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1 Materials and methods

1.1 Strains and growth conditions

Escherichia coli strain DH5α was used as a host for gene manipulation and *A. tumefaciens* strain LBA4404 was used as a T-DNA donor for fungal transformation. These bacterial strains were maintained on LB medium at 37℃ and on YEB medium at 28℃, respectively. HDF-68, a taxol producing fusant strain was used as a recipient. Fungal strains were cultured on potato dextrose agar (PDA) medium at 28℃.

1.2 Construction of the T-DNA binary vector pBI121-43

The T-DNA binary vector pBI121-43 carrying the hygromycin B phosphotransferase (*hph*) gene cassette (the *hph* gene under control of *Aspergillus nidulans* trpC promoter and the terminator) between the right and left borders was constructed on the backbone of pBI121. The 2.4kb *hph* gene cassette was derived from pCSN43 (kindly provided by the Fungal Genetics Stock Center (FGSC), Kansas City, USA) by *Sal* I digestion and introduced into the *Sal* I site of pUC18 to make pUC18-43. The *Sma* I/*Hin*d III fragment containing the *hph* gene cassette was excised from pUC18-43 after its *Dra* I digestion and ligated with the 11.7kb backbone from pBI121, after *Eco*R I/*Hin*d III double-digestion and blunting the *Eco*R I site, resulting in the binary vector pBI121-43.

1.3 ! "#$%&'()#*+, -mediated transformation of HDF-68

The ATMT protocol was a modification of a method described previously[8]. Conidia of HDF-68 were freshly prepared and adjusted to about 10^6/mL. A single colony of *A. tumefaciens* containing the binary vector was grown overnight at 28℃ under agitation (200rpm) in YEB medium supplemented with 250μg/mL spectinomycin and 50μg/mL kanamycin. The culture was diluted to an optical density at 660 nm (OD_{660}) of 0.15 in induction medium (IM) either in the presence (IM + AS) or in the absence (IM-AS) of 200μM acetosyringone (AS)[15]. The cells were grown for additional several hours until the OD_{660} of the culture reached 0.24 ~ 1.53. Subsequently, 100μL of bacterial and 100μL of conidial suspension were mixed and spread onto nylon membranes on IM agar plates with or without AS. Following 24 ~ 96h of co-cultivation under different temperatures, the membranes were transferred onto solid PDA selection medium containing 200μM cefotaxim to kill the bacteria and 30μg/mL hygromycin B (Invitrogen) to select transformants.

Mitotic stability of the integrated T-DNA was tested by subculturing transformants on PDA medium for five times without hygromycin B. Subsequently, resistance of these transformants to hygromycin B was tested by transferring them to PDA medium containing 100μg/mL hygromycin B.

1.4 Molecular analysis of transformants

Fungal genomic DNA was extracted from mycelia by the CTAB method. PCR amplification for detection of the presence of *hph* gene in the putative transformants was carried out using primers: hph-F (5'-GAGCCTGACCTATTGCATCTC-3') and hph-R (5'-CCGTCAACCTTGCTCTGATAG-3'). The PCR program used was one cycle of 1min denaturation at 94℃, followed by 30 cycles of 30s denaturization at 94℃, 30s of annealing at 55℃ and 1min extension at 72℃, followed by a final 7min elongation at 72℃. DNA from untransformed wild-type HDF-68 served as a negative control.

For the hybridization analysis, DNA (about 5μg) from transformed strains and untransformed wild-type HDF-68 was digested for 16h with *Hin*d III, of which the restriction site was not presented inside the T-DNA. The internal 550bp *hph* fragment was used as a probe. The 550bp PCR product that was amplified using hph-F and hph-R as primers and plasmid pCSN43 as template served as a positive control. Hybridization was performed under conditions recommended for the digoxigenin (DIG) hybridization system by Roche (Mannheim, Germany).

2 Results

2.1 Hygromycin B sensitivity of HDF-68

The sensitivity of HDF-68 to hygromycin was tested by bringing mycelium onto PDA agar plates with different concentrations (0, 5, 10, 15, 20 and 30μg/mL) of hygromycin. The results showed that the growth of mycelium was totally inhibited on 15μg/mL (Fig.1). Therefore, 30μg/mL hygromycin B was chosen for the selection of resistant colonies in the following transformation experiments to prevent the growth of false transformants.

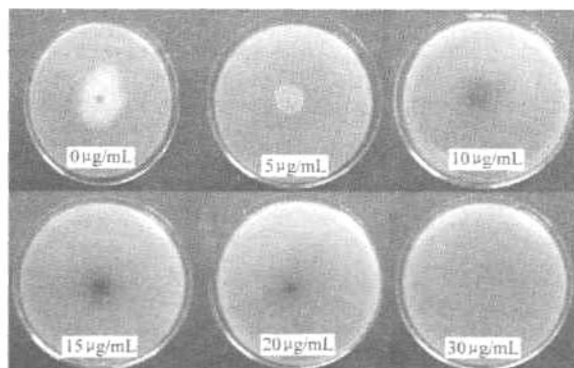

Fig.1 Effect of different concentrations of hygromycin B on HDF-68 mycelium growth

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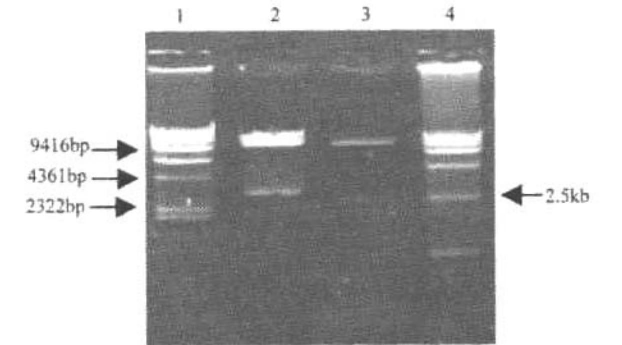
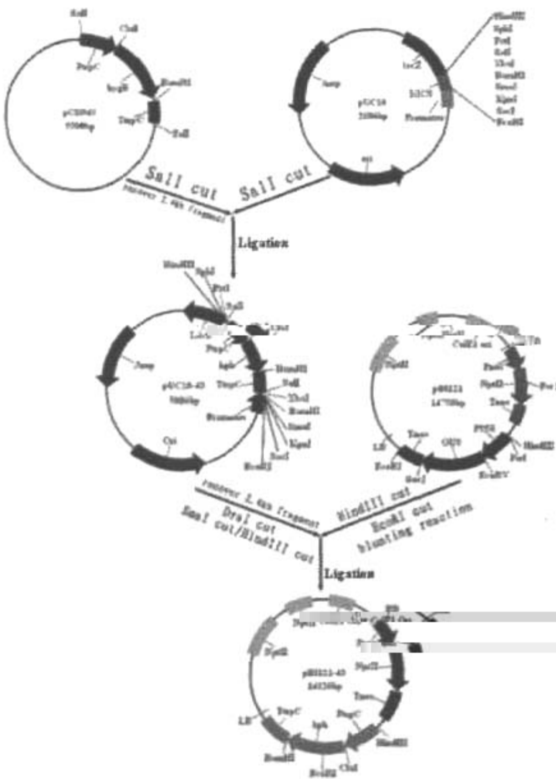
Enzyme digestion was performed to identify recombinant pBI121-43. Fragments (11.7kb and 3kb) appeared after the original plasmid pBI121was digested with *Eco*R I and *Hin*d III. The 2.4kb fragment was obtained when the recombinant pBI121-43 was digested with *Sal* I, suggesting that T-DNA binary vector, pBI121-43, had been successfully constructed (Fig.2(a), and Fig.2(b)).



Lane 1 : λ *Hin*d III molecular marker ; Lane 2 : pBI121 *Eco*R I / *Hin*d III digestion ; Lane 3 : pBI121-43 Sal I digestion ; Lane 4 : DL15000 molecular marker

(a) Digestion identification of binary vector pBI121-43



(b) Construction flow of binary vector pBI121-43

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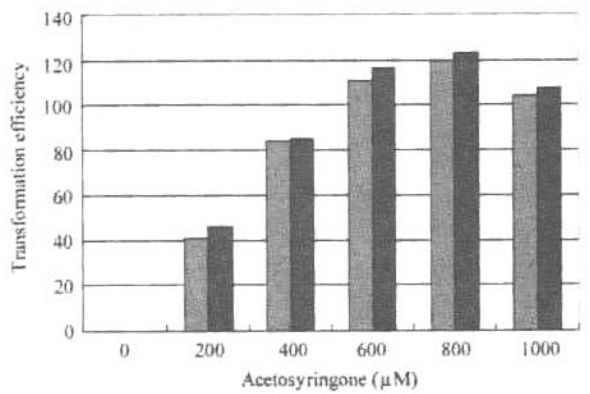
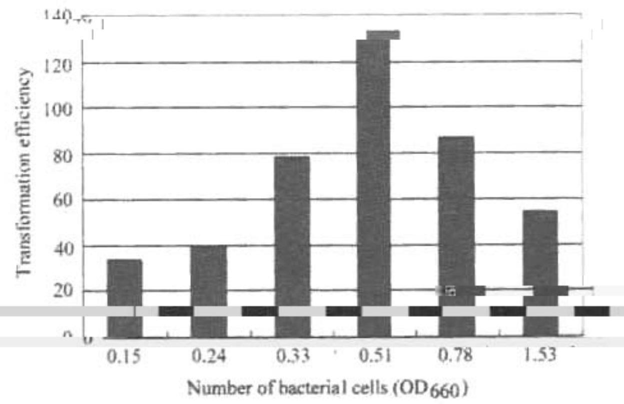
Hygromycin-resistant transformants were obtained after 7 ~ 10 days cultivation on selection medium.

First, we determined whether AS, which is necessary for the induction of *vir* genes[10, 16-18], was essential for the transformation of HDF-68. No transformants were detected when AS was omitted from the co-cultivation treatment. Furthermore, we examined the effect of AS prior to co-cultivation. The number of hygromycin-resistant colonies was not associated with the induction of bacterial cells with AS prior to co-cultivation. The preincubation of *A. tumefaciens* cells with AS displayed little effect on transformation efficiency (Fig.3(a)). Therefore, in the



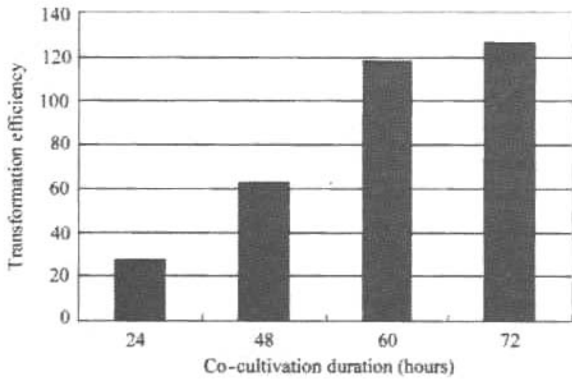
Bacterial cells were incubated in the presence (the left bar/column) of 200 μM AS or absence (the right bar/column) of AS prior to co-cultivation. The OD_{660} of bacterial culture was 0.5. Conidial suspension was adjusted to 10^6/mL conidia. Various concentrations of AS was added to IM plates. The bacterium culture (100μL) was mixed with the same volume of conidial suspension and incubated on each IM plate at 25℃ for 60h.

(a) Effect of AS on transformation efficiency



The OD_{660} of bacterium culture (non-preincubated with AS) was 0.15-1.53. The conidial suspension was adjusted to 10^6/mL conidia. The bacterium culture (100μL) was mixed with the same volume of conidial suspension and incubated on each IM plate containing 800 μM of AS for 60h at 25℃

(b) Effect of the number of *A. tumefaciens* cells initially applied for co-cultivation on transformation efficiency



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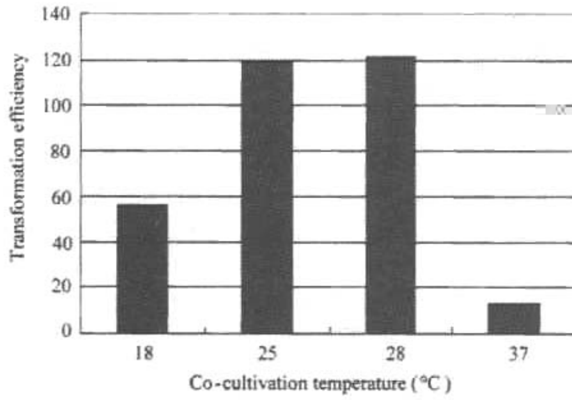

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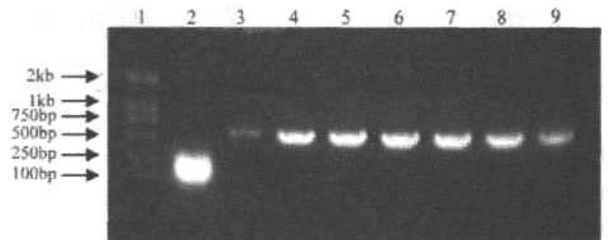

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