results clearly demonstrate that, upon floral dip transformation, almost 100% of the transferred T-DNAs are integrated in the genome of transformation competent gametophytes. Therefore, especially the formation of a

successful *Agrobacterium*-plant cell interaction resulting in T-DNA transfer seems to limit the transformation of competent target cells. This observation is completely different from the one observed upon *Arabidopsis* root transformation (De Buck et al., 2000, and this analysis). There, 50% of the transformants with a first T-DNA showed transient expression of a second nonselected T-DNA. 50% of these cotransferred T-DNAs are integrated into the plant genome, suggesting that both T-DNA transfer and T-DNA integration are limiting the transformation efficiencies. Recently, we could demonstrate that the transformed target cell determines the complexity of the T-DNA integration pattern (De Buck et al., 2009). Here, our results suggest that the efficiency of T-DNA integration is also determined by the transformed target cell. Indeed, our data indicate that once a T-DNA entered the female gametophyte, it is efficiently integrated in the genome.

During floral dip transformation, T-DNA transfer occurs at the end of the female haploid phase and before fertilization (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000; Bechtold et al., 2003). After fertilization and precisely one hour after karyogamy, the first nuclear division takes place (Faure et al., 2002). This means that the chromatin should condense into chromosomes at the prophase stage and all DNA, present in the nucleus, should “come together”. During the time in-between T-DNA transfer and the beginning of cell division, the T-DNA might become double stranded and integrated (De Neve et al., 1997; Kapila et al., 1997; Krizkova and Hrouda, 1998; De Buck et al., 1999; Chilton and Que, 2003; Tzfira et al., 2003). Moreover, the T-DNA is frequently replicated prior to integration resulting in multi-copy T-DNA integration patterns (De Buck et al., 2009). It is then tempting to speculate that the cellular transcription machinery in the gametophyte is limited by the short time between the karyogamy and the start of nuclear division to have genes transiently expressed during floral dip transformation. However, we could detect *CRE* transient expression in 3 floral dip transformants. Furthermore, in our assay, determination of the T-DNA transfer frequency was based on *CRE* activity. Hence there might be an underestimation of the T-DNA transfer if, the level of CRE was not sufficient for the deletion of the lox flanked sequence.

Unlike the T-DNA transfer and integration frequencies, the cotransformation frequencies are very similar after floral dip and root transformation. The

cotransformation frequencies vary between 8 to 34% upon floral dip transformation and 11 and 20% for the root transformation in 16h light/8hours dark conditions. These frequencies are in the same range as previously reported (Poirier et al., 2000; De Buck et al., 2000; Stuitje et al., 2003; Radchuk et al., 2005; De Buck et al., 2009). Furthermore, for both transformation methods, the cotransformation frequencies are at least 10-fold higher than the transformation frequencies. Indeed, upon floral dip, the transformation frequencies are comprised between 0.03% and 1.37% for the *CRE* containing T-DNA and between 0.38% and 1.05% for the K T-DNA. Upon root transformation, the transformation efficiency is in general below 1%. Therefore, both female gametophytes and root cells, which are able to stabilize a first T-DNA, have at least a 10 fold higher chance to stabilize a second incoming T-DNA. It is clear that accessibility of the target plant cell for transformation is a very important parameter. Previously, we hypothesized that accessibility might be an important parameter for stable transformation to occur upon root transformation (De Buck et al., 1998). The same might be true for the floral dip transformation. Additionally, the window of competence, the time period in which the ovules are susceptible for *Agrobacterium* transformation, has been described to be short. Both Desfeux et al., (2000) and Bechtold et al., (2000) reported that transformants were only obtained from developmentally young flowers that were still 5 or more days from anthesis at the time of inoculation. Similarly, Martinez-Trujillo et al., (2004) concluded that dipping should occur on closed floral buds, and not on opening flowers whereas Wiktorek-Smagur et al., (2009) showed that unopened 1-1,5 mm long flower buds could be transformed, whereas opened 3 mm long flower buds could not. Thus the 99% seedlings in the T1 seedstocks obtained from our floral dipped plants are most likely the result of developed seeds from flowers that were not in the correct time window for *Agrobacterium* entry and transformation. The remaining 1% T1 seedlings are transformed with one T-DNA and thus derived from an ovule from a flower that was accessible. As about 70 to 90% of those transformants were not transformed with a second T-DNA from another *Agrobacterium* also present in the cocultivation mixture, we can derive that also the formation of an *Agrobacterium*-plant cell interaction is limiting the transformation frequency. At the same time we can conclude once the T-DNA is transferred to the plant cell, T-DNA integration occurs very efficiently.

In 0% to 16% of the cotransformants with the K T-DNA and the *CRE* T-DNA, P35S-*GUS* cassette between lox sites was not deleted. This failure in inducing the deletion occurred even slightly more frequently in FD4 (16%) and FD5 (5%) than inducing the deletion (13% and 3% respectively). This could be due to the presence of 2 out of frame ATGs upstream the *CRE* gene leading to low activity of the *CRE* recombinase in the HSC-SERK1 T-DNA (Van Ex et al., 2009). However, in transformation FD6 and FD7, where the HSCL-PSERK1 T-DNA was used, the failure of inducing the deletion lies in the same range (10% and 7%). We also observed that the failure of deletion induction occurred more frequently with the T-DNAs where *CRE* was under the control of PSERK (5% to 16%) than when it was driven by the 35S promoter (0% to 3%). This difference could be due to the low activity of the SERK promoter in *Arabidopsis* leaves (Hecht et al., 2001) in contradiction to the 35S promoter (Odell et al., 1985). Indeed, the PCR screening for the deletion was performed using DNA prepared from leaves and thus the *CRE* mediated excision might have occurred during growth with higher probability when *CRE* was under the control of the P35S then of the PSERK. The failure in inducing the deletion was not observed before in the transformants obtained after root transformation (De Buck et al., 2000), and also here, only one root transformant, obtained after cocultivation in continuous light, did not contain the deletion. Several reasons can explain the lack of CRE mediated deletion of the 35S-*GUS* lox cassette. First of all, the lox sequences may be inaccessible to the *CRE* recombinase due to heterochromatinization of complex T-DNA loci (De Buck et al., 2007; Marjanac et al., 2008; De Paepe et al., 2009). Secondly, as floral dip gives rise to the integration of multiple T-DNA copies, the *CRE* recombinase might be post-transcriptionally silenced (De Buck et al., 2004; De Buck et al., 2009).

Here we confirmed that during root explants transformation in the absence of selection for transformation competent cells, T-DNA transfer into *Arabidopsis* cells is enhanced by the application of a continuous light regime. Also, we identified the interaction between *Agrobacterium* and the plant cell, and therefore the T-DNA transfer, as a limiting step for the commonly used floral dip transformation method.

4. Material and Methods

4.1. Plasmids and constructs

All T-DNAs used in this study are presented in Figure1. K and C T-DNAs were previously described by De Buck et al., (1998) and De Buck et al., (2000). The K T-DNA harbored the neomycin phosphotransferase II (*NPTII*) gene under control of the nopaline synthase promoter (*Pn*) and a β-glucuronidase (*GUS*) expression cassette driven by the cauliflower mosaic virus promoter (P35S) and surrounded by two *loxP* sequences (Dale and Ow, 1990, 1991). The C T-DNA harbored the phosphinothricin phosphotransferase gene (*BAR*) driven by the small subunit promoter of *Arabidopsis* (PSSUARA) and a *CRE* recombinase expression cassette under control of the P35S. Deletion of the P35S-*GUS* cassette, due to the *CRE* recombinase activity, results in the formation of the Kd T-DNA (Figure1).

HSLC-SERK1 and HSC-SERK1 T-DNAs harbored the hygromycin phosphotransferase gene (*HPT*) driven by the Pn promoter and a *CRE* expression cassette under the control of the somatic embryogenesis receptor kinase 1 (SERK1) promoter. The difference between both T-DNAs is the presence in the HSC vector of a suboptimal leader sequence: two ATG codons; both in frame with each other but out of frame with the *bona fide* start codon of the *CRE* gene (Van Ex et al., 2009). All constructs were introduced in the *Agrobacterium* C58C1^{Rif} (pMP90) strain via electroporation.

Plasmid KGWFS7-SERK1 was obtained by transferring the SERK1 promoter into the pKGWFS-7 Destination^{TM} vector (Karimi et al., 2002) upstream of the *GUS;EGFP* reporter genes. Seeds of generated transformants - kindly provided by Frederic Van Ex and Geert Angenon - were used for the histochemical staining of flowers.

4.2. Histochemical GUS staining

To check the activity of the P35S and the SERK1 promoter in the ovules, a histochemical *GUS* staining of flowers collected before anthesis stage from pKGWFS-SERK1 and FK24 plants was performed (De Buck et al., 2004). The pistil length of the examined flowers was approximately 2 mm. The latter is measured at 1 day after anthesis as shown in our laboratory (results not shown). The flowers were washed three times with 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7; Jefferson et al., 1987)) and incubated overnight at 37ºC in 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7) containing 10mM EDTA, 0.5mM K3[$Fe(CN)_6$)], 0.5mM K_4[$Fe(CN)_6$)], 1% DMSO and 50mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) dissolved in DMSO. After that, the seedlings were washed 3 times with 0.1M Na_2HPO_4/NaH_2PO_4 buffer (pH7), destained in 90% ethanol and stored in 70% ethanol. Samples mounted in lactic acid (90%) were examined using a differential interference contrast microscope (Leica).

4.3. *Arabidopsis* floral dip transformation and transgenic plants generation

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col-0) and two transgenic FK24 plants, with a single resident K T-DNA in homozygous conditions (De Buck et al., 2004), were transformed via floral dip as described by Clough and Bent, (1998) (see also Chapter 3 of this thesis, section material and methods). For the cotransformation experiments, the *Agrobacterium* strains were separately grown overnight in YEB medium and mixed before dipping. Comparable inocula of both strains (10^8 bacteria/ml) were used for cocultivation. Seeds from dipped plants were harvested, and 25 mg (≈1000 seeds) were sown on Murashige and Skoog agar medium supplemented with Nystatin (50 mg/l) and Vancomycin (700 mg/l) to kill the agrobacteria and with the appropriate selection (kanamycin (50 mg/l), hygromycin (20 mg/l), or phosphinothricin (10 mg/l).

4.4. *Arabidopsis thaliana* root explants transformation

Transformation of root explants was performed as described by (Valvekens et al., 1988). Experiments were done with either root explants of KH15 (carrying a single copy of the K T-DNA in homozygous conditions (De Buck et al., 1998)) or wild type *A. thaliana* (L.) Heynh. ecotype C24 plants. KH15 was supertransformed using an *Agrobacterium* strain harboring the C T-DNA while C24 roots were cocultivated using a mixture of two *Agrobacterium* strains, one harboring the K T-DNA and the other harboring the C T-DNA (Figure 1). The *Agrobacterium* strains were separately grown overnight in LB medium (without selection) and mixed before cocultivation. Comparable inocula of both strains ($5x10^7$ bacteria/ml) were used for cocultivation. Green calli and shoots were obtained approximately 6 to 8 weeks after cocultivation. These shoots were put on non selective (KH15 roots) and kanamycin (50 mg/l) germination medium after KH15 and C24 transformation respectively. Single shoots from individual calli were placed in separate Falcon (Becton Dickinson, Bedford, MA) tubes to ensure the scoring of independent transformation events.

4.5. Plant DNA preparation and PCR reactions

DNA was prepared from 5 to 6 weeks old *Arabidopsis* leaf material using the DNeasy Plant Mini Kit (Westburg, the Netherlands) or the Gentra PuregeneTM DNA purification system (http://www1.qiagen.com/) according to the manufacturer's protocol. Deletion of the P35S-*GUS* cassette in the K T-DNA was revealed by PCR using primer combinations P3+4 and P3+5 (Figure1). The presence of the T-DNA carrying the *CRE* gene was analyzed by PCR using P25+26 (Figure1). Approximately 70 ng of DNA was incubated with 250 ng of each primer in 1× *Taq* polymerase incubation buffer (Roche Diagnostics, Brussels, Belgium) and 2.5 U of *Taq* polymerase in a final volume of 50 µl. Samples were heated to 94℃ for 5 min before PCR. For all primer combinations, denaturation was performed at 94℃ for 45 s. For P3+4 and P3+5, annealing occurred at 55℃ du ring 1 min and the extension reaction at 72℃ for 5 min. For P25+26, the anneali ng was done at 57℃ during 1 min and the extension reaction at 72℃ for 1 min. Seque nce of the primers from 5' to 3' is as follows: primer 3, TGATCCTGTTTCCTGTGTGAAATT; primer 4,

TTGTAAGGAGATGCACTGATTTAT; primer 5, ATTTGCGGCCGCTTTAATAGTAAATTGTAATGTTGT; primer 25, TCCTTAGCGCCGTAAATCAATCGAT; and primer 26, GATCGCCAGGCGTTTTCTGAGCATA.

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References

Arencibia AD, Carmona ERC, Tellez P, Chan MT, Yu SM, Trujillo LE, Oramas P (1998) An efficient protocol for sugarcane (Saccharum spp. L) transformation mediated by *Agrobacterium tumefaciens*. Transgenic Res **7:** 213-222

Banta LM, Montenegro M (2008) *Agrobacterium* and plant biotechnology. *In* T Tzfira, V Citovsky, eds, *Agrobacterium*: From Biology to Biotechnology. Springer, New York, pp 73-148

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Paris, Life Sciences **316:** 1194-1199

Bechtold N, Jaudeau B, Jolivet S, Maba B, Vezon D, Voisin R, Pelletier G (2000) The maternal chromosome set is the target of the T-DNA in the in planta transformation of *Arabidopsis thaliana*. Genetics **155:** 1875-1887

Bechtold N, Jolivet S, Voisin R, Pelletier G (2003) The endosperm and the embryo of *Arabidopsis thaliana* are independently transformed through infiltration by *Agrobacterium tumefaciens*. Transgenic research **12:** 509-517

Bent A (2006) *Arabidopsi thaliana* floral dip transformation method. *In* K Wang, ed, Methods in Molecular Biology: *Agrobacterium* Protocols, Vol 1. Humana Press, Iowa

Boyko A, Matsuoka A, Kovalchuk I (2009) High frequency *Agrobacterium tumefaciens*-mediated plant transformation induced by ammonium nitrate. Plant cell reports

Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. In Vitro Cell. Dev. Biol. **40:** 31-45

Chilton MD, Que Q (2003) Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. Plant Physiol **133:** 956-965

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant journal **16:** 735-743

Dale EC, Ow DW (1990) Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase. Gene **91:** 79-85

Dale EC, Ow DW (1991) Gene transfer with subsequent removal of the selection gene from the host genome. Proceedings of the National Academy of Sciences of the United States of America **88:** 10558-10562

De Buck S, De Wilde C, Van Montagu M, Depicker A (2000) Determination of the T-DNA transfer and the T-DNA integration frequencies upon cocultivation of *Arabidopsis thaliana* root explants. Molecular plant-microbe interactions **13:** 658-665

De Buck S, Jacobs A, Van Montagu M, Depicker A (1998) *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Molecular plant-microbe interactions **11:** 449-457

De Buck S, Jacobs A, Van Montagu M, Depicker A (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. The Plant journal **20:** 295-304

De Buck S, Peck I, De Wilde C, Marjanac G, Nolf J, De Paepe A, Depicker A (2007) Generation of Single-copy T-DNA *Arabidopsis* Transformants by the CRE/loxP Recombination-mediated Resolution System. Plant Physiol **145:** 1171-1182

De Buck S, Podevin N, Nolf J, Jacobs A, Depicker A (2009) The T-DNA integration pattern in Arabidopsis transformants is highly determined by the transformed target cell. The Plant journal **60:** 134-145

De Buck S, Windels P, De Loose M, Depicker A (2004) Single-copy T-DNAs integrated at different positions in the *Arabidopsis* genome display uniform and comparable beta-glucuronidase accumulation levels. Cellular and molecular life sciences **61:** 2632-2645

De Clercq J, Zambre M, Van Montagu M, Dillen W, Angenon G (2002) An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius A. Gray* (tepary bean). Plant Cell Rep. **21:** 333-340

De Neve M, De Buck S, Jacobs A, Van Montagu M, Depicker A (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. The Plant journal **11:** 15-29

De Paepe A, De Buck S, Hoorelbeke K, Nolf J, Peck I, Depicker A (2009) High frequency of single-copy T-DNA transformants produced by floral dip in CRE-expressing Arabidopsis plants. The Plant journal

Desfeux C, Clough SJ, Bent AF (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. Plant physiology **123:** 895-904

Dillen W, De Clerq J, Kapila J, Zambre M, Van Montagu M, Angenon G (1997) The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant J **12:** 1459-1463.

Faure JE, Rotman N, Fortune P, Dumas C (2002) Fertilization in *Arabidopsis thaliana* wild type: developmental stages and time course. The Plant journal **30:** 481-488

Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SE, Li B, Nettleton DS, Pei D, Wang K (2002) *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol **129:** 13-22

Francis KE, Spiker S (2005) Identification of *Arabidopsis thaliana* transformants without selection reveals a high occurrence of silenced T-DNA integrations. The Plant journal **41:** 464-477

Gelvin SB (2000) *Agrobacterium* and Plant Genes Involved in T-DNA Transfer and Integration. Annu Rev Plant Physiol Plant Mol Biol **51:** 223-256

Gelvin SB (2003) Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiology and molecular biology reviews **67:** 16-37, table of contents

Gelvin SB (2008) *Agrobacterium*-mediated DNA transfer, and then some. Nature biotechnology **26:** 998-1000

Gelvin SB (2009) Agrobacterium in the genomics age. Plant Physiol **150:** 1665-1676

Hashizume F, Tsuchiya T, Ugaki M, Niwa Y, Tachibana N, Kowyama Y (1999) Efficient *Agrobacterium*-mediated transformation and the usefulness of a synthetic GFP reporter gene in leading varieties of japonical rice. Plant Biotechnol. **16:** 397-401

Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt ED, Boutilier K, Grossniklaus U, de Vries SC (2001) The *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol **127:** 803-816

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant journal **6:** 271-282

Horsch RB, Fry GE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A Simple and General Method for Transferring Genes into Plants. Science (New York, N.Y **227:** 1229-1231

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO journal **6:** 3901-3907

Jenik PD, Irish VF (2000) Regulation of cell proliferation patterns by homeotic genes during *Arabidopsis* floral development. Development (Cambridge, England) **127:** 1267-1276

Kapila J, Rycke RD, Montagu MV, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. Plant science **122:** 101-108

Karimi M, Inze D, Depicker A (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci **7:** 193-195

Kim SI, Veena, Gelvin SB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. The Plant journal **51:** 779-791

Krizkova L, Hrouda M (1998) Direct repeats of T-DNA integrated in tobacco chromosome: characterization of junction regions. The Plant journal **16:** 673-680

Liu CN, Li XQ, Gelvin SB (1992) Multiple copies of virG enhance the transient transformation of celery, carrot and rice tissues by *Agrobacterium tumefaciens*. Plant molecular biology **20:** 1071-1087

Marjanac G, De Paepe A, Peck I, Jacobs A, De Buck S, Depicker A (2008) Evaluation of CRE-mediated excision approaches in *Arabidopsis thaliana*. Transgenic research **17:** 239-250

Martinez-Trujillo M, Limones-Briones V, Cabrera-Ponce JL, Herrera-Estrella L (2004) Improving Transformation Efficiency of *Arabidopsis thaliana* by Modifying the Floral Dip Method Plant Molecular Biology Reporter **22:** 63-70

Maximova SN, Dandekar AM, Guiltinan MJ (1998) Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. Plant molecular biology **37:** 549-559

McCullen CA, Binns AN (2006) *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annual review of cell and developmental biology **22:** 101-127

Nam J, Matthysse AG, Gelvin SB (1997) Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. Plant Cell **9:** 317-333

Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313:** 810-812

Opabode J (2006) *Agrobacterium*-mediated transformation of plants: emerging factors that influence efficiency. Biottechnology and Molecular Biology Review **1:** 12-20

Poirier Y, Ventre G, Nawrath C (2000) High-frequency linkage of co-expressing T-DNA in transgenic *Arabidopsis thaliana* transformed by vacuum-infiltration of *Agrobacterium tumefaciens*. Theor Appl Genet **100:** 487-493

Potrykus I (1991) Gene transfer to plants: assessment of published approaches and results. Annual review of plant physiology and plant Molecular biology and evolution **42:** 205-225

Radchuk VV, Thi Van D, Klocke E (2005) Multiple gene co-integration in Arabidopsis thaliana predominantly occurs in the same genetic locus after simultaneous in planta transformation with distinct Agrobacterium tumefaciens strains. Plant Sci **168:** 1515-1523

Salas MC, Park SH, Srivatanakul M, Smith RH (2001) Temperature influence on stable T-DNA integration in plant cells. Plant cell reports **20:** 701-705

Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan BS (1992) Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. Planta **188:** 439-456

Sangwan RS, Bourgeois Y, Sangwan-Norreel BS (1991) Genetic transformation of *Arabidopsis thaliana* zygotic embryos and identification of critical parameters influencing transformation efficiency. Molecular & general genetics **230:** 475-485

Shen WH, Escudero J, Schlappi M, Ramos C, Hohn B, Koukolikova-Nicola Z (1993) T-DNA transfer to maize cells: histochemical investigation of beta-glucuronidase activity in maize tissues. Proceedings of the National Academy of Sciences of the United States of America **90:** 1488-1492

Stuitje AR, Verbree EC, van der Linden KH, Mietkiewska EM, Nap JP, Kneppers TJ (2003) Seed-expressed fluorescent proteins as versatile tools for easy (co)transformation and high-throughput functional genomics in Arabidopsis. Plant Biotechnol J **1:** 301-309

Tzfira T, Citovsky V (2000) From host recognition to T-DNA integration: the function of bacterial and plant genes in the *agrobacterium*-plant cell interaction. Mol. Plant Pathol **1:** 201-212

Tzfira T, Citovsky V (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol **12:** 121-129

Tzfira T, Frankman LR, Vaidya M, Citovsky V (2003) Site-specific integration of *Agrobacterium tumefaciens* T-DNA via double-stranded intermediates. Plant physiology **133:** 1011-1023

Tzfira T, Vaidya M, Citovsky V (2002) Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. Proceedings of the National Academy of Sciences of the United States of America **99:** 10435-10440

Valvekens D, Montagu MV, Lijsebettens MV (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci **85:** 5536-5540

Van Ex F, Verweire D, Claeys M, Depicker A, Angenon G (2009) Evaluation of seven promoters to achieve germline directed Cre-lox recombination in Arabidopsis thaliana. Plant cell reports

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Sangwan-Norreel BS (1997) Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of Petunia: Evidence of an S-phase control mechanism for T-DNA transfer Planta **201:** 160-172

Wiktorek-Smagur A, Hnatuszko-Konka K, Kononowicz AK (2009) Flower Bud Dipping or Vacuum Infiltration—Two Methods of *Arabidopsis thaliana* Transformation Russian Journal of Plant Physiology **56:** 560-568

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. The Plant journal **19:** 249-257

Zambre M, Terryn N, De Clercq J, De Buck S, Dillen W, Van Montagu M, Van Der Straeten D, Angenon G (2003) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta **216:** 580-586

Chapter 5

Stability of the T-DNA flanking regions in transgenic *Arabidopsis thaliana* plants under influence of abiotic stress and cultivation practices

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Contribution to this work: Rim Ghedira performed the amplification of the 2kb fragments, the RFLP analysis, contributed to the sequencing reactions and the writing of the manuscript.

Abstract

Genetic transformation is often associated with different rearrangements of the plant genome at the site of insertion. Therefore the question remains whether these T-DNA insertion sites are more prone to genotoxic stresses. Here, we studied the impact of propagation through generations, the influence of gene stacking and of photo oxidative stress caused by high light intensity on the stability of the transgene flanking regions in the model plant *Arabidopsis thaliana*. Conformational Sensitive Capillary Electrophoresis (CSCE), Restriction Fragment Length Polymorphism (RFLP) and sequencing were deployed in this analysis in order to study the proximal 100 bp and the long range T-DNA flanking sequences. By screening seven transgenic lines no evidence for occurrence of mutation events were found, implying that the flanking regions of the studied T-DNA insertion events are relatively stable.

1. Introduction

The stability of the transgene and the sequences flanking the T-DNA is an important asset for the approval of genetically modified plants. The stable inheritance of the transgene throughout several generations is a concern for risk assessment, food labeling, traceability and post-release monitoring. Special attention is given to the sequences flanking the T-DNA, which are specific for each transgenic event and based on these sequences, PCR assays are designed to identify these events. The latter is implemented in the EU GMO legislation (EC, 2004).

Integration of the T-DNA into the plant genome is a process usually accompanied by small or large rearrangements at the insertion locus. Complex rearrangements such as duplications of large regions (Tax and Vernon, 2001), large scale deletions (Nacry et al., 1998), reciprocal translocations (Forsbach et al., 2003), presence of filler DNAs (Windels et al., 2003) or insertion of organel DNA (Szabados et al., 2002) were observed in *Arabidopsis thaliana*. Rearrangements associated with the transformation process were also reported for other species such as aspen and rice (Kumar and Fladung, 2002; Kim et al., 2003) and some commercial transgenic events. For instance, the risk assessment dossiers of maize events Mon863 and NK603 reported respectively an insertion of chloroplast (EFSA, 2003) and mitochondrial DNA (EFSA, 2004) close to the integrated transgenic DNA.

Post-transformation changes in the nucleotide sequence of the transgenic plants also can not be excluded. Point mutations such as small deletions and base pair substitutions can occur as a result of the adaptive response of the plants to the environmental conditions (Koorneef et al., 2004). For instance, Ogasawara et al., (2005) showed that point mutations did occur in the coding region of Roundup Ready soybean transgenic insert.

The impact of point mutations can be significant and depends on the region in which they occur. Mutation events occurring in particular motifs in the promoter regions can lead to a change in promoter activity. Point mutations in the coding sequence can result in inactivation of the coding sequence or cause amino acid substitutions leading to leaky or inactive transgene products (Ogasawara et al.,

2005). Mutations occurring in the plant-T-DNA junctions could interfere with event-specific PCR based assays and thus would cause a problem with detection and/or quantification of a transgenic event in GM plants or derived products. Our previous study showed that the plant-T-DNA junctions in *Arabidopsis* transgenic lines subjected to callus induction and regeneration are not prone to an increase of point mutations, although these conditions are considered to be highly mutagenic (Papazova et al., 2006). However, mutation frequencies (Kovalchuk et al., 2000; McCallum et al., 2000) could increase depending on environmental growth conditions. For example photo-oxidative stress caused by overexposure to UV light significantly increases the point mutation frequency in *Arabidopsis thaliana* transgenic sequences (Kovalchuk et al., 2000).

In the present study, we analyze the stability of the proximal flanking regions of *Arabidopsis thaliana* transgenic lines after propagation through 4 generations or subjected to the production of stacked transgenic lines or exposed to photo oxidative stress after high light intensity. Detection of the genetic variation can be done by means of different methods and recently several new technologies for mutation detection working in a sensitive and high throughput manner were developed (for review see Yeung et al., 2005). Here we used a combination of Conformational Sensitive Capillary Electrophoresis (CSCE), Restriction Fragment Length Polymorphism (RFLP) and sequencing in order to detect point mutations and larger rearrangements in the T-DNA flanking regions.

2. Results

2.1. Detection of point mutations and large scale re-arrangements in long range T-DNA flanking regions in transgenic lines at fourth generation

Four transgenic single copy *Arabidopsis* lines (CK2L7, CK2L36, FH16 and FH14; Theuns et al., 2002; Windels et al., 2003; De Buck et al., 2004) were self pollinated and grown until fourth generation. Approximately 2 kb fragment up- and/or downstream the T-DNA/plant junction of the analyzed events was amplified (material

and methods). The presence of large insertions/deletions in the regions upstream and downstream from the transgenic insert was investigated by comparison of the length of 2 kb amplicons. Further, to improve the sensitivity of the analysis to detect small changes in the nucleotide structure, the obtained amplicons were subjected to restriction enzyme analysis. Vector NTI (Invitrogen) mapping and *in silico* gel patterns were used as pilots for our experiments.

The lengths of experimentally obtained amplicons from the tested transgenic lines (left and right junctions for CK2L7 and CK2L36, left junctions for both FH14 and FH16) were compared with the expected PCR fragment length. The comparative analysis revealed no changes in the length of the flanking regions of the transgenic lines compared with the length of the corresponding locus in the original non-transformed ecotype (Col0 or C24) (data not shown). These results indicate the absence of rearrangements such as large insertions or deletions that can be detected as a change in the length of the amplified nucleotide sequence.

To analyze the presence of small nucleotide changes in the 2kb flanking regions, the experimentally obtained RFLP patterns of the studied transgenic lines were compared on the one hand with an *in silico* predicted pattern and on the other hand with the experimentally obtained patterns of the wild type ecotypes. An example of comparison is shown in Figure 1.

Considering the used enzymes, the number of the recognition sites and the number of nucleotides per restriction sites we analyzed 3.28% of CK2L7, 7.33% of CK2L36, 3.85% of FH14 and 1.89% of FH16 flanking sequences. The comparative analysis showed that the digestion patterns of amplified DNA from wild-type were similar to those from the flanking regions in the transgenic lines and both confirmed the expected in silico pattern (Figure 1). These results indicate that no small rearrangement (deletions, insertions or base pair substitutions) leading to loss of restriction site occurred neither in the DNA surrounding the transgenic insert nor in the pre-integration locus of the corresponding original ecotypes Col0 and C24. As the analyzed plants were grown in the presence of the appropriate selective marker only the plants carrying the transgene were analyzed.

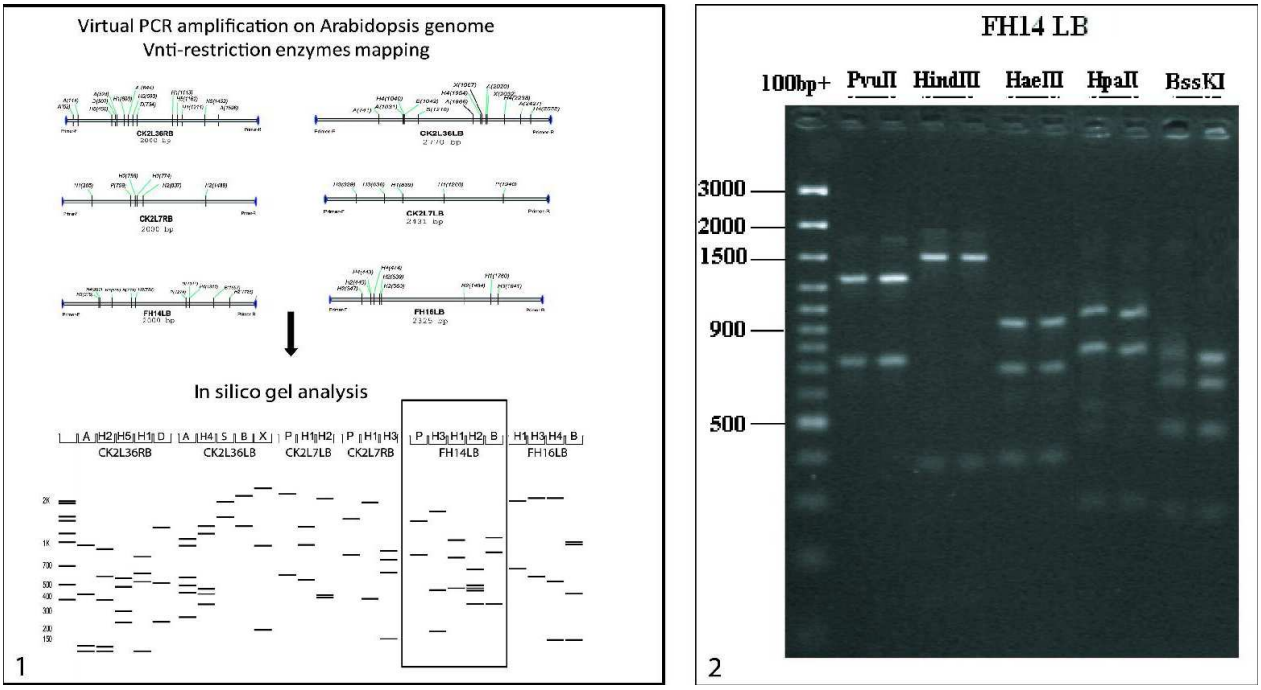

Figure 1: Comparative analysis of the *in silico* obtained RFLP patterns with the experimentally obtained patterns of the left border flanking sequence in line FH14. 1. *In silico* predicted restriction patterns for the flanking regions of each event. The pattern of the FH14 left flanking sequence is indicated in a rectangle. 2. Experimentally obtained pattern after digestion with *Pvu*II, *Hind*III, *Hae*III, *Hpa*II, *BssK*I of the FH14 left flanking region. After digestion reactions, the wild type DNA was loaded (lane a) followed by the correspondent transgenic event DNA (lane b).

2.2. Optimization of a heteroduplex based procedure for SNP detection

In a heterozygous individual, the presence of single or multiple SNPs can be detected as follows: during a PCR using specific primers two alleles will be amplified in a 1:1 ratio. After denaturation and re-annealing different duplex DNA molecules will be formed: the amplicons with the same sequence will only form homoduplex which are specific for the ‘‘wild-type’’ and the molecule containing mismatch and the amplicons with different sequences will form heteroduplexes containing mismatch base pair(s). When the re-annealed PCR products are injected to the capillary array containing CAP® polymer, the homoduplexes will migrate as single major electrophoretic peaks, while the heteroduplexes under semi-denaturating conditions should show an altered mobility and will migrate as a minor electrophoretic peaks.

We simulated this situation using the *Arabidopsis thaliana* ecotypes Col0 and C24 for which SNPs have been described. The ability to detect one single base pair

substitution was tested using the 447bp fragment described in Table 1. PCR reactions were carried out on Col0 and C24 independently. These PCR products were either subjected to CSCE as such or in mixture. The homoduplexes of Col0 and C24 fragments were visualized as single fluorescent peaks with different mobilities (not shown). In the pattern of the mixed PCR product multiple peaks were observed. The peaks correspond to the homoduplexes of Col0 and of C24 and the two possible heteroduplexes (Figure 2).

Table 1: Optimization of the CSCE – characteristics of the primers and amplicons.

Primer pair	Primers (5'-3')	SNP Col/C24 (position of the SNP in the amplicon (bp))	BAC clone/ Position of the SNP in the BAC clone	Amplicon size (bp)
SNP1aFor SNP1Rev	attgcaaagaagatgagacttggcaattgg tgaaactgggttcttgtgattg (FAM)	A/C (315)	T9J23/19921	447
SNP2For SNP2Rev	ccggaaacactttcagtttca tcgaatccatacctcttcgag (FAM)	A/C (178) TA/CC (185, 186)	T9I1 69665/69672-69673	452

Similarly, the detection of more complex patterns such as multiple SNPs was tested on a 452 bp fragment containing three base pairs substitutions (Table 1). For this fragment multiple peaks were detected on the basis of either Col0 or C24, perhaps due to the presence of more than one base pair substitution located close to each other. However, the mixed amplicons gave a different pattern than the homoduplexes as additional peaks corresponding to the heteroduplex molecules were observed (not shown).

To determine the detection limit of a SNP in pooled samples, PCR products were generated using DNA extracted from plant materials in which the two ecotypes Col0 and C24 had been mixed according to different ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 and 1:9 Col0:C24). After CSCE on the 447 bp DNA fragment, a multi-peaks pattern displaying the Col0 and C24 homoduplexes and the two extra heteroduplexes peaks was observed. The number and the position of the peaks remained unaltered in all the pools and the same pattern was detectable even in the

highest dilution ratio (1:9), although the height of the heteroduplex and Col0 homoduplex peaks was very low (Figure 2).

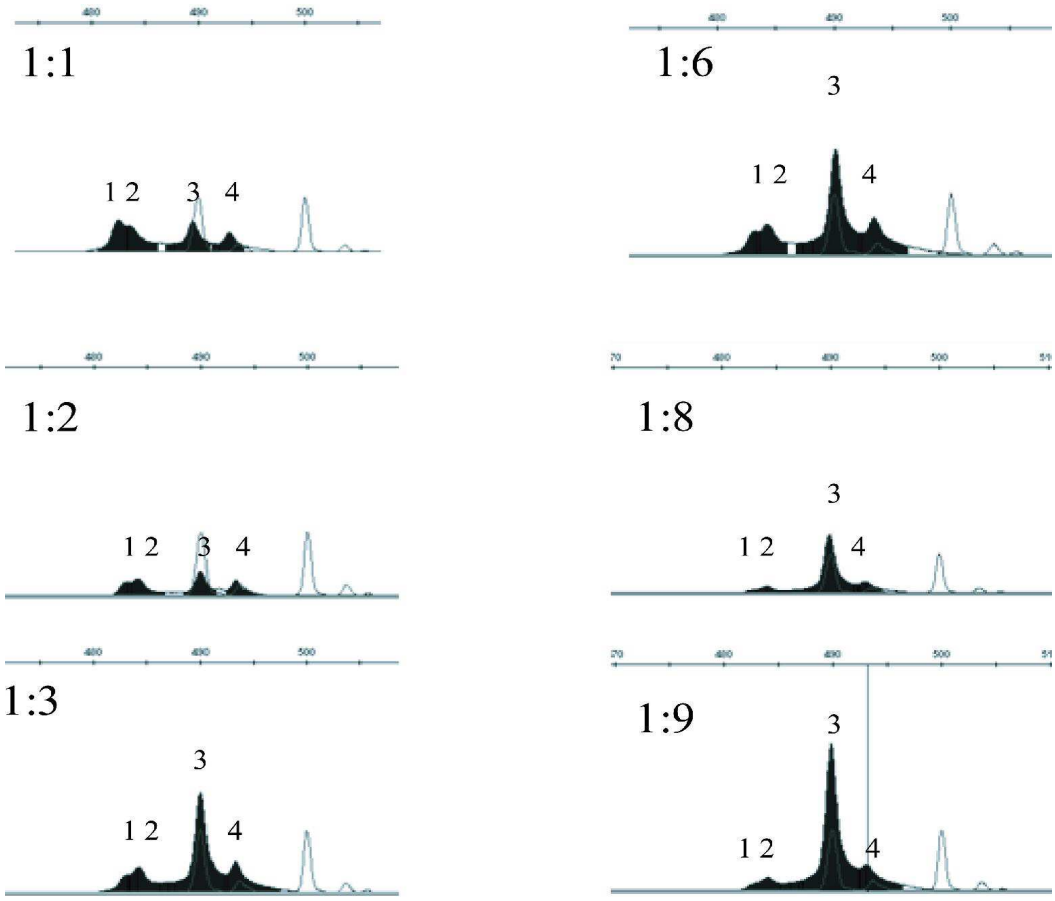

Figure 2: Optimization of SNP detection procedure. Plotted are the fluorescence patterns after separation in the capillary array. Peaks 2 and 3 correspond to the homoduplexes of Col0 and C24 respectively. Peaks 1 and 4 correspond to the heteroduplexes. Note that one of the heteroduplexes has a mobility close to this of Col0 homoduplex. The peaks height changed according to the proportion of the different molecules present in the mixture: as expected the height of the C24 homoduplex peak increased as the pooling ration increased and was the highest in ratio 1/9. Accordingly, the heights of the Col0 homoduplex and of the heteroduplex peaks decreased.

2.3. SNPs detection in the plant T-DNA junctions of gene stacks

The F1 progeny of three transgenic lines (FH14, FH21, FH33) pollinated with one pollinator (FK24) was analyzed either by sequencing or by CSCE (Table 2). The left junction of line FH14 and both left and right junctions of the pollinator line FK24 were sequenced. The junction regions of FH33 and FH21 were scanned for SNPs by

means of CSCE by pooling of six or seven plants. The sequence analysis of the left and right junction of line FK24 used as pollinator showed uniformity in all of the analyzed plants from the three F1 crosses. The same was observed for the left junction of line FH14. No polymorphisms were detected either by sequencing or by CSCE analysis.

Table 2: Summary of the analyzed nucleotide sequence of plant-T-DNA junctions in *Arabidopsis* transgenic lines with stacked genes

Line/Junction	Length of the reference[a] (bp)	Length of the characterized plant junction[b] (bp)	Total No of analyzed individuals	Total size of the analyzed sequence (bp)	Analytical method
FK24 left	430	350	32	11200	sequencing
FK24 right	286	286	30	8580	
FH14 left	61	61	5	305	sequencing
FH21 left 1	342	342	11	3762	CSCE
FH21 left 2	229	229	11	2519	
FH33 left	30	9	10	90	CSCE
FH33 right	28	28	14	392	

[a] Length of the plant DNA flanking the transgenic insert junction as it is determined in the primary transformants; [b] Length of the plant junction region amplified and characterized either by sequencing or CSCE

2.4. Stability of the plant-T-DNA junctions in transgenic lines grown under oxidative stress conditions

The occurrence of nucleotide variability in the plant T-DNA junctions of the transgenic plants grown under high light intensity (material and methods) was investigated by means of a direct sequencing approach (lines CK2L7 and FK24) and the optimized procedure for CSCE (line CK2L148) (Table 3).

Table 3: Summary of the analyzed nucleotide sequence of plant-T-DNA junctions in *Arabidopsis* transgenic lines exposed to photo oxidative stress

Line/Junction	Length of the reference[a] (bp)	Length of the characterized junction[b] (bp)	Number of progenies	Total individuals per F1 progeny	Total size of the analyzed sequence (bp)	Analytical method
CK2L7 left	95	95	3	45	4275	sequencing
CK2L7 right	210	95		45	9450	
FK24 left	430	350	3	40	14200	sequencing
FK24 right	286	286		40	11400	
CK2L148 left	140	139	3	45	6255	CSCE
CK2L148 right	34	34		45	1530	

[a] Length of the plant DNA flanking the transgenic insert junction as it is determined in the primary transformants; [b] Length of the plant junction region amplified and characterized either by sequencing or CSCE

In the totally sequenced 13,725 and 25,600 bp junction regions respectively in 45 CK2L7 and in 40 FK24 plants derived from three different treated plants no alteration of the nucleotide sequence was observed.

The amplified left and right junction fragments of CK2L148 individual plants were pooled together and subjected to heteroduplex formation. No additional peaks were observed in any of the DNA pools and we can therefore conclude that within the total scanned 7,785 bp of CK2L148 junction regions no alteration of the nucleotide sequence has occurred (Table 3).

3. Discussion

Many studies show that different rearrangements are associated with the application of both natural and physical plant transformation methods in the model plant *Arabidopsis thaliana* and some crops (Gorbunova and Levy, 1997; Szabados et al., 2002; Forsbach et al., 2003; Kohli et al., 2003; Windels et al., 2003; Nakano et al., 2005). These rearrangements were separated into two types: those introduced at the site of transgene insertion, called *insertion-site mutations*, and those introduced

at other random locations, which are *genome wide mutations* (Latham et al., 2006). All the available data in the literature describe these numerous mutations and rearrangements which result as a consequence of the transformation itself, but less is known about the post transformation stability of the T-DNA insert and its flanking regions. Here we aimed at studying the post-transformation stability of the insertion locus in transgenic lines through successive generations and when exposed to abiotic stress conditions such as oxidative stress caused by high light intensity or in the situation of gene stacking.

Self-pollination is a practice often used to transmit the transgenic insert through subsequent generations. Our results show that also in the fourth generation, the proximal flanking regions remain unchanged up to 2kb upstream and downstream of the T-DNA. Also comparison of the pre-insertion locus and the flanking regions in the self-pollinated transgenic lines did not reveal any evidence of pre-existing locus instability arising during plant self-propagation.

Also cross-pollination, used to combine two or more transgenic inserts in one genome, did not have an effect on the stability of the stacked events. The transgenic T-DNA junctions of the combined T-DNA loci did not show any sequence alteration as result of interaction at DNA level.

Finally, we screened the plant-T-DNA junctions of 3 transgenic lines grown under oxidative stress conditions. No evidence for the occurrence of point mutations in the T-DNA flanking regions was obtained. Thus, it is unlikely that high light intensity dramatically increases the mutation frequency in the flanking sequences of a transgenic event. Similar observations were made in our previous study in which the stability of the plant T-DNA junctions of the same events after tissue culture stress was investigated (Papazova et al., 2006). Tissue culture is known to induce somaclonal variation in plant genome (Larkin and Scowcroft 1981). Indeed, tissue culture induced mutations are reported for plant species such as maize and sugarcane (Brettel et al., 1986; Dennis et al., 1987; Brown et al., 1991; Zucchi et al., 2002; Carmona et al., 2005). Variations comprise chromosomal rearrangements, single gene mutations, activation of transposons and retrotransposons, alteration of the methylation pattern, base pair substitutions and small deletions (reviewed in Phillips et al., 1994; Madlung and Comai 2004). In Papazova et al., (2006), five single

locus transgenic *Arabidopsis* lines and their respective wild type ecotypes were subjected to callus induction as described by Valvekens et al. (1988). After sequencing of the T-DNA plant junction fragments in the obtained regenerants, no evidence for nucleotide sequence alteration was found (Papazova et al., 2006).

Our results show that the application of normal cultivation practices or enhancing the intensity of abiotic factors in most cases will not lead to dramatic changes in the stability of the transgene flanking sequences.

The spontaneous mutation frequency detected in *Arabidopsis* genome ranges 10^{-6}–10^{-7} bp in coding regions (Kovalchuk et al., 2000; McCallum et al., 2000). With the applied methodology, 39325bp were sequenced and we could not detect mutation rate lower than 1/40 000. Thus, the mutation frequency tested in this study was not increased above 1/40000.

In addition we optimized the CSCE technique as a high throughput SNP screening technique which has potential application for detection of genetic variability in the plant-T-DNA junctions. The optimized heteroduplex based procedure for SNP detection is sensitive and allows high throughput, comparable with other heteroduplex-based methods such as denaturing high-performance liquid chromatography (dHPLC) or CelI assay. A homozygous mutation is detectable in pool of 10 wild type plants in *Arabidopsis* by dHPLC (McCallum et al., 2000), while by the CelI heteroduplex assay, a mutant can be detected in a pool of 8 plants (Colbert et al., 2001). The CSCE method can be applied to analyze small genomic amplified PCR fragments with a length up to 450-500 bp. The optimized method can be alternatively used to monitor the sequence stability of the transgenic construct or junction fragments in a high throughput manner.

In conclusion, the combination of methods used in this study provides an approach that can be applied to analyze stability of the transgene and its flanking regions in function of risk assessment and monitoring purposes in commercial transgenic events.

4. Materials and Methods

4.1. Plant materials propagation and treatments

Arabidopsis thaliana ecotypes Col0 and C24, from which the transgenic lines studied were originally derived, were used as controls.

All seven transgenic events used in this study are derived from single copy *Arabidopsis* lines (Theuns et al., 2002; Windels et a., 2003; De Buck et al., 2004) and were produced via *Agrobacterium* mediated transformation either of Col0 or C24 ecotypes with plasmids pK2L610 and pH610 (De Buck et al., 1998). The plasmids contain neomycin phosphotransferase (*nptII*) (pK2L610) and hygromycin phosphotransferase gene (*Hpt*) (pH610) used as selectable markers for the transformation.

4.1.1. Self pollination

Four subsequent generations from four transgenic *Arabidopsis* lines (CK2L7, CK2L36, FH16 and FH14) were grown as follows: 15 plants from homozygous seed stocks from each transgenic line were grown. Each plant was separately grown and self pollinated up to second generation. Then the seed stocks of all 15 plants for each transgenic line were combined and further 15 plants were grown and self pollinated. The same scheme was applied to produce the seed stocks of the fourth generation of each transgenic line, which was further used for the analysis.

4.1.2. Production of stacked gene lines

The stacked gene lines were produced by cross pollination of three transgenic lines (FH14, FH33, and FH21) with one pollinator (FK24). The hybrid seeds were harvested and further grown on standard germination medium containing 50mg/l kanamycin and 20mg/l hygromycin in order to ensure the presence of both T-DNA inserts. These F1 plants were transferred in a green house after 4-6 weeks and grown four weeks more.

4.1.3. Highlight stress

Seeds from each transgenic line were sown on a germinating medium and grown to a stage before flowering then the plants were transferred to a phytotron where they were grown for one day at light intensity 300µmol/m^2/s, and subsequently exposed for seven days to get a higher light intensity (600 µmol/m^2/s). During this period the plants flowered. Ten plants per line were allowed to form their seeds under normal light conditions. The seeds from each treated plant were harvested. Ten to fifteen seeds from each line were sown on standard germinating medium containing 50mg/l kanamycin (for the lines transformed with pK2L610: CK2L7) and 20mg/L hygromycin (for the lines transformed with pH610: FK24). After 4-6 weeks the plants were transferred to a green house and leaf samples for DNA preparation were harvested 4 weeks later.

4.2. Sampling and DNA preparation

Individual leaf samples were taken from Col0, C24 and the different transgenic *Arabidopsis* plants. Four to five leaves were lyophilized and ground to fine powder. For reconstruction experiments, leaves with equal size from Col0 and C24 were harvested and pooled in ratios from 1/1 to 1/9 (Col0/C24). DNA was extracted according to the method described by Dellaporta et al., (1983).

4.3. RFLP analysis

For the RFLP experiments and based on the sequence data of the different junctions (Theuns et al., 2002; Windels et al., 2003; De Buck et al., 2004), primers were designed using Primer express software (Applied Biosystems). Each primer pair was designed to amplify approximately 2 kb fragment up- and downstream the T-DNA/plant DNA junction of each event (Table 4).

Table 4: Sequence of the primers used to amplify ~2kb plant DNA flanking the left or the right T-DNA borders.

Line	Border	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon length (bp)
CK2L7	Right	cctctcttgctttatctgaatgctg	ggtttgcacgtttagaagttaatcaa	2,000
	Left	tgtccgcagttagtccgatgc	ttaaacgttgtcacatcacgcatt	2,431
CK2L36	Right	cggagaagtataaatcaaagccgc	cacttggcctcagacctatgacc	2,000
	Left	cttcaagaacgtcatgggggttag	tggcaagatgagtggaacaggta	2,770
FH14	Left	tgagctgcatagtcatccaaatggt	ggattcaaacccattggcaaaga	2,000
FH16	Left	gggtagtgtcgaacgggtattcttg	tttggcaactttggcttttaatgc	2,325

4.3.1. *In silico* analysis

The specificity of the primers was first tested by means of the VPCR 2.0 (Lexa and Valle, 2003). Amplified sequences were introduced into Vector NTI 9.0 (Invitrogen) for *in silico* analysis. Restriction mapping was done on each sequence using the following restriction enzymes *Alu*I, *BssK*I, *Dra*I, *Hha*I, *Hpa*II, *Hind*III, *Hae*III, *Pvu*II, *Sal*I, and *Xho*II.

4.3.2. Long range PCR reactions

The PCR reactions were conducted in 20 µl containing 200ng of the extracted plant DNA, 1X PCR-buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl and 1.5 mM MgCl2), 200 µM dNTPs mix, 0.25 µM of each forward and reverse primer, 2 units AmpliTaq polymerase (Applied Biosystems). The reactions were run on PE9700 thermocycler (Perkin Elmer), using the following program: 4 min at 94℃ initial step followed by 35 cycles 30 s at 94℃, 45 s at 58℃, 2 min at 72℃, o ne terminal cycle of 10 min at 72℃ and holding step at 4℃.

4.3.3. Restriction enzyme digestion and agarose gel analysis

All amplified fragments were subjected to *Alu*I, *BssK*I, *Dra*I, *Hha*I, *Hpa*II, *Hind*III, *Hae*III, *Pvu*II, *Sal*I, *Xho*II digestion (Invitrogen). The reactions were performed in final volume of 10µl containing 5 *µl* of the PCR product, 1.5 units of each restriction enzyme, 1X compatible reaction buffer. The mixture was incubated for 2 hours at 37℃. The fragments were separated by agarose gel e lectrophoresis under the following conditions: 1.5% agarose gel, 1 hour at 100V. The separated fragments

were visualized after ethidium bromide staining by means of Kodak UV-Camera (Kodak).

4.4. Optimization of the Conformational Sensitive Capillary Electrophoresis (CSCE)

4.4.1. Primers

To optimize the CSCE a 447bp fragment containing a single base pair substitution between the Col0 and C24 ecotypes and a 452 bp fragment containing multiple base pair substitutions between Col0 and C24 were chosen. These sequences were identified from *Arabidopsis* Information Resource (TAIR) database (www.arabidopsis.org) (Table 1).

For each transgenic line a primer pair amplifying a part of the T-DNA and the flanking plant DNA was designed. One of the primers was universal and bound the T-DNA close either to the left or to the right border. The T-DNA primers had the following sequence: KHLB+277 5'–CCCCCATTTGGACGTGAATGTAG–3', KHLB+47 5'–ACTCATTGGTGATCCATGTAGATTT–3', for the left border; and KHRB+87 5'-ACCTCAATTGCGAGCTTTCTAATTT-3' for the right border. A second primer binding the plant DNA flanking the T-DNA insert was used in combination with a transgenic primer (Table 5). These primers were designed using the primer3 program (Rozen and Skaletsky, 2000). The same primers were used to sequence the target amplicons.

Table 5: Primer pairs used to amplify plant-T-DNA junctions in the transgenic *Arabidopsis* lines

Line	Junction	T-DNA primer	Plant primer	Amplicon length (bp)
CK2L7	Left	KHLB+277	tgatcgttgaaagcctagctcgt	406
	Right	KHRB+87	cctccgaggttttacaaacattca	541
CK2L148	Left	KHLB+277	tgatcaccatcgaaattggtta	419
	Right	KHRB+87	tacctctggatccgtcactgtatct	207
FK24	Left	KHLB+47	ggacccattcaacaacccttttg	487
	Right	KHRB+87	cttgaccaacaccgatagttac	416
FH33	Left	KHLB+277	ttagtatcatcaactgatgcaatttt	289
	Right	KHRB+87	aggccataacaggctatgacaaaat	296
FH14	Left	KHLB+277	ggtcgtgaagtctgagaatgg	416
FH21	Left1	KHLB+47	cattaaacgcaactgataggtgtt	418
	Left2	KHLB+47	gcccaaataaaggcctaatagtg	299

4.4.2. PCR conditions

PCR was performed in a 25 µl volume containing 30-50ng DNA from either transgenic lines or wild type *Arabidopsis* ecotypes Col0 and C24, 300µM from both forward and reverse primers, 200µM dNTPs, 1U AmpliTaq DNA polymerase (Applied Biosystems). Thermal cycling was performed on GenAmp® PCR system 9700 (Applied Biosystems) under the following conditions: initial denaturation at 94℃ for 3 min, followed by 35 cycles of denaturation at 94℃ – 30 sec; primer annealing at 57℃ or 59℃ (depending on the primer pair) for 40s , and elongation at 72℃ for 40s to 1 min depending on the amplicon size. The final extension reaction was performed at 72℃ for 10 min, after which the reaction was he ld at 4℃. The PCR products were used either for sequencing or for CSCE. Before sequencing, the PCR products were purified by using QIAquick® PCR purification kit (QIAGEN).

4.4.3. CSCE

After the PCR amplification, heteroduplexes were formed under the following conditions: 96℃-10 min; 72 cycles (96 ℃ $\rightarrow$ 60 ℃ (-0.5 ℃/cycle) 20 seconds), 60℃-30 min, then cooled down to room temperature. The homo- and heteroduplexes were electrophoretically separated at 18 ℃ for 250 0 sec, at voltage 10 KVolt using an ABI3100-*Avant* Genetic Analyzer and its upgraded version ABI3130 Genetic Analyzer (PE Applied Biosystems). The instrument has a capacity of 96 samples automatically injected at 10kV for 10 sec to a 60 cm long diameter (ø) 50µm capillary

filled with the sieving matrix Conformational Analysis Polymer (CAP®, Applied Biosystems). This polymer is composed of 5% native polymer diluted with TTE glycerol tolerant buffer, 4M urea, 4% sucrose. The PCR fragments were 5' FAM labeled. The relative position of the fluorescent peaks was determined by comparison with an internal 500 ROX Standard (Applied Biosystems). The level of fluorescence was monitored by diluting the samples from 120 to 1000 times. The fluorescent peak patterns were analyzed by using GeneMapper 3.7. (Applied Biosystems).

4.5. Sequencing

Sequencing reactions were done using the BigDye™ Deoxy Terminator Sequencing Kit v.1.1 (Applied Biosystems) according to the manufacturer's instructions. Sequencing reactions were denaturated at 96℃ for 1 min; and injected to 30 cycles: 96℃ – 10s, 50℃ – 5s, 60℃ – 4 min. Excess dye-terminators are removed by ethanol precipitation. The sequencing products are dissolved in formamide and denaturated for 8 min. Then the samples are loaded and separated on ABI3130-*Avant* Genetic analyzer system. For the lane tracking and first pass base calling was used Sequencing Analysis software v.5.2. (Applied Biosystems). For the single nucleotide polymorphism (SNP) detection, the sequences are assembled and aligned against reference sequence by means of SeqScape software v.2.5 (Applied Biosystems).

References

Brettel RIS, Dennis ES, Scowcroft WR, Peacock WJ (1986) Molecular analysis of a somaclonal mutant of maize alcohol dehydrogenase. Mol Gen Genet **202:** 235-239

Brown PTH, Gobel E, Lortz H (1991) RFLP analysis of *Zea mays* callus cultures and their regenerated plants. Theor Appl Genet **81:** 227-232

Carmona ER, Arencibia AD, Lopez J, Simpson J, Vargas D, Sala F (2005) Analysis of genomic variability in transgenic sugar cane plants produced by *Agrobacterium* mediated transformation. Plant Breed **124:** 33-38

Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S (2001) High-throughput screening for induced point mutations. Plant Physiol **126:** 480-484

De Buck S, Jacobs A, Van Montagu M, Depicker A (1998) Agrobacterium tumefaciens transformation and cotransformation frequencies of Arabidopsis thaliana root explants and tobacco protoplasts. Mol Plant Microbe Interact **11:** 449-457

De Buck S, Windels P, De Loose M, Depicker A (2004) Single-copy T-DNAs integrated at different positions in the Arabidopsis genome display uniform and comparable beta-glucuronidase accumulation levels. Cell Mol Life Sci **61:** 2632-2645

Dellaporta S, Wood J, Hicks JB (1983) A plant DNA mini preparation: version 2. Plant Mol Biol Rep **1:** 19-22

Dennis ES, Brettel RIS, Peacock WJ (1987) A tissue culture induced *Adh1* null mutant of maize results from a single base pair change. Mol Gen Genet **210:** 181-183

EC (2004) on detailed rules for implementation of Regulation (EC) No1829/2003 of the European Parliament and the Council as regards the application and authorization of new genetically modified food and feed, the notification of existing products and adventitious and technically unavoidable presence of genetically modified material which has benefited from a favorable risk evaluation. *In* No641/2004. Off J Eur Com

EFSA (2003) Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the Notification (Reference CE/ES/00/01) for the placing on the market of herbicide-tolerant genetically modified maize NK603, for import and processing, under Part C of Directive 2001/18/EC from Monsanto. *In*. EFSA J, pp 1-13

EFSA (2004) Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation. *In*, Vol 102. OJ L, pp 14-25

Forsbach A, Schubert D, Lechtenberg B, Gils M, Schmidt R (2003) A comprehensive characterization of single-copy T-DNA insertions in the Arabidopsis thaliana genome. Plant Mol Biol **52:** 161-176

Gorbunova V, Levy AA (1997) Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. Nucleic Acids Res **25:** 4650-4657

Kim SR, Lee J, Jun SH, Park S, Kang HG, Kwon S, An G (2003) Transgene structures in T-DNA-inserted rice plants. Plant Mol Biol **52:** 761-773

Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P (2003) Transgene integration, organization and interaction in plants. Plant Mol Biol **52:** 247-258

Koorneef M, Alonso-Blanco C, Vreugdenhil D (2004) Naturally Occurring Genetic Variation in *Arabidopsis thaliana*. Annu Rev Plant Biol **55**

Kovalchuk I, Kovalchuk O, Hohn B (2000) Genome-wide variation of the somatic mutation frequency in transgenic plants. EMBO J **19:** 4431-4438

Kumar S, Fladung M (2002) Transgene integration in aspen: structures of integration sites and mechanism of T-DNA integration. Plant J **31:** 543-551

Larkin PJ, Scowcroft WR (1981) Somaclonal variation-a novel source of variability from cell cultures for plant improvement. Theor Appl Genet **60:** 197-214

Latham JR, Wilson AK, Steinbrecher RA (2006) The mutational consequences of plant transformation. J Biomed Biotechnol **2005:** 25376

Lexa M, Valle G (2003) PRIMEX: rapid identification of oligonucleotide matches in whole genomes. Bioinformatics **19:** 2486-2488

Madlung A, Comai L (2004) The effect of stress on genome regulation and structure. Ann Bot **94:** 481-495

McCallum CM, Comai L, Greene EA, Henikoff S (2000) Targeted screening for induced mutations. Nat Biotechnol **18:** 455-457

Nacry P, Camilleri C, Courtial B, Caboche M, Bouchez D (1998) Major chromosomal rearrangements induced by T-DNA transformation in Arabidopsis. Genetics **149:** 641-650

Nakano A, Suzuki G, Yamamoto M, Turnbull K, Rahman S, Mukai Y (2005) Rearrangements of large-insert T-DNAs in transgenic rice. Mol Genet Genomics **273:** 123-129

Ogasawara T, Chikagawa Y, Arakawa F, Nozaki A, Itoh Y, Sasaki K, Umetsu H, Watanabe K, Akiyama H, Maitani T, Toyoda M, Kahemada H, Goda Y, Ozeki Y (2005) Frequency of mutations, which might resulting the loss of the glyphosate-tolerant phenotype, was lowered in Roundup Ready® soybeans. J Health Sci **51:** 197-201

Papazova N, Windels P, Depicker A, Taverniers I, Roldan-Ruiz I, Milcamps A, Van Bockstaele E, Van Den Eede G, De Loose M (2006) Sequence stability of the T-DNA - plant junctions in tissue culture in Arabidopsis transgenic lines. Plant Cell Rep **25:** 1362-1368

Phillips RL, Kaepler SM, Olhoft P (1994) Genetic instability of plant tissue culture: breakdown of normal controls. Proc Natl Acad Sci USA **91:** 5222-5226

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. *In* S Krawetz, S Misener, eds, Bioinformatics methods and protocols: methods in molecular biology. Humana, Totowa, pp 365-386

Szabados L, Kovacs I, Oberschall A, Abraham E, Kerekes I, Zsigmond L, Nagy R, Alvarado M, Krasovskaja I, Gal M, Berente A, Redei GP, Haim AB, Koncz C (2002) Distribution of 1000 sequenced T-DNA tags in the Arabidopsis genome. Plant J **32:** 233-242

Tax FE, Vernon DM (2001) T-DNA-associated duplication/translocations in Arabidopsis. Implications for mutant analysis and functional genomics. Plant Physiol **126:** 1527-1538

Theuns I, Windels P, De Buck S, Depicker A, Van Bockstaele E, De Loose M (2002) Identification and characterization of T-DNA inserts via T-DNA fingerprinting. Euphytica **123:** 75-84

Valvekens D, Van Montagu M, Van Lisebettens M (1988) *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants using kanamycin selection. Proc Natl Acad Sci USA **85:** 5536-5540

Windels P, De Buck S, Van Bockstaele E, De Loose M, Depicker A (2003) T-DNA integration in Arabidopsis chromosomes. Presence and origin of filler DNA sequences. Plant Physiol **133:** 2061-2068

Yeung AT, Hattangadi D, Blakesley L, Nicolas E (2005) Enzymatic mutation detection technologies. Biotechniques **38:** 749-758

Zucchi MI, Arizono H, Morais VA, Fungaro MHP, Vieira MLC (2002) Genetic instability of sugarcane plants derived from meristem cultures. Genet Mol Biol **25:** 91-96

Chapter 6

Assessment of primer/template mismatch effects on real-time PCR amplification of target taxa for GMO quantification

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Contribution to this work: Rim Ghedira conducted all practical work. Statistical analysis were performed by Marnik Vuylsteke. The body of this chapter was entirely written by Rim Ghedira.

Abstract

GMO quantification, based on real-time PCR, relies on the amplification of an event-specific transgene assay and a species-specific reference assay. The uniformity of the nucleotide sequences targeted by both assays across various transgenic varieties is an important prerequisite for correct quantification. Single nucleotide polymorphisms (SNPs) frequently occur in the maize genome and might lead to nucleotide variation in regions used to design primers and probes for reference assays. Further, they may affect the annealing of the primer to the template and reduce the efficiency of DNA amplification. We assessed the effect of a minor DNA template modification, such as a single base pair mismatch in the primer attachment site, on real-time PCR quantification. A model system was used based on the introduction of artificial mismatches between the forward primer and the DNA template in the reference assay targeting the maize starch synthase (*SSIIb)* gene. The results show that the presence of a mismatch between the primer and the DNA template causes partial to complete failure of the amplification of the initial DNA template depending on the type and location of the nucleotide mismatch. With this study we show that the presence of a primer/template mismatches affects the estimated total DNA quantity to a varying degree.

1. Introduction

The release and use of genetically modified organisms (GMOs) are regulated in the European Union (EU) and worldwide. In the EU, the traceability and labeling legislation currently applied established a labeling threshold level of 0.9% for the presence of genetically modified (GM) material in food and feed products (EC, 2003 a and b). As part of the authorization dossier of any new GMO, an analytical method for GMO analysis has to be provided (EC, 2003a; EFSA, 2004). The method is evaluated and validated by the Community Reference Laboratory (CRL). The European Commission (EC) recommends to express a relative (%) GMO content on the basis of haploid genome equivalents (HGE) of the GM-target relative to the taxon or species target (EC, 2004). The recommended % GMO unit is calculated as the quantity of GM target divided by the total quantity of the species-specific target, i.e. reference sequence (EC, 2004). Therefore, generally a GMO content is measured at the DNA level and real-time polymerase chain reaction (real-time PCR) is the accepted and widely used technique for this purpose.

Real-time PCR GMO quantification is based on two separate amplification assays: an event-specific assay amplifying the target GMO event and a reference assay amplifying a plant taxon specific region. The latter is thus used to estimate the number of haploid genome equivalents of the plant species or ingredient. Real-time PCR amplification plots show a lag phase, an exponential phase and a plateau phase. Quantification occurs during the exponential phase of amplification when the fluorescence signal exceeds the detection threshold value, commonly referred to as crossing threshold cycle (C_T). At an appropriate point in the exponential phase of amplification, C_T values are measured for both the event-specific and the reference assay. Quantification can be done using a direct comparison of C_T values (ΔC_T method) or by calculating the absolute DNA target quantities, derived from a calibration curve consisting of calibrants or standards in absolute DNA target quantities (standard curve method) (Lie and Petropoulos, 1998; Holst-Jensen et al., 2003; Bubner and Baldwin, 2004).

Globally, maize (*Zea maize)* is an economically important crop. To date, several GM maize events have been developed and are used for food and feed

production (www.agbios.com/dbase.php). Maize is the second major biotech crop after soybean with a cultivation area of 37.3 million hectares representing 30% of the global biotech area in 2008 (James, 2008).

The maize genome is highly diverse and variations at the nucleotide level are commonly observed between different maize inbred lines (Buckler et al., 2006). Single base pair substitutions occur more frequently than insertions/deletions (indels) of one or more nucleotides. Indeed, Vroh Bi et al., (2006) reported that SNPs and indels occur in maize coding genes every 73 bp and 309 bp, respectively. Bhattramakki et al., (2002) identified 655 indels by sequencing 502 loci across eight maize inbred lines. Single-base indels accounted for more than half of the identified indels and occurred with a frequency of 44.28 indel per 10 kb followed by two- and three-nucleotide indels which occurred with a frequency of 11.22 and 10.48 indels per 10 kb respectively (Bhattramakki et al., 2002). The analysis of the sequence diversity in 21 loci distributed along maize chromosome 1 showed that two randomly selected sequences have, on average, one single base pair substitution every 104 bp (Tenaillon et al., 2001). More detailed studies on single nucleotide polymorphisms (SNPs) present in 18 maize genes in 36 maize inbred lines, representing the genetic diversity in the U.S.'s elite maize breeding pool, demonstrated the high rate of nucleotide variation in maize: 1 polymorphism per 31 bp in non-coding regions and 1 per 124 bp in coding regions (Ching et al., 2002).

SNPs may interfere with GMO quantification. If a SNP occurs in the sequence targeted by the primers used for the quantitative real-time PCR assay, a mismatch with the DNA template will be formed. This mismatch will destabilize the primer-DNA template duplex and will lead to a less efficient amplification of the PCR product (Kwok et al., 1990; Huang et al., 1992; Day et al., 1999; Ayyadevara et al., 2000), resulting in either a decrease in the estimated template quantity or even a complete failure of the amplification. Consequently, the inaccurate estimation of the DNA template quantity amplified by the reference assay will lead to inaccurate quantification of the GMO content. If the mismatch occurs in the reference gene sequence, this will likely lead to an overestimation of the GMO content, while if it occurs in the event-specific sequence, this will lead to an underestimation. As allelic variation is more likely to occur in endogenous genomic sequences than in transgenic sequences, GMO content overestimation may occur more often than

GMO content underestimation. This, in turn, might affect the labeling of the product especially in cases where the GMO content is near the labeling threshold of 0.9%.

In this study we evaluate the effect of single base pair substitutions on the real-time PCR amplification in a reference assay commonly used for GMO quantification in maize. Our choice for single base pair substitutions arises from the fact that they represent the most frequent sequence variants, compared to multiple base pair substitutions or indels, in the maize genome. In principle, two approaches could be followed: (1) testing a series of DNA templates (genomic DNA or cloned fragments) carrying different single base pair substitutions in the primer attachment site, in combination with a fixed primer pair, or (2) testing a fixed DNA template in combination with a series of primers carrying single base substitutions. Bru et al., (2008) showed that a single mismatch carried either by the template sequence or by the primer sequence will have a similar effect on the real-time PCR. For two reasons we choose to mimick the presence of single base pair substitutions in the genomic DNA template by replacing base pairs at different positions of the forward primer targeting the maize reference assay *SSIIb*, while using a fixed reverse primer. First, a series of maize genomic DNA carrying different single base pair substitutions specifically located in the primer attachment site of the *SSIIb* sequence is not available to conduct our study. Second, using a single common genomic DNA extract as input for all reactions allows direct comparison of quantitative data, and circumvents the need for any additional normalization for the template quantity input across such a series of independent DNA extracts.

A set of plasmid DNA calibrator solutions, each containing a precisely known number of DNA target copies, were used to establish real-time standard curves for absolute quantification. We quantified the *SSIIb* gene in two types of biological material, the conventional B73 maize inbred line and the certified reference material (CRM) 5% Mon810, using 16 different primer combinations representing various types and positions of the base pair mismatch in the primer attachment site. The effects of the mismatch type and position on the measured C_T values and estimated DNA template quantities were assessed.

2. Results

To assess the effect of a primer/template mismatch on real-time PCR amplification, we used 15 alternative forward primers for the *SSIb* reference assay used for GMO quantification in maize. These primers carry in their sequence a single base pair substitution compared to the standard forward primer described in Kuribara et al., (2002) (Table 1, Material and methods) and were used in combination with the standard reverse primer (also described in Kuribara et al., (2002)). Real-time PCR reactions were performed in three independent runs for both maize lines B73 and 5% Mon810 (material and methods).

Table 1: Sequence of the forward primers. The substituted base is indicated in bold

No	Forward primer sequence 5'-3'
1	CTC CCA ATC CTT TGA CAT CTG C
2	C**A**C CCA ATC CTT TGA CAT CTG C
3	CTC CCA ATC C**A**T TGA CAT CTG C
4	CTC CCA ATC CTT TGA CAT C**A**G C
5	C**C**C CCA ATC CTT TGA CAT CTG C
6	CTC CCA ATC C**C**T TGA CAT CTG C
7	CTC CCA ATC CTT TGA CAT C**C**G C
8	C**G**C CCA ATC CTT TGA CAT CTG C
9	CTC CCA ATC C**G**T TGA CAT CTG C
10	CTC CCA ATC CTT TGA CAT C**G**G C
11	CTC CCA ATC CTT TGA CAT CT**T** C
12	CTC CCA ATC CTT TGA CAT CT**A** C
13	CTC CCA ATC CTT TGA CAT CT**C** C
14	CTC CCA ATC CTT TGA CAT CTG **T**
15	CTC CCA ATC CTT TGA CAT CTG **A**
16	CTC CCA ATC CTT TGA CAT CTG **G**

The amplification efficiency and the specificity of the different primer pairs were evaluated using LinReg PCR 7.5 software and dissociation curve analysis after SYBR Green I amplification respectively (material and methods). The data (C_T values and target DNA quantities) collected after each run were statistically analyzed using Analysis of Variance (ANOVA; material and methods). For primers 2-16, the substituted base is numbered based on its proximity to the elongation start point (3' end of the primer sequence). For instance, the G replacing a C in primer 16 is considered as a substitution at position 1.

2.1. A mismatch near the 3' end can lead to low amplification efficiency

The LinReg analysis shows that most primer pairs have a high amplification efficiency. The values varied between 1.85 and 1.93 for primer pairs 1-14. Primer pairs number 15 and 16 display the lowest amplification efficiencies: 1.58 and 1.76, respectively (Figure 1).

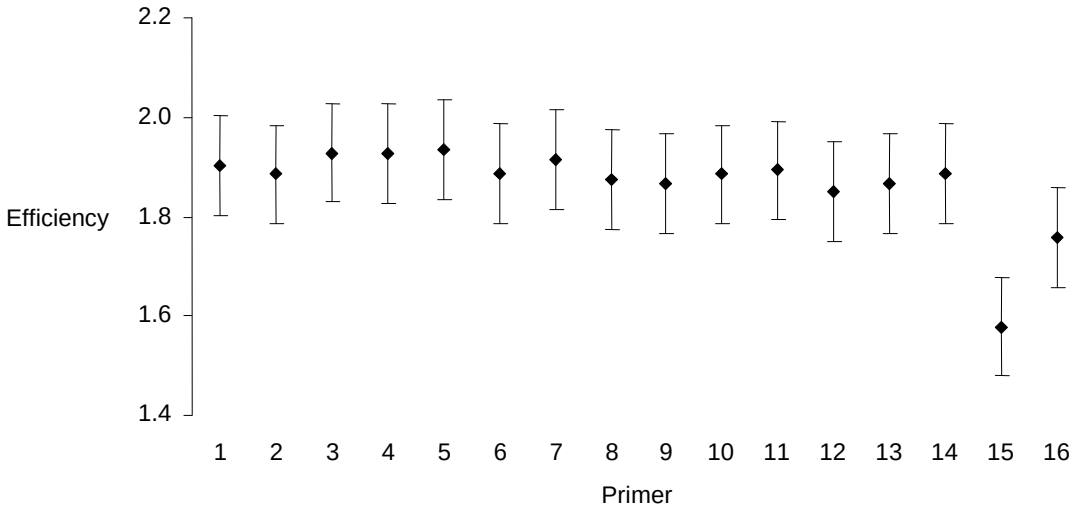

Figure 1: Comparison of the primer pairs amplification efficiencies. Vertical bars denote error bars.

To check if the lower efficiency is due to low primer specificity we performed dissociation curve analysis after SYBR Green I amplification. Each PCR product displayed a single melting curve peak at 87°C except the melting curve obtained with primer pair 15, where a double peak curve was obtained with maxima at 87°C and 77°C (results not shown). The peak observed at 77°C was higher and broader than the peak observed at 87°C, indicating primer-dimer formation. Inefficient amplification using primer pair 15 was confirmed by analysis of TaqMan real-time PCR amplicons on agarose gel, where the amplicon with the expected length was present for all primer pairs except for primer pair 15 (results not shown).

2.2. A single internal mismatch along the forward primer sequence results in small C_T value shifts

For all performed reactions, a single calibration curve was generated using plasmid DNA (material and methods). The effect of primer/template mismatch was assessed by comparing the C_T values derived from the altered primers with those derived from the standard primer.

Primer pairs 2-10 (Table 1), harboring a single mismatch at different positions along the forward primer, show a shift in C_T values compared to the standard primer pair 1. Two-way ANOVA analysis shows a highly significant effect of the mismatch type on the C_T values in B73 ($p < 0.001$) and 5% Mon810 ($p = 0.001$). In contrast, the effect of the mismatch position was less significant (B73: $p < 0.05$) or not significant (5% Mon810: $p = 0.15$). The shift in the C_T varied between +0.25 and +1.25 for B73 and between -0.28 and +0.45 for 5% Mon810 (Figure 2). The largest shift was observed with primer 10 where T was replaced by a G at position 3, for both B73 and 5% Mon810 (Figure2).

The shift of C_T values results in an incorrect estimation of the *SSIIb* quantity. The measured C_T values were converted to quantities to estimate the fold-change in under- and/or overestimation as a result of a primer mismatch (see materials and methods). The absolute quantity in each reaction was calculated using the standard curve. This approach is analogous to what would occur during GMO routine analysis when an unknown sample carrying a polymorphism in the primer attachment site would be quantified using a standard curve derived from a reference material containing a perfect primer attachment site. Subsequently the absolute quantities were converted to relative quantities by setting the standard primer to 100%. So, all estimated quantities obtained with the respective mismatch primer combinations are expressed relative to the standard primer.

Two-way ANOVA was used to assess the effect of the mismatch type and its position on estimated *SSIIb* quantities. The analysis showed a highly significant effect ($p < 0.001$) of the mismatch type on the estimated *SSIIb* quantities in both B73 and 5% Mon810. For B73, the primer 10 (T replaced by G; position 3) derived *SSIIb* quantity decreased to 45% in comparison with primer pair 1. The shift was less

pronounced for 5% Mon810: it decreased to 78% of the *SSIIb* quantity obtained with primer pair 1. Overall, we observed that the substitution of a T with a G at positions 3, 12 or 21 resulted in a more pronounced decrease in the *SSIIb* quantities in comparison to the substitution of a T with a C or an A at the same positions (Figure 3).

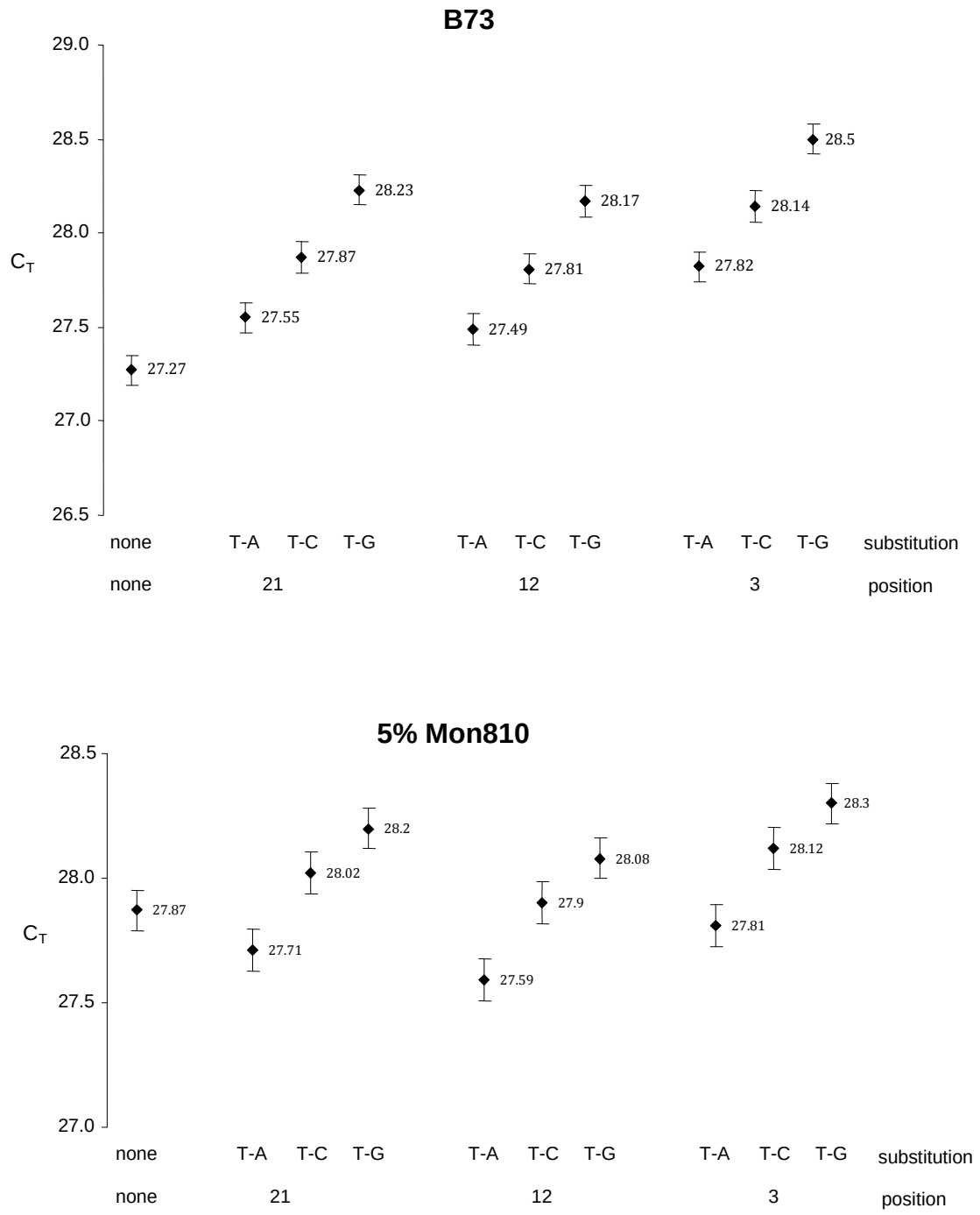

Figure 2: C_T means for substitution types at different positions, compared to the reference primer, in the B73 and 5% Mon810 matrices. Bars represent the standard error.

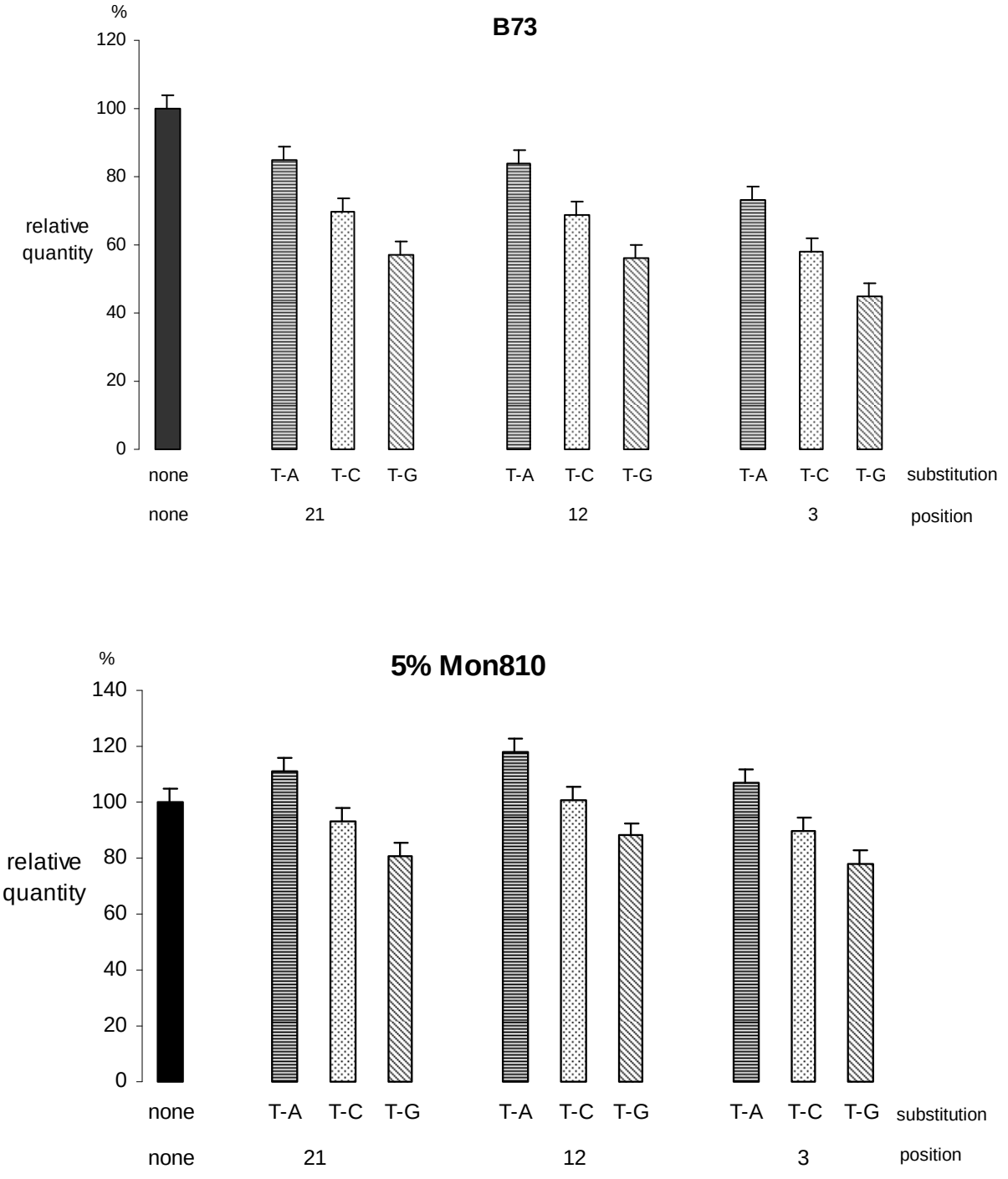

Figure 3: Relative *SSIIb* quantity means for substitution types at different positions, expressed as percentage of the quantity measured by means of the standard primer pair (indicated as none on the graph) for the B73 and 5% Mon810 matrix. Bars represent the standard error.

2.3. A single mismatch near the 3' end has larger effect on the real-time PCR amplification and on *SSIIb* quantification

As mismatches at position 3 show the largest shifts in C_T and *SSIIb* quantities , we were interested in studying the effect of mismatches when they occur at the 3' penultimate and ultimate positions of the primer attachment site. We analyzed the data from the second set of experiments (using primers 1, 4, 7 and 10-16 in Table 1) by one-way ANOVA to assess the effect of the mismatch type and position. The mismatch type shows a significant effect (p <0.001) on both C_T and *SSIIb* quantity in both B73 and 5% Mon810.

Primers 4, 7 and 10 were used in the second set of experiments to confirm the repeatability of the results obtained in the first set of experiments. A shift in the obtained C_T values in comparison with primer pair 1 was observed. The C_T shift varied between -0.17 and -0.92 for B73 and between -0.09 and -0.68 for 5% Mon810 (Figure 4). C_T values obtained in the first data set slightly differed from those observed in the second data set. However, the magnitude of C_T shifts was the same in both experimental data sets.

The C_T shift caused by the primers harboring a mismatch at the penultimate position (number 11, 12 and 13) were between -0.74 and +0.76 for B73 and between -0.60 and +0.66 for 5% Mon810 (Figure 4). The derived *SSIIb* quantities represented 60% to 164% for B73, and 64% to 145% for 5% Mon810 of the *SSIIb* quantity obtained using the standard primer pair (Figure 5).

For primer 14, with a C by T substitution at the ultimate position, the C_T shift was relatively small (-0.14 for B73 and -0.28 for 5% Mon810). The shift in C_T values was more pronounced for other mismatch types at the same position. Indeed, when the 3' ultimate C was replaced by a G (primer 16), a C_T value that is 7 C_T's higher compared to the standard primer pair, was obtained for both matrices (Figure 4). A deviation in the estimated *SSIIb* quantity was also observed. The *SSIIb* quantity estimated with primer pair 14 was 8% (for B73) and 17% (for 5% Mon810) higher than the *SSIIb* quantity derived from the standard primer pair. Using primers 15 and 16 and for both B73 and 5% Mon810, the obtained *SSIIb* quantities represented a

maximum of 1% of the standard primer pair derived *SSIIb* quantity (Figure 5). This large decrease is due to the low efficiency of primer pairs 15 and 16: 1.58 and 1.76, respectively (Figure 1).

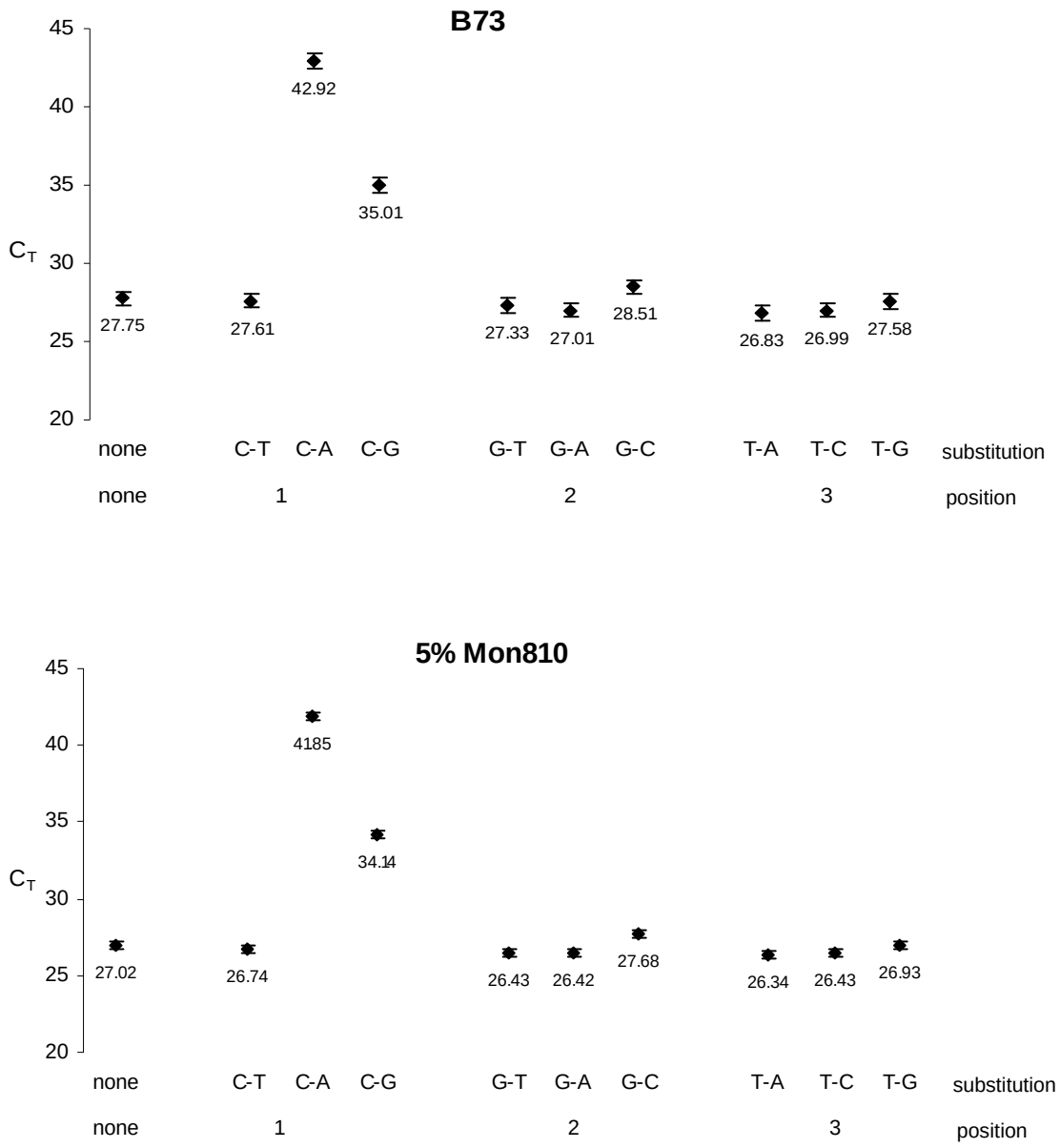

Figure 4: Mean C_T values for substitution type at different 3' end positions for B73 and 5% Mon810 matrices Bars represent the standard error.

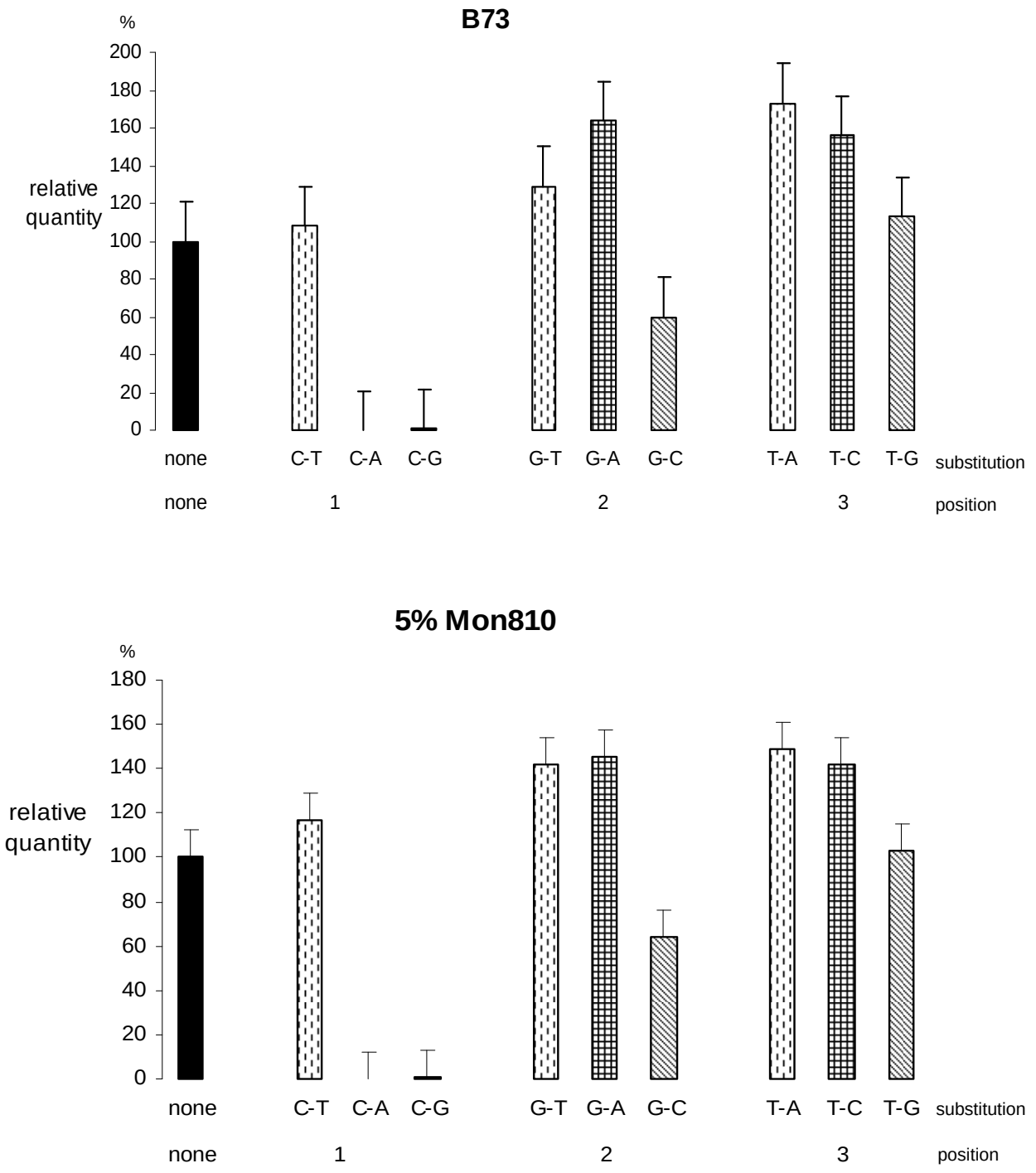

Figure 5: Relative *SSIIb* quantity means for substitution type at different 3' end positions for B73 and 5% Mon810 matrices, expressed as percentage of the of the standard primer pair quantity (indicated as none on the graph). Bars represent the standard error.

3. Discussion

In this study we showed that a primer/template mismatch at different positions of the forward primer attachment site leads to a shift in the real-time PCR C_T values measured during maize *SSIIb* endogene quantification. Genomic DNA extracts from two matrices were used: B73 and the certified reference material 5% Mon810. Several mismatch types, located along the forward primer attachment site and at its 3' end were tested. The significance of the type and location of the single base pair substitution was assessed by evaluating the shift in measured C_T value as well as in the estimated *SSIIb* target quantities.

The type of the mismatch significantly influences the C_T values, leading to shifts ranging from +0.25 to +1.25 C_T for B73 and from -0.28 to +0.45 C_T for 5% Mon810. Due to the exponential nature of the real-time PCR, small fluctuations in C_T values translate into large variability in the measured DNA target quantity (Bubner and Baldwin, 2004). In our study the target DNA quantities calculated and derived from the C_T measurements with primers harboring a mismatch, were 15% to 55% lower than the *SSIIb* quantities obtained in the reactions using the standard primer pair for B73. For 5% Mon810, the *SSIIb* quantity varied between -22% to +11% in comparison with the standard primer pair. Although the influence of the mismatch position was not statistically significant, the C_T shift was higher when the mismatch was located towards the 3' end of the primer. Therefore, we additionally assessed the effect of mismatches located at the last two positions at the 3' end of the primer sequence. Our findings reveal that when the mismatch is located at the penultimate position, the C_T shifts were still relatively small, ranging from -0.74 to +0.76 C_T for B73 and from -0.60 and +0.66 C_T for 5% Mon810. The influence of a mismatch located at the ultimate 3' end was dependent on the substitution type. Substitution of a C by a T at the ultimate position did not lower the amplification efficiency. The observed C_T shift was ~ -0.1 and -0.3 C_T for B73 and 5% Mon810, respectively. Substitution of a C by a G or an A at the same position, yielded 1.76 and 1.58 amplification efficiencies, respectively. The C_T values obtained using these primers were very high compared to the standard primer pair (7 to 15 C_T higher) and the *SSIIb* quantities represented only 1% of that obtained with the standard primer pair. From these results we can conclude that the position of the mismatch within the

primer sequence is important for the stability of the primer annealing. In addition, our results show that mismatches located more towards the 3' end of the primers are more critical and affect the PCR more dramatically than mismatches at other positions, which is consistent with the findings in several other studies (Petruska et al., 1988; Kwok et al., 1990; Ayyadevara et al., 2000; Bru et al., 2008).

Broothaerts et al., (2008) reported a 2- to 4-fold decrease in the estimated *adh1* endogene quantity compared to the *hmg* endogene quantity in different non-GM and GM maize varieties. The decrease was due to the presence of a mismatch created by the substitution of a T by a C in the *adh1* genomic sequence in some maize varieties. The mismatch was located in the middle of the *adh1* reverse primer. In our study, using forward primers where the mismatch was located in the middle, the maximal obtained decrease in the *SSIIb* quantity was 55%. The difference between both studies regarding the extent to which a mismatch located in the middle of the primer is influencing the target DNA quantity estimation can be explained by several factors. Primer annealing temperature was set at 60ºC in our amplification reactions while it was 56ºC in the study reported by Broothaerts et al., (2008). Mismatch effects were described to be dependent on the annealing temperature (Kwok et al., 1990; Ishii and Fukui, 2001). However, it was shown that the bias due to the presence of mismatches was reduced at lower annealing temperature (Ishii and Fukui, 2001). Primer length is also slightly different: 18 bp in Broothaerts et al., (2008) study compared to 22 bp in our studies. The mismatch described in Broothaerts et al., (2008), was located at position 10, while it was at position 12 in our primers.

GMO content is determined by the ratio between the GM event-specific DNA target quantity and the species- or taxon-specific DNA target quantity. We show that the presence of a primer/template mismatch during quantification of the endogenous reference gene will lead to a C_T and a DNA quantity deviation, hence leading to a deviation in the estimated GMO content. This deviation is dependent on the position of the mismatch within the primer attachment site. If the mismatch is located at the 3' ultimate base, a high shift in the C_T and DNA quantities can be expected (except when a C was replaced by a T in the primer sequence). The high C_T shift will be immediately recognized and will indicate that the amplification reaction was severely

hampered. If the primer/template mismatch is not located at the 3' ultimate base of the primer, the deviation in the C_T values will be small (-1 to +1.25 C_T). Variations in measured C_T values within the range of one C_T are considered as normal variations within one run or between runs performed by the same operator, within a short time period, in the same laboratory (under so-called "repeatability conditions"). A difference of one C_T corresponds to a 2-fold difference in the estimated target quantity (+1 C_T equates to two times fewer targets while -1 C_T equates to two times more targets). Thus, the taxon-specific DNA target quantification will be affected, leading to an under- or overestimation of the GMO content. For maize, genes involved in metabolic pathways such as alcohol dehydrogenase (*adh*), chromatin-associated high mobility group (*hmg*), invertase (*ivr*) and *zein* genes are commonly used as taxon-specific reference genes for GMO quantification (Hernandez et al., 2004). Primers used for quantification are designed at coding sequences or intron/exon borders of these genes (Hernandez et al., 2004). SNP are single base pair positions in genomic DNA at which different alleles exist in individuals in a population, wherein the least frequency allele has an abundance of at least 1% (Jehan and Lakhanpaul, 2006). After examining 18 genes in 36 maize inbred lines representing the genetic diversity of the U.S.`s elite maize breeding pool, it has been shown that the frequency of SNP occurrence in maize coding sequences (on average one polymorphism per 124 bp) is lower than in non-coding regions (on average one polymorphism per 31 bp) (Ching et al., 2002). However, a SNP will only interfere with GMO quantification if it is located within the primer attachment sequence of the reference gene and/or transgene. The sequence length of the validated primers used for maize reference gene quantification is ranging from 18 to 23 bp (Hernandez et al., 2004) which reduces the chance of a SNP occurrence in these regions of the DNA template sequence (36 to 46 bp including both primers attachment sites). Moreover, according to our results, a SNP that will severely hamper the amplification reaction and be noticed by the investigator, must be located at the 3' end of the primer attachment site, which is even more unlikely to occur.

Studying SNP frequency occurring in the reference genes sequences of commercially available maize varieties, which number is increasing and reached more than 1000 varieties registered in the EU (EC, 2006), will be helpful to estimate the incidence of a SNP in a primer attachment site. Recently in our laboratory, eight endogenous maize reference gene assays, amplifying sequences ranging in length

from 70 up to 136 bp, were analyzed for their nucleotide sequence variation in a worldwide collection of different maize varieties and certified reference materials (Papazova et al.). One SNP in 11 out of more than 100 tested varieties was identified in the primer attachment site of the *Adh1* target sequence (136 bp). In another, *zein* specific reference target (110 bp), a SNP was identified in 48 out of more than 150 tested varieties. However, this SNP was not located in the primer nor in the probe attachment sites of this reference assay (Papazova et al.). Hence, deviations in the calculated GMO content due to the presence of a base pair substitution in the genomic DNA are not likely to occur frequently. However, the impact of a primer/template mismatch might result in an inaccurate GMO content estimation. Thus, special attention should be given to the selection of species- or taxon-specific reference gene sequences for GMO quantification. In this selection, priority should be given to conserved (within a taxon) genomic regions with absence of SNPs and showing high nucleotide stability among the different cultivars.

For maize, reference assays for GMO quantification have been developed and validated based on genes encoding for high mobility group protein gene (*Hmg*), alcohol dehydrogenase 1 gene (*Adh1*), invertase (*Ivr*), *zein* and the starch synthase type B (*SSIIb*) gene (Kuribara et al., 2002; Shindo et al., 2002; Hernandez et al., 2004; Yoshimura et al., 2005). Hence, it is possible to quantify multiple reference genes in parallel in order to overcome the risk of endogene quantification deviation due to the presence of a primer-template mismatch. A difference in one of the amplified genes quantity compared to the other(s) will be indicative of possible presence of a mismatch in one of its primer attachment sites. Amplifying two or more reference genes in parallel will, however, increase the workload and cost of the analysis. These can be reduced by using duplex and multiplex amplification methods (Leimanis et al., 2006; Nadal et al., 2006; Yamaguchi et al., 2006; Xu et al., 2007; Chaouachi et al., 2008), but multiplex reference assays have yet to be optimized.

4. Materials and Methods

4.1. Materials

Grains from maize inbred line B73 and the certified reference material (CRM) powder containing 5% mixture of transgenic event Mon810 and conventional maize variety were used. 5% Mon810 is commonly used in GMO analysis to generate standard curves. B73 is frequently used in breeding programs. These two independent biological materials are equivalent with respect to quantification of the reference genes.

4.2. Methods

4.2.1. DNA extraction

B73 grains were ground to fine powder using a mixer mill (Retsch MM301) for 1 min at 30 Hz. DNA was isolated from 100 mg of each sample by means of DNeasy Plant Mini Kit (Westburg, the Netherlands) according to the manufacturer's protocol with the incubation time of the sample in lysis buffer extended to 30 minutes. DNA extracted from five samples for each matrix were pooled. The DNA concentration was measured by means of GeneQuant spectrophotometer (Amersham Pharmacia Biotech, The Netherlands).

4.2.2. Oligonucleotides

A primer pair amplifying a 151 bp fragment of maize starch synthase (*SSIIb*) was used for real-time PCR (Kuribara et al., 2002). A single base at different positions in the forward primer was substituted resulting in 15 alternative primers containing a single base pair mismatch in their sequence. In primers 2 to 10, the thymine (T) at positions 3, 12 and 21 starting from the 3' end, was substituted either by adenine (A), guanine (G) or cytosine (C) (Table 1). In primers 11 to 13, the 3' penultimate base (position 2; G) was substituted by either C, T or A. In primers 14 to 16, the 3' ultimate base (position 1; C) was substituted by either G, T or A (Table 1).

All reactions were performed with the same reverse primer (5'-TCG ATT TCT CTC TTG GTG ACA GG-3') and probe (5′-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3′).

4.2.3. TaqMan real-time PCR

All reactions were performed in a total volume of 25 µl using 96-well microwell plates and an ABI Prism 7000 High Throughput Sequence Detection System (Applied Biosystems). The mixture contained 1x GMO MasterMix (Diagenode, Luik, Belgium), 300 nM of each primer, 200 nM of *SSIIb*-Taq probe and 37 ng of genomic DNA (approximately 13700 maize genome copies per reaction on the basis of the maize genome size) (Arumuganathan and Earle, 1991). Thermal conditions were as follows: 2 min at 50℃, 10 min at 95℃, and 45 cycl es of 15 s at 95℃ and 1 min at 60℃. The real-time PCR data were processed using A BI Prism 7000 SDS software 1.0; Applied Biosystems).

Standard curves were prepared using plasmid DNA containing the *SSIIb* fragment (Nippongene) in quantities of 20, 125, 1500, 20000 and 250000 copies per reaction. Each standard was run in triplicate.

In a first set of experiments, using primers 1 to 10 (Table 1), four and three independent runs were carried out for B73 and 5% Mon810, respectively. In a second set of experiments, using primers 1, 4, 7 and 10 to 16, three independent runs were carried out for both B73 and 5% Mon810. Within each run, the reactions were performed in triplicate for each primer combination with either B73 or 5% Mon810. Absolute quantities of target DNA were obtained by interpolating the measured C_T values to the generated standard curve. Relative quantities, used to generate Figures 3 and 5, were determined relatively to the *SSIIb* quantity obtained with the standard primers. This *SSIIb* quantity was set as 100%. For instance, using primer 2 (where a T was replaced by an A at position 21) the obtained target quantity was 11645 (data not shown), equivalent to 85% of the 13700 maize genome copies obtained with the standard primer and loaded as template input (Figure 3). The purity of the real-time PCR amplicons was analyzed by separating the reactions on a 2% agarose gel and ethidium bromide staining and visualization.

4.2.4. SYBR Green I real-time PCR

Per reaction, 37 ng of genomic DNA, 12.5 µl SYBR green I PCR Master Mix (containing a uracil N-glycosylase - Applied BioSystems), and 300 nM of primers were added to each well in a total volume of 25 µl. Reaction thermal conditions were as follows: 50℃ for 2 min (UNG erase reaction) and 95℃ for 10 min, followed by 40 cycles at 95℃ for 15 s and 55℃ for 30 s and a final melt-curve analysis (60℃ to 95℃; ramp time, 19.59 min). Melting curve analysis was performed in order to verify the specificity of the reaction.

4.3. Data analysis

4.3.1. Analysis of the amplification efficiency

The amplification efficiency of each reaction was determined using LinReg PCR 7.5 (Ramakers et al., 2003). Linear regression analysis of TaqMan dye fluorescent signal rate emission in the exponential phase of the PCR amplification was performed for each individual reaction well to determine the primer pair specific amplification efficiency (*E*). Further, the mean of the amplification efficiency values was calculated for each primer pair. The mean amplification efficiency per primer pair was calculated based on variable number of data points depending on the number of runs performed and varies between 18 and 51 observations.

4.3.2. Analysis of C_T values and estimated copy numbers

To assess the effects of the mismatch type and/or position on the C_T values and the estimated DNA quantities generated by primer pairs 1-10, the data was analyzed as a two-way design (with runs set as blocking factor) using analysis of variance (ANOVA). Entries were calculated as an average of three or four technical replicates (runs) for 5% Mon810 and B73, respectively. To assess the effects of mismatch type on the C_T values and the estimated *SSIIb* DNA quantities generated by primer pairs 1, 4, 7, 10 -16, the data was analyzed as a one-way ANOVA (with runs set as blocking factor). Entries were calculated as an average of three technical replicates for 5% Mon810 and B73. In both analyses, *F*-statistics were calculated and significance was

assigned to the main terms mismatch type and position effects and to their interaction, if relevant. The one-way and two-way ANOVA were performed separately for B73 and 5% Mon810. The analyses were performed using Genstat (Payne and Lane, 2005).

References

Arumuganathan K, Earle ED (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol. Biol. Rep. **9:** 208-218

Ayyadevara S, Thaden JJ, Shmookler Reis RJ (2000) Discrimination of primer 3'-nucleotide mismatch by taq DNA polymerase during polymerase chain reaction. Anal Biochem **284:** 11-18

Bhattramakki D, Dolan M, Hanafey M, Wineland R, Vaske D, Register JC, 3rd, Tingey SV, Rafalski A (2002) Insertion-deletion polymorphisms in 3' regions of maize genes occur frequently and can be used as highly informative genetic markers. Plant Mol Biol **48:** 539-547

Broothaerts W, Corbisier P, Schimmel H, Trapmann S, Vincent S, Emons H (2008) A single nucleotide polymorphism (SNP839) in the adh1 reference gene affects the quantitation of genetically modified maize (Zea mays L.). J Agric Food Chem **56:** 8825-8831

Bru D, Martin-Laurent F, Philippot L (2008) Quantification of the detrimental effect of a single primer-template mismatch by real-time PCR using the 16S rRNA gene as an example. Appl Environ Microbiol **74:** 1660-1663

Bubner B, Baldwin IT (2004) Use of real-time PCR for determining copy number and zygosity in transgenic plants. Plant Cell Rep **23:** 263-271

Buckler ES, Gaut BS, McMullen MD (2006) Molecular and functional diversity of maize. Curr Opin Plant Biol **9:** 172-176

Chaouachi M, Chupeau G, Berard A, McKhann H, Romaniuk M, Giancola S, Laval V, Bertheau Y, Brunel D (2008) A high-throughput multiplex method adapted for GMO detection. J Agric Food Chem **56:** 11596-11606

Ching A, Caldwell KS, Jung M, Dolan M, Smith OS, Tingey S, Morgante M, Rafalski AJ (2002) SNP frequency, haplotype structure and linkage disequilibrium in elite maize inbred lines. BMC Genet **3:** 19

Day JP, Bergstrom D, Hammer RP, Barany F (1999) Nucleotide analogs facilitate base conversion with 3' mismatch primers. Nucleic Acids Res **27:** 1810-1818

EC (2003a) Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed 2001/18/EC. *In*. Off. J. Eur. Commun., pp 24-28

EC (2003b) Regulation (EC) No. 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending directive 2001/18/EC. . *In*, Vol *Legis.* 2003, L268. Off. J. Eur. Commun., pp 24-28

EC (2004) EC/787/2004 of 04.10.2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisns as or in products in the context of Regulation (EC) No 1830/2003. . *In*, Vol L348. Off. J. Eur. Union, pp 18-26

EC (2006) The common catalogue of varieties of agricultural plant species. Off J Eur Union **C**

EFSA (2004) Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation. *In*, Vol 102. OJ L, pp 14-25

Hernandez M, Duplan MN, Berthier G, Vaitilingom M, Hauser W, Freyer R, Pla M, Bertheau Y (2004) Development and comparison of four real-time polymerase chain reaction systems for specific detection and quantification of Zea mays L. J Agric Food Chem **52:** 4632-4637

Holst-Jensen A, Ronning SB, Lovseth A, Berdal KG (2003) PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal Bioanal Chem **375:** 985-993

Huang MM, Arnheim N, Goodman MF (1992) Extension of base mispairs by Taq DNA polymerase: implications for single nucleotide discrimination in PCR. Nucleic Acids Res **20:** 4567-4573

Ishii K, Fukui M (2001) Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. Appl Environ Microbiol **67:** 3753-3755

James C (2008) Global Status of Commercialized Biotech/GM Crops: 2008. *In*, Vol Brief No. 39. ISAAA, Ithaca, NY.

Jehan T, Lakhanpaul S (2006) Single nucleotide polymorphism (SNP) - Methods and applications in plant genetics: a review. Indian Journal of Biotechnology **5:** 435-459

Kuribara H, Shindo Y, Matsuoka T, Takubo K, Futo S, Aoki N, Hirao T, Akiyama H, Goda Y, Toyoda M, Hino A (2002) Novel reference molecules for quantitation of genetically modified maize and soybean. J AOAC Int **85:** 1077-1089

Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky JJ (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res **18:** 999-1005

Leimanis S, Hernandez M, Fernandez S, Boyer F, Burns M, Bruderer S, Glouden T, Harris N, Kaeppeli O, Philipp P, Pla M, Puigdomenech P, Vaitilingom M, Bertheau Y, Remacle J (2006) A microarray-based detection system for genetically modified (GM) food ingredients. Plant Mol Biol **61:** 123-139

Lie YS, Petropoulos CJ (1998) Advances in quantitative PCR technology: 5' nuclease assays. Curr Opin Biotechnol **9:** 43-48

Nadal A, Coll A, La Paz JL, Esteve T, Pla M (2006) A new PCR-CGE (size and color) method for simultaneous detection of genetically modified maize events. Electrophoresis **27:** 3879-3888

Papazova N, Zhang D, Gruden K, Vojvoda J, Yang L, Buh M, Blejec A, Fouilloux S, De Loose M, Taverniers I Evaluation of the reliability of maize reference assays for GMO quantification. submitted to Analytical and Bioanalytical Chemistry

Payne RW, Lane PW (2005) GenStat® Release Reference Manual , Part3, Oxford, UK.

Petruska J, Goodman MF, Boosalis MS, Sowers LC, Cheong C, Tinoco I (1988) Comparison between DNA melting thermodynamics and DNA polymerase fidelity (base pairing free energy differences/DNA polymerase kinetics). Proc. Natl. Acad. Sci. **85:** 6252-6256

Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett **339:** 62-66

Shindo Y, Kuribara H, Matsuoka T, Futo S, Sawada C, Shono J, Akiyama H, Goda Y, Toyoda M, Hino A (2002) Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. J AOAC Int **85:** 1119-1126

Tenaillon MI, Sawkins MC, Long AD, Gaut RL, Doebley JF, Gaut BS (2001) Patterns of DNA sequence polymorphism along chromosome 1 of maize (Zea mays ssp. mays L.). Proc Natl Acad Sci U S A **98:** 9161-9166

Vroh Bi I, McMullen MD, Sanchez-Villeda H, Schroeder S, Gardiner J, Polacco M, Soderlund C, Wing R, Fang Z, Coe EHJ (2006) Single Nucleotide Polymorphisms and Insertion-Deletions for Genetic Markers and Anchoring the Maize Fingerprint Contig Physical Map. Crop Sci **46:** 12-21

Xu J, Zhu S, Miao H, Huang W, Qiu M, Huang Y, Fu X, Li Y (2007) Event-specific detection of seven genetically modified soybean and maizes using multiplex-PCR coupled with oligonucleotide microarray. J Agric Food Chem **55:** 5575-5579

Yamaguchi A, Shimizu K, Mishima T, Aoki N, Hattori H, Sato H, Ueda N, Watanabe T, Hino A, Akiyama H, Maitani T (2006) Detection method for genetically modified papaya using duplex PCR. Shokuhin Eiseigaku Zasshi **47:** 146-150

Yoshimura T, Kuribara H, Matsuoka T, Kodama T, Iida M, Watanabe T, Akiyama H, Maitani T, Furui S, Hino A (2005) Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. J Agric Food Chem **53:** 2052-2059

Chapter 7: Summary

Agrobacterium-mediated transformation has revolutionized agriculture as well as basic research in plant molecular biology, by enabling the genetic modification of a wide variety of plant species. This technology has yielded a stunning array of transgenic plants with novel properties ranging from enhanced agronomic performance, nutritional content and disease resistance to the production of pharmaceuticals and industrially important compounds (Newell, 2000; Halford, 2006; Banta and Montenegro, 2008). Transgenic plants can have a positive impact on both human and environmental health which require the increase of their production. Using *Agrobacterium*, two main methods can be applied: regeneration dependent methods (*in vitro*) such as root explants transformation (Valvekens et al., 1988) or regeneration independent methods (*in planta*) such as floral dip (Clough and Bent, 1998). *In planta* transformation methods are preferred over *in vitro* methods due to the simplicity of their protocols and their low cost (**Chapter 1**).

In the first part of this thesis, we focused on *Agrobacterium* transformation efficiencies improvement of the model plant *Arabidopsis thaliana* using floral dip method. The influence of 12 different *Agrobacterium* strains, with different chromosomal backgrounds or *vir* plasmid, on the floral dip transformation frequency was evaluated (**Chapter 3**). The commonly used *Agrobacterium* strains C58C1Rif^{R} and LBA4404 were included in our study. Floral dip was carried out in two, three or four biological repeats using 5 *Arabidopsis* Col0 or C24 plants for each bacterial strain. 2000 seeds from each T1 seedstock were sown on appropriate selective medium and the number of transgenic plants was scored. We observed a high intra- and inter repeat variability. Within one biological repeat, the number of produced transgenic plants varied up to 33 fold between the 5 dipped plants although they were grown in the same conditions (light, temperature, watering) and were dipped using the same bacterial culture at the same time. Hence, the variability is due to the plant characteristics. Most probably, the number of flowers competent for the T-DNA transfer and integration is the main limiting factor as it has been shown that the female tissue and more precisely the ovule of *Arabidopsis thaliana* represent the *Agrobacterium* target during floral dip transformation (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000).

To compare the transformation performance of the used bacterial strains, we plotted for each strain how many of the 15 floral dipped plants yielded no transformants in 2000 seeds, 1 to 10 transformants, 11-40 transformants or more then 40 transformants per 2000 seeds. From this classification we concluded that *Agrobacterium* strains LMG201 Rif^R (pMP90,pTJK136), C58C1 Rif^R (pMP90,pTJK136) and LMG62 Rif^R (pMP90,pTJK136) have a higher transformation performance after *Arabidopsis* Col0 floral dip compared to the commonly used *Agrobacterium* strain LBA4404 Rif^R (pTJK136).

The same bacterial strains were used for floral dipping of *Arabidopsis thaliana* ecotype C24. The obtained transformation frequencies were consistently low (0% to 0.33%) compared to *Arabidopsis* Col0 ecotype transformation efficiencies (0% to 2.4%). This result indicates that, *Arabidopsis* C24 ecotype is, to some extent, recalcitrant to *Agrobacterium* floral dip transformation.

Cotransformation experiments allowed to overcome the plant to plant variation and the bacterial strain C58C1Rif^R (pMP90, pTJK136) was used in every cotransformation experiment as reference. The results confirmed that plant characteristics highly influence the transformation frequencies. Additionally, it was clearly demonstrated that LBA4404 Rif^R (pTJK136) was less efficient than C58C1 Rif^R (pMP90,pAAVS1) while LMG201 Rif^R (pMP90,pTJK136) was more efficient than C58C1 Rif^R (pMP90,pAAVS1) for *Arabidopsis* Col0 floral dip transformation.

We further investigated the properties of *Agrobacterium* LMG201 Rif^R (pMP90,pTJK136) strain as it showed better transformation frequencies than the commonly used C58C1 Rif^R (pMP90,pTJK136). Pathogenicity tests showed that LMG201 Rif^R was no longer oncogenic after the introduction of the pMP90 *vir* plasmid. Moreover, segregation analyses, GUS activity measurements and DNA blot experiments showed that in transgenic plants, obtained with the *Agrobacterium* LMG201 Rif^R (pMP90,pTJK136) strain, the percentage of T-DNA integration in one locus, the T-DNA integration pattern and the T-DNA expression profiles were comparable to those observed in transgenic plants transformed with the *Agrobacterium* C58C1 Rif^R (pMP90,pTJK136) strain.

In **Chapter 4,** we first assessed the influence of the applied light regime on T-DNA stable integration frequencies after *Arabidopsis* root explants transformation. This environmental factor was shown to promote T-DNA transfer from *Agrobacterium* to plant cells (Zambre et al., 2003). Two light regimes were applied during root cocultivation: continuous light and 16 hours light/8 hours dark. Experiments were carried out either with or without selection for transformation competent cells. We concluded that light promotes T-DNA transfer; however, no T-DNA stable integration enhancement could be observed after *Arabidopsis* root explants transformation.

Second, we investigated the extent to which T-DNA transfer and/or T-DNA integration is limiting the *in planta* floral dip *Agrobacterium* transformation method. Experiments performed in the absence of selection for transformation competent cells showed that the transformation frequency determined by selection or by PCR screening is identical, demonstrating that no or only few T-DNAs integrated within regions that are disfavored by selection such as heterochromatin which is contradictory with the results described for *Arabidopsis* cell culture (Kim et al., 2007). The results obtained from experiments performed with selection for transformation competent cells by selecting for the presence of a first T-DNA, showed that the cotransformation frequency was rather low being below 35% and that almost none of the transformants witnessed transient expression of a second unselected T-DNA. This means that the integration of the incoming T-DNA is not limiting the transformation frequency. Rather, we could conclude that the formation of a productive *Agrobacterium*-plant cell interaction is crucial for *Arabidopsis* floral dip transformation. Similar to root transformation, the cotransformation frequencies obtained after floral dip transformation were always higher than the transformation frequencies which suggested that the plant cell accessibility might be an important parameter for stable transformation to occur upon both floral dip and root transformation.

The use of genetic engineering for agricultural benefit is increasing through the years and some transgenic plants were introduced on the market in different countries as a source of food and feed (James, 2008). In Europe, several regulations and legislations are governing the marketing of these biotech crops. As the T-DNA is inserted at random location within the genome of the host, DNA sequences at the

transition between the plant genomic DNA and the newly introduced exogenous transgene are unique for every transgenic event. Based on these unique sequences, methods are developed for the identification and analysis of each event. Hence, the stability of the inserted sequence and of the insertion locus are crucial parameters for the development of GMOs analytical methods (**Chapter 1**).

In **Chapter 5**, we studied the impact of propagation through generations, the influence of gene stacking and photo oxidative stress caused by high light intensity on the stability of the transgene flanking regions in transgenic *Arabidopsis thaliana* plants. In order to detect point mutations and larger rearrangements in the T-DNA flanking regions, we used restriction fragment length polymorphism analysis (RFLP), sequencing and conformational sensitive capillary electrophoresis (CSCE). We did not observe any instability of the transgene insertion locus and in the transgene flanking sequences after propagation through generations, in stacked events and events submitted to a photo oxidative stress showing that T-DNA insertion sites are stable and not more prone to genotoxic stresses.

Before release on the market of a food or a feed product containing GMOs or GMO traces, a quantification step is required and the acceptance threshold is different according to the country where the product will be marketed. In Europe, the GMO content (%) is expressed on the basis of haploid genome equivalents (HGE) of the GM-target relative to the taxon or species target (EC, 2004). The recommended % GMO unit is thus a percentage of GM DNA quantity, relative to the number of target taxon-specific DNA quantity (EC, 2004). The DNA quantity of both the taxon specific and the transgene are determined using real-time PCR. Maize is the second major biotech crop (James, 2008). The maize genome is highly diverse and single base pair substitutions occur more frequently than insertions/deletions (indels) of one or more nucleotides (Vroh Bi et al., 2006). If a SNP is present at the primer attachment site used during taxon-specific DNA quantification, it will create a mismatch and might interfere with GMO quantification. In **Chapter 6** and in order to assess the influence of a SNP on the real-time PCR quantification, we mimicked the presence of a single base pair substitution in maize genomic DNA by replacing base pairs at different positions in the forward primer. The C_T values and DNA quantities obtained using the primers harbouring a base pair substitution showed a deviation

compared to the values obtained using the non altered primers. The deviation was more pronounced and amplification was even abolished when the substitution was located at the ultimate 3'end of the primer. When the primer/template mismatch is not located at the 3' ultimate base of the primer, the deviation in the C_T values was rather small (-1 to +1.25 C_T) and will be probably considered normal due to "repeatability conditions". As a difference of one C_T corresponds to a 2-fold difference in the estimated target DNA quantity, the taxon-specific DNA target quantification will be affected, leading to an under- or overestimation of the GMO content. This, in turn, might affect the labeling of the product especially in cases where the GMO content is near the labeling threshold of 0.9%. Hence, special attention should be given to the selection of species- or taxon-specific reference gene sequences for GMO quantification. In this selection, priority should be given to conserved (within a taxon) genomic regions with absence of SNPs and showing high nucleotide stability among the different cultivars.

References

Banta LM, Montenegro M (2008) *Agrobacterium* and plant biotechnology. *In* T Tzfira, V Citovsky, eds, *Agrobacterium*: From Biology to Biotechnology. Springer, New York, pp 73-148

Bechtold N, Jaudeau B, Jolivet S, Maba B, Vezon D, Voisin R, Pelletier G (2000) The maternal chromosome set is the target of the T-DNA in the in planta transformation of *Arabidopsis thaliana*. Genetics **155:** 1875-1887

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant journal **16:** 735-743

Desfeux C, Clough SJ, Bent AF (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. Plant physiology **123:** 895-904

EC (2004) EC/787/2004 of 04.10.2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisns as or in products in the context of Regulation (EC) No 1830/2003. . *In*, Vol L348. Off. J. Eur. Union, pp 18-26

Halford NG (2006) Plant Biotechnology: current and future applications of genetically modified crops. Wiley, U.K.

James C (2008) Global Status of Commercialized Biotech/GM Crops: 2008. ISAAA Briefs **No. 39**

Kim SI, Veena, Gelvin SB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. The Plant journal **51:** 779-791

Newell CA (2000) Plant transformation technology. Developments and applications. Molecular biotechnology **16:** 53-65

Valvekens D, Montagu MV, Lijsebettens MV (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci **85:** 5536-5540

Vroh Bi I, McMullen MD, Sanchez-Villeda H, Schroeder S, Gardiner J, Polacco M, Soderlund C, Wing R, Fang Z, Coe EHJ (2006) Single Nucleotide Polymorphisms and Insertion-Deletions for Genetic Markers and Anchoring the Maize Fingerprint Contig Physical Map. Crop Sci **46:** 12-21

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. The Plant journal **19:** 249-257

Zambre M, Terryn N, De Clercq J, De Buck S, Dillen W, Van Montagu M, Van Der Straeten D, Angenon G (2003) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta **216:** 580-586

Chapter 8: Samenvatting

Transformatie met behulp van *Agrobacterium* maakt het mogelijk om een hele reeks plantensoorten genetisch te modificeren, en heeft hierdoor een revolutie veroorzaakt in de landbouw en in het fundamenteel onderzoek in de planten moleculaire biologie. Deze transformatie technologie levert namelijk een hele variëteit aan transgene planten met nieuwe eigenschappen, gaande van planten met verbeterde landbouwkundige eigenschappen, verhoogde voedingswaarde en resistentie tegen ziektes, tot planten die industrieel belangrijke componenten produceren (Newell, 2000; Halford, 2006; Banta en Montenegro, 2008). Transgene planten kunnen een positieve invloed hebben op het welzijn van de mens en zijn omgeving, en dit vereist een stijging in hun productie. Transformatie met *Agrobacterium* kan via 2 methoden gebeuren: de regeneratie afhankelijke (*in vitro*) methoden, zoals worteltransformatie (Valvekens et al., 1998) en de regeneratie onafhankelijke (*in planta*) methoden zoals floral dip (Clough en Bent, 1998). Deze *in planta* transformatiemethoden genieten de voorkeur boven de *in vitro* transformatiemethoden wegens hun eenvoudige protocol en hun lage kostprijs (**Hoofdstuk 1**).

In het eerste deel van dit proefschrift onderzochten we de transformatie-efficiënties na *Agrobacterium* transformatie via floral dip van de modelplant *Arabidopsis thaliana*. De invloed van 12 verschillende *Agrobacterium* stammen, met een verschillende chromosomale oorsprong of een verschillend *vir* plasmide, op de transformatie-efficiënties na floral dip werden geëvalueerd (**Hoofdstuk 3**). Ook de veelvuldig gebruikte stammen C58C1Rif^{R} en LBA4404 werden in deze studie geanalyseerd. Voor elke bacteriële stam werden 2, 3 of 4 floral dip transformaties uitgevoerd (biologische herhalingen). Hierbij werden per floral dip transformatie en per bacteriële stam telkens 5 *Arabidopsis* planten, met ecotype Col0 of C24, gedipt. Vervolgens werden 2000 zaden van elke T1 zaadstock op selectief medium uitgezaaid en werden het aantal transformanten per T1 zaadstock bepaald. Per *Agrobacterium* stam observeerden we een hoge variabiliteit in de transformatie-efficiënties tussen de biologische herhalingen, en zelfs tussen de 5 planten gebruikt tijdens één floral dip transformatie. Inderdaad, ondanks het feit dat de gedipte planten in dezelfde condities (licht, temperatuur, bevochtiging) werden opgegroeid

en in dezelfde bacteriële cultuur werden gedipt, kon zelfs een 33-voudige variatie in de transformatie-efficiëntie tussen de 5 planten van één floral dip experiment waargenomen worden. Deze resultaten suggereren dat de hoge variatie in transformatie-efficiëntie hoofdzakelijk te wijten is aan karakteristieken van de gedipte plant. Vermoedelijk is het aantal bloemen die competent zijn voor T-DNA transfer en T-DNA integratie de limiterende factor. Studies toonden immers aan dat het vrouwelijke weefsel, en meer bepaald de eicel van de *Arabidopsis thaliana* getransformeerd wordt tijdens floral dip (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000).

Om de transformatie capaciteit van de verschillende bacteriestammen te evalueren, werden de 15 gedipte planten per stam ingedeeld volgens het aantal bekomen transformanten per 2000 zaden, nl. geen transformanten, 1 tot 10 transformanten, 11 tot 40 transformanten en meer dan 40 transformanten per 2000 zaden. Uit deze classificatie bleek dat de *Agrobacterium* stammen LMG201 Rif^R (pMP90,pTJK136), C58C1 Rif^R (pMP90,pTJK136) en LMG62 Rif^R (pMP90,pTJK136) een hogere transformatie capaciteit vertonen dan de frequent gebruikte *Agrobacterium* stam LBA4404Rif^R(pTJK136).

Dezelfde bacteriële stammen werden ook gebruikt voor een floral dip transformatie van *Arabidopsis thaliana* planten met het ecotype C24. De geobserveerde transformatie-efficiënties waren zeer laag (0% tot 0.33%) in vergelijking met de transformatie-efficiënties bekomen voor *Arabidopsis thaliana* planten van het Col0 ecotype (0% tot 2.4%). Deze resultaten impliceren daarom dat *Arabidopsis* planten van het C24 ecotype minder gevoelig zijn voor floral dip transformatie.

De plant tot plant variatie binnen één floral dip experiment werd omzeild door een cotransformatie uit te voeren met een mengsel van 2 *Agrobacterium* stammen, waarin telkens de *Agrobacterium* stam C58C1 Rif^R (pMP90,pAAVS1) als referentiestam werd gebruikt. De resultaten bevestigden dat de planteneigenschappen een zeer sterke invloed hadden op de transformatie efficiënties. Daarenboven bleek de stam LBA4404 Rif^R (pTJK136) minder efficiënt te zijn dan de stam C58C1 Rif^R (pMP90,pAAVS1) tijdens floral dip transformatie van

Arabidopsis planten van het Col0 ecotype terwijl de stam LMG201 Rif^R (pMP90,pTJK136) resulteerde in een hogere transformatie-efficiëntie dan de stam C58C1 Rif^R (pMP90,pAAVS1).

Vervolgens werden de eigenschappen van de *Agrobacterium* stam LMG201 Rif^R (pMP90,pTJK136) nog wat verder onderzocht aangezien deze stam een hogere transformatie-efficiëntie vertoonde dan de veelgebruikte stam *Agrobacterium* stam C58C1 Rif^R (pGV2260,pTJK136). Testen toonden aan dat de stam LMG201 Rif^R niet langer pathogeen was na introductie van het pMP90 *vir* plasmide. Verder toonden segregatie analyses, GUS activiteitsmetingen en DNA gel blot analyses aan dat de transgene planten, bekomen na floral dip met de *Agrobacterium* stam LMG201 Rif^R (pMP90,pTJK136) stam, zeer vergelijkbaar waren met de planten bekomen na transformatie met de *Agrobacterium* stam C58C1 Rif^R (pMP90,pTJK136) op het vlak van het percentage planten met 1 T-DNA locus, het T-DNA integratie patroon, en het transgen expressie profiel.

In **hoofdstuk 4** bestudeerden we de invloed van licht tijdens T-DNA transformatie bij *Arabidopsis* worteltransformatie. Deze omgevingsfactor bleek immers eerder een positief effect te hebben op T-DNA transfer van *Agrobacterium* naar de plantencel (Zambre et al., 2003). Twee licht condities werden tijdens worteltransformatie onderzocht: continu licht (24u) en 16 uren licht / 8 uren donker. Verder werd er tijdens de transformatie experimenten zowel wel als niet geselecteerd voor transformatie competente cellen. Uit onze resultaten konden we concluderen dat licht een positief effect heeft op T-DNA transfer, maar dat cocultivatie tijdens continu licht geen positief effect heeft op stabiele T-DNA integratie tijdens *Arabidopsis* worteltransformatie.

Ten tweede bestudeerden we in welke mate T-DNA transfer en/of T-DNA integratie de *in planta* floral dip transformatie methode limiteren. In tegenstelling tot de resultaten uitgevoerd op *Arabidopsis* celculturen (Kim et al., 2007), toonden onze experimenten, uitgevoerd zonder selectie voor transformatie competente cellen, aan dat de transformatiefrequentie bepaald via selectie of via PCR screening gelijk is, wat aanduidt dat tijdens floral dip geen of slechts een beperkt aantal T-DNA's integreren

in gebieden die een negatieve invloed hebben op genexpressie, zoals heterochromatine.

Na selectie voor transformatie competente cellen, door te selecteren voor de aanwezigheid van een eerste T-DNA, bleek de cotransformatie-frequentie met een tweede T-DNA steeds lager dan 35%. In bijna geen enkele transformant kwam het tweede niet-geselecteerde T-DNA transiënt tot expressie. Dit betekent dat de T-DNA integratie de transformatie-efficiëntie niet limiteert. Net zoals bij worteltransformatie, zijn de cotransformatiefrequenties bekomen na floral dip, steeds hoger dan de transformatiefrequenties. Dit suggereert dat de toegankelijkheid van de plantencel een belangrijke parameter is voor het bekomen van stabiele transformatie na zowel floral dip als na worteltransformatie.

Het gebruik van genetische modificatie voor landbouwkundige toepassingen steeg doorheen de jaren en sommige transgene planten kwamen in verschillende landen op de markt als voedsel- of voedergewas (James, 2008). In Europa bepalen verschillende regels en wetgevingen de productie en marketing van deze biotechnologische gewassen. Aangezien de T-DNA's op willekeurige plaatsen in het gastheergenoom integreren, zijn de DNA sequenties ter hoogte van de overgang tussen genomisch plant DNA en het nieuwe geïntroduceerde exogene transgen uniek voor elk transgen event. Op basis van deze unieke sequenties werden methodes ontwikkeld voor de identificatie en de analyse van al deze events. Hierbij zijn de stabiliteit van de geïntegreerde sequentie en de insertieplaats cruciale parameters **(Hoofdstuk 1)**.

Daarom analyseerden we in **Hoofdstuk 5** de impact van vermeerdering over generaties, de invloed van "gene stacking" en foto oxidatieve stress, veroorzaakt door hoge licht intensiteiten, op de stabiliteit van de regio's die het transgen flankeren in *Arabidopsis thaliana*. Om in staat te zijn puntmutaties en grote veranderingen te detecteren in deze flankerende regio's, gebruikten we RFLP ("restriction fragment length polymorphism analysis"), sequenering en CSCE ("conformational sensitive capillary electrophoresis"). We observeerden geen instabiliteit, noch in het trangen locus, noch in de flankerende sequenties na vermeerdering over generaties. Ook in de stacked events en in de events onderworpen aan foto oxidatieve stress werd

geen instabiliteit waargenomen. Deze observaties tonen aan dat de T-DNA integratieplaatsen stabiel zijn en niet onderhevig zijn aan genotoxische stress.

Voordat een product van GMO oorsprong of een product welke sporen van GMO gewassen bevat op de markt kan gebracht worden, is een kwantificatie analyse vereist. De drempelwaarde voor aanvaarding is afhankelijk van het land waar het product zal verhandeld worden. In Europa wordt het GMO gehalte (%) uitgedrukt op basis van het aantal haploid genoom equivalenten van het GMO target (HGE) ten opzichte van de gastheer (EC, 2004). De aanbevolen % GMO eenheid is dus een percentage van de hoeveelheid GMO DNA, relatief ten opzichte van de hoeveelheid gastheer DNA (EC, 2004). De DNA hoeveelheid van zowel de gastheer als van het transgen wordt bepaald door real-time PCR. Maïs is het tweede belangrijkste biotechnologisch gewas (James, 2008). Het maïs genoom is zeer divers en één basepaar substituties (SNPs) komen frequenter voor dan inserties/deleties (indels) van één of meerdere nucleotiden (Vroh Bi et al., 2006). Wanneer een SNP aanwezig is in de aanhechtingsplaats van de primer, gebruikt tijdens gastheer-specifieke DNA kwantificatie, zal dit een “mismatch” creëren en kan dit interfereren met de GMO kwantificatie. In **Hoofdstuk 6** onderzoeken we de invloed van een SNP op real-time PCR kwantificatie. Daarom bootsten we de aanwezigheid van een basepaarsubstitutie na in het genomisch DNA van maïs door een basepaar te wijzigen op verschillende posities in de forward primer. De C_T waarden en de DNA hoeveelheden, bekomen door gebruik te maken van de primers met de basepaarsubstituties aan het 5’uiteinde, vertoonden een afwijking die vergelijkbaar was met de waarden bekomen met de niet gewijzigde primers. Wanneer de primer/template fout niet gelegen was in de uiterste 3’ base van de primer, was de afwijking in C_T waarden eerder klein (-1 tot +1.25 C_T). De afwijking werd groter en amplificatie werd zelfs onmogelijk wanneer de substitutie helemaal op het 3’uiteinde van de primer gelegen was. Dit kan dan vermoedelijk toegeschreven worden aan herhaaldelijke condities en als normaal aanschouwd worden. Maar aangezien een verschil van 1 C_T overeenkomt met een 2-voudig verschil in de geschatte target hoeveelheid, zal de gastheer specifieke DNA kwantificatie beïnvloed worden, wat tot een onder- of overschatting van het GMO gehalte zal leiden. Dit op zijn beurt zal een invloed hebben op de labeling van het product, zeker in het geval dat het GMO

gehalte dicht bij de drempelwaarde van 0.9% ligt. Daarom moet speciale aandacht gegeven worden aan de selectie van gastheer of soort-specifieke referentie gensequenties voor GMO kwantificatie. Tijdens deze selectie dient prioriteit gegeven te worden aan de geconserveerde (in eenzelfde taxon) genomische regio's zonder SNPs die een hoge nucleotidestabiliteit vertonen tussen de verschillende cultivars.

Referenties

Banta LM, Montenegro M (2008) *Agrobacterium* and plant biotechnology. *In* T Tzfira, V Citovsky, eds, *Agrobacterium*: From Biology to Biotechnology. Springer, New York, pp 73-148

Bechtold N, Jaudeau B, Jolivet S, Maba B, Vezon D, Voisin R, Pelletier G (2000) The maternal chromosome set is the target of the T-DNA in the in planta transformation of *Arabidopsis thaliana*. Genetics **155:** 1875-1887

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant journal **16:** 735-743

Desfeux C, Clough SJ, Bent AF (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. Plant physiology **123:** 895-904

EC (2004) EC/787/2004 of 04.10.2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisns as or in products in the context of Regulation (EC) No 1830/2003. . *In*, Vol L348. Off. J. Eur. Union, pp 18-26

Halford NG (2006) Plant Biotechnology: current and future applications of genetically modified crops. Wiley, U.K.

James C (2008) Global Status of Commercialized Biotech/GM Crops: 2008. ISAAA Briefs **No. 39**

Kim SI, Veena, Gelvin SB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. The Plant journal **51:** 779-791

Newell CA (2000) Plant transformation technology. Developments and applications. Molecular biotechnology **16:** 53-65

Valvekens D, Montagu MV, Lijsebettens MV (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci **85:** 5536-5540

Vroh Bi I, McMullen MD, Sanchez-Villeda H, Schroeder S, Gardiner J, Polacco M, Soderlund C, Wing R, Fang Z, Coe EHJ (2006) Single Nucleotide Polymorphisms and Insertion-Deletions for Genetic Markers and Anchoring the Maize Fingerprint Contig Physical Map. Crop Sci **46:** 12-21

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. The Plant journal **19:** 249-257

Zambre M, Terryn N, De Clercq J, De Buck S, Dillen W, Van Montagu M, Van Der Straeten D, Angenon G (2003) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta **216:** 580-586

Annex

E.U. Directives and regulations (main points and comments) for GMOs [Varzakas, 2007 #165]

Directive—Title	Main points	Comments
E.U. 90/219/EEC (entry into force 23/10/1991) Contained use of G.M. Micro-organisms	• Measures for limited use of G.M. Micro-organisms. • Not applicable to certain techniques of genetic modification. • Measures for avoidance of adverse effects in human health and environment. • Emergency plan in case of an accident and regular inspections.	Amendments – Directive E.U. 98/81/EC • Additional elements for the articles. (entry into force 5/12/1998)
E.U. 90/220/EEC (entry into force 23/10/1991) Deliberate release into the environment of GMOs	• Protective measures for human health and environment. • Not applicable to certain techniques of genetic modification. • Measures for avoidance of adverse effects. • Activities of Member States for deliberate release into the environment of GMOs for research, development and market placing purposes.	Amendments – Directive E.U. 97/35/EC • Additional elements for the disposal on the market of products which contain GMOs. – Regulation (EC) No. 258/97 and Regulation (EC) No. 1139/98 • Labelling of food containing proteins or DNA derived from genetic modification. (entry into force 1/1/2002 and enforcement for 10 years)
E.U. 2001/18/EC (entry into force 17/4/2001) Deliberate release into the environment of GMOs	• Measures of authorization of the release and disposal on the market of GMOs. • Obligatory controls after the disposal of GMOs on the market. • Consultations with the public and labelling of GMOs.	Repeal – Directive E.U. 90/220/EEC since 17/10/2002 Amendment – Regulation (EC) No. 1830/2003 (entry into force 7/11/2003)
E.U. 2004/204/EC (entry into force 23/3/2004) Arrangements for the operation of the registers for recording information on genetic modifications in GMOs	• Lists of information of genetic modification in GMOs. • Lists should contain detailed report of documents. • Lists are public available.	
E.U. 2004/643/EC	• Product should be as safe as conventional (equivalence	

	principle).	
Placing on the market of a maize product (Zea mays L. line NK603) GM for glyphosate tolerance	• Handling, packaging and protection as conventional. • Obligatory recordation of the code MON-00603-6 (unique). • Measures for labelling and traceability in all stages of the market promotion.	
E.U. 2004/657/EC Placing on the market of a sweet corn from GM maize line Bt11 as a novel food or novel food ingredient	• Product should be as safe as conventional. • Obligatory labelling as “GM sweet corn”. • Obligatory recordation of the code SYN-BTø11-1 (unique). • No more controls after placing on the market.	Replacement – Directive E.U. 90/220/EC.
Regulation (EC) No. 258/97 (entry into force 14/5/1997) Novel food and novel food ingredients	• Placing on the market within the Community of foods and food ingredients which have not been used for human consumption to a significant degree within the Community before. • Not applicable to food additives, flavorings and extraction solvents. • Specific requirements for labelling. • Specific procedure for foodstuffs containing GMOs.	
Regulation (EC) No. 1139/98 (entry into force 1/9/1998) The compulsory indication of the labelling of certain foodstuffs produced from GMOs	• Application to food and food ingredients which are produced from GM soybean or GM corn. • No application to food additives and condiments. • No application to products which are legally produced, labelled and imported, and commercialized, in the Community.	Replacements *Regulation (EC) No. 1813/97. Amendments *Regulation (EC) No. 49/2000 (entry into force 31/1/2000) *Regulation (EC) No. 50/2000 (entry into force 31/1/2000) • Additional elements for certain articles of the Regulation.
Regulation (EC) No. 1829/2003 (entry into force 7/11/2003) GM food and feed	• Measures for human and animal health protection, Community procedures of approval, inspection and labelling of GM food and feed. • Approvals are applicable for	Replacements *Regulation (EC) No. 1139/98 *Regulation (EC) No. 49/2000 *Regulation (EC) No. 50/2000

	10 years with the potential of renewal.
Regulation (EC) No. 1830/2003 (entry into force 7/11/2003) Traceability and labelling of GMOs and traceability of food and feed products produced from GMOs	• Traceability of products consisting of, or containing GMOs and foodstuffs, feed produced from GMOs. • Application for all stages of disposal on the market. • Specific demands on labelling. • Inspection, control measures and sanctions in case of infringement.
Regulation (EC) No. 65/2004 (entry into force on the date of its publication in the Official Journal of the European Union) Establishment of a system for the development and assignment of unique identifiers for GMOs	• Unique identifier for each GMO which is placed on the market. • Not applicable to pharmaceuticals intended for human and veterinary use.
Regulation (EC) No. 641/2004 (entry into force 18/4/2004) The authorization of new GM food and feed, the notification of existing products and adventitious or technically unavoidable presence of GM material which has benefited from a favorable risk evaluation	• Transformation of applications and statements in the applications. • Requirements of input on the market of certain products. • Transitional measures for adventitious or technically unavoidable presence of GM material which has benefited from a favorable risk evaluation.
Proposal for a Regulation COM/2002/0085–COD 2002/0046 (entry into force 27/10/2002) The trans-boundary movement of GMOs	• Establishment of a notifying system and exchanging information on the exports of GMO to third countries. • No application for pharmaceuticals for human use. • Surveillance, submission of reports, and imposition of sanctions for any infringement.

Dankwoord
Thanks
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A Mehdi, la version Tunisienne de Geert

A la communauté Tunisienne de Gand : Riadh et sa famille, Moez, Hichem et Ahmed

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Rim

Curriculum vitae

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Education

2004- 2009
PhD student. Gene Regulation group. Department of Plant Systems Biology, Ghent, Belgium
Topic: T-DNA transfer, integration, stability and quantification in transgenic plants
Promoter: Prof. Dr. Ann Depicker and Prof. Dr. Marc De Loose

2002-2004
Master Biology and Health. Institut Superieur de Biotechnologie, Monastir - Tunisia
Thesis: β-tropomyosin characterization from skeletal muscle in *Liza aurata*
Promoter: Prof. Dr. Aghleb Bartegi

1998-2002
Bachelor Biology Sciences. Institut Superieur de Biotechnologie, Monastir - Tunisia
Thesis: proportioning of paracetamol
Promoter: Prof. Dr. Fadhel Najjar

Other studies

2009- Effective oral presentations course organized by VIB (Vlaams Interuniversitair Instituut voor Biotechnologie), Ghent, Belgium

2006- IPBO (Institute of Plant Biotechnology for Developing Countries) course: Biosafety Assessment and Regulation of Agricultural Biotechnology, Ghent, Belgium

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Publications

In Preparation:

Rim Ghedira, Sylvie De Buck and Ann Depicker
T-DNA transfer and T-DNA integration frequencies after *Arabidopsis thaliana* floral dip and root explant cocultivation

Rim Ghedira, Sylvie De Buck, Anni Jacobs and Ann Depicker
Comparative study of *Arabidopsis thaliana* floral dip transformation mediated by 12 different *Agrobacterium* strains

Published:

1) Rim Ghedira, Nina Papazova, Marnik Vuylsteke, Tom Ruttink, Isabel Taverniers and Marc De Loose (2009) Assessment of primer/template mismatch effects on real-time PCR amplification of target taxa for GMO quantification. Journal of Agricultural and Food Chemistry: 57:9370-9377.

2) Nina Papazova*****, Rim Ghedira*****, Sabine Van Glabeke, Aghleb Bartegi, Pieter Windels, Isabel Taverniers, Isabel Roldan-Ruiz, Erik Van Bockstaele, Anne Milcamps, Guy Van Den Eede, Ann Depicker and Marc De Loose (2008) Stability of the T-DNA flanking regions in transgenic *Arabidopsis thaliana* plants under influence of abiotic stress and cultivation practices. Plant Cell Rep. 27:749-757.
***** Equal Contribution

3) Skhiri Lamia, Ghedira Rim, Bouaziz Aicha, Barbouche Rim and Bartegi Aghleb (2007) Dégradation protéique des muscles des animaux marins au cours de la conservation. Microbiol. Hyg. Alim. 19: 26-31.

4) Aicha Bouaziz, Rim Ghedira, Patrick Chaussepied, Lamia Skhiri, Jean Derancourt, Ahmed Noureddine Helal and Bartegi Aghleb (2005) Specific β-Tropomyosin release from skeletal muscle in the early post-mortem storage of the *Liza aurata* fish. Biologia. N3:28-34.

Attendance at national and international meetings

Oral Communications

- 16^{éme}journées Nationales de Biologie (Hammamet, Tunisia - November 2006) Etude de la stabilité des transgènes et analyse de la quantification des OGM par PCR en temps réel.
Ghedira R., Chaouachi M., Papazova N., Taverniers I., Depicker A., Helal A.N., Van Bockstaele E., Bartegi A., De Loose M.

- 16^{éme}journées Nationales de Biologie (Hammamet, Tunisia - November 2006) Détection des OGM par approche matricielle: du 'MULTIPLEXING' au 'SNPLEXING'
Chaouachi M., Ghedira R., Bartegi A., Brunel D., Bertheau Y.

- Deuxième Journée de Biotechnologie de l'ISBM (Monastir, Tunisia - September, October 2005) Stabilité des OGM : application d'une nouvelle technique.
Ghedira R., Papazova N., Taverniers I., Roldan-Ruiz I., Depicker A., Van Bockstaele E., Bartegi A., De Loose M.

- 16^{éme}journées Biologiques (Hammamet, Tunisia - March 2005) Caractérisation de la β-tropomyosine du muscle squelettique chez le mulet dore (*Liza aurata*) au cours de la conservation.
Ghedira R., Skhiri L., Chaussepied P., Helal A.N., Bartegi A.

- 15^{éme}journées Biologiques (Hammam Sousse, Tunisia - March 2004) Désorganisation sarcomérique des muscles squelettiques de poisons et des crustaces au cours de la conservation.
Ghedira R., Skhiri L., Jabeur C., Bartegi A.

- Première journée de Biotechnologie Médicale. (ISBM-Monastir, Tunisia - February 2004) Désorganisation sarcomérique des muscles squelettiques de poisons, des mollusques et des crustacés au cours de la conservation.
Skhiri L., Ghedira R., Jabeur C., Bartegi A.

Poster Communications

- FEBS workshop: Adaptation potential in plants (Vienna, Austria – 19-21 March 2009) T-DNA transfer and T-DNA integration efficiencies upon *Agrobacterium*-mediated transformation
Ghedira R., De Buck S. and Depicker A.

- VIB seminar (Blankenberge, Belgium – 12-13 March 2009) T-DNA transfer and T-DNA integration efficiencies upon *Agrobacterium*-mediated transformation
Ghedira R., De Buck S. and Depicker A.

- Benelux qPCR Symposium (Belgium - October 2008) Effects of primer/template mismatches on real-time PCR quantification of GMOs.
Ghedira R., Papazova N., Ruttink T., Vuylsteke M., De Loose M., Taverniers I.

- 1st Global Conference on GMO Analysis (Villa Ebra, Como, Italy – June 2008) Influence of point mutations on real-time PCR efficiency – relevance for GMO quantification.
Ghedira R., Papazova N., Ruttink T., De Loose M., Taverniers I.

- Seconde Conférence Internationale sur la Coexistence entre Filières de Production Agricole (Montpellier, France - November 2005) Monitoring the stability of the transgenic sequences – application of a new approach.
Papazova N., Ghedira R., Taverniers I., Van Glabeke S., Milcamps A., Roldan-Ruiz I., Depicker A., Van Den Eede G., De Loose M.

- Société des Sciences Naturelles de Tunisie, 4^{éme} Colloque « Sciences & Environnement »: section du Nord (Bizerte, Tunisia - April 2004) Analyze des activites endocriniennes des micropolluants par des lignees cellulaires bioluminescentes.
Mnif W., Ghedira R., Pillon A., Balaguer P., Bartegi A.

Teaching Activities

2004- 2005: Biochemistry assistant at High Institute of biotechnology-Monastir (ISBM) for 6 months.
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