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Recent Progress in Cucumber (*Cucumis sativus*) Transformation

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ABSTRACT

Genetic transformation is vital to the transfer of novel genes into vegetable plants as well as the emerging area of functional genomics. However, the successful genetic transformation of cucumber still remains time-consuming and genotype dependent. This paper updates the progress made in recent years toward developing a genetic transformation system for cucumbers. *Agrobacterium*-mediated cucumber transformation offers advantages, such as single copy gene insertion, minimal rearrangement of DNA, low cost and comparatively high efficiency. The recent developments in cucumber transformation could lead to an increased efficiency of a cucumber breeding program. The most exciting recent progress has been in the production of elevated levels of an anti-aging superoxide dismutase in transgenic cucumber fruits, since cucumber fruits were considered to be one of the most promising economical plant bioreactors which can produce edible pharmaceutical proteins.

Keywords: *Agrobacterium tumefaciens*, genetic transformation, particle bombardment
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **6-BA**, 6-benzylaminopurine; **CFMMV**, *Cucumber fruit mottle mosaic tobamovirus*; **CGMMV**, *Cucumber green mottle mosaic virus*; **IBA**, indole-3-butyric acid; **KGMMV**, Kyuri green mottle mosaic virus; **NAA**, 1-naphthyleneacetic acid; **PPT**, phosphinothricin; **SOD**, superoxide dismutase; ***trp* promoter**, tryptophane promoter; **ZGMMV**, *Zucchini green mottle mosaic virus*

CONTENTS

INTRODUCTION

The cucumber (*Cucumis sativus* L.) is one kind of major cucurbitaceous fruit, bred all over the word especially in Southeast Asia (Esquinas-Alcazar *et al*. 1983). By the year 2002, the total planting area of cucumber was 1.253 million hm^2 and the total yield was 44.537 million tons (30% of the world total yield) in China. Moreover, cucumber is planted in almost every province except Tibet of China (Huang *et al*. 2007). The process of breeding a new variety and improvement of characters, referring directly to national basket shopping, has become very important. At present, abiotic factors, including low temperature and drought, and biotic factors, including *Green mottle mosaic virus*, angular leaf spot, fusarium wilt and cucurbit scab seriously influence crop yield and quality of cucumber. Anti-disease, anti-insect and anti-adversity genes that exist in nature are difficult to transform into cucumber through traditional means due to their interspecific affinity. It is very hard to breed a new cucumber cultivar with profitable properties from other species. Plant gene engineering technology conquers that limit and all kind of genes, cloned from either plants, animals or even microorganisms, can be transferred into cucumber, creating new properties. This is a new approach for the genetic improvement of *Cucurbitaceae* (Compton *et al*. 2004).

A stable and highly efficiency cucumber regeneration system offers a stable basis for genetic transformation. The time spent on cucumber transformation can be greatly shortened by direct regeneration from cotyledons, hypocotyls,

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Invited Mini-Review

Table 1 Cucumber transformation studies.

Genotype	Transgene construct	Method	TE	Explant	TRP	Expression Level	*A*grobacterium strain	Selection	Plasmid vector	Reference
cv. Poinsett 76	pnos::*npt*II	A.T.	10%	cotyledon	ND	Pr	A. T. C58Z707.	Km	pGA482	Chee *et al*. 1990a
Cucumis sativus	ptrp::*nos*	A.T.	-	hypocotyl	ND	Pr	A. T. A519	-	pVOL2	Gafni *et al*. 1990
cv. Straight 8	pNOS::*npt*II Ri T-DNA	A.R.	3.2%	hypocotyl	ND	DNA	A.R. A4	Km	pARC8	Trulson *et al*. 1986
cv. Marketmore	Ms-*Gus* Icl-*Gus*	A.R.	-	hypocotyl	Mediation glyoxylate cycle	Pr	A.R. A4	-	pBIPCOR pBIN2900	Ismail *et al*. 1997
cv. JinYan No. 4	p35S::*cgh* I	A.R.	-	cotyledon	Cardenolide 16-O-glucohydrolase	Pr	A.R. ATCC15834	Km	pBI121cgh.	Shi *et al*. 2006
cv. Libelle	p35S::*npt*II p35S::*uid*A	B.T.	4 P/B	EC	ND	DNA	-	Km	pRT99-GUS	Schulze *et al*. 1995
cv. Poinsett 76	pnos::*npt*II	B.T.	16%	EC	ND	Pr	-	Km	pGA482 pUC19	Chee *et al*. 1992b
cv. Winter Long	pASO::mSODI pnos::*bar*	A.T.	4%	cotyledon	ND	Pr	A.T. LBA4404	bialaphos	pGPTV-Bar	Lee *et al*. 2003
pure line 1021	pnos::*npt*II p35S::I-*gus* p35S::*bpt*	A.T.	1.4 %	hypocotyl	ND	Pr	A.T. EHA105	Km¥ Hyg	plG121-Hm	Nishibayashi *et al*. 1996
cv. Jinyan no.7	p35S::*pmi*	A.T.	23%	cotyledon hypocotyl	ND	Pr	A.T. EHA105	Mannose	pMAN1300	He *et al*. 2006
Hybrid Bambina	pnos::*npt*II p35s::*luc*II	A.T.	-	leaf	ND	Pr	A.T. LBA4404	Km	pAL4404 pAQ2	Sapountzakus *et al*. 1996
pure line 1021	p35S::CMV-O *cp* p35S::*npt*II p35S::I*gus* p35S::*hpt*	A.T.	-	hypocotyl	CMV^R	RNA	A.T. EHA101	Hyg	pIG121-HmCP	Nishibayashi *et al*. 1996
cv. Shimoshirazu	p35S::*RCC2*	A.T.	-	petiole	gray mold resistence	Pr	——	Km	pBI121-RCC2	Kishimoto *et al*. 2004
cv. Shimoshirazu	pnos::*npt*II p35S::*RCC2*	A.T.	-	cotyledon	gray mold resistence	Pr	A.T. LBA4404	Km	pBI121-RCC2	Tabei *et al*. 1998
cv. Endeavor	pnos::*npt*II p35S::chitinase gene	A.T.	-	petiole	Fungal tolerence	Pr	A.T. EHA105 MOG101 MOG301	Km	pMOG196 pMOG198 pGA492	Raharjo *et al*. 1996
cv. Borszczagowski	pGT::*Dhn*10	A.T.	4%	-	chilling tolerence	RNA	A.T. LBA4404	Km	pBI121	Yin *et al*. 2004b
cv. Borszczagowski	pGT::*Dhn*24	A.T.	-	-	chilling tolerance	Pr	A.T. LBA4404	Km	pBI121-pGT::Dhn24	Yin *et al*. 2006c
cv. Shimoshirazujibai	rolA rolB rolC ORF13 p35S::*uid*A	B.T.	-	cotyledon	ND	Pr	-	-	pBI221 pE7.4	Kodama *et al*. 1993
Inbred line 672,3676 (Gy-14)	pnos::*npt*II p35s::*npt*II	A.T.	9%	petiole leaf	ND	Pr	A.T. LBA 4404	Km	pCGN783 pBIN19	Sarmento *et al*. 1992
cv. Borszczagowski	pPR-2d::*uid*A	A.T.	1.52%	-	ND	Pr	A.T. LBA4404	Km	pGA482	Yin *et al*. 2004a
cv. Ilan	pSVBV::54-kDa replicase gene pSVBV::*npt*II	A.T.	-	cotyledon	CFMMV resistance	Pr	A.T. EHA105	Km	pCAMSV54-kDa	Gal-On *et al*. 2005
cv. Borszczagowski	pnos::*npt*II p35s::thaumatin II	A.T.	-	leaves	sweet-tasting plant proteins	Pr	A.T.LBA4404	Km	pUR528	Szwacka *et al*. 2002
cv. Poinsett 76	pnos::*npt*II p35S::*gus* p35S::*bar*	A.T.	6.6%	cotyledon	ND	DNA	A.T. EHA105	PPT	pGA492GL	Vasudevan *et al*.2007

Abbreviations: A.T., *Agrobacterium tumefaciens*-mediated transformation; A.R., *Agrobacterium rhizogenes*-mediated transformation; *bar*, bialaphos-resistant gene; *bpt*, hygromycin phosphotransferase gene; B.T., biolistic transformation; CMV, *Cucumber mosaic virus*; CFMMV, *Cucumber fruit mottle mosaic tobamovirus*; $CFMMV^R$, CFMMV resistance; CMV^R, CMV resistance; CMV-O *cp*, CMV-O coat protein gene; Dhn 10, *Solanum sogarandinum* dehydrin (10 kDa) gene; Dhn 25, *Solanum sogarandinum* dehydrin (25 kDa) gene; EC, embryogenic callus; *gus*, β-D-glucuronidase (GUS) gene; Hyg, hygromycin; Km, kanamycin; *Luc*, firefly luciferase gene; *mSODI*, cytosolic CuZnSOD cDNA from cassava; ND, not determined; *npt*II, neomicin phosphotransferase II gene; p35S, *Cauliflower mosaic virus* 35S promoter; P.B., particle bombardment; pnos, nopaline synthase promoter; PPT, phosphinothricin; pPR-2d, tobacco β-1,3-glucanase promoter; Pr, protein level; *RCC2*, a rice chitinase cDNA; RNA, RNA level; *rolA*, *rolB*, *rolC*, ORF13, genes involved in hairy root induction from plasmid pRi1724 of *A. rhizogenes* stain MAF 03-01724; TE, transformation efficiency confirmed by PCR and Southern hybridization; TRP, transgene related phenotype; *uid*A, β-D-glucuronidase (GUS) gene.

leaves or petioles. Transformation had first been achieved through either *Agrobacterium*-mediated or direct gene transfer. Root-inducing (Ri) plasmids were used earlier than tumor-inducing (Ti) plasmids, but were less effective. Microprojectile bombardment and pollen-tube pathway were also appropriate means for introducing transgenes into cucumber. In addition to selector, reporter and marker genes, various types of transgenes with agronomic potential have been introduced into cucumbers, such as fungi-resistant genes, antifreezing genes and genes that result in improvement of quality. These were transferred into cucumbers, being expressed and stably inherited in progenies, as summarized in **Table 1**.

In this review we highlight the achievements to date in cucumber genetic transformation. Stable regeneration systems and methods of transgene integration are also discussed. We also attempt to evaluate the prospects and problems inherent to cucumber genetic transformation.

CUCUMBER GENETIC TRANSFORMATION SYSTEMS

Explants used for gene transformation

An efficient and stable plant regeneration system is the most important requirement for plant transformation. The hormone combination and concentration are the main factors effecting plant regeneration while the source (leaf microexplants, leaf petiole, cotyledon and hypocotyls) and size of explants can also influence regeneration. Incubation with bacteria can extend the period in which mature plants are obtained. Regeneration systems can be divided into two classes based on different pathways.

Explants for cucumber organogenesis

Adventitious buds, which can grow into plantlets, can be obtained when cotyledon or hypocotyl explants are stimulated by exogenous cytokinins such as 6-BA and IBA. Organogenesis is a good method to directly regenerate large number of buds without the differentiation from somatic embryos and takes less time. Thus, it is the most efficient method in cucumber genetic transformation (Burza *et al*. 1995). Cucumber regeneration through organogenesis has been extensively reported throughout the world. Gambley *et al*. (1990) obtained adventitious buds, 50 per callus at most, from cotyledon segments of cucumber seedlings. Colijn-Hooymans *et al*. (1994b) discovered that regeneration frequency from 3- to 5-days-old cotyledons was highest (up to 100%) but that over 7 days there was an obvious decrease (only about 0.9 shoots per explant or less). They also reported polyploidization or increase in the number of chromosomes in seedlings. Selvaraj *et al*. (2007b) claimed that it takes about 120 to 140 days to get regenerated plantlets when subculturing once every 20 days. Callus dedifferentiated from hypocotyls could also be stimulated by 8.88 μM 6-BA to redifferentiate into buds at frequency of 80.3% over a period of four months (Selvaraj *et al*. 2006a).

Cucumber protoplasts

Protoplasts are cells without a cell wall and can be induced to form regenerated plantlets under appropriate culture conditions. They appear to be better suited for genetic transformation since they can easily take up and integrate exogenous genes without being hampered by the cell wall. The frequency of transformation is high and the incidence of chimeras is low an thus is one of the efficient ways to overcome plant chimerism in genetic transformation. This system is suitable for every kind of transgenic method. However, its inheritance is unstable because somatic mutants are introduced in growth and development progress. Despite much work and a complex operation, it is still difficult to obtain plantlets and this disadvantage restricts its applications in genetic transformation.

Cucumber protoplasts, following the enzymatic degradation of cell walls by 1.25% pectinase for 16 hours digestion, can be obtained within 16 days (Punja *et al*. 1990). Shoot buds or somatic embryos formed when calli were subcultured onto MS medium containing low concentrations (0.05-0.01 μM) of 2,4-D/6-BA or NAA/6-BA. Colijn-Hooymans *et al*. (1988a) discovered that only in the case of leaf protoplasts were morphologically normal plants obtained but with only 20% diploid (normal ploidy level) and tetraploid or even octaploid were detected. However, there was no evidence that this could influence plant regeneration negatively.

METHODS OF TRANSGENE INTRODUCTION

Agrobacterium-mediated transformation

The genus *Agrobacterium* is a kind of plant pathogen that has been divided into five species based on disease symptomology and plant host range (Otten *et al*. 1996; Gelvin *et al*. 2003). Thus far, only two of them, *A. tumefaciens* and *A. rhizogenes*, have been used for plant transformation. T-DNA, a specific DNA fragment from the Ti plasmid of *A. tumefaciens*, could integrate into the plant genome regulated by the *Vir* gene and express this gene (Xu 1990). Foreign genes less than 50 kb can be transferred into the cucumber genome by a Ti plasmid in a single copy.

The first report of successful cucumber (cv. 'Poinsett 76') genetic transformation mediated by *A. tumefaciens* was accomplished by Chee *et al*. (1990a). The neomycin phosphotransferase II (*ntp*II) gene, in binary vector plasmid pGA482, was confirmed to have been integrated into the cucumber genome by kanamycin selection (100 mg/l) and Southern blot analysis, and positive R_1 generants. This demonstrated that foreign genes mediated by *A. tumefaciens* can be transfer into cucumber and inherited stably. The nopaline synthase gene (*nos*) under the *Escherichia coli trp* promoter was transformed into cucumber in the same year (Gafni 1990). The *npt*II and phosphinothricin resistance (*bar*) genes harbored in strain EHA105 were simultaneously transferred into cv. 'Greenlong' cotyledon successfully and five different plants (i.e. independent lines) (1.1%) were demonstrated to be transgenic following Southern hybridization (Vengadesan *et al*. 2005).

The Ri plasmid causes "hairy roots" in plant sections incubated with *A. rhizogenes* and shoots can be regenerated from transgenically positive roots. However, it was so laborious to regenerate plants from hairy roots that this limited their used in cucumber transformation (Tang *et al*. 2001). Trulson *et al*. (1986) obtained the first transgenic cucumbers in this way. The *npt*II gene in vector pARC8 of *A. rhizogenes* induced hairy roots on hypocotyl sections. The transgenic plants were positive for the *npt*II gene. The *Icl* and *Ms* gene promoters (Ismail *et al*. 1997) in transgenic hairy roots were simply transcribed and isocitrate lyase (ICL) and malate synthase (MS) were regulated at the transcription level in both transgenic and control cucumbers. The expression of *Icl* and *Ms* genes in the roots of whole plants was controlled in the presence and absence of 25 mM sucrose, respectively. Transgenic plants can potentially be used as a system for the large-scale production of recombinant proteins for industrial and pharmaceutical uses (Austin *et al*. 1994; Horn *et al*. 2004). The coding sequence for cardenolide 16'-*O*-glucohydrolase was inserted into the cucumber genome by plant expression vector pBI121*cgh* of *A. rhizogenes* strain 15834 (Shi *et al*. 2006). Glycolytic activity of transgenic cucumber roots was detected by HPLC using lanatoside glycosides as substrate. It was a valuable model for biotransformation of natural compounds by recombinant enzymes.

Microprojectile bombardment

Microprojectile or particle bombardment is a direct gene transformation method not often used in cucumber gene

transfer since multiple copies are inserted into the cucumber genome. Transgenic cucumber (cv. 'Poinsett 76') harboring the nos-*npt*II (Chee *et al*. 1992b) gene and the *npt*II and *uid*A genes (Schulze *et al*. 1995) were obtained by bombarding embryogenic callus suspension cultures. Nos-*npt*II gene (Chee *et al*. 1992b) transformation frequency was about 16% analyzed by Southern blot but only 25% of these transcripts expressed the gene. The authors concluded that multiple copies of the transferred gene inserted into the cucumber genome, as demonstrated by PCR and Southern blot hybridization analyses, was the main reason transgene silencing. Twenty-eight vigorously developed and rooted cucumbers (cv. 'Libelle'), regenerated 9 months after bombardment from kanamycin-resistant callus, were obtained and 67% of them showed β-glucuronidase (GUS) activity (Schulze *et al*. 1995). Multicopy integration and rearrangement were also confirmed by Southern blot analysis with *uid*A and *npt*II probes. The *uid*A gene in the transgenics still had a high and stable activity about one year after bombardment providing a new route for non-selectable genetic transformation. Hairy roots from cotyledons were obtained after transformation of *rol*A, B and C genes which are located on the T-DNA of the Ri plasmid A4 (Kodama *et al*. 1993). The stable integration of *rol* genes into the cucumber (cv. 'Shimoshirazujibai') genome was confirmed by Southern blot analysis.

Pollen tube pathway method

There has only been one report of cucumber transformation using the pollen tube pathway. Li *et al*. (2000) reported a genetic transformation system of cucumber (cv. 'Jingyan No. 4') with high efficiency by injecting exogenous DNA with concentration of 100 mg/kg into the ovary 12 hours after pollination, and transgenic plants, derived from seeds, were obtained. Using this approach authors could obtain the highest transformation frequency but the lowest fruit setting ratio and number of seeds per fruit. Three positive plants detected by GUS analysis and Southern blot hybridization were obtained and showed *npt*II and *uid*A gene integration.

Selective marker genes in transgenic cucumbers

The most and earliest chimeric gene studied in cucumber transformation was the *npt*II gene for kanamycin resistance when used as a selectable marker. The *npt*II gene inserted into *A. tumefaciens* binary vector plasmid pGA482 (Chee *et al*. 1990a) and into vector pARC8 of *A. rhizogenes* (Trulson *et al*. 1986) was transferred in cucumber (cv. 'Straight Eight') cotyledons and hypocotyls, respectively. Transformed R_0 and R_1 cucumber plants, proved by hybridization with a 2.0 kb (*Bam*HI and *Hind*III restriction) *npt*II gene-containing fragment, appeared normal and were tested positive for NPTII enzyme activity (Chee *et al*. 1990a). Even DNA fragment from Ri plasmid was detected in transcripts (Trulson *et al*. 1986). Following the success of these two cases, the *npt*II gene was selected as the preferred method in the field of cucumber transgenics. The *uid*A gene in pRT99-GUS (Schulze *et al*. 1995), *bar* in strain EHA105 (Vengadesan *et al*. 2005), *gus* and *bph* in plG121-Hm (Nishibayashi *et al*. 1996b) co-fused with the *npt*II gene were studied under different genetic backgrounds (**Table 1**).

Gus is another important and promising reporter gene for cucumber genetic transformation. The *gus* gene fused to the CaMV 35S promoter harbored in plG121-Hm of EHA101 strain of *A. tumefaciens* was transferred into a pure line 1021 of a *C. sativus* cultivar (pure line 1021) and was highly expressed in the young leaves of 12 regenerated plantlets (Nishibayashi *et al*. 1996a). Eight GUS-positive R_0 plants examined by Southern blot analysis had either a single or a few positive bands which showed multiple copies of the inserted transgene. R_1 progeny, which inherited the *gus* gene from R_0, also showed stable GUS activity in the cells of all tissues. The expression and segregation ratio for the CMV-O coat-protein gene (*cp* gene), which provides resistance to *Cucumber mosaic virus* (CMV), were obtained directly from GUS assays and PCR analysis (Nishibayashi *et al*. 1996a). GUS activity was low in the presence of 25 mM sucrose and high in transgenic roots when sucrose was absent, demonstrating that sugar supply could control the expression of *Icl* and *Ms* genes when fused to a GUS reporter gene in cucumber roots of whole plants (Ismanizan *et al*. 1997).

Five other reporter or selector genes including *pmi* (encoding phosphomannose-isomerase, He *et al*. 2006), *hph* (hygromycin phosphotransferase gene, Nishibayashi *et al*. 1996b), *bar* (phosphinothricin resistance genes conferring resistance to PPT (Vengadesan *et al*. 2005), *uid*A (β-glucuronidase gene fused with 35S promotor in pRT99GUS transmitted via particle gun, Schulze *et al*. 1995), *luc* (the firefly luciferase gene in pAL4404 and pAQ2, Sapountzakus *et al*. 1996) were introduced into different lines. These genes were controlled under a constitutive or tissue-specific promoter. Selection using PPT or hygromycin B rather than kanamycin or the presence of 50 μM acetosyringone was found to enhance transformation frequency as evidenced by transient expression of GUS or hygromycin phosphotransferase (Nishibayashi *et al*. 1996b; Vengadesan *et al*. 2005) (**Table 1**).

PRACTICAL EVALUATIONS

Disease resistance

Agricultural yield tends to be reduced constantly by various plant diseases which are generally caused by phytopathogenic fungi, bacteria and viruses. Thus, breeding for disease resistance has become one of the most crucial objectives in cucumber cultivation. Four kinds of chitinase genes including the rice class I chitinase gene exhibited resistance to *Phytophthora* rot (Kishimoto *et al*. 2003a), an acidic chitinase gene from petunia and basic chitinase genes from tobacco and bean respectively (Raharjo *et al*. 1996), a class III chitinase gene against *Botrytis cinerea* (Kishimoto *et al*. 2004b) and a rice chitinase cDNA for resistance against gray mold (Tabei *et al*. 1998) were isolated from different plants and transformed into several cultivated varieties of cucumbers using molecular biology. The transgenic cucumbers showed varying levels of disease resistance and high expression and intracellular localization of rice chitinase, which might be involved in enhancing the resistance of transgenic plants to gray mold (Kishimoto *et al*. 2002c). However, *Fusarium oxysporum* f. sp. *cucumerinum* located in petiole vessels could avoid antifungal substance and resulted in *Fusarium* wilt in transgenic cucumber (Kishimoto *et al*. 2003a). The highly resistant transgenic cucumber strains were inheritable with a segregation ratio of 3:1 (resistant: susceptible) and can serve as good breeding materials for disease resistance (Tabei *et al*. 1998).

The CMV irus coat protein gene, CMV-O *cp* (Nishibayashi *et al*. 1996b) and the putative 54-kDa replicase gene of CFMMV (Gal-On *et al*. 2005) were transformed into cucumber genome with the aim of producing cucumber plants with CMV and CFMMV resistance. The RNA transcripts from the CaMV 35S-*cp* gene were detected in the leaves of R_0 transgenic cucumber plants and in the epicotyls containing two cotyledons of transgenic progeny plants by Northern blot analysis but protein signals were absent following Western blot analysis. Nishibayashi *et al*. (1996a) also discovered that cucumber agronomic characteristics of CMV resistance could be enhanced by the combination of cucumber genotypes and the CMV-O *cp* gene. Line I44 (Gal-On *et al*. 2005), homozygous for the putative 54-kDa replicase gene, was immune to CFMMV infection and a substantial delay of disease symptoms, CGMMV, ZGMMV and KGMMV, was observed at 14, 14 and 20 days post inoculation respectively, compared with the control cucumbers at 2, 8 and 8 days post inoculation (**Table 1**).

Chilling tolerance

The pGT::*Dhn*10 (Yin *et al*. 2004b) and pGT::*Dhn*24 (Yin *et al*. 2006c) gene fusions, which encode dehydrin proteins and have a potential role in chilling tolerance, were transmitted into the cucumber genome. mRNA of the Dhn10 gene was detected in the leaves, cotyledons, hypocotyls and roots by RT-PCR, but no Dhn10 protein by alkaline phosphatase conjugated antibody. The pGT::*Dhn*10 gene fusion regulated cold tolerance in transgenic lines at the transcriptional level. SK3-type DHN24 dehydrin encoded by the *Solanum sogarandinum* pGT::*Dhn*24 gene was detected in leaves, cotyledons, stems and roots of transgenic seedlings by RT-PCR and Western blot analysis, with particularly high expression in roots. Different expression levels of pGT::*Dhn*24 gene among transgenic lines had no correlation with the degree of chilling tolerance. The chilling tolerance of cold-sensitive species such as cucumber can be improved through genetic technology by transmitting pGT::*Dhn*10 and pGT::*Dhn*24 gene fusions.

The *Arabidopsis thaliana* CBF 3 gene (CRT/DRE binding factor gene), a kind of antifreeze gene, fused with CaMV 35S promoter to construct plasmid pBINP-35S-CBF3 was successfully induced into cucumber and confirmed by PCR analysis (Deng *et al*. 2004). However, the chilling tolerance of the transgenic line was not reported.

Production of new traits

Superoxide dismutase (SOD) has an anti-aging function in cellular defense against oxidative stress in aerobic organisms and has been added to many cosmetics products. The CuZnSOD cDNA (mSOD1) from cassava fused with the ascorbate oxidase promoter in plasmid ASOp::mSODI/ pGPTV-Bar was introduced into cucumber cv. 'Winter Long' (Lee *et al*. 2003). The mSOD1 gene was integrated into the nuclear genomes of three cucumber plants confirmed by Southern blot, and was highly expressed in transgenic cucumber fruits three times more than the control, but levels were low in leaves since the ASO promoter was expressed more dominantly in cucumber fruits than in other tissues. The elevated SOD activity in transgenic fruits was a consequence of the introduced mSOD1 chimeric gene and two additional induced CuZnSOD genes.

Thaumatins, produced by the fruits of a West African perennial plant *Thaumatococcus daniellii* Benth, represent a unique class of the sweet-tasting plant proteins and are approximately 100,000 times sweeter than sucrose on a molar basis (van der Wel *et al.* 1980). Transgenic *C. sativus* cv. 'Borszczagowski' plants containing thaumatin II cDNA driven by the CaMV 35S promoter in the binary plasmid pRUR528 were produced (Szwacka *et al*. 2002). Thaumatin might be controlled at the levels of both transcription and translation demonstrated by the lack of correlation between protein and mRNA levels. The positive correlation between thaumatin accumulation levels and sweet taste intensity was noticed in transgenic fruits with a sweet phenotype. It was also reported that transgenic protein levels of thaumatin which belonged to the pathogenesis-related (PR) protein family had no relationship with the increased tolerance for pathogenic fungus *Pseudoperonospora cubensis.*

Transgenic cucumbers can also play an important role in the development of potential systems for large-scale production of recombinant proteins for industrial and pharmaceutical uses. The *cgh*I gene, encoding the *Digitalis lanata* EHRH cardenolide 16'-*O*-glucohydrolase, which catalyzes the deglucosylation of lanatosides, inserted downstream of the 35S promoter in plant expression vector pBI121*cgh*, was transformed in cucumber plants using *A. rhizogenes* 15834 (Shi *et al*. 2006). The CGH I activity in cucumber hairy roots was detected by HPLC.

Inheritance of transgenes

The stable transmission of important agronomic traits expressed by a transgene in cucumber plants from R_0 to progeny was documented following both Mendelian and non-Mendelian transmission. The CMV-O *cp* gene was induced into cucumber (pure line 1021) plants by *Ti-Aorobacterium*-mediated transformation and the segregation ratio for the presence and absence of the *cp* gene was 1:1 by PCR and GUS analysis (Nishibayashi *et al*. 1996a). Transgenic cucumber line CR32 with the rice chitinase cDNA (RCC2) confirmed by PCR, Southern analysis and ELISA analysis showed segregation of disease resistance following the predicted Mendelian ratio of 3:1 (resistant: susceptible), as tested by χ^2 analysis ($\chi^2 = 0.078$, $P < 0.01$) (Tabei *et al*. 1998). A phenotypic segregation of kanamycin resistance in transgenic cucumber with the construct *PR-2duidA-nosnpt*II and *thaumatin*II-*nosnpt*II was demonstrated and 78% of the hemizygous progenies exhibited a segregation ratio consistent with Mendelian inheritance while 22% were non-Mendelian (Yin *et al*. 2004a). He *et al*. (2006) also discovered that the *pmi* gene had a 3:1 segregation ratio in six transgenic lines. A 3:1 Mendelian ratio (kanamycin-resistance: kanamycin-sensitivity) was documented in T_1 progeny (Szwacka *et al*. 2002; Gal-On *et al*. 2005).

FUTURE PERSPECTIVES

Six kinds of agronomically important genes including *cghI* (the *Digitalis lanata* EHRH. cardenolide 16'-O-glucohydrolase), *mSODI* (superoxide dismutase, Lee *et al*. 2003), CMV-O *cp* (CMV resistance), *RCC2* (the rice chitinase cDNA; Tabei *et al*. 1998), *Dhn* (chilling tolerance; Yin *et al*. 2004a, 2006c), and the *Dhn24* 54-kDa replicase gene (CMV and CFMMV resistance; Nishibayashi *et al*. 1996a) were thus far successfully transmitted to cucumber. The following expression and inherence of these genes were reported in cucumber transformation. There were still several serious problems for biotechnological transformation.

Marker or selectable genes induced with the target gene in transformation will be problematical because in the European Union the registration of genetically modified organisms (GMO) containing the antibiotic or herbicide resistance genes will be banned from 2008 (Kuiper *et al*. 2001; http://www.gmo-compass.org/eng/regulation/ regulatory_process/). Two strategies had been reported for transgenic plants regeneration without the marker or selectable genes. One way was taking the *pmi* gene (encoding for phosphomannose-isomerase that converts mannose-6-phosphate to fructose-6-phosphate) as selectable method (He *et al*. 2006). Only the regeneration plants with the *pmi* gene could take the mannose as a carbon source while the control can not. This gene was capable to replace the *npt* II gene with selectable function. Another approach was excise or segregate marker gene from the genome of transgenic plantlets. Hohn *et al*. (2001) and David *et al*. (2001) proved this method of removing marker genes by the site-specific expression of transgene sequence.

Some problems containing significant low frequency of transformation, unable to generate directly from explants, some DNA sequence of plasmid harboring in host genome, chromosome doubling in progenies, multiple gene copy in host plant, were hampered the cucumber gene transformation. Proper transgenic methods as well as vectors with highly harboring and expression capability should be chosen for different cucumber lines.

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