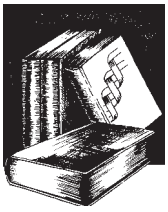

Cucumber transformation methods – the review

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Cucumber transformation methods – the review

Summary

Several aspects of cucumber transformation including the ways of transgene introduction, factors influencing the transformation efficiency and the fate of the introduced genes were reviewed. Various transgenes have been introduced into the cucumber genome mostly *via* the *Agrobacterium*-mediated transformation. The frequency of *Agrobacterium*-mediated transformation ranged from 0.8 to 10% and was influenced by the selection agent, the regeneration efficiency, activation of *vir* genes expression, the explant size, bacteria cell density, the length of exposure and the co-cultivation period. The transgenes were integrated mostly as single copy in the *Agrobacterium*-mediated transformation and as multiple copies in direct transformation. Variable levels of the transgene expression were observed. The transmission of the transgenes as well as the transgenic phenotype follow the Mendelian, and rarely non-Mendelian, ratio. The production of marker-free transgenic cucumber and use of an alternative transformation method are recommended.

Key words:
Agrobacterium, transformation, *Cucumis sativus*, marker gene.

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1. Introduction

Cucumber (*Cucumis sativus* L.) belongs to the group of the most popular vegetables in the world and the development of transgene introduction methods is very desirable for its biotechnology. Cucumber tissue culture systems are well defined (1).

Plants could be regenerated from various culture types in two ways: indirect regeneration from cotyledon explants (2,3) or leaf callus (4,5) and directly from leaf microexplants (6) or protoplast (7). The direct regeneration methods are fast allowing for regeneration of the plants without a distinguishable callus phase, and the leaf microexplant procedure is free of somaclonal variation. This can be good prerequisite for the use of leaf microexplant for transformation experiments.

Nearly two decades ago, transformation of cucumber began through an *Agrobacterium rhizogenes*-mediated transformation system (8,9) and direct gene transfer (10). At the same time, the *Agrobacterium tumefaciens* was also used (11,12). To date, many procedures have been developed with several new details. In this paper, we present the more reliable ways for transgene introduction into the cucumber genome and indicate the most important factors influencing the transformation efficiency and the fate of the introduced constructs. The agronomical properties of the transgenic cucumber plants were summarized elsewhere (13).

2. Ways of transgene introduction

The transgenes were introduced into cucumber genome mostly by *A. tumefaciens*-mediated transformation, rarely *via* a direct gene transfer, and in two cases by *A. rhizogenes*. Up to now, all agronomically important genes were transferred into the cucumber genome by *A. tumefaciens*-mediated transformation (13).

2.1. *Agrobacterium tumefaciens*-mediated transformation

The *Agrobacterium* genus has been divided into five species based on disease symptomology and plant host range (14,15). So far, *A. tumefaciens* and *A. rhizogenes* have been used for cucumber transformation (Tab. 1, 2 and 3). In cucurbits, susceptibility to crown gall disease has genetic basis (36). Hence, to some extent the differences in the transformation frequency depend on the genotype.

2.1.1. Genotype and explant sources

Various genotypes, including non-hybrid and hybrid cultivars, pure line and inbred lines of different origins, were successfully used for *Agrobacterium*-mediated transformation (Tab. 1, 2 and 3). The agronomically important genes: coat protein gene from cucumber mosaic virus (CMV-*cp*), superoxide dismutase gene from cassava (*mSOD1*), chitinase genes from different plant species (petunia, tobacco, bean), coat protein gene from zucchini green mottle mosaic virus (ZGMMV-*cp*), and chitinase gene from rice (*RCC2*) were introduced into different cucumber genotypes.

Moreover, eight different constructs were introduced into one genotype, the inbred line Borszczagowski, using the same procedure (Tab. 3). All of them contained the gene of interest and the marker gene. However, the marker or selectable genes in plant transformation constructs have become very problematical, because in the European Union the registration of GMO containing the antibiotic or herbicide resistance genes will be not allowed starting from 2006.

There are two main sources of explants – directly excised from the plants or seedlings and tissues cultured *in vitro*. In the first case, leaf microexplants, leaf petiole, cotyledons and hypocotyls were inoculated. The *in vitro* growing tissue was a leaf- or cotyledon-derived embryogenic callus and a meristem-derived protoplast. According to our experience, the most promising explant is a leaf microexplant, previously described as being free of somaclonal variation and able to quickly and prolifically regenerate into the plants (6). A simple modification of the medium makes possible its application in various genotypes. Bacteria inoculation step, incorporated into this regeneration procedure, doubled the time of obtaining mature plants, i.e. 9-12 weeks, depending on the kind of the construct and the period of the year.

2.1.2. Some transgene elements

The total number of plasmid vectors used for cucumber transformation is 18 (Tab. 1, 2 and 3). Most frequently, a chimeric gene for kanamycin resistance (*npt*II) was used as the selectable marker. In four cases, a hygromycin resistance *hpt* gene and in only one, a herbicide resistance *bar* gene, were used. The β-D-glucuronidase gene (*uidA*) and luciferase gene (*luc*), mostly under the 35S cauliflower mosaic virus promoter (35S CaMV), were utilized as reporter genes. The constructs with agronomically important genes were most often driven by the 35S promoter and included the CMV coat protein gene (12,24), coat protein gene of ZGMMV (22), chitinase genes (16,18) and thaumatin II cDNA (25). In several cases, tissue specific promoters were used, such as *mSOD1* gene under the ascorbate oxidase promoter (pASO) (21), thaumatin II cDNA driven by tomato polygalacturonase (PG) promoter (Szwacka unpubl.), and tryptophan monooxigenase gene (*iaaM*) under the control of *Antirrhinum majus* ovule-specific *Deficiens* homologue 9 promoter (p*Def*H9) (35).

Table 1

The genotypes, *Agrobacterium* strains and plasmid vectors used for cucumber transformation

Genotype	Explant	*Agrobacterium* strain*	Plasmid vector	Transgene construct	Literature
1	2	3	4	5	6
cv. Endeavor	petiole	EHA105 MOG101 MOG301	pMOG196 pMOG198 pGA492-CHN	pnos::*npt*II p35S::chitinase gene	(16)
cv. Poinsett 76	cotyledon	C58Z707	pGA482	pnos::*npt*II	(11)
		C58Z707	pGA482GG/ cpCMV19	pnos::*npt*II p35S::*uidA* p35S::CMV-C *cp*	(12)
		EHA105	pME524	*npt*II, *uidA* *bar*	(17)
cv. Shimoshirazu	cotyledon	LBA4404	pBI121-RCC2	pnos::*npt*II p35S::*RCC2*	(18)
cv. Shinhokusei No. 1	cotyledon	GV3101 LBA4404	pGV3850 HPT pBI121	p35S::*hpt* pnos::*npt*II	(19)
cv. Spring Swallow	cotyledon	GV2260	p35S-GUSINT	*npt*II *gus*	(20)
cv. Winter Long	cotyledon	LBA4404	pGPTV-Bar	pASO::*mSOD1* pnos::*bar*	(21)
var. Chungjang	–	–	pGA748-ZGMMV	ZGMMV *cp* *npt*II	(22)
Pure line 1021	hypocotyl	EHA101	pIG121-Hm	pnos::*npt*II p35S::I-*gus* p35S::*hpt*	(23)
	hypocotyl	EHA101	pIG121-Hm CP	p35S::CMV-O *cp* pnos::*npt*II p35S::I-*gus* p35S::*hpt*	(24)
Inbred line Borszczagowski	leaf	LBA4404	pRUR528s	p35S::*thaumatin II cDNA* pnos::*npt*II	(25)
	leaf	LBA4404	pGA482	pPR-2d::*uidA* pnos::*npt*II	(26,27)
	HECSC	LBA4404 EHA105	pCAMBIA1301 pGPTVhpt	*hpt* *uidA*	(28)
Inbred line 3672 3676 (Gy14)	petiole, leaf	LBA4404	pCGN783 pBIN19	pnos::*npt*II p35S::*npt*II	(9,29)
Inbred line Gy14A	cotyledon	LBA4404	pCIB10	Km^R gene	(30)
Hybrid Bambina	cotyledon	LBA4404	pAL4404 pAQ_2	pnos::*npt*II p35S::*luc*	(31)
Hybrid Brunex Hybrid Bambina	cotyledon, hypocotyl	C58C1	pGKB5	*npt*II *bar* promoterless::*uidA*	(32)
cv. Straight Eight	hypocotyl	A4 (*A.r.*)	Ri plasmid pARC8	Ri T-DNA pnos::*npt*II	(8)

1	2	3	4	5	6
Inbred line Gy3 cv. Straight Eight	hypocotyl	LBA9402 (*A.r.*)	Ri plasmid	Ri T-DNA	(33)

Abbreviations:

*-*A. tumefaciens* strains
A.r.-A. rhizogenes strains
bar-bialaphos resistance gene
CMV-C *cp*-Cucumber Mosaic Virus-C coat protein gene
CMV-O *cp*-Cucumber Mosaic Virus-O coat protein gene
gus-β-D-glucuronidase (GUS) gene
HECSC-highly embryogenic cell suspension culture
hpt-hygromycin phosphotransferase gene
I-first intron of catalase gene from castor bean
luc-firefly luciferase gene
mSOD1-cytosolic CuZnSOD cDNA from cassava
*npt*II-neomycin phosphotransferase II gene
p35S-cauliflower mosaic virus 35S promoter
pASO-ascorbate oxidase promoter
pnos-nopaline synthase promoter
pPR-2d-tobacco β-1,3-glucanase promoter
RCC2-a rice chitinase cDNA
uidA-β-D-glucuronidase (GUS) gene
ZGMMV *cp*-coat protein gene of zucchini green mottle mosaic virus

Table 2

The plant genotypes, special treatment application and the efficiency of *Agrobacterium*-mediated cucumber transformation

Genotype	Transgene construct	Selection	Special treatment	TC	TE	ITE	Literature
1	2	3	4	5	6	7	8
cv. Endeavor	pnos::*npt*II p35S::chitinase gene	Km	suspension culture, acetosyringone	ND	ND	9, 32, 44 plants	(16)
cv. Poinsett 76	pnos::*npt*II	Km	5 weeks in dark	ND	10%[A]	100 plants	(11)
	pnos::*npt*II p35S::*uidA* 35S::CMV-C *cp*	Km	5 weeks in dark	ND	ND	100 plants	(12)
	*npt*II *uidA* *bar*	PPT	BAP acetosyringone	ND	ND	ND	(17)
cv. Shimoshirazu	pnos::*npt*II p35S::*RCC2*	Km	–	ND	ND	200 strains	(18)
cv. Shinhokusei No. 1	p35S::*hpt* pnos::*nptII*	Km G418 Hy	liquid culture for selection	ND	ND	6 plants	(19)
cv. Spring Swallow	*npt*II *gus*	–	acetosyringone	ND	ND	ND	(20)
cv. Winter Long	pASO::*mSOD1* pNOS::*bar*	bialaphos	–	ND	4%[A]	4 plants	(21)
var. Chungjang	ZGMMV *cp* *npt*II	Km				3 plants	(22)
Pure line 1021	pnos::*npt*II p35S::I-*gus* p35S::*hpt*	Km Hy	acetosyringone	4-6 months	1.4%[A]	12 plants	(23)

1	2	3	4	5	6	7	8
Pure line 1021	p35S::CMV-O *cp* pnos::*npt*II p35S::I-*gus* p35S::*hpt*	Hy	3', 5'- dimethoxy-4'--hydroxy--acetophenone	4-6 months	ND	4 plants	(24)
Inbred line Borszczagowski	p35S::*thaumatin II cDNA* pnos::*npt*II	Km	–	ND	2.9--6.3%[B]	16 ITE / 63 plants	(25)
	pnos::*npt*II pPR-2d::*uidA*	Km	–	ND	1.52%[B]	7 ITE	(26,27)
Inbred line 3672 3676 (Gy14)	pnos::*npt*II p35S::*npt*II	Km	acetosyringone	ND	9%[A]	21 plants	(9,29)
Inbred line Gy14A	Km^R gene	–	tobacco nurse culture	ND	ND	ND	(30)
Hybrid Bambina	pnos::*npt*II p35S::*luc*	Km	–	ND	ND	ND	(31)
Hybrid Brunex Hybrid Bambina	*npt*II *bar* promoterless::*uidA*	Km	BA	ND	ND	ND	(32)
cv. Straight Eight	Ri T-DNA pnos::*npt*II	medium without hormone	–	10 weeks	3.2%[A]	22 plants	(8)
Inbred line Gy3 cv. Straight Eight	Ri T-DNA	medium without hormone	–	ND	ND	0	(33)

Abbreviations:

bar-bialaphos resistance gene

CMV-C cp – Cucumber Mosaic Virus-C coat protein gene

CMV-O cp – Cucumber Mosaic Virus-O coat protein gene

G418-geneticin

gus-β-D-glucuronidase (GUS) gene

hpt-hygromycin phosphotransferase gene

Hy-hygromycin

ITE-independent transformation event

Km-kanamycin

luc-firefly luciferase gene

mSOD1 – cytosolic CuZnSOD cDNA from cassava

ND-not determined

*npt*II-neomycin phosphotransferase II gene

p35S-Cauliflower Mosaic Virus 35S promoter

pASO-ascorbate oxidase promoter

pnos-nopaline synthase promoter

PPT-phosphinothricin

pPR-2d-tobacco β-1,3-glucanase promoter

RCC2-a rice chitinase cDNA

TC-time consumption, referred to as the duration between bacteria inoculation and the transgenic plantlets ready to transfer into the soil

TE-transformation efficiency. A: percent of the inoculated explants producing regenerated shoots. B: percent of the inoculated explants produced transgenic plants

uidA-β-D-glucuronidase (GUS) gene

ZGMMV cp-coat protein gene of zucchini green mottle mosaic virus

Table 3

Some information concerning the cucumber transformation experiments using a highly inbred line of *Cucumis sativus* L. cv. Borszczagowski and various constructs

Construct	*A. tumefaciens* strain	Plamid vector	Selection	TE	ITE	Literature
p35S::*thaumatin II cDNA*	LBA4404	pRUR528s	Km	6.3%	16	(25)
pPG::*thaumatin II cDNA*	LBA4404		Km	1%	3	Szwacka, Jankowska unpubl.
pPR-2d::*uidA*	LBA4404	pGA482	Km	1.52%	7	(26,27)
pPR-2d::*uidA*	LBA4404	pGA482	Km	1.4%	5	Yin unpubl.
mldh	LBA4404	pBinAR	Hy	1.4%	14	Yin unpubl.
apinv	LBA4404	pCAMBIA	Km	1.3%	9	Yin unpubl.
pGT::*Dhn10*	LBA4404	pBI121	Km	0.8%	11	(34), Yin unpubl.
pGT::*Dhn10*	LBA4404	pBI121	Km	4%	21	(34), Yin, Ziółkowska unpubl.
pGT::*Dhn25*	LBA4404	pBI121	Km	2.3%	17	Yin, Ziółkowska unpubl.
pDefH9::iaaM	GV2260	pPCV002	Km	1.5%	8*	(35)
hpt *uidA*	LBA4404 EHA105	pCAMBIA1301 pGPTVhpt	Hy	1000 ITE / 1ml of PCV	29	(28), Zuzga et al. unpubl.

Abbreviations:

* 50% of them were tetraploid

apinv-Saccharomyces cerevisiae apoplastic invertase gene

Dhn10–Solanum sogarandinum dehydrin (10 kDa) gene

Dhn25-Solanum sogarandinum dehydrin (25 kDa) gene

hpt-hygromycin phosphotransferase gene

Hy-hygromycin

iaaM- tryptophan monooxigenase gene

ITE-independent transformation events, referred to as the single transformation event produced rooted transgenic plants

Km-kanamycin

mldh-Brassica napus cytosol malate dehydrogenase gene

p35S-Cauliflower Mosaic Virus 35S promoter

PCV-packed cell volume

p*Def*H9-*Antirrhinum majus Deficiens* homologue 9 promoter

pGT-*Solanum sogarandinum* glucosyl transferase promoter

pPG-tomato fruit-specific polygalacturonase promoter

pPR-2d-tobacco β-1,3-glucanase promoter

TE-transformation efficiency, percent of inoculated explants that produced transgenic plants

uidA-β -D-glucuronidase (GUS) gene

2.1.3. Explant size, bacteria cell density and length of exposure

Sarmento et al. (29) mentioned the following factors as influencing the frequency of petiole callus development on kanamycin (75 mg/l)-containing medium: explant size, bacteria cell density and the length of exposure, co-cultivation period, and the presence of acetosyringone. The optimal procedure involved exposing the segments of petiole (4-6 mm length) or leaf (0.5 cm^2) to a bacterial suspension (10^8 cells/ml) containing 20 μM acetosyringone for 5 min, followed by 48 hr co-cultivation period on a tobacco feeder layer. The frequency of callus formation ranged between 0 and 40%.

2.1.4. *Vir* genes induction

The processing and transfer of T-DNA from *Agrobacterium* to plant cell are regulated by the activation of the *vir* genes. The *vir* gene expression can be induced by phenolic compounds such as acetosyringone and related molecules released by the wounded tissue (14). Using acetosyringone at the concentration of 50-200 μM during co-cultivation is sufficient to increase the transformation frequency. Nishibayashi et al. (23) used 100 μM acetosyringone during 5 days co-cultivation. Lower (50 μM) concentration enhanced the transformation efficiency of cotyledon explants and additional wounding treatment with a hollow needle pierced through the surface of the proximal end of the cotyledon enhanced the stable transfer of T-DNA into plant cells (17). Acetosyringone (200 μM) is a component in the procedure of leaf microexplants transformation used in our laboratory (Tab. 3).

2.1.5. Selection system

A proper selection procedure should reduce the occurrence of "escapes". The selection agents used are kanamycin, hygromycin, phosphinothricin (PPT), and geneticin (G418). Kanamycin, in the concentration of 50-150 mg/l, applied for four to six weeks, was a commonly used and efficient agent (11,17,19,23-25). In our laboratory, both kanamycin and hygromycin were used. According to our experience, kanamycin is a much better selective antibiotic compared to hygromycin. The use of kanamycin allows plants to be much more vigorous following the transfer into the soil, whereas, hygromycin disturbs plant development considerably and makes seed production extremely difficult. By contrast, Tabei et al. (19) demonstrated that the growth suppression of non-transgenic callus was more efficient with the use of G418 or hygromycin than kanamycin in the liquid culture system. Similarly, Nishibayashi et al. (23) demonstrated that kanamycin in the concentration of 50-100 mg/l is much less efficient than hygromycin at 20-30 mg/l for the selection of transgenic callus. We supposed that such difference might result from the genotype and/or type of culture.

2.1.6. Regeneration efficiency

An efficient and stable plant regeneration procedure is the most important requirement for reliable plant transformation. However, bacteria inoculation may considerably change some of the relations, mostly concerning time and regeneration efficiency. Sometimes, an incorporation of new stimulatory substances is necessary. Tabei et al. (18) demonstrated that the addition of abscisic acid (ABA) into the shoot induction medium increased the efficiency of shoot organogenesis and induced

multiple shoots. Low concentration (1 mg/l) of 6-benzylaminopurine (BAP) stimulated the production of a higher number of shoots and roots (17). Sapountzakis and Tsaftaris (32) reported that the BAP level, on which the highest number of shoots was obtained, is 0.5 mg/l. In our procedure (Tab. 3), the concentration of the growth regulator is stable at each step, as experimentally estimated. In accordance with our experience (Malepszy et al. unpubl.), incorporation of the bacteria inoculation step into the leaf microexplants procedure (6) usually diminished the regeneration efficiency by a factor of 20, and delaying the occurrence of regenerants. However, some remarkable differences may occur, depending on the construct. This was most contrasting with p*Def*H9::*iaaM* chimeric transgene (*iaaM* gene from *Pseudomonas syringae* pv. *savastanoi*, under the control of the regulatory sequences of the ovule-specific *Def*H9 gene from *Antirrhinum majus*), where a lot of abnormal shoot structures and high amount of tetraploids (50%) were observed (35).

2.1.7. Transformation efficiency and time consumption

The transformation efficiency is represented as the percentage of explants producing regenerated shoots or transgenic plants. It remains low, ranging from 1.5 to 6.3% for regenerated shoots and 1.4-10%, as the ratio between the number of the obtained transgenic plants and the number of the total explants inoculated (Tab. 2). It takes 10 weeks (8) to 6 months (23,24) from the inoculation of the explants to the moment the plantlets are ready to be transferred into the soil, not considering the time required to obtain the seeds. In our transformation procedure, the length of this period is comparable with that needed for a plant derived from a regeneration procedure without bacteria inoculation. However, considerable differences have occurred for some constructs. In case of R_0 plants harbouring p*Def*H9::*iaaM* construct, a longer adaptation time following the transfer into the greenhouse was observed (35). This has prolonged, by 2-4 weeks, the time required for seed harvest.

2.2. *Agrobacterium rhizogenes*-mediated transformation

Two papers (8,33) reported the use of *A. rhizogenes* mediated transformation but only one (8) described a successful regeneration of transgenic plants within 10 weeks (Tab. 1 and 2). Plantlets were regenerated from 64 out of the total 691 roots harvested from the inoculated hypocotyl sections. Twenty-two plantlets were neomycin phosphotransferase II (*npt*II) positive. The addition of 25 mg/l kanamycin to the embryo-inducing medium did not affect the regeneration from the transformed tissue and did not prevent regeneration of some *npt*II-negative plants. The other report demonstrated that opine synthesis was detected in 20% of the 25 fast growing root clones tested (33).

2.3. Direct gene transfer

There were only a few reports of direct cucumber transformation (Tab. 4). Chee and Slightom (10) obtained a total number of 107 independently regenerated plants from ten different batches of embryogenic callus tissues bombarded with plasmid pGA482. Among them, 16% were transformed with pnos::*npt*II chimeric gene (*npt*II gene from *E. coli* under the control of the regulatory sequences of the nopaline synthase gene from *A. tumefaciens*). Schulze et al. (37) reported the production of transgenic plants by biolistic transformation of highly embryogenic cell suspension culture. After 6 months of *in vitro* culture, 189 structures were formed and 34 of them developed into plantlets finally resulting in 28 vigorously developed and rooted plants. The transformation frequency was four plants per bombardment (0.5 ml packed cell volume). All selected plants were proved to be *npt*II-positive and no "escape" could be detected. Co-integration efficiency for the linked unselectable *uidA* gene was 67%. Kodama et al. (38) obtained the transformed roots from cotyledon tissues bombarded with gold particles coated with plasmid pE7.4.

Table 4

Some characteristics of direct cucumber transformation

Genotype	Target tissue	Method	Plasmid vector	Transgene construct	Selection	TC	TE	Primary transgenic plant	Literature
cv. Libelle	HECSC	biolistic transformation	pRT99-GUS	p35S::*npt*II p35S::*uidA*	Km	6 months	4 plants/ bombardment	28	(37)
cv. Poinsett 76	EC	microprojectile bombardment	pGA482 pUC19	pnos::*npt*II	–	ND	16%*	17	(10)
cv Shimoshirazujibai	cotyledon	particle bombardment	pE7.4 pBI221	*rolA* *rolB* *rolC* ORF13 p35S::*uidA*	–	ND	ND	0	(38)
Inbred line Borszczagowski	protoplast	electroporation	pBI121	p35S::*gus* pnos::*npt*II	–	ND	ND	0	(39)

Abbreviations:

*: percent of the regenerated plants transformed with pnos::*npt*II

EC-embryogenic callus

gus- β-D-glucuronidase (GUS) gene

HECSC- highly embryogenic cell suspension culture

Km- kanamycin

ND- not determined

*npt*II- neomycin phosphotransferase II gene

p35S- Cauliflower Mosaic Virus 35S promoter

pnos- nopaline synthase promoter

rolA, *rolB, rolC,* ORF13- genes involved in hairy root induction from plasmid pRi1724 of *A. rhizogenes* strain MAF 03-01724

TC- time consumption, referred to as the duration between bacteria inoculation and the transgenic plantlets ready to transfer into the soil

TE- transformation efficiency

uidA -β -D-glucuronidase (GUS) gene

3. Fate of the introduced transgene

The integration and expression of transgenes was summarized in Table 5. The transgenes can be stably integrated into the cucumber genome, however, the copy number of the integrated transgene may vary depending on the choice of the transformation method. Variable levels of the transgene expression were observed. The expression of the transgene at RNA and/or protein level was positively correlated with the transgene-related phenotype, but with some exceptions.

Table 5

Integration and expression of transgenes in cucumber

Method	Transgene	Copy number	Transgene expression tissue			Transgene related phenotype	Literature
			RNA	Protein expressed	Protein not expressed		
1	2	3	4	5	6	7	8
A.t.	pnos::*npt*II	single	ND	leaf	ND	ND	(11,12,40)
	p35S::*uidA*	ND	ND	leaf	10% NPT-positive plants	ND	
	p35S::CMV-C *cp*	ND	ND	leaf	ND	CMV^R	
A.t.	p35S::I-*gus*	single or few	ND	young leaf, root meristematic region, cotyledon, ovule, pollen, another	mature leaf, old root, petiole, tendril	ND	(23,24)
	p35S::CMV-O *cp*	single	leaf		leaf, cotyledon	$CMV\text{-}Y^R$ $CMV/ZYMV^T$ $ZYMV^S$	
A.t.	pnos::*npt*II p35S::*npt*II	10	ND	callus, shoot, cotyledon, leaf	ND	ND	(29)
A.t.	pnos::*npt*II	single or two	ND	ND	ND	ND	(16,41)
	p35S::chitinase genes	ND	ND	leaf, callus	ND	No increase in $fungal^T$	
A.t.	p35S::*RCC2*	ND	ND	epidermal cells of leaves	ND	H^R, I^R or S to gray mold	(18,42)
A.t.	p35S::*thaumatin II cDNA*	single, 2 or 5	leaf, fruit	leaf, fruit	ND	Changing in fruit taste	(43-45)
A.t.	pPR-2d::*uidA*	single or 2	ND	leaf	ND	ND	(27)
A.t.	pASO::*mSOD1*	single or 2	leaf, fruit	leaf, fruit	ND	Elevated SOD activity	(21)
D	pnos::*npt*II	single or multiple	ND	25% of the transgenic plants	75% of the transgenics	ND	(10)

1	2	3	4	5	6	7	8
D	p35S::*npt*II	multiple	ND	leaf	ND	ND	(37)
	p35S::*uidA*						

Abbreviations:

A.t -A. tumefaciens-mediated transformation
CMV-Cucumber Mosaic Virus
CMV^R-CMV resistance
$CMV\text{-}Y^R$-CMV-Y resistance
$CMV/ZYMV^T$-CMV/ZYMV tolerance
CMV-C *cp*-Cucumber Mosaic Virus C coat protein gene
CMV-O *cp*-Cucumber Mosaic Virus O coat protein gene
D-direct gene transfer
$fungal^T$-fungal tolerance
gus-β-D-glucuronidase (GUS) gene
H^R-high resistance
I-first intron of catalase gene from castor bean
I^R-intermediate resistance
mSOD1-cytosolic CuZnSOD cDNA from cassava
ND-not determined
*npt*II-neomycin phosphotransferase II gene
pnos-nopaline synthase promoter
p35S-Cauliflower Mosaic Virus 35S promoter
pPR-2d-tobacco β-1,3-glucanase promoter
pASO-ascorbate oxidase promoter
RCC2-a rice chitinase cDNA
S-susceptibility
uidA-β -D-glucuronidase (GUS) gene
$ZYMV^S$-ZYMV susceptibility

3.1. Integration of the transgene

The transgenes were integrated mostly as a single copy in the *Agrobacterium*-mediated transformation and multiple copies in the direct transformation.

3.1.1. *Agrobacterium tumefaciens*-mediated transformation

The *npt*II gene was the commonly used selectable gene in *Agrobacterium*-mediated transformation. Single or multiple *npt*II gene was integrated and stably transmitted to R_1 progeny (11,12). In some cases, a copy number of the *npt*II gene was ten per haploid genome, including the multiple insertions (29). Raharjo et al. (16) reported single or two copies of *npt*II gene integration and Szwacka et al. (25,45) suggested single integration. The copy number of the introduced *uidA* gene was either single or few (23). Yin et al. (27) reported one or two copies of the integrated *uidA* gene.

With regard to the agronomically important genes, a single copy of CMV-*cp* gene was detected (24). Lee et al. (22) demonstrated that 3 out of 20 selected R_0 lines contained the ZGMMV-*cp* gene. The *RCC2* gene was transmitted to the T_1 progeny (18). Szwacka et al. (45) reported that the copy number of the thaumatin II gene varied in T_1 plants, appearing as one in most cases and as two or five in others. The chromosome location of the p35S::*thaumatin II cDNA*-pnos::*npt*II gene construct was determined by fluorescent *in situ* hybridization (FISH) method. The transgenes were preferentially located in the euchromatic region of chromosomes 1, 2, 3 and 4 (46).

3.1.2. *Agrobacterium rhizogenes*-mediated transformation

Among the plants tested for the integration of the Ri-plasmid T-DNA, two plants did not contain any Ri-plasmid T-DNA fragment, one possessed a 5.7 kb fragment of the TR-DNA and two others had the TL-DNA of a different length (8). Southern blot analysis showed that each transgenic plant appeared to contain a single copy of the integrated T-DNA.

3.1.3. Direct gene transfer

In case of the direct transfer method, preferentially multiple copies of the transgenes integrated. For 19 R_0 plants transformed with pnos::*npt*II, six contained a single copy and the remainder multiple copies (10). The plant with a single copy of the *npt*II gene transmitted it into 60-80% of the progeny. In the other case of *npt*II gene, single- as well as multiple-copy integration and rearrangement occured (37).

3.2. Expression of the transgenes

After the integration into the cucumber genome, the transgenes can be expressed at transcriptional and/or translational level, and the expression pattern of the transgenes may be further influenced by the transgene-dependent, recipient-dependent as well as environment related factors (47). Usually, expression of the transgenes was positively correlated with the transgene related phenotype, but with some exceptions.

3.2.1. *Agrobacterium tumefaciens*-mediated transformation

Expression of the marker/reporter genes *uidA*, *luc* and *npt*II was analysed on the protein level. A strong β-D-glucuronidase (GUS) expression occurred in very young leaves, root meristematic regions, ovule cells and cotyledons of R_0 plants (23,24). However, GUS expression was not detected in mature leaves, which had displayed strong GUS expression in very young leaves, nor in old roots, petioles, tendrils, and various tissues of male and female flowers of some plants. This was rather surprising, because the gene was driven by a strong constitutive promoter. Other authors observed GUS expression in leaves (17,32) and roots (17). However, the expression of the *uidA* gene driven by an inducible tobacco β-1,3-glucanase promoter (PR-2d) was pathogen-, salicylic acid (SA)-, and development-dependent (27). An exogenous SA treatment increased GUS activity from 2 to 11 fold over the control, whereas the inoculation with *Erysiphe polyphage* increased GUS activity from 4 to 44 fold. Under cold stress, the

PR-2d promoter was induced up to 624 fold. The elevated level of GUS activity was detected in floral organ. Furthermore, the expression level of the pPR-2d::*uidA* varied between/within homozygous lines following the SA treatment (48). The GUS activity varied between the lines from 1.7 to 18-fold, as well as between the sibling lines from 1.0 to 5.3 fold. Considerably higher variability in GUS expression levels, from 1.0 up to 56-fold, existed within the lines. The expression of firefly luciferase (*luc*) gene was confirmed by leaf luminescence recorded on the x-ray film (31). Chee (11) reported that 100 transformed kanamycin resistant R_0 plants showed the presence of neomycin phosphotransferase (NPT II) enzyme activity in their protein extracts.

The expression of agronomically important genes and the transgene-related phenotype were studied. The expression of CMV-*cp* gene, either on RNA or protein level, was positively correlated with resistance to CMV infection (12,24). The expression of rice chitinase gene enhanced resistance to gray mold (18), whereas the expression of chitinase genes from petunia, tobacco or bean did not offer resistance to the inoculation with fungal pathogens: *Colletotrichum lagenarium*, *Alternaria cucumerina*, *Botrytis cinerea* and *Rhizoctonia solani* (41). Variable levels of thaumatin II transcript as well as thaumatin II protein accumulation were observed in leaves and fruits; and there was lack of correlation between protein and mRNA accumulation (44,45). For the expression of *mSOD1* driven by the tissue specific pASO promoter, accumulation of *mSOD1* transcript was much higher in fruits of all transgenics, but with lower levels in leaves (21). SOD specific activity (approximately 150 units/mg total cellular protein) in transgenic cucumber fruits was about three times higher than in non-transformed control plants. However, it was much lower (15 units) in leaves, almost on the same level as in non-transgenic plants.

3.2.2. *Agrobacterium rhizogenes*-mediated transformation

For the *npt*II gene introduced by *A. rhizogene*s, NPT II assay showed that 22 plantlets were NPT II positive in the *in vitro* test and after potting into the soil (8).

3.2.3. Direct gene transfer

The expression level of the *npt*II and *uidA* gene, introduced by direct transfer, was studied. The NPT II enzyme activity was detected in only 25% of the 17 transgenic plants containing the *npt*II gene (10). Schulze et al. (37) demonstrated GUS activity in 67% of the kanamycin-resistant plants. Histochemical staining revealed GUS activity in leaves, stems, roots and petals of *npt*II-positive plants. There were transformants with a strong NPT II signal and only low or no GUS activity at all, as well as with a weak NPT II signal and strong GUS activity. Thus, the expression level from each of the two genes located on the transgene may differ considerably.

4. Genetic analysis

Inheritance of marker and reporter genes was analysed in different lines and varieties. Both Mendelian and non-Mendelian inheritance was observed. The segregation of the *npt*II gene occurred at the expected ratio for a single locus in R_1 progeny (9,10). The segregation of kanamycin resistance in 7 out of 9 independent transformants was consistent with the predicted ratio in the T_1 generation as expected for a single locus, while deviated from the expected ratio for the other two events (45). Furthermore, the segregation of kanamycin resistance was investigated in two groups of transgenic lines, one containing pPR-2d::*uidA*-pnos::*npt*II and the second p35S::*thaumatin II cDNA*-pnos::*npt*II, up to the third and fifth generation (49). In case of PR-2d transformants, 78% of the progeny exhibited segregation ratio consistent with Mendelian ratio, whereas in case of the other construct, Mendelian segregation was observed in 46% of the progeny. Segregation ratio for 2 and 3 independent *loci* appeared for each construct.

However, the transmission of the agronomically important transgene and its expression were rarely studied. The single copy of the CMV-*cp* gene was detected (24). Lee et al. (22) demonstrated that 3 out of 20 selected R_0 lines contained the ZGMMV-*cp* gene. Tabei et al. (18) demonstrated a transmission of the *RCC2* chitinase gene to the T_1 progeny together with disease resistance against gray mold. The segregation of disease resistance among the progeny was in accordance with the predicted Mendelian ratio of 3:1 (resistant : susceptible). The integration of *RCC2* gene was confirmed in 7 out of 13 progeny of the CR33 line, exhibiting resistance. Szwacka et al. (45) reported that the copy number of the thaumatin II gene in 16 T_1 plants was one for the majority of cases, and single cases each with a copy number of two and five. No truncation or rearrangement of the thaumatin II expression cassette were detected. For the multiple copy events, the changes in the transgene copy number were observed in the T_2 generation.

5. Cucumber transformation experiments using one plant genotype and different plasmid vector carrying various transgenes

We have developed an *A. tumefaciens*-mediated leaf microexplant transformation system using a highly inbred line of *C. sativus* L. cv. Borszczagowski. Eight different transgene constructs including p35S::*thaumatin II cDNA* (25), pPG::*thaumatin II cDNA* (Szwacka and Jankowska, unpubl.), pPR-2d::*uidA* (26,27, Yin unpubl.), *mldh* (*Brassica napus* cytosol malate dehydrogenase gene, Yin unpubl.), *apinv* (*Saccharomyces cerevisiae* apoplastic invertase gene, Yin unpubl.), pGT::*Dhn10* (*Solanum sogarandinum* 10 kDa dehydrin gene under the control of the regulatory sequences of the S. *sogarandinum* cold-induced glucosyl transferase *Ssci17* gene; 34, Yin unpubl., Yin and Ziółkowska unpubl.), pGT::*Dhn25* (*S. sogarandinum* 25 kDa dehydrin gene under the

control of the regulatory sequences of the *S. sogarandinum* cold-induced glucosyl transferase *Ssci17* gene, Yin and Ziółkowska unpubl.), p*Def*H9::*iaaM* (35, Yin and Ziółkowska unpubl.) were introduced (Tab. 3). They contained target genes or marker genes under the control of constitutive or tissue-specific promoters. The transformation efficiency, represented as the percentage of inoculated leaf explants that give rooted transformed plants, ranged from 0.8 to 6.3%. Independent transformation events (ITE, regenerable independent kanamycin resistant calli) ranged from 3 to 21, and the number of regenerated plantlets obtained from one independent transformation event was from 2 to 17. Some transformation events regenerated quicker and were more prolific than others. The first shoots can be recovered within 6 weeks and most of them appeared between 8 to 10 weeks after inoculation. The well-rooted plantlets are ready for transfer into the soil within 3 months. Usually, fertile transgenic plants without morphological changes were produced. The method proved reproducibility and reliability. However, some differences in time consumption and plant regeneration, which depended on the construct, were observed (p*Def*H9::*iaaM*). Acetosyringone was used during the inoculation and co-cultivation stage.

6. Future perspectives

For biotechnological progress, the development of a new transformation strategy is necessary. New transformation method should be improved in three main aspects: alternative ways of selecting for transformed plants with the use of marker-free gene constructs, omitting the *in vitro* regeneration step, and increasing the transformation efficiency. Nowadays, such procedures are not yet ready for the application in cucumber, but some good prospects seem to appear (28,50). Additionally, there is no example of organellar transformation in the cucumber, except for Havey et al. (51) proposing a system for mitochondrial transformation using MSC mutant.

6.1. Marker free transformation

There are two main strategies to generate the transgenic plants free of selectable or marker genes. One approach is to excise or segregate marker genes from the host genome after regeneration of transgenic plants. Among many attempts for such marker gene removal, the site-specific expression of a transgenic DNA sequence containing the marker gene is commonly used (52,53). The most recent example uses a chemical regulation of the Cre/lox DNA recombination system (54). Other methods include the use of co-transformation, transposase/transposable element systems, and intra-chromosomal recombination. The second approach is

based on the so-called 'marker free' transformation. Zuo et al. (55) proposed the possible strategies for generating transgenic plants without a selectable marker by appropriate manipulation of the regeneration-promoting gene. In such a system, only transformed cells can regenerate in the absence of key growth regulators. In the cucumber, green autofluorescence (GAF), a simple and visible marker of embryogenic capacity, was utilized as a reporter gene for embryogenic suspension transformation (28). This procedure makes it possible to identify the transformation events on the cellular level and gives rather high efficiency of transformation.

6.2. Use of alternative transformation method

The alternative methods such as infiltration, electroporation of cells and tissues, electrophoresis of embryos, microinjection, pollen-tube pathway, silicon carbide- and liposome-mediated transformation have been suggested (56-61). Most of these methods were used for the transformation of some recalcitrant species. Among them, infiltration is used as the main transformation method for *Arabidopsis*. The adaptation of these systems to a broad spectrum of plant species, including the cucumber, should enhance the frequency of transformation events with simple transgene integration without an *in vitro* culture step and interference of somaclonal variation.

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