ISSN : 0974 - 7435 Volume 10 Issue 17

2014

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 10(17), 2014 [9781-9789]

Study on agrobacterium-mediated genetic transformation of soybean with the resistance gene

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ABSTRACT

Soybean is an important oil crop in China, and is also an important source of food and industrial ingredients. One of the important factors affecting china's soybean production is soybean diseases. The traditional method of disease prevention will make serious damage to the ecological balance. In this study, we utilize transgenic technology, carrying out agrobacterium-mediated genetic transformation of soybean with the resistance gene caAMP1, to obtain transgenic soybean positive material.

KEYWORDS

Agrobacterium-mediated method; Genetic transformation; Soybean.

INTRODUCTION

Soybean is an important oil crop in China, and is also an important source of food and industrial ingredients. One of the important factors affecting china's soybean production is soybean diseases. Various diseases are causing a 5-10% decrease in the yield of soybean every year, or the decline of grain protein and oil content of soybean. Classified by pathogens, soybean diseases mainly include fungal diseases, bacterial diseases and viral diseases. Currently, the main method of disease prevention is still the high-dose use of pesticides, which not only increases the cost of soybean production, but also makes serious damage to the ecological balance. What's more, long-term use of pesticides will cause increased resistance of pathogens, forming a vicious spiral. By contrast, breeding of resistant soybean is one of the fundamental measures to reduce disease, especially breeding of broad-spectrum resistance soybean.

Transgenic technology as an effective means of breeding technology, has effectively broken reproductive isolating barriers between species, thus provides abundant gene resources for crop breeding. Meanwhile, it can greatly shorten the breeding cycle, conducive to breeding of disease-resistant soybeans and other crops. The broad-spectrum resistance gene caAMP1 (capsicum annuum antimicrobial protein 1) used in this study is isolated from capsicum annuum leaves inoculation with Xanthomonas campestris. CaAMP1 has resistance function to a variety of fungi and bacteria, so it is a qualified exogenous resistance gene.

In this study, we utilize transgenic technology, carrying out agrobacterium-mediated genetic transformation of soybean with the resistance gene caAMP1, to obtain transgenic soybean positive material. After verification, antibacterial peptide gene caAMP1 has broad-spectrum antimicrobial function, so the choice of resistance gene caAMP1 is proactive. For genetic transformation methods, agrobacterium-mediated transformation is currently the most mature and preferred transformation method of soybean.

GENETIC TRANSFORMATION TECHNOLOGY OVERVIEW

Agrobacterium-mediated method

When the agrobacterium infect the receptors, bacteria enter the host tissue by receptor original lesion or wound. However, bacteria themselves do not enter the host plant cells, but insert the Ti plasmid DNA fragment into the genome of a plant cell. The agrobacterium used in this method mainly includes agrobacterium tumefaciens and agrobacterium rhizogenes. While Agrobacterium-mediated transformation methods mainly include whole plant inoculation coinfection method and Leaf disc transformation method.

Agrobacterium-mediated method was the method to obtain the first transgenic plants in history. In 1988, Hinchee reported the first successful examples of agrobacterium transformation of soybean. He took cotyledon as acceptor material, *gus* (β-glucuronidase gene) as reporter gene, to screen *npt II* gene. Such genetic transformation process comprises at least four steps:

(1) Agrobacterium accumulation;
(2) Agrobacterium toxicity system induction;
(3) T-DNA transfer complex formation;
(4) T-DNA integrated into the plant genome.

In addition, using cotyledon node, cotyledon, hypocotyl as explant, to carry out Agrobacterium-mediated genetic transformation of soybean with *Bt* gene, chitinase gene, maize transposon *Ac* gene, etc. had made success.

Resistance Gene caAMP1

In recent years, pioneers of plant genetic engineering have completed separation and functional verification of large number plant disease-resistance genes. Various antibacterial peptides are isolated from plants, and there are signs that the peptides are participate in compositing or inducing defense mechanisms to various pathogenic bacteria. The principle of strengthening plant disease resistance by transgenic method is to make defense-related pathway genes, fungal hydrolase encoding gene, antibacterial protein encoding gene work.

The gene caAMP1 is one of them, which is able to express antimicrobial peptides. caAMP1 is isolated from capsicum annuum leaves inoculation with Xanthomonas campestris. Its cDNA contains an open reading frame consist of 185 amino acids. The mature caAMP1 molecular weight is 21,152Da. After being infected by pathogens or inactivated fungal elicitor, caAMP1 will abundantly express in pepper leaves. The purified caAMP1 shows broad-spectrum resistance to plant pathogenic bacteria and fungi. Studies have shown that, even though at concentrations of less than 30 micrograms per milliliter, caAMP1 exhibits inhibitory to the growth of botrytis cinerea, phytophthora capsici, candida albicans, ugly wine yeast and bacillus subtilis. This shows that, caAMP1 can directly inhibit the growth of pathogenic bacteria.

MATERIALS AND METHODS

Expression vector construction

(1) Experimental materials

a) Main reagents: Taq plus DNA polymerase, DNA fragments rapid purification/ recovery kit, T_4 DNA ligase, nucleic acid molecular weight marker, kanamycin, etc.

b) Experimental instruments: 2400 and 9700 type PCR instrument, DYY-III2 electrophoresis apparatus, DOC2000 gel imaging system, TGL-16G high speed tabletop centrifuge, CR22F high speed refrigerated centrifuge, PB303-E electronic balance, DHP-9052 heated incubators, HZQ-F full temperature oscillation incubator, SW-CJ-2FD clean bench, etc.

c) Medium: i) Escherichia coli medium (LB medium) (g/L): peptone 10g; yeast extract 5g; NaCl 5g; agar 15g; pH=7. ii) Agrobacterium medium (YEP medium) (g/L): peptone 10g; yeast extract 10g; NaCl 5g; agar 15g; pH=7.2.

(2) Experimental methods

a) Target gene caAMP1 amplification and recovery: according to caAMP1 gene sequence design the primer. Respectively introduce restriction enzyme sites *XbaI* and *SacI* into 5' terminal and 3' terminal of the primer. The upstream primer is: 5'-GCTCTAGAATGAATGCTAATGGATTTAGCGGTG-3'; the downstream primer is: 5'-GAGAGCTCTTAGACCTGATCAATGGGTTCTGTC-3'. The PCR reaction system and reaction condition is respectively shown in TABLE 1 and TABLE 2:

TABLE 1 : PCR reaction system

Reaction components	Amount
Buffer (10×)	5μl
dNTP (10mmol/L)	1μl
Upstream primer (10μmol/L)	1μl
Downstream primer (10μmol/L)	1μl
Pfu DNA Polymerase (5u/μl)	0.5μl
pGH-caAMP1 Plasmid DNA	1μl
H_2O	add to 50μl

TABLE 2: PCR reaction condition

Conditions	Time
94℃	5μl
94℃ 30s→56℃ 45s→72℃ 1min	30 cycles
72℃	10min
4℃	constant

b) pCAMBIA3300 Plasmid DNA Extraction

We use alkaline lysis method to extract pCAMBIA3300 plasmid DNA. The process is as follow:

Provoke the transformants on the LB plate, and vaccinate in 5ml kanamycin-contained LB medium, overnight fostered at 37°C;

Take 1.5ml broth, centrifugal for 30s at 12000rpm, and then discard the supernatant, and resuspend the precipitate with 100μl solution I, stirring;

Add 200μl new solution II, and take an ice-bath for 5-10min after gently mixing hook;

Centrifugal for 5min at 12000rpm, suck out the supernatant, equal volume of age / chloroform for 1 times, then centrifugal for 10min at 12000rpm;

Take out the supernatant, and precipitate it with twice volume anhydrous ethanol at -20°C for 30min;

Centrifugal for 5min at 12000rpm, take out the supernatant, dissolve in 20μl RNase-contained Sterile water, for 1-2h at 37°C;

Take small amount of solution, subjected to agarose gel electrophoresis and observe.

c) Digestion System

Respectively use *XbaI* and *SacI* enzyme to digest pCAMBIA3300 plasmid DNA and caAMP1 amplified recovery products, subjected to 1% agarose gel electrophoresis, and then recover PCR fragment and the vector fragment. The digestion system is shown in TABLE 3:

TABLE 3 : Digestion system

Component	Amount
10×Buffer	10μl
XbaI	5μl
SacI	5μl
DNA	20μl
Add water up to 100μl, water bath for 3h at 37°C	

d) Connection and Transformation

Connect the two digested products above. Connection system of target fragment and vector fragment is shown in TABLE 4:

TABLE 4 : Connection system

Component	Amount
10×T4 ligase Buffer	1μl
T4 DNA ligase	1μl
Target Fragment	7μl
Vector Fragment	1μl
Total Volume	10μl

e) Recombinant Enzyme Verification System

Extract recombinant plasmid by alkaline lysis method, then through recombinant enzyme verification system to appraisal whether the target gene successfully connect with the vector pCAMBIA3300. The PCR digestion system is shown in TABLE 5:

TABLE 5 : Connection system

Component	Amount
Template DNA	1μl
Buffer (10×)	5μl
dNTP (10mmol/L)	1μl
Upstream primer (1μmol/L)	1μl
Downstream primer (1μmol/L)	1μl
Taq DNA Polymerase (5u/μl)	1μl
H_2O	40μl
Total Volume	50μl

After enzyme verification, select Positive clone samples for sequencing. The expression vector is named pCAMBIA3300-caAMP1.

Agrobacterium-mediated Transformation of Soybean

(1) Experimental Materials

a) Acceptor: williams82, 03-3.

b) Medium: the mediums are shown in TABLE 6 ~ Table

TABLE 6 : Germination medium

Components	Amount
B5 (20×)	50mL
B5 (200×)	5mL
Fe salt (200×)	5mL
B5 Vitamin (100×)	10mL
Sucrose	20g
Phytagel	3g

TABLE 7 : Co-culture medium (1L, PH=5.4)

Components	Amount
B5 powder	0.321g
Sucrose	30g
MES	3.9g
BAP (1mg/mL)	1.67mg
Difco glue	4.8g
GA_3 (1mg/mL)	0.25mL
Cys (200mg/mL)	2mL
DTT (154.2mg/mL)	1mL
As (200μ mol/L)	0.03924g

TABLE 8 : Rooting medium (1L, PH=5.6)

Components	Amount
MS powder	4.43g
Sucrose	20g
MES	0.59g
Phytagel	3g
Asp (10mg/ml)	5ml
L-Glu (10mg/ml)	5ml
Tic (250mg/ml)	0.5ml
Cef (100gm/ml)	0.5ml
IBA (1mg/ml)	1ml

(2) Experimental Methods

Select the seed coat smooth and no lesion, no crack, no mildew full mature soybean seeds, and place them in the sealed container, fumigating and sterilizing for 3-4h with chlorine.

a) Germination: place the soybean seed hilum down after sterilization, uniformly embedded in colloidal-state GM medium. Seal it with plastic wrap, and then carry out seed germination for about 18h at 23 ° C in a dark environment.

b) Infection: pick a single colony containing vector pCAMBIA-caAMP1 agrobacterium, inoculated in 5ml YEP liquid medium that contains Kanamycin 50mg/L and Rifamycin 50mg/L. Incubate it overnight at 28 ° C shaker. Then take out the germinated seeds and cut them into two parts evenly. Dipped into the CCM liquid culture medium with scalpel, and infect the seed tissue. And then place the watercress in CCM liquid culture medium. Seal and rock it for 30min in shaker.

c) Co-culture: place a layer of filter paper after sterilization on the petri dishes containing solid medium CCM. Transfer the infected seeds in last step to the filter paper, with the section up, so that allow sufficient space for growth of the epicotyl. Then place it in 23 ° C dark environment, fostering for 5 days.

d) Rooting induction: Dipped into a small amount of IBA with soybeans that meet the conditions of rooting, to promote the growth of adventitious roots. Then transfer it to rooting culture medium, fostering in light tissue culture room.

e) Transplanting: When transfer the 3cm soybean young lotus into rooting culture medium for 15-30 days, place it in acclimatization chamber. Add 1cm-high sterile water, at 25-28° C, light intensity 10000lx or above, humidity 60-70%, for 3-4 days.

RESULT

Verification of expression vector

(1) Recombinant plasmid pGH-caAMP1

According to caAMP1 gene design the primer, and respectively introduce restriction enzyme sites *XbaI* and *SacI* into 5' terminal and 3' terminal of the primer.

Upstream primer: 5'-GCTCTAGAATGAATGCTAATGGATTTAGCGGTG-3';

Downstream primer: 5'-GAGAGCTCTTAGACCTGATCAATGGGTTCTGTC-3'

The DNA template is pGH-caAMP. After being PCR amplified and agarose gel electrophoresis, we obtain a 585bp segment which length is the same as camp. The electrophoresis result is shown in Figure 1.

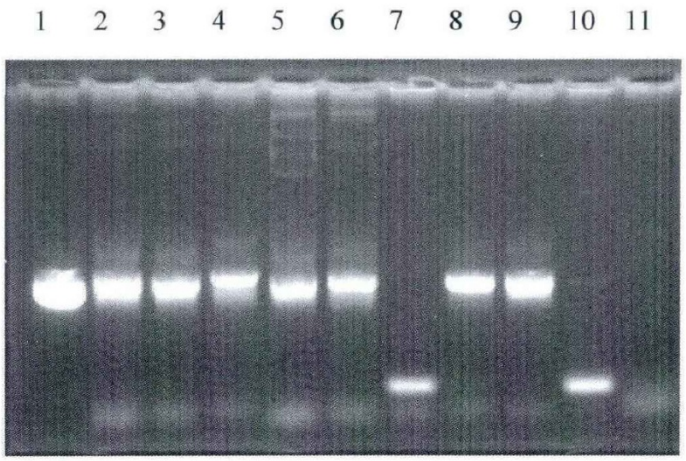

Figure 1 : The Electrophoresis result of pGH-caAMP

The gene sequence of caAMP1 is as follow:
1 atgatgaatg ctaatggatt tagcggtgtg gagaaagagt atataaggaa acatcatctt
61 catcagccta aggagaatca atgcagttcg tttltagtca agcacattcg agcacctgtt
121 catcttgttt ggtcattggt taggcggttt gatcagccac aaaagtacaa gccctttgtt
181 agcaggtgca tcgcgcaagg agatcttgag attggtagtc ttagggaagt tgatgtcaag
241 tctggcctcc ctgcaacaac gagtaccgag agattggagc ttcttgatga tgaagagcac
301 attttgagtt tcagaattat tggcggggat cacagactta ggaactactc ctcaatcata
361 tctctccacc ccgaggtgat cgatggaaga cctgggacct tagtaattga atcatttgtg
421 gtggacgtgc cccaaggaaa caccaaagac gagacgtgtt attttgttga agcattgatc
481 aattgcaacc taaaatcact tgctgatgtc tcagagaggc ttgctgtgca ggacaggaca
541 gaacccattg atcaggtcta a

(2) Recombinant expression vector pCAMBIA3300-caAMP1

For the recovery products of pCAMBIA3300 plasmid DNA and caAMP1, after *XbaI* and *SacI* double digestion, do 1% agarose gel electrophoresis, and then recover the PCR digestion segment and vector segment. Connect the two segments, with PCR and digestion verification, and select positive samples for sequencing, so as to construct the expression vector pCAMBIA3300-caAMP1.

Extract the recombinant plasmid pGH-caAMP form pCAMBIA3300-caAMP1 vector, and detect the accuracy of transferring into agrobacterium by PCR. Take gf-L and gf-R as the primer, recombinant plasmid as template, PCR products of recombinant plasmid in E. coli as positive control, PCR products with water template as negative control. After agarose gel electrophoresis, if obtain a 585bp band, we can verify that the recombinant plasmid has been transferred into Agrobacterium tumefaciens.

In our experiment, the PCR result show a 585bp band, the fragment size consistent with the positive control group, while there is no any band in negative control group. The PCR detection result is shown in **Figure 2**.

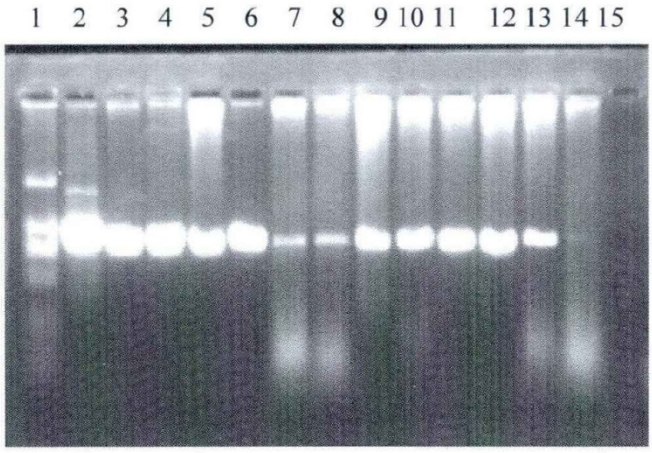

Figure 1 : The PCR detection result of the transformation

Transformation record

The transformation record is shown in TABLE 9-TABLE 10.

TABLE 9 : Transformation record table

Transformation Batch	1		2			3		
Subculture Process	Explants Number	Subculture Date	Subculture Process	Explants Number	Subculture Date	Subculture Process	Explants Number	Subculture Date
CCM	305	9.7	CCM	359	9.8	CCM	354	9.9
SIM	297	9.12	SIM	350	9.13	SIM	340	9.14
SIM	188	9.26	SIM	324	9.27	SIM	238	9.28
SEM	175	10.11	SEM	285	10.11	SEM	218	10.12
SEM	161	10.24	SEM	261	10.25	SEM	189	10.26
SEM	157	11.7	SEM	236	11.8	SEM	175	11.9
SEM	140	11.21	SEM	219	11.22	SEM	168	11.23
SEM	140	12.5	SEM	217	12.6	SEM	165	12.7
SEM	139	12.19	SEM	217	12.2	SEM	165	12.22
SEM	132	1.4	SEM	196	1.5	SEM	158	1.6
SEM	132	1.16	SEM	161	1.17	SEM	119	1.17
SEM	56	2.3	SEM	119	2.3			

……

TABLE 10 : Transformation record table

Transformation Batch	10	
Subculture Process	Explants Number	Subculture Date
CCM	231	4.19
SIM	206	4.24
SIM	147	5.9
SEM	119	5.23
SEM	102	6.6
SEM	97	6.2
SEM	91	7.5
SEM	80	7.19

Integrated with emergence results and transplanting record, when SEM subculture reaches the third batch, it will be seedling; when about the fourth or fifth batch, the seeding number will get most. The total subculture batch should better not exceed 6, or it will bring too much cost pressure, and the seeding rate is not ideal.

Soybean growth state

The Growth State records of soybean in transformation process are shown in Figure 3~Figure 7.

Figure 3 : Soybean after sterilization

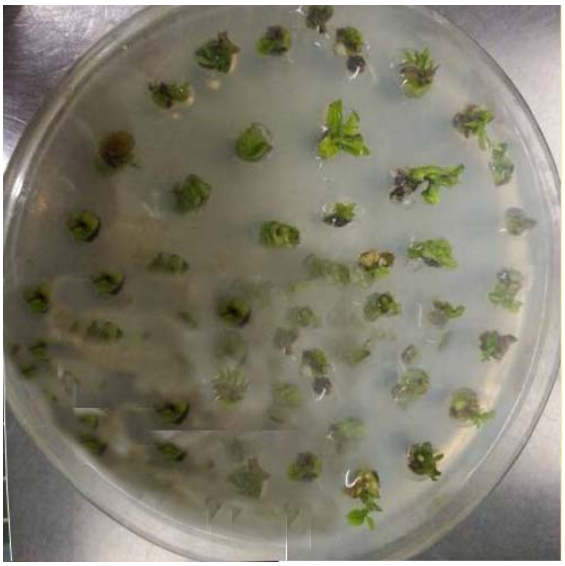

Figure 4 : Induced bud growth

Figure 5 : Plants in RM

Figure 6 : Growth state acclimatization stage

Figure 7 : Growth state after transplanting

DISCUSSION

In this study, we utilize agrobacterium-mediated method, which is one of the most widely used genetic transformation methods of soybean. This method has simple and quick operations, without seasonal restrictions. Only by the technique of cultivating a certain time after infection of the tissue, we can obtain regenerated transgenic plant. What's more important, after research and improvement for many years, the whole process of agrobacterium-mediated method has already been quite mature. The optional range of explants is very broad, including soybean cotyledons, cotyledon node, mature embryos, immature embryos, hypocotyl, leaf and other tissues, which provides more flexible space exploration for transformation of resistant gene caAMP1.

ACKNOWLEDGEMENTS

This work was supported by the grants from National Natural Science Foundation of China（Nos.31260482 and 31060263）

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