Oct 08, 2019

The laboratory protocol: Agrobacterium tumefaciens-mediated transformation of a hevein-like gene into asparagus leads to stem wilt resistance

PLOS One

Helong Chen[1]

[1]Institute of Tropical Agriculture and Forestry, Hainan University/Institute of Tropical Bioscience and Biotechnology/Hainan Academy of Tropical Agricultural Resource, Chinese Academy of Tropical Agricultural Sciences

1 Works for me dx.doi.org/10.17504/protocols.io.68bhhsn

Helong Chen

ABSTRACT

The laboratory protocol's aim is to develop a genetic transformation system for asparagus and apply transgenic technology to obtain transgenic plantlets through *A. tumefaciens*-mediated transformation. Analysis of disease resistance and determination of physiological indexes confirmed that the transgenic lines represented a new germplasm source for breeding high yielding, disease-resistant Asparagus varieties.

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0223331

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Chen H, Guo A, Lu Z, Tan S, Wang J, Gao J, Zhang S, Huang X, Zheng J, Xi J, Yi K (2019) *Agrobacterium tumefaciens*-mediated transformation of a hevein-like gene into asparagus leads to stem wilt resistance. PLoS ONE 14(10): e0223331. doi: 10.1371/journal.pone.0223331

MATERIALS TEXT

The diploid asparagus cultivar “JingKang 701” was used as the sample for the test. The pBI121 and pCAMBIA3300 expression vectors and the pUC57-hevein-like were provided by the laboratory; and the bacterial strains for the test were EHA105 and DH5α. The ligase, incision enzyme, and polymerase were purchased from New England Biolabs (Beijing), ThermoFisher, and Takara, respectively. The kits (Gel recovery kit, DNA purification recovery kit, Plasmid extraction, purification kit, DIG High Prime DNA Labeling and Detection Starter Kit I and so on) were purchased from OMEGA. Hybond-N membrane was purchased from Pharmacia USA. Synthesis and sequencing were handled by Sangon Biotech. The hevein-like target gene sequence is as follow (or sees GenBank Accession No: U40076.1.):
ATGAAATACTGTACTATGTTTATTGTTCTCTTGGGTTTAGGCAGCTTGTTGTTGACACCAACAACAATAATGGCACAACAGTGCGGGAGACAAG
CCAGTGGGCGTCTGTGCGGCAACGGCCTTTGCTGCAGCCAGTGGGGCTACTGTGGCTCCACTGCAGCCTACTGTGGAGCTGGTTGCCAGAGC
CAATGCAAATCTACTGCTGCTTCTTCCACCACCACTACCACTGCAAACCAATCAACCGCTAAGTCGGATCCCGCCGGCGGTGCCAACTGA
Main instruments: Takara TP600 gradient PCR instrument, German Eppendorf Mastercycler gradient PCR instrument, agar-sugar gel electrophoresis instrument, German Eppendorf 5810R high-speed freezing centrifuge, American Thermo Nanodrop 2000 trace ultraviolet spectrophotometer, tanon-4500 gel imaging system, sterilizer, oven, refrigerator (-4°C, -20°C and -70°C), water bath pot, dry bath pot, incubator, shaker and microscope.

1 Construction of Vector

(1) The fragments cloned of pBI121-hevein-like vector was shown in Fig 1. Extraction and purification of pBI121 and the hevein-like carrying plasmids were performed using kits according to manufacturer's instructions. We used *Xba*I and *Sac*I for enzyme digestion and performed gel extraction using a kit (OMEGA). The P1 primer was used to add *Xba*I and *Sac*I cleavage sites to the ends of the hevein-like target gene.

F: 5' TGCTCTAGAATGAAATACTGTACTATGTTTAT 3'

R: 5' CGAGCTCTCAGTTGGCACCGC 3'

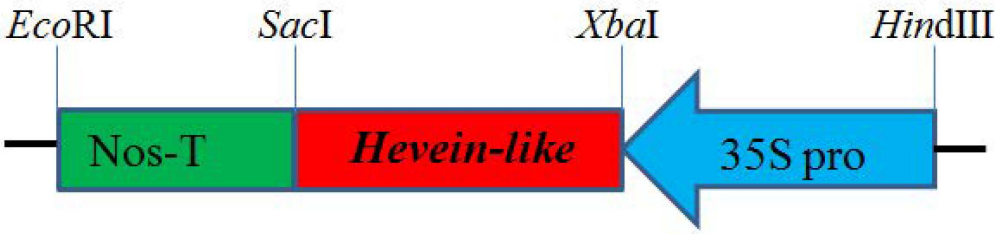

Fig 1 The fragments cloned of pBI121-hevein-like vector

(2) pCAMBIA3300-35S-hevein-like-NOS vector (Fig 2) was constructed following the steps of constructing pBI121-hevein-like. The P2 primer below was used for PCR verification, and the construction was completed upon verification.

F: CCCAAGCTTCATGGAGTCAAAGATTCAAATAG (32 bp)

R: GGAATTCCCCGATCTAGTAACATAGATGAC (30 bp)

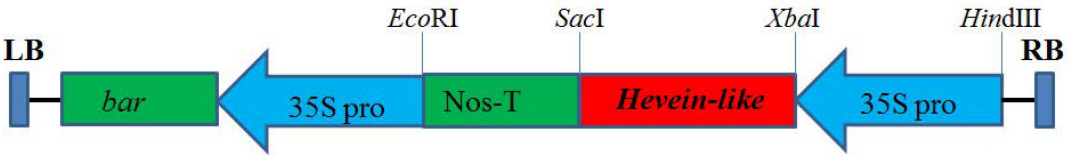

Fig 2 The fragments cloned of the plant expression vector-pCAMBIA3300-35S-hevein-like-NOS

2 Glufosinate （PPT）Resistance Observation

A total of 20 units of 0.6 cm^2 blocks of asparagus embryos were placed in different concentrations of transformation media (0, 5, 10, 20, 40, and 80 mg/l PPT), and the survival rate of asparagus embryos was recorded after 20 days.

3 Preculture

The asparagus embryos measuring 0.6 cm^2 each were placed in the preculture medium for light culturing for 2 days at 26 °C. The formula of the culture medium was: Murashige and Skoog (MS) culture medium, 4% sucrose, 800 mg/l Gln, 500 mg/l acid-hydrolyzed casein, 0.70 mg/l ancymidol, 0.10 mg/l naphthaleneacetic acid (NAA), and 0.50mg/l kinetin, of which the ph value is 5.8.

4 Preparation of Transformation Bacterium Solution

A total of 20 µL *Agrobacterium tumefaciens*containing pCAMBIA3300-35S-hevein-like-NOS plasmid was applied on yeast extract peptone (YEP) solid medium (containing 50 m/l Kanamycinand 100 mg/l Rifampin). The medium was placed in stationary position and turned upside down for 30 min to initiate cultivation at a temperature of 28 °C for 2 days. A single colony was inoculated in 50 mg/l Kanamycin (Km) and 100 mg/l Rifampin(Rif) containing the YEP fluid medium, and shake cultivation was performed at 250 rpm in 28 °C. Centrifugation (OD600 about 0.55) was done for 8 min at a rotation rate of 5500 rpm, and the liquid transformation medium was adopted for the suspension culture after centrifugation.

5 Inoculation

The light absorption values of the transformation bacterium solution at wavelength of 600 nm was adjusted, namely 0.2, 0.4, 0.6, and 0.8. The cultivated embryos were placed in a pasteurized 150 mL Erlenmeyer flask. The 50 mL Acetosyringone (AS) liquid media of different concentrations (0, 50, 100, and 200 µmol/l) were used to invade the embryo for 5, 10, 20, and 40 min (when the medium contained transformation *A. tumefaciens*). Shake cultivation was performed at a temperature in 26 °C at a frequency of 150 rpm.

6 Co-culturing

The invaded embryo was wiped dry and transferred to a solid medium for dark culturing at 26 °C for 2, 4, 6, and 8 days.

7 Removal of bacteria and selection of transformed embryos

The co-cultured embryos were treated with 200 mg/l Timentin for 10 min at an oscillation frequency of 100 rpm to remove the bacteria. The embryos were washed with distilled water, wiped dry, and transferred to a solid transformation medium containing glufosinate (PPT) and Timentin for light culturing at 26 °C. The growth situation of the embryos was observed and statistically analyzed. The test was done thrice (80 embryos were used each time).

Transformation percentage of resistant embryos = number of resistant embryos/total number of embryos *100%

8 DNA Extraction and confirmation of transformation

Newly growing embryos were placed in a seedling culture medium to become seedlings. The seedlings were transplanted to the prepared nutrition substrate after induction, and the asparagus seedlings were managed and held for detection. The kit method was adopted to extract transgenic asparagus seedling DNA. The P2 primer mentioned in vector construction was used for PCR verification. Moreover, integration of the hevein-like gene was further confirmed by southern blot hybridization. Genomic DNA of T1 generation transgenic plant leaves was used as template, genomic DNA of non-transgenic plant leaves was used as negative control, and plasmid pCAMBIA3300-35S-hevein-like-NOS DNA was used as positive control. Genomic DNA was digested overnight at 37°C using *EcoR*I and *Hind*III separated by 0.8% agarose gel electrophoresis and transferred onto a Hybond-N membrane (Pharmacia, USA). The membrane was hybridized with the hevein-like-CDS DNA probe, which was random primed, labeled with Digoxigenin-11-dUTP using DIG-High Prime (Roche). Hybridization (at 42°C) and detection were performed following the instruction manual of the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, USA).

9 Disease resistance identification and physiological indexes determination of transgenic plants

The stem wilt disease resistance of transgenic asparagus plants was calculated using the disease index described by Yang[13]. The infective strain was the virulent strain BT05 screened in the laboratory. BT05 was prepared as 10^6/mL bacterial suspensions for spraying with the bacterial suspension to inoculate. Three transgenic plants were treated and three non-transgenic plants of the same seedling age were compared. Three replicates were carried out. Inoculated samples were left under high humidity for 48 hours, and then the plastic cover was removed, allowing natural infection at a temperature of 25 °C to 28 °C. On the 8th day after inoculation, the disease incidence of each treatment was observed and the disease level of each replicate was determined according to disease level criteria, and the DI (disease index) was calculated according to the disease level.

Disease level criteria

Grade 0: no disease; Grade 1: the disease area accounts for less than 10% of the canopy area Grade 2: the disease area accounts for 11%-30% of the canopy area; Grade 3: the disease area accounts for the crown area 31%-50%; Grade 4: The disease area accounts for more than 50% of the canopy area.

$DI = \sum$ (plant number of disease grades × disease level value) / (number of total plants per replicate × 4) × 100;

Immunity: DI= 0; High resistance: 0<DI≤10; Medium resistance 10<DI≤30; Resistance: 30<DI≤50; Susceptibility: DI>50

Presence of Malondialdehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT) and Phenylalanine ammonia lyase (PAL) level were determined in inoculated leaves by the method of Han [14].

10 Data Analysis

All the experiments were carried out in triplicate using a completely randomized design asparagus embryos number of PPT resistance test was 20 in each repeat, whereas embryos number of each repeat was 80 in other tests. SPSS19.0 and Excel 2007 were employed for statistical analysis [15] and significantly different levels of disease resistance and physiological index between transgenic and control lines were calculated using SPSS19.0 software.