Transformation of Arabidopsis thaliana ESB1 Gene into Agrobacterium tumefaciens and Its Identification

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Abstract: Botrytis cinerea is a major disease in crop production. Chrysanthemum gray mold is easy to occur during chrysanthemum growing season. It can cause a lot of defoliation when serious, affecting plant flowering and reducing ornamental value. In this study, Arabidopsis thaliana ESB1 gene introduced into Agrobacterium tumefaciens was introduced into Agrobacterium tumefaciens and its identification after induction, and the sensitivity of chrysanthemum to antibiotics. It is expected that Agrobacterium tumefaciens will be transformed into chrysanthemum, result in transgenic chrysanthemum new lines resistance to disease, high quality, and high yield to lay the technical foundation. In this study, Arabidopsis thaliana ESB1 gene was introduced into Agrobacterium tumefaciens by freeze-thawing method. The PCR reaction was used to determine whether the ESB1 gene was successfully transferred into Agrobacterium tumefaciens and the susceptibility test of chrysanthemum to hygromycin was carried out to determine the critical tolerance concentration, Establishment of Chrysanthemum Transformation Receptor System. The result of PCR showed that the ESB1 gene was successfully transferred into Agrobacterium tumefaciens. The critical concentration of chrysanthemum to hygromycin was 30mg / L.

Key words:ESB1 gene; Agrobacterium tumefaciens; Genetic transformation; Cut flower chrysanthemum.

Chapter 1 Introduction

1.1 Advances in Genetic Engineering of Cut Chrysanthemum

1.1.1 Introduction to chrysanthemum

Chrysanthemum (Chrysanthemum morifolium) is one of the perennial half-lignified herbs, is chrysanthemum after years of artificial cultivation, choose to cultivate the valuable ornamental flowers, also known as art chrysanthemum. Chrysanthemum originated in China, the ancient also called Ju, Huang Hua, Jin Rui, flowers [1]. Chrysanthemum cold, like deep fertile, well-drained sandy loam, bogey waterlogging. Its cultivation has a long history, many varieties of precious, leaves, flower type, petal type has changed greatly, is color, fragrance, pose, rhyme and superb Chinese traditional flower, is one of the world's famous ornamental flowers, High ornamental value and broad application prospects [2]. With the development of the flower industry, there is a growing demand for high quality chrysanthemum flowers and cut flowers. Therefore, breeders seek different breeding methods and adopt new methods and techniques to cultivate more ornamental flowers new varieties of chrysanthemum. Chrysanthemums in a variety of forms, according to their application classification, can be divided into cut chrysanthemum, potted chrysanthemum, ground chrysanthemum and chrysanthemum.

1.1.2 History and status quo of cut flower chrysanthemum

Chrysanthemum is native to our country's traditional flower, it is not only colorful, gestures thousands, but also with cold arrogance, proud frost in full bloom, Ling Han not withered character, so with the plum, blue, bamboo and called the four gentlemen [3]. China Chrysanthemum since the Zhou Dynasty has documented, so far has been a long history of more than 3,000 years. AD 729 - 748, China Chrysanthemum first introduced to Japan, by the Japanese people highly appreciated, and become one of Japan's most noble flowers, while chrysanthemum was also designated as the Japanese royal national flower. In 1688, China's chrysanthemum by the Dutch businessmen into Europe in the mid-19th century, and from United Kingdom into North America after China's chrysanthemums all over the world.

Chrysanthemum originated in China. There are variety of chrysanthemums, such as chrysanthemum, potted specimens of chrysanthemum, Dali chrysanthemum, cliff chrysanthemum, chrysanthemum chrysanthemum, chrysanthemum, chrysanthemum, and chrysanthemum bonsai [4]. Its flower culture long, rich in content and impress people, but cut flower chrysanthemum production in Europe and United States, Japan and other countries have been developed and grown. International cut flower cultivation is divided into three plates, supply of European market in Africa, supply of Americas market in Colombia and Ecuador planted and supply Asian market in China. In recent years, cut flower chrysanthemum has developed into the highest value of international commodity flower output. The 20th century, the eighties, the domestic cut chrysanthemum industry is also a sudden emergence of the trend, flourish.

1.1.3 Cut flower chrysanthemum development prospects

The flower industry is one of the most vibrant industries in the world. Today, the world's flower industry to an average annual growth rate of 25%, faster than the average speed of world economic growth. Japan is the world's largest producer of cut flower chrysanthemum, accounting for more than 50% of the world's chrysanthemum production, but the production is not enough with its demand due to higher labor costs, lack of land resources, the low temperature during winter in Japan. The production gradually shifted to those developing countries with suitable climate and also have low production costs. The steady growth of the world's flowers and industrial transfer, for the further development of China's flower industry to create a good external environment.

1.1.4 Advances in Genetic Engineering of Cut Flower Chrysanthemum

Genetic engineering breeding is a technology developed in the early 1970s. That is, the use of molecular biology techniques, a variety of foreign genes into biological cells, tissues or organs to achieve the desired effect [5]. Modern biotechnology has created new genetic variants and breeding resources for plant breeders whose purpose is to use genetic engineering to integrate the exogenous genes of economic value into the genome of the recipient cells and to be effectively expressed in order to achieve directional improvement plant traits, to create new varieties, to improve plant yield and quality. Since the 1980s, this technique has been gradually applied to the improvement of higher plant species and the cultivation of new varieties. Compared with traditional breeding methods, genetic engineering has its unique

advantages: can be modified to modify one or some of the target traits and retain their original traits; through the introduction of foreign genes can expand its gene pool. The development of transgenic technology provides a new way for chrysanthemum breeding. Chrysanthemum transgenic research is devoted to change the color, flower, flowering, plant type and anti-pests and other aspects [10].

1985 Hayh and other first leaf disc transformation method [11], 1987 Sanford and other use of gene gun technology to create a new breeding methods, so as to further use of new genetic resources to improve chrysanthemum varieties provide a new means and ways. Since Lemieux's first successful use of Agrobacterium tumefaciens-mediated production of transgenic chrysanthemum, with the improvement of the genetic transformation system of chrysanthemum, the expression of exogenous genes in plant cells and the improvement of transformation methods. Some of the target traits and retain the original traits, so that in the color, resistance, pattern, flowering and other aspects of a great improvement and development [14]. Lc, CHS, resistance gene bt, NP-I, TSWV, altered morphological genes GAI, Rolc, PHYB-1, flowering gene LFY, FT, etc. [9,10]. Some of these transgenic seedlings showed the purpose of traits and entered the field experiment, but a large part of the emergence of gene silencing.

Fu Rongzhao and other rabbit defensin NP-1 gene into chrysanthemum, obtained anti-kanamycin plant [5]. Mitouchkina and so on using Agrobacterium-mediated method, the rolC gene into the chrysanthemum variety 'White Giant', the use of rolC gene on the chrysanthemum morphological changes, obtained the plant type, branching, flowers and petals are changed chrysanthemum new varieties [11,13]. To the Taihe and so on using chrysanthemum leaves into the rolC gene, get dwarf chrysanthemum transgenic plants [2]. To improve resistance is an important goal of chrysanthemum breeding, Y. Takatsu and other rice chitinase gene RCCZ into chrysanthemum, improve the resistance to bacteria. Takatsu et al. transferred the rice chitinase gene into Agropyron glauca and obtained three strains which had strong resistance to Botrytis cinerea by ELISA. The CPRO-DLO in the Netherlands has successfully isolated a promoter from chrysanthemums to ensure a high expression of the genes of chrysanthemum transformed plants. The promoter of the toxin gene with Baciilus thubergiensis was then introduced into chrysanthemum to develop a new type of chrysanthemum against beet army worm. Yepes wrapped the potato dotted wilting virus TSWV (N) core-shell gene and NPTII dichotomous plasmid PBIN19 on the surface of tungsten particles to attack the leaves of the chrysanthemum cultivars 'Blush', 'Dark Bronze Charm', 'Iridon', 'Tara' Stem explants, obtained a high conversion efficiency (89%) [12]. Mitiouchkina and so on will be isolated from the golden fish CHS gene in the antisense direction into the chrysanthemum variety 'Parliament', access to flowers lighter color of the new varieties [13].

1.2 The Principle and Research Progress of Agrobacterium - mediated Transformation of Plants

Agrobacterium tumefaciens is considered to be the only organism with internal gene transfer [8]. Is a gram-negative Agrobacterium, which contains Ti plasmid, can induce infected cells to form tumors, that is, induced crown gall tumor. Ti plasmid (including Ri plasmid) has a transfer of DNA, Agrobacterium infection in the host plant, this DNA can be transferred into the plant cells, and stably retained in the plant cell chromosome, into a new plant cells of a gene , And ultimately through the sexual generation to the offspring.

In the 1950s, the Braun-AC study found that the plant crown gall tumors caused by Agrobacterium tumefaciens in the soil had mechanisms for transformation due to their infinite growth characteristics [6]. Subsequent discovery of opal in cells demonstrates that Agrobacterium's genes have been integrated into plant cells, making it possible to transform Agrobacterium.

1.3 Overview of Arabidopsis thaliana ESB1 gene

Botrytis cinerea is considered to be an important plant pathogenic fungus in the world. It can infect more than 200 crops and cause gray molds, causing serious economic losses to agricultural production worldwide. Arabidopsis thaliana and Botrytis cinerea as a plant-pathogen interaction model to provide a theoretical basis for studying the resistance mechanism of plant resistance to saprophytic fungi infection. In the previous study, we used the genetic screening system to obtain a T-DNA insert mutant of Arabidopsis thaliana resistant to Botrytis cinerea, named esb1 (Enhanceimetic to Botrytis1), and the corresponding mutant gene named ESB1. Mutation of ESB1 gene resulted in increased susceptibility to plant blight and high concentration of NaCl stress. The transformation of the ESB1 gene into the mutant plant completely restored its resistance to Botrytis cinerea infection and NaCl stress, indicating that the ESB1 gene is essential for Arabidopsis thaliana and other biological and abiotic resistance to Botrytis cinerea and high salt.

1.4 Arabidopsis thaliana ESB1 gene and chrysanthemum gray mold

Botrytis cinerea is a major disease in crop production. The pathogen is Botrytis cinerea, which belongs to the subfamily, subspecies, of the saprophytic pathogens. Botrytis cinerea is considered to be an important plant pathogenic fungus in the world. It can infect more than 200 kinds of crops to cause gray mold, causing serious economic losses to agricultural production in the world.

According to the observation of chrysanthemum gray mold in the chrysanthemum growing period prone to the main damage to chrysanthemum leaves, stems, flowers and other parts; serious can cause a lot of leaves, affecting plant flowering, reduce ornamental value; leaves at the edge of the leaves will appear brown lesions. The surface slightly pulsating wave wrinkles, petiole and flower handle first softened after the skin rot, flowers affected when the flowering and seed ripening; high temperature and rain, planting too close, too much nitrogen application, soil texture sticky weight will lead to disease. When the bacteria infected with flowers, will produce water-like brown lesions, humidity, the disease department of light gray black mold, that is, bacteria conidia and conidia.

Therefore, the ESB1 gene, which is found and cloned from Arabidopsis thaliana, can be introduced into chrysanthemum by Agrobacterium tumefaciens-mediated method, which provides a theoretical basis for studying the resistance mechanism of chrysanthemum resistance to saprophytic fungi.

1.5 The Purpose and Significance of the Study

Flower as a sunrise industry in Yunnan Province, is also one of the pillar industries of the province. Yunnan Province, the flower production since 1994 has been ranked first in the country, the current national more than 60% of the flowers from Yunnan, while cut chrysanthemum in the international and domestic markets are very popular, is one of the world's four major cut flowers. Accounting for 23% of the total amount of cut flowers in the flower industry occupies a very important position [8]. Strengthen the chrysanthemum breeding has a high economic and social benefits. With the development of flower industry, the demand for cut chrysanthemum is getting bigger and bigger. The methods of how to prevent and control chrysanthemums from various diseases, to increase production, are the important issues that the scientific research and production needs to be solved.

In order to further understand the function of the gene, 4x CaMV35S :: ESB1 cDNA (cauliflower mosaic virus) was constructed in the early stage of this study, and the promoter of ESB1 was driven by super promoter. The cDNA sequence of the gene was transferred

into the chrysanthemum by agrobacterium tumefaciens-mediated inflorescence infestation into the wild type Arabidopsis thaliana and leaf disc. The plant was screened out and the homozygous transformants were screened. Some plants were selected for ESB1 and the results showed that the overexpression of ESB1 gene had an effect on the resistance of Botrytis cinerea to the control of the plant.

Chapter 2 Arabidopsis thaliana ESB1 gene Transformation Agrobacterium tumefaciens

2.1 Materials and methods

2.1.1 Experimental materials

2.1.1.1 Strain

Agrobacterium tumefaciens strain GV3101, expression vector pCAMBIAN1300-221, which contains ESB1 gene for transformation, both of which are laboratory-conserved.

2.1.1.2 Reagents

Kanamycin (Gen), rifampicin (Rif), solution P1, P2, P3, yeast extract and peptone were produced by Sigma and purchased from Beijing Baird Biotechnology Company. Rinse solution WB (including anhydrous ethanol), elution buffer EB, other reagents for domestic analysis of pure.

2.1.1.3 Medium

YEB medium (for Agrobacterium tumefaciens)

YEB medium formulation (Agrobacterium cultured at 28 ° C)

Yeast extract 1.0 g / L

Peptone 5.0 g / L

Beef 5.0 g / L

Glucose 5.0 g / L

Magnesium sulfate 0.5 g / L

Adjust the pH to 7.0. To match the solid medium, add 7 ~ 8g agar.

2.1.1.4 Instruments and equipment

High speed refrigerated centrifuge: Eppendorf centrifuge 5804 R

Thermostat incubator: TS-100B Shaker incubator (Shanghai Star Experimental Instrument Manufacturing Co., Ltd.)

TS-111B Shaker incubator (Shanghai Star Experimental Instrument Manufacturing Co., Ltd.)

Refrigerator (Haier), ultra-low temperature refrigerator 368 liters (Czech Republic JOUAN SA)

Ice machine: SANYO ice maker SIM-F140AY65

Ultra pure water purification system: Milli-Q, Resistivity monitor (Biocel)

Pressure steam sterilizer: SYQ-DSX-280B (Shanghai Shen'an Medical Equipment Factory)

Clean bench: Air Tech (manufactured by Su-Group)

Electronic Balance: METTLER TOLEDO AL204 (METTLER TOLEDO Instruments Shanghai Co., Ltd.)

Magnetic stirrer, centrifugal adsorption column, 722 UV spectrophotometer.

2.1.2 Experimental methods

2.1.2.1 High-purity plasmid preparation kit (centrifugal column type)

(1) Take 1 to 5 mL of the overnight culture solution (Escherichia coli solution containing the plasmid), add to the centrifuge tube and centrifuge for 1 min using a conventional benchtop centrifuge at 12000 rpm.

(2) Add 250 μL of solution P1 (containing RNase A) to the centrifuge tube leaving the cell pellet and shake to complete suspension.

(3) to the centrifuge tube by adding 250 μL solution P2, gently flip up and down 6 to 10 times, so that the bacteria fully cracked (at this time the bacteria should be clear thick particles, the time should not exceed 5min).

(4) Add 400 μL of solution to the centrifuge tube P3, immediately and gently flip up and down 6 to 10 times, room temperature for 5min, room temperature 13000rpm, centrifugation 10min, carefully take the supernatant.

(5) The supernatant collected in the previous step was transferred to the adsorption column AC using a pipette, centrifuged at 13000 rpm for 1 min, and the filtrate was discarded.

(6) Add 500 μL of rinse solution WB (containing anhydrous ethanol) to the adsorption column AC and centrifuge at 13000 rpm for 30 to 60 s to discard the filtrate.

(7) Repeat step 6 once, 13000rpm centrifuge 30 ~ 60s, discard the filtrate.

(8) the adsorption column AC into the collection tube, 13000rpm, centrifugation 2min, then the adsorption column AC open, room temperature for 3 ~ 5min, remove the residual ethanol.

(9) Remove the adsorption column AC, into a clean centrifuge tube, in the middle of the adsorption membrane plus 60 ~ 100μL elution buffer EB (65 ~ 70 ℃ warm), room temperature for 1min. 13000 rpm, centrifuged 1 min, eluted DNA, stored at -20 ° C.

2.1.2.2 Preparation of Agrobacterium-competent cells

(1) The retained GV3101 original strain was screened on a YEB plate containing rifampicin [Rif (15 g / L)] and gentamicin [Gen (15 g / L)] at 28 ° C, 200 rpm oscillating culture for 1 to 2 days.

(2) A single colony was picked on a plate and inoculated into 3 to 5 mL of YEB liquid medium containing Rif and Gen, cultured at 28 ° C and shaken at 250 rpm overnight to shake the bacteria to orange.

(3) The shredded bacteria were added to the YEB liquid medium containing Rif and Gen in a ratio of 1: 100 (take 2000 μL of bacterial solution to 200 mL of YEB liquid medium) and incubated at 28 °C and 250 rpm concentration reached the bacteria was orange (OD600 = 0.6 when the conversion efficiency of the highest).

(4) To take fresh culture of fresh culture 1.5mL, 10000 r / min centrifugal 1min, set bacteria, discard the supernatant.

(5) The cell pellet was resuspended in 100 μL TSS buffer. Agrobacterium tumefaciens competent cells.

2.1.2.3 Recombinant Plasmid Transformation Agrobacterium

10 μL of purified recombinant plasmid DNA was added and 100 μL of competent cells were added. The cells were beaten, mixed with ice for 5 min, frozen into liquid nitrogen for 8 min (5 min), rapidly at 37 ° C (28 °C) for 5 min, and 800 μL of YEB liquid medium. This step was carried out on a clean bench) at 28 ° C and 250 rpm / min for 4 to 5 hours. The bacteria were transferred to the surface of the YEB solid medium containing kanamycin (Kan), coated with the entire plate, 28 ℃ shaking culture 1 to 2 days, until the growth of plaque.

Note: In the above experimental steps, liquid nitrogen was frozen for 8 min (5 min) and rapidly at 37 °C (28 °C). The two groups were controlled, i.e., a group of liquid nitrogen was frozen for 8 min and rapidly 37 °C water bath. One group is liquid nitrogen frozen 5min, rapid 28 ℃ water bath, other conditions remain unchanged.

2.2 Results and Analysis

Arabidopsis thaliana ESB1 gene was transformed into Agrobacterium tumefaciens by liquid nitrogen freezing and thawing. Single colonies were randomly selected and seeded on YEB solid medium. After incubation at 28 ℃ for 2 days, Agrobacterium tumefaciens was grown on YEB solid medium. Colony. (Figure 2.1, this figure shows the expansion of single colonies.)

Figure 2.1 Agrobacterium transformation

2.3 Discussion

Plant genetic engineering development has become one of the effective ways to improve plant genetic breeding and quality traits. Today, there are many ways to use plant transgenic technology, mainly: Agrobacterium-mediated method, chemical method, gene gun method, electric shock method, laser method, ultrasonic method and other methods. Among them, Agrobacterium tumefaciens-mediated method has been widely used and the most successful and effective method for its simple operation, low cost, high conversion efficiency, few copy number and long gene fragments. Agrobacterium tumefaciens is a natural plant gene transformation system. At present, 85% of the transgenic plants are obtained by Agrobacterium-mediated method. However, Agrobacterium-mediated transformation of plant genetic transformation is still a problem to be solved.

In this study, Arabidopsis thaliana ESB1 gene was introduced into Agrobacterium tumefaciens, and Agrobacterium tumefaciens colonies were successfully grown on the surface of YEB solid medium. Can be used for subsequent experimental studies.

Chapter 3 Identification of Agrobacterium tumefaciens by PCR

3.1 Materials and methods

3.1.1 Experimental materials

3.1.1.1 Materials

Agrobacterium tumefaciens as a template.

3.1.1.2 Instruments and equipment

General Thermal Cycling PCR: BIO-RAD Ordinary thermal Cycler 582BR

Bio-rad level electrophoresis equipment and Wealtec Corp.ELITE 300 PLUS power supply

Wealtec Corp. GES cell complete system

Gel imaging system: UVP Bioimaging system G6

Ice machine: SANYO ice maker SIM-F140AY65

High speed refrigerated centrifuge: Eppendorf centrifuge 5804 R

Ultra Low Temperature Refrigerator: Thermo scientific Reco VALUE series ULT1386-3-V

Pipettes and pipettes, silanized PCR tubules.

3.1.1.3 Reagent

(1) PCR reaction system required reagents:

10 × PCR reaction buffer: 500 mmol / L KCl, 100 mmol / L Tris-Cl, pH 9.0 at 25 ° C,

1.0% Triton X-100.

MgCl2: 25 mmol / L.

4 dNTP mixtures: 2.5 mmol / L each.

Taq DNA polymerase 5U / μL.

T4 DNA ligase and ligation buffer.

(2) Reagents required for electrophoresis:

Agarose, EDTA, EB, liquid nitrogen, glycerol

3.1.2 Experimental methods

The PCR primers were used as primers for the transformation of Agrobacterium tumefaciens using the Forward Primer (ESB1CDS200): 5'-CTGCAGATGC TTCGGAAGTCGGTTCTCG-3 'and Reverse Primer (ESB1CDS200): 5'-ACTAGTTGAT GGCATGTCCGGCTTAGCT-3' The reaction system is as follows (see Table 3.1) (unit: μl)

Table 3.1 PCR Reaction System

Components	The volume of each component（μL）
Master Mix	12.5
Forward Primer	1
Reverse Primer	1
Water	8.5
Template	2
Total Vol.	25

PCR reaction conditions:

Temperature (℃)	Duration	Process
94	3 min	Pre-denaturation
94	30 s	Denaturation
56	45 s	Annealing 35 cycle
72	1 min 30 s	Extension
72	5 min	Extension
4	∞	Save

3.2 Detection of PCR reaction products

3.2.1 Preparation of 1.0% agarose gel

Weigh 80g of agarose in 250mL Erlenmeyer flask, add 80mL EDTA, shake, placed in a microwave oven for 2min until the agarose melted, remove, then add 10μLEB, mix, that is 1.0% agarose gel.

3.2.2 Preparation of rubber sheet

Select the appropriate level of electrophoresis tank and install, check the power supply and positive and negative lines. Select the appropriate size of the point of the comb, the comb vertical frame in the electrophoresis tank fixed position. The agarose gel solution cooled to about 65 ° C was carefully poured into the electrophoresis tank and the glue was allowed to spread slowly until a homogeneous layer was formed and allowed to stand at room temperature until the gel was completely coagulated and the comb was pulled out gently.

3.2.3 Loading

Add 10 μL of the microtiter to separate the sample into the sample cell of the plate. Each sample should be replaced. A sample should be replaced to prevent contamination. Do not touch the gel surface around the sample hole. (Note: before loading the first note of the order of loading)

3.2.4 Electrophoresis

The sample was transferred from negative (black) to positive (red) and electrophoresed for 30 min.

3.2.5 observe the camera

Using DNA marker DL5000 as the molecular weight reference, the band was observed under UV gel imaging system and the size of the target fragment was determined.

3.2.6 Save

The correct bacteria into the total volume of 20% glycerol, liquid nitrogen quick-frozen, a 80 ℃ ultra-low temperature refrigerator to save spare.

3.3 Results and Analysis

The ESB1 gene was amplified by using the primers of the ESB1 gene as primers and the recombinant plasmid DNA as template. PCR amplification was carried out using the same primers to screen the Agrobacterium single colony as a template. The PCR amplification product was then loaded onto an agarose gel plate for electrophoretic analysis.

In the three electrophoresis results, 1 lane for the positive control, 2, 3 lanes need to identify the Agrobacterium samples. Three samples were amplified by a specific band of about 600 bp. The lanes of lane 2 were consistent with the size of the control bands, indicating that the ESB1 gene was successfully introduced into Agrobacterium and could be used for subsequent experimental studies (see Figure 3.1).

Figure 3.1 PCR analysis of ESB1 gene in Agrobacterium tumefaciens GV3101

M: DNA Maker5000 1: Positive Control 2-3: Sample

Chapter 4 Sensitivity of Callus to Hygromycin

In Agrobacterium-mediated genetic transformation, kanamycin and hygromycin are often used as resistance selection markers. The target gene ESB1 and the resistance screening marker hygromycin phosphotransferase gene Hyg were constructed on the plasmid vector used in this experiment. Thus, hygromycin is used as a selectable marker. If the transformed cells are resistant to hygromycin, they will survive on the selection medium containing hygromycin, while the non-transformed cells will die and be eliminated. In the process of transformation of the target gene, the hygienic sensitivity of the untransformed receptor material was tested to study the effect of hygromycin on the chrysanthemum of the recipient material, the appropriate concentration of hygromycin was determined, and the genetic transformation research is of great significance.

4.1 Materials and Methods

4.1.1 Experimental materials

4.1.1.1 Plant material

To 20 ~ 25d growth of healthy chrysanthemum sterile seedlings in the upper part of the full start, uniform and consistent leaves for the material.

4.1.1.2 Biochemical Reagents

(6-BA), Naphthalene Acetic Acid (NAA), sucrose, agar powder, hygromycin (Hyg), other inorganic and organic reagents for imported sigma or domestic analytical grade products.

4.1.1.3 Major instruments and equipment

Ultra pure water purification system: Milli-Q, Resistivity monitor (Biocel)

Pressure steam sterilizer: SYQ-DSX-280B (Shanghai Shen'an Medical Equipment Factory)

Clean bench: Air Tech (manufactured by Su-Group)

Thermostat incubator: TS-100B Shaker incubator (Shanghai Star Experimental Instrument Manufacturing Co., Ltd.)

TS-111B Shaker incubator (Shanghai Star Experimental Instrument Manufacturing Co., Ltd.)

Digital acidity meter: PHS-3C (Hangzhou Aoli Long Instrument Co., Ltd.)

HPG-400H artificial climate box, induction cooker, 1000mL Erlenmeyer flask, glass bottle, pipette, surgical scissors, tweezers.

4.1.1.4 Medium

The culture medium for hygromycin screening was SH basic medium (SH + 0.5 mg / L 6-BA + 1.0 mg / L NAA + 30 g / L sucrose + 6.3 g / L agar powder) 5.8.

4.1.2 Experimental methods

4.1.2.1 Preparation of media (see Table 4.1)

Table 4.1 SH Media Formulation

Ingredients	Amount	Remarks	Amount	Remarks
A lot of elements			500μL	
Trace elements			1000μL	
Organic material			Set to 1L	
Inositol				
Iron salt				
6-BA				
NAA				
Distilled water	100mL	100mL		
	10mL			
	10mL			
	10mL			
	10mL			

Adjust the pH to 5.8

Note: The composition of the media composition of the appendix

30 g of sucrose and 6.3 g of agar powder were added to the above-mentioned liquid, and the mixture was heated and boiled in an induction cooker. The flask was heated in a glass bottle and 80 mL of each flask was sealed with a sealing film. After sealing all the bottles and newspapers wrapped in tweezers, surgical scissors and other tools, together into the high-pressure steam sterilization pot, 121 ℃ sterilization 30min.

4.1.2.2 Preparation of SH medium with different concentrations of antibiotics Hyg

Will be just finished sterilization of the inverted SH medium bottles and tweezers, surgical scissors together with the removal, tweezers and surgical scissors into the high temperature oven spare, inverted SH medium bottle quickly placed on the clean bench, UV light irradiation 10 ~ 15min. After the UV irradiation, open the ion wind, with 75% alcohol wipe his hands, ignite the alcohol lamp. Then, immediately open a part of the glass bottle sealing film, quickly with the pipette to add a different preparation of different concentrations of hygromycin, and then cover the sealing film seal is good. Then shake the glass bottle, so that antibiotics fully and evenly contact the medium, and finally labelled with a marker on the glass wall date, antibiotic name and concentration.

4.1.2.3 Chrysanthemum sterile vaccine antibiotic Hyg susceptibility test

ESB1 gene overexpression vector is hygromycin resistance, so need to choose the first resistance to the concentration of antibiotics Hyg before transformation by using different concentration of chrysanthemum leaves explants on the regeneration of the former. Based on the study, Hyg was treated with 0, 15, 20, 30, 35, 40 mg / L 6 gradients. SH + 0.5mg / L 6-BA + 1.0mg / L NAA as the basic medium, in the clean bench, with tweezers take the expand fully and uniformly leaves from the upper part of 20 ~ 25d growth strong sterile growing chrysanthemum. The leaves were cut into 0.5 cm2 size and cut into 15 different explants of SH in different concentrations of antibiotic Hyg. The experiment was repeated three times. To observe the effect of Hyg on the regeneration of chrysanthemum leaves, to determine the sensitivity of chrysanthemum leaves to antibiotic Hyg, and to determine the Hyg critical tolerance of chrysanthemum genetic transformation according to the degree of inhibition of leaf regeneration by different concentrations of antibiotics Hyg.

4.2 Results and Analysis

In this experiment, 0, 15mg / L l, 20mg / L, 30mg / L, 35mg / L, 40mg / L6 concentration gradient, vaccination is as follows (see Table 4.2, Figure 4.1):

Table 4.2 Results of screening for Hyg concentrations of different antibiotics

Treatment	Hyg add concentration (mg / L)	Number of explants (tablets)	Number of deaths (tablets)	Growth condition
C0	0	15	0	Good
C1	15	15	8	Leaves turn yellow
C2	20	15	9	Leaves turn yellow

C3 30	15	9	Death
C4 35	15	11	Death
C5 40	15	13	Death

A.0 mg / L B.15mg / L C.20mg / L D.30 mg / L E.35 mg / L F.40mg / L

Figure 4.1 Growth of chrysanthemum explants under different concentrations of hygromycin

The results showed that the leaves of the chrysanthemum turned yellow from the 5th day, and only the leaves of C0 could grow well after 10 days. During the treatment of C1 ~ C2, the growth was inhibited obviously. Most of the leaves turn yellow, the treatment of C3 and after the chrysanthemum leaves were all dead. It can be seen that chrysanthemum leaves are more sensitive to Hyg and choose 30mg / LHyg as chrysanthemum transformants for screening pressure.

4.3 Discussion

Chrysanthemum genetic transformation is mainly mediated by Agrobacterium tumefaciens. In the subculture of transgenic processes, the corresponding antibiotics need to be added to ensure high conversion efficiency. The screening of antibiotics has two purposes: first is the antibiotic selection marker, whose role is to screen for transformants. If the concentration of antibiotics is too high, it will lead to the death of the transformant cells are poisoned, and thus inhibit the activity of adjacent cells, and ultimately difficult to obtain transgenic plants; if the concentration is too low, it is likely to lead to a large number of chimeric and false-positive plants, post - molecular work. The second purpose is to act as a bacteriostatic agent to prevent the propagation of Agrobacterium and cause the explants to die. It should be noted that the determination of the concentration of antimicrobial agents should not only be able to completely inhibit the growth of Agrobacterium, but also can not affect the normal growth of plant cells. In addition, different plants on the antibiotic sensitivity is also a big difference. For example: hygromycin for the genetic transformation of corn, the use of the concentration range is generally 5 ~ 20 mg / L, some up to 50 mg / L. Even the same plant, different varieties of hygromycin sensitivity is also different. Therefore, the correct use of antibiotics is one of the key factors affecting chrysanthemum transformation.

At present, Hyg is one of the most widely used screening agents in plant genetic transformation. In order to find a suitable antibiotic screening concentration, we designed 0, 15, 20, 30, 35 and 40 mg / L concentration gradient. In the study, Hyg was used at a concentration of 30 mg / L, and the transgenic chrysanthemum leaves were gradually killed under Hyg 30 mg / L. This also shows that the sensitivity of different materials to hygromycin is very different. Therefore, it is necessary to study the sensitivity of the receptor to antibiotics before genetic transformation, which can provide the theoretical basis for the screening concentration after transformation.

Conclusion

In this study, the study of Arabidopsis thaliana ESB1 gene transformation chrysanthemum and its transformation, as well as hygromycin resistance screening study, the following conclusions:

1. The Arabidopsis thaliana ESB1 gene was successfully introduced into Agrobacterium tumefaciens by freeze-thawing method.

Using the PCR technique to detect Agrobacterium tumefaciens, a specific band appeared at about 600bp, which was consistent with the expected results, indicating that the ESB1 gene was successfully introduced into Agrobacterium tumefaciens.

3. Screening of hygromycin resistance to untransformed chrysanthemums, the screening concentration of 30 mg / L Hyg as chrysanthemum transformants was determined.

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