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Full Length Article

Establishment of a High-efficiency Genetic Transformation System of Cucumber (*Cucumis sativus*) using *Csexpansin* 10 (*CsEXP*10) Gene

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Abstract

Cucumber is one of the most important vegetable crops in the world. To establish an efficient genetic transformation system in cucumber, the role of different factors such as pre-cultivation time, acetosyringone concentration, infection time and co-cultivation time that can influence the transformation, was evaluated. In addition, *Csexpansin* 10 (*CsEXP*10) gene was transformed into the cucumber genome to produce transgenic lines. The results showed that various optimal parameters such as 100 mg L^{-1} of kanamycin concentration for selection of transformants, 2 d of pre-cultivation time, 100 µmol L^{-1} of acetosyringone concentration, 15 min of infection time and 2 d of co-cultivation time were obtained using cotyledonary node explants. The rooting frequency observed on Murashige and Skoog (MS) medium supplemented with 0.2 mg L^{-1} indole acetic acid and 400 mg L^{-1} cefotaxime was found to be 100.00%. The positive transgenic cucumber lines were identified using PCR analysis and GUS staining assay. It suggests that the genetic transformation system developed using cotyledonary node explants is efficient and successful in cucumber. © 2017 Friends Science Publishers

Keywords: Genetic transformation; Cucumber (*Cucumis sativus* L.); *Csexpansin* 10 (*CsEXP*10); Transformation efficiency

Introduction

Cucumber (*Cucumis sativus* L.) is an important vegetable crop and widely cultivated throughout the world. It is very rich in nutrients such as carbohydrates, amino acids, minerals and vitamins. In recent years, there has been a dramatic increase in the cultivation area of cucumber, especially under protected cultivation in China. However, under protected cultivation in the winter, environments stresses such as salt stress, low temperature, and weak light often affect cucumber growth and development, leading to high fruit deformation frequency and low yield.

Genetic engineering, an important technology for studying plant molecular biology, can be used to analyze gene functions and culture genetically modified plants, to improve the crop yield and quality. In addition, genetic engineering has some clear advantages such as a short breeding time and no changes in the desirable characteristics of plants, therefore, it is particularly valuable in genetic improvement of cucumber (Wang *et al.*, 2015). During the last three decades, cucumber transformation techniques have undergone advances. The most effective method to transfer the desired genes into the cucumber genome is to use an *Agrobacterium*-mediated transformation approach (Trulson *et al*., 1986; Kose and Koç, 2003; Lin *et al*., 2011a). However, the genotype dependency, low-regeneration efficiency, and low reproducibility of this transformation system limit the use of transformation techniques in the genetic improvement of cucumber. Therefore, it is very essential to develop an efficient genetic transformation system for cucumber.

Expansin genes take part in the process of plant growth and development, and play an important role in morphogenesis, root hair growth (Lin *et al.*, 2011b), pollination, flower development (Lee *et al.*, 2001; Choi *et al*., 2006), fruit development (Rose *et al.*, 1997; Brummell *et al.*, 1999) and abscission. *Csexpansin*10 (*CsEXP*10) gene was identified and cloned from young cucumber fruits by Sun *et al.* (2005), and was helpful for tomato fruits development in our previous study (Sun *et al.*, 2016). In current study, an efficient and stable *Agrobacterium*-mediated genetic transformation approach using cotyledonary node explants in cucumber with *CsEXP*10 gene has been developed, which can be successfully used for the introduction and functional analysis of desired genes.

Materials and Methods

Plant Material

Cucumber inbred lines ‘Cs0601’ was used as the experimental material. ‘Cs0601’ has only female flowers in the stem, seeds were supplied by School of Horticulture and

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Landscape Architecture, Henan Institute of Science and Technology, Xinxiang, China. Cucumber seeds were soaked for 4 h in water, surface-sterilized for 1 min with 70% (v/v) ethanol followed by 20 min sterilization with 0.1% mercuric chloride. After rinsed in sterilized water for five times, surface sterilized seeds were cultured on the Murashige and Skoog (MS) medium supplemented with 7 g L^{-1} agar and 30 g L^{-1} sucrose for 48 h at 28 ±1°C under darkness to promote seeds germination. Thereafter, germinated cucumber seeds were cultivated at 25 ±1°C for 5 d under a 16-h photoperiod in a culture room. Cotyledonary nodes excised from 5-d-old sterilized cucumber seedlings without fully expanded cotyledons were used as explants for pre-cultivation, according to the techniques described by Li *et al.* (2016).

Determination of Kanamycin Concentration

In order to obtain the optimal kanamycin concentration for selection of transformants, cotyledonary nodes of cucumber were placed on MS medium supplemented with 2.0 mg L^{-1} 6-benzylaminopurine (6-BA) and 2.0 mg L^{-1} $AgNO_3$, containing different concentrations of kanamycin (0, 50, 75, 100 or 125 mg L^{-1}) for 30 d. Regeneration frequency and number of shoots were recorded.

Pre-cultivation

Cotyledonary node explants were pre-cultivated in the plates containing pre-cultivation medium (MS + 2.0 mg L^{-1} 6-BA + 2.0 mg L^{-1} $AgNO_3$). The plates were kept in growth chamber (25 ±1 °C) under darkness for 0, 1, 2, 3, 4 or 5 d.

Plasmid Vector

Agrobacterium tumefaciens strain GV3101 harboring pCAMBIA-1301-CaMV35S constitutive promoter with GUS reporter gene was used for genetic transformation. *CsEXP*10 gene coding sequence (827 bp) was constructed downstream of the CaMV35S promoter as pCAMBIA-1301-*CsEXP*10 (Fig. 1) described by Sun *et al.* (2015).

Inoculum Solution Preparation

A single colony of *A. tumefaciens* strain GV3101 harboring pCAMBIA-1301-*CsEXP*10 was grown on Yeast Extract Peptone (YEP) medium supplemented with 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin on incubator shaker (28 ±1°C, 250 rpm) for 16 h. Then the *A. tumefaciens* solution was diluted 1: 100 with YEP medium and was cultivated in YEP medium on incubator shaker (28 ±1°C, 250 rpm) for 4 h.

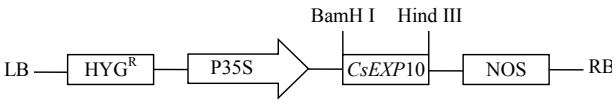

Fig. 1: Plant expression vector pCAMBIA1301-*CsEXP*10 used for genetic transformation
LB: T-DNA left border; HYG^R: hygromycin; P35S: promoter from cauliflower mosaic virus; NOS: nos terminator; RB: T-DNA right border

Co-cultivation and Selection

Pre-cultivated cotyledonary node explants were immersed in inoculum solution of *A. tumefaciens* with different concentrations of acetosyringone (0, 100, 200, 300, 400 or 500 μmol L^{-1}) and kept gentle shaking for 0–30 min. Then cotyledonary node explants were removed, dried with sterilized filter paper and placed to co-cultivation medium (MS + 2 mg L^{-1} 6-BA + 2 mg L^{-1} $AgNO_3$ + 100 μmol L^{-1} acetosyringone) under darkness for 0, 1, 2, 3, 4 or 5 days. After co-cultivation, cotyledonary node explants were transferred to selective medium (MS + 2 mg L^{-1} 6-BA + 2 mg L^{-1} $AgNO_3$ + 300 mg L^{-1} cefotaxime + 50 mg L^{-1} kanamycin). Regenerated putative transgenic cucumber shoots were cut and transferred to the different rooting mediums (Table 6). Rooted plantlets were then transferred to pots containing a sterile media of 1 turf : 1 vermiculite (v/v).

PCR Detection of Transgenic Cucumber Lines

Genomic DNA of the putative transgenic cucumber lines was extracted from the young leaves using Plant Genomic DNA Extraction Kit, SUN SHINE BIO. A pair of primer sequences: forward: 5'-ATGGCTTCTTCTCTTTCTCTCC-3' and reverse: 5'-TGTAAAACGACGGCCAGT-3' were designed for PCR amplification. PCR amplification was carried out at 94°C for 3 min; then 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 1 min, with final extension at 72°C for 10 min. PCR products of the expected size (500 bp) were separated on a 1.0% agarose gel by electrophoresis.

GUS Activity Assay

Expression of GUS gene in the young leaves of putative transgenic cucumber lines was analyzed with X-glucuronide, as described by Sun *et al.* (2015).

Data Analysis

The data were analyzed by the Duncan's multiple range test at $P \leq 0.05$, to determine significant differences. The statistical analyses were carried out with SPSS 17.0 software package. The data are expressed as mean ± standard error.

Results

Kanamycin Concentration

Regeneration frequency and number of shoots from

Fig. 2: Rooting of regenerated transgenic shoots

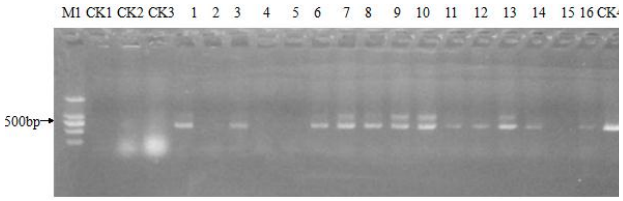

Fig. 3: PCR analysis of transgenic cucumber lines with a pair of primer sequences: forward: 5'-ATGGCTTCTTCTCTTTCTCTCC-3' and reverse: 5'-TGTAAAACGACGGCCAGT-3'

M1: DL2000^{TM} DNA Marker; CK1: Negative control; CK2-CK3: *Agrobacterium tumefaciens* solution; CK4: Plasmid DNA; 2, 4, 5, 15: Non-transgenic lines; 1, 3, 6-14, 16: Transgenic lines

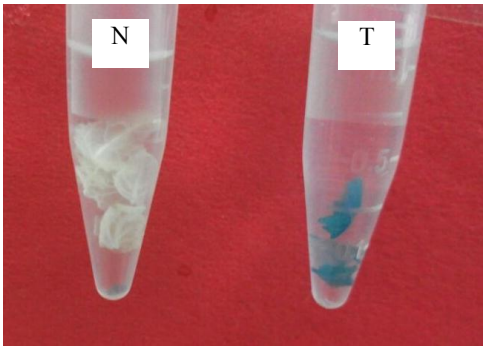

Fig. 4: GUS staining analysis of young leaves from cucumber

N: Non-transgenic line; T: Transgenic line

cotyledonary node explants were significantly inhibited by different kanamycin (Table 1). The inhabited frequency was increasing with the increasing concentrations of kanamycin. Regeneration frequency and number of shoots were 70.61% and 1.16, respectively, when cotyledonary node explants were grown on medium without kanamycin. While, 20.29% of regeneration frequency and 0.14 of number of shoots were observed with 100 mg L^{-1} of kanamycin. Cotyledonary node explants showed complete bleaching and no regenerated shoots at 125 mg L^{-1} kanamycin. Based on these observations, 100 mg L^{-1} of kanamycin was considered optimal for the selection of transformants.

Pre-cultivation Time

Regeneration frequency and number of shoots varied with different pre-cultivation times (Table 2). Both regeneration frequency (90.01%) and number of shoots (1.72) were the highest when the cotyledonary node explants were pre-cultivated for 2 d. Therefore, 2 d of pre-cultivation was considered optimal for the cotyledonary node explants.

Acetosyringone Concentration

Acetosyringone has been used to enhance *Agrobacterium*-mediated genetic transformation (Mishra *et al.*, 2013; Manickavasangam *et al.*, 2013; Gupta and Rahman, 2015). In this invesitgation, we observed the effect of different concentrations of acetosyringone in the *A. tumefaciens* inoculum solution on the transformation efficiency of the cucumber cotyledonary node explants (Table 3). Both regeneration frequency (72.77%) and number of shoots (1.5) were the highest with 100 µmol L^{-1} of acetosyringone, significantly different from those with other concentrations. Therefore, 100 µmol L^{-1} of acetosyringone was essential for successful and high-efficiency transformation.

Infection Time

For efficient plant transformation, optimal *A. tumefaciens* infection time is one of the most important factors. Suitable infection time dramatically increases the efficiency of cucumber transformation. In our experiment, regeneration frequency and number of shoots were seen with different infection time (0, 5, 10, 15, 20, 25 or 30 min). Regeneration frequency and number of shoots varied with the infection time. No regeneration frequency and number of shoots were observed with 0 min of infection time. The optimal infection time for obtaining the highest regeneration frequency (99.31%) and number of shoots (1.90) for the cotyledon node explants were 15 min (Table 4).

Co-cultivation Time

Different co-cultivation times (0, 1, 2, 3, 4 or 5 d) were also used as a parameter to investigate the transformation efficiency. We observed that the highest transformation efficiency was 2 d of co-cultivation. After 2 d of co-cultivation, the maximum regeneration frequency (100%) and number of shoots (1.88) were observed. With the longer co-cultivation time, transformation efficiency decreased drastically. The lower regeneration frequency (82.76%) and the lowest number of shoots (0.55) were observed after 5 d of co-cultivation (Table 5).

Table 1: Effects of kanamycin on uninfected cotyledon node explants of cucumber

Kanamycin (mg L^{-1})	Number of explants	Regeneration frequency	Number of shoots/explant
0	24	70.61 a	1.16 a
50	24	50.45 ab	0.81 b
75	24	42.83 bc	0.57 c
100	24	20.29 cd	0.14 d
125	24	0 d	0 d

Note: Lower case letters indicate significant differences at $p < 0.05$

Table 2: Effects of pre-cultivation time on the regeneration frequency and number of shoots

Pre-cultivation time (d)	Number of explants	Regeneration frequency	Number of shoots/explant
0	24	0 c	0 c
1	24	86.61 a	0.36 bc
2	24	90.01 a	1.72 a
3	24	88.29 a	0.34 bc
4	24	86.03 a	0.44 bc
5	24	80.31 a	0.56 b

Note: Lower case letters indicate significant differences at $p < 0.05$

Table 3: Effects of acetosyringone on the regeneration frequency and number of shoots

Acetosyringone concentration (μmol L^{-1})	Number of explants	Regeneration frequency	Number of shoots/explant
0	24	32.83 ab	0.43 ab
50	24	45.13 ab	0.60 ab
100	24	72.77 a	1.50 a
150	24	22.44 b	0.62 ab
200	24	48.61 ab	0.71 ab
300	24	48.44 ab	0.36 b
500	24	47.63 ab	1.00 ab

Note: Lower case letters indicate significant differences at $p < 0.05$

Table 4: Effects of infection time on the regeneration frequency and number of shoots

Infection time (min)	Number of explants	Regeneration frequency	Number of shoots/explant
0	24	0 c	0 d
5	24	88.40 a	0.92 b
10	24	91.17 a	1.10 b
15	24	99.31 a	1.90 a
20	24	93.35 a	0.89 b
25	24	50.31 b	0.42 c
30	24	23.08 bc	0 d

Note: Lower case letters indicate significant differences at $p < 0.05$

Table 5: Effects of co-cultivation time on the regeneration frequency and number of shoots

Co-cultivation time (d)	Number of explants	Regeneration frequency	Number of shoots/explant
0	24	80.25 b	1.12 b
1	24	75.24 b	1.15 b
2	24	100.00 a	1.88 a
3	24	98.16 ab	1.20 b
4	24	83.97 ab	0.88 bc
5	24	82.76 a	0.55 c

Note: Lower case letters indicate significant differences at $p < 0.05$

Table 6: Effects of different mediums on the rooting frequency

Mediums	Rooting frequency (%)
MS	0c
MS+300 mg L^{-1} cefotaxime	0c
1/2MS+300 mg L^{-1} cefotaxime	0c
MS+0.2 mg L^{-1} IAA+400 mg L^{-1} cefotaxime	100.00 a
1/2MS+0.2 mg L^{-1} IAA+400 mg L^{-1} cefotaxime	83.13 b
MS+0.2 mg L^{-1} NAA+400 mg L^{-1} cefotaxime	89.50 b

Note: Lower case letters indicate significant differences at $p < 0.05$

Rooting Medium

For rooting, regenerated transgenic cucumber shoots were placed on various mediums (Table 6). Rooting was observed on three mediums. The highest rooting frequency (100.00%) was observed on MS medium supplemented with 0.2 mg L^{-1} indole acetic acid (IAA) and 400 mg L^{-1} cefotaxime. The rooting frequencies observed on MS medium, supplemented with 0.2 mg L^{-1} α-naphthaleneacetic acid (NAA) and 400 mg L^{-1} cefotaxime, and on 1/2 MS medium, supplemented with 0.2 mg L^{-1} IAA and 400 mg L^{-1} cefotaxime, were 89.5% and 83.13%, respectively. Roots obtained on the MS medium supplemented with 0.2 mg L^{-1} IAA and 400 mg L^{-1} cefotaxime were thicker and longer than those on the other two mediums (Fig. 2); therefore, it was considered as the optimum rooting medium.

Identification of Transgenic Plants

To prove the genetic transformation system, PCR amplification of genomic DNA from putative transgenic cucumber lines with the specific primer pair was conducted. PCR products of the expected size (500 bp) were amplified from 12 transgenic cucumber lines, which gave the same size PCR products with the positive control (plasmid DNA), none was detected in the negative control and non-transgenic cucumber lines (Fig. 3). Young leaves from transgenic cucumber lines showed a dark blue colouration, indicating GUS gene expression, whereas the leaves from non-transgenic cucumber lines did not show positive results (Fig. 4).

Discussion

Functional analysis of genes underlying agricultural traits depends on an efficient genetic transformation system. In general, target genes are transferred into dicotyledonous plant genomes using the *Agrobacterium*-mediated transformation approach (Wang *et al.*, 2015). Success of *Agrobacterium*-mediated transformation depends upon the regeneration frequency and number of shoots, which, in turn, depend upon the explant types, pre-cultivation time, acetosyringone concentration, infection time and co-cultivation time. It is essential to optimize the above factors to establish an efficient genetic transformation system.

To evaluate the effects of pre-cultivation time and co-cultivation time on the efficiency of genetic transformation, different times of pre-cultivation and co-cultivation were investigated using cotyledonary node explants. It was observed that 2 d of pre-cultivation and 2 d of co-cultivation were very effective in producing the highest cucumber regeneration frequency and number of shoots in cucumber. These findings of present study are in agreement with those found by Cardoza and Stewart (2003), Jonoubi *et al*. (2005), Khan *et al*. (2010), Wang *et al.* (2013) and Khan *et al.* (2013). The explants co-cultured for 2 d showed the highest regeneration frequency and number of shoots in current study. Previously, co-cultivation for 2 d was reported by Wang *et al.* (2013). In contrast, Mishra *et al.* (2013) reported that explants co-cultivated for 5 d showed the maximum transformation efficiency in rice. The dissimilarity in co-cultivation time may be due to the differences in type and age of the explants used. In addition, Infection time is another essential factor in genetic transformation. Cotyledonary node explants infected for 15 min obtained the highest transformation efficiency in this study. In contrast to this result, Mashayekhi *et al*. (2008), Khan *et al*. (2010) and Tang *et al*. (2011) reported that 5–10 min of infection showed the maximum transformation efficiency.

Acetosyringone has been previously used to increase *Agrobacterium*-mediated transformation efficiency during infection (Mishra *et al.*, 2013; Subramanyam *et al.*, 2013). These studies reported that 20–200 μmol L^{-1} of acetosyringone were optimal for transformation and different concentrations of acetosyringone were used for different explants. In our experiment, maximum transformation efficiency was found to be 100 μmol L^{-1} of acetosyringone.

Kanamycin has been previously used for selection of transformants in cucumber; however, different concentrations were used for different explants. 150 mg L^{-1} of kanamycin was used to select transformants by Wang *et al.* (2013), 200 mg L^{-1} of kanamycin was used by Oliveira *et al.* (2011), Mishra *et al.* (2013), and Alvarez and Ordás (2013). In present study, transformed cucumber lines were successfully selected at 100 mg L^{-1} of kanamycin.

Regenerated transgenic lines were identified using PCR analysis and GUS gene expression, non-transgenic cucumber lines did not show the positive results. These results indicated that plasmid vector had succeeded into the genome of the transgenic cucumber lines, demonstrating that this system could be successfully used for cucumber transformation.

Conclusion

An efficient *Agrobacterium*-mediated genetic transformation system using cotyledonary node explants in cucumber has been established by modulating of the different factors such as pre-cultivation time, acetosyringone concentration, infection time and co-cultivation time. And *CsEXP*10 gene was successfully transformed into the cucumber genome. The genetic transformation system developed was successful in cucumber and may be used for the functional analysis of desired genes in cucumber.

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