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Optimization of the Genetic Transformation System of Lettuce

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Abstract

Lettuce (*Lactuca sativa* L.) is an annual vegetable crop of the family Asteraceae. It is the most consumed leaf vegetable in the world and is highly valued for its edible and medicinal value. Using transgenic technology, introducing functional genes into plants can shorten the breeding time and improve the quality of lettuce. However, in the genetic transformation of lettuce, the application of transgenic technology is limited by the low conversion rate. In this experiment, using 'S39' cotyledons as the test material, to establish a stable genetic transformation system. The results showed that when the leaf regeneration medium was 0.01 mg/L 6-BA and 0.4 mg/L NAA, the highest regeneration rate reached 97.9%, which was the best leaf regeneration hormone concentration. When the infection fluid was used in the OD_{600} value of 0.2, the infected leaves were in good condition and controllable Agrobacterium was the most suitable infection fluid concentration. When the infection time was 15 min, the infection effect was the best, the leaves grew well, and the resistant plants could grow. The screening in a medium containing 30 mg/L of Kana concentration and 250 mg/L of Cef was the suitable medium formulation. NAA 0.1 mg /L and 6-BA 0.2 mg /L were selected as the optimal concentrations in the rooting medium.

Keywords: genetic transformation, Agrobacterium-mediated, lettuce

1. Introduction

1.1 Problem

With the demand of plant breeding and the development of genetic transformation, in order to obtain new varieties of good quality and high yield and overcome the problems existing in traditional breeding, people have started the research of plant genetic transformation, and the continuous progress and development of transgenic technology have broken the restrictions of conventional breeding (Kang et al., 2022). Genetic transformation is becoming increasingly important as a tool for plant breeding, and it is widely used in plants, such as Chinese cabbage (*Brassica rapa*), Rape (*Brassica napus* L.) developed excellent varieties through genetic transformation, overcoming the limitations of conventional breeding (Wang et al., 2023; Sohn et al., 2022). As an important method for genetic improvement of lettuce, genetic engineering can overcome many problems existing in traditional breeding. With the rapid development of genetic transformation technology, plants acquire excellent traits that they do not possess by being introduced into foreign genes (Gerszberg et al., 2018).

1.2 Justification

Plant gene transformation is a powerful biotechnology that allows us to improve plant traits and yield by introducing new or improved genes (Toledo-Ortiz et al., 2023). Among many gene transformation methods, Agrobacterium-mediated transformation is one of the earliest and most effective ones (Puite et al., 1996). Agrobacterium is a bacterium that naturally lives in soil and is able to insert segments of its DNA, called T-DNA, into the DNA of plant cells to introduce new genes into the plant. The Agrobacterium-mediated genetic transformation system depends on the repair mechanism of plant cells after injury (Gelvin, 2017). When a plant is injured, cells at the wound site release a chemical that attracts Agrobacterium. Agrobacterium inserts the target gene into the DNA of the plant cell through its T-DNA. Using the Agrobacterium transformation method, scientists have successfully introduced target genes into a variety of important crops, such as corn, tomatoes, and lilies (Azizi-Dargahlou et al., 2023). These genetically modified plants play an important role in agricultural

production by increasing yields, increasing resistance to pests and diseases, and increasing adaptability to environmental stresses (Singh et al., 2016). However, obtaining these genetically modified plants is only part of the process. In order to confirm the successful insertion and expression of the target gene, scientists also need to perform a series of verification and screening work. This usually involves molecular biology techniques and specific screening media. In the screening medium, only those cell lines that successfully integrated the target genes were able to grow and form new plants (Antonio et al., 2004). In this way, scientists can obtain transgenic lines with specific good traits (Wang et al., 2023). In addition to the Agrobacterium transformation method, scientists have also developed other genetic transformation methods, such as gene guns and particle beam methods. Each approach has its own unique advantages and scope of application (Ramkumar et al., 2023). However, the Agrobacterium transformation method is still widely used in the field of plant gene transformation because of its high efficiency and low cost. Overall, plant gene transformation is a complex but very promising field. Not only does it improve our understanding of plant genetics, but it also provides powerful tools for improving crop traits and increasing yields.

The in vitro culture of different plants requires different hormone species, and their content and proportion are also different, so different treatments are needed to determine the appropriate proportion. Plants require the catalytic influence of various hormones during growth, the most important of which are phytoauxin and cytokinin, and their concentration is also one of the influencing factors of leaf regeneration (Xi et al., 2012). The proportion of hormones in the culture medium is necessary, because the leaves are long and the buds differentiate less when the cytokinin is excessive (Yang et al., 2009). When cell auxin is too much, the leaves are easy to turn yellow and not conducive to bud differentiation; improper ratio will also lead to regeneration defects such as vitrification and deformity (He et al., 2019). Therefore, it is particularly important to determine the hormone concentration for leaf regeneration during the early experimental period. In the tissue culture of alfalfa, adventitious buds of seedlings germinated on the initial medium were used as explants, and these explants were cultured on MS medium by adding different parts of hormones. The results showed that the appropriate initial medium MS +6-BA 0.5 mg L^{-1} + NAA 0.2 mg L^{-1} could make them multiply rapidly (Liu et al., 2011).

During Agrobacterium infection, the bacterial fluid concentration closely followed the transformation efficiency (Sandhya et al., 2022). In the process of potato Agrobacterium transformation, research has found that the transformation concentration of Agrobacterium had a certain range, and the low concentration of bacterial liquid was not conducive to the proliferation of Agrobacterium; with the high concentration of bacterial liquid, the excessive propagation of Agrobacterium caused harm to the growth of explants, thus reducing the transformation efficiency (Vanet al., 2019). During the process of Agrobacterium infection, the sugar and phenolic substances produced by the injured tissue have a strong attraction to Agrobacterium, and these substances are concentrated in the wound site of the plant, providing an ideal living environment for Agrobacterium (Gelvin et al., 2006). Dicotyledonous plants were found to be more susceptible to agrobacterium infection than monocotyledonous plants (Gao et al., 2005). This is mainly because dicotyledonous plants are prone to producing sugars and phenols, as well as substances known as phenolic inducers, which can stimulate agrobacterium activity. In proper concentration, an acetyl syringe can significantly promote conversion efficiency. In addition, the pH value of the solution also has a greater impact on the activation, when the pH value is 5.2~6.8, the induction rate is the highest. In practice, scientists typically control for these environmental factors to optimize the process by which Agrobacterium transforms plants. For example, they will adjust the pH value of the solution, the concentration of acetyl syringe, and other parameters to ensure that Agrobacterium is in the best living environment to maximize the conversion efficiency (Manfroi et al., 2015). However, it should be noted that these conditions do not apply to all plants, and different plants have different sensitivities to Agrobacterium infection, so specific adjustments need to be made according to the characteristics of each plant.

Co-culture is the time of Agrobacterium also explants in the absence of antibiotics, which allows Agrobacterium to fully contact the explants and enter the cell for transformation. Previous studies proved that the most appropriate co-culture time was from 2 to 3 day (Rahman et al., 2023). If the culture time is short, the chance of Agrobacterium entering the cells is small, and it is easy to be screened out. For the long culture time, the excessive growth of Agrobacterium on the leaf disc surface will cause sterile leaves and culture media, antibiotics cannot effectively inhibit Agrobacterium and the exant cannot grow (Rojo et al., 2021).

1.3 Background

Transgenic technology provides a favorable way to study lettuce leaves in vitro under the premise of establishing an efficient leaf regeneration system and a stable genetic transformation system and studies some factors affecting the genetic transformation process, thus laying a good foundation for the successful construction of its genetic transformation system. Transgenic technology is mediated by Agrobacterium tumefaciens to improve the

genetic characteristics of plants by transforming foreign genes. In order to improve the conversion efficiency, an efficient leaf regeneration system and a stable genetic transformation system were established, which would help to better control the transgenic process, so as to study the genetic transformation of lettuce leaves in vitro. In order to obtain efficient and stable genetic transformation, these factors need to be further studied and discussed. In summary, transgenic technology provides a favorable way to study lettuce leaves in vitro, and some factors affecting the genetic transformation process are studied. The establishment of an efficient leaf regeneration system and stable genetic transformation system can provide a certain theoretical and technical basis for the breeding of excellent new varieties of lettuce.

2. Method

2.1 Materials Treatment

The plant material in the experiment was 'S39' raw vegetable seedling, which was kept in the group culture Center of Beijing University of Agriculture. In order to ensure the consistency of genetic basis, S39 lettuce selected leaves with good growth, no disease deformity, consistent growth period and size for infection. Temperature 23±2 °C in long day at 16 h/8 h with 60% relative humidity.

2.2 Vectors and Bacterial Strains

Reagent materials can be used in a variety of biotechnology experiments, including gene cloning, transformation, and expression studies. Including *E. coli*: This is a common bacterium that is widely used in gene cloning and expression studies. Escherichia coli has the advantages of easy culture, rapid propagation, and high safety, so it is a commonly used genetic engineering host bacteria in laboratories. Trans5α chemically active cell: This is a mutant strain of *E. coli* that has sensitivity to certain chemicals (such as IPTG) and resistance to specific antibiotics (such as kanamycin). Such cells are often used in gene expression experiments as host bacteria to monitor and screen for gene expression products. Asy-Blunt Simple Cloning Kit: This is an efficient and easy-to-use gene cloning kit for fast and accurate gene cloning. The kit adopts Blunt terminal digestion mode, which avoids the problem of low flat terminal conversion efficiency when the target gene is connected to the carrier and also simplifies the operation process. Agrobacterium Agrobacterium: This is a soil bacterium that performs transformation and transduction. LBA4404 is a strain of Agrobacterium Tumefaciens, which has high transformation ability and is often used in plant genetic engineering to introduce target genes into plant cells. Overexpressed vector pRI101-eGFP vector: This is a commonly used eukaryotic expression vector, which can bind to the target gene and introduce it into the host cell to achieve efficient expression of the target gene. Together, these materials and reagents constitute an important toolbox for laboratory experiments in genetic engineering and molecular biology, providing powerful means for researchers in basic and applied research.

2.3 Culture of Explants

During aseptic operation, ensure the absolute cleanliness of the operating environment, including the laboratory bench, laboratory equipment, and hands. All blades and other tools used to cut blades need to be high-temperature sterilized before each use to prevent contamination. When removing the leaves, it is necessary to select the lettuce cotyledon that is growing robustly and free of pests and diseases. These leaves are usually bright green in color, free of disease spots on the surface, and in good condition. After removing the leaves, remove the leaves from the mother and make 3 transverse cuts with a scalpel perpendicular to the main vein, immediately placing them in a new, clean medium to avoid dehydration or contamination of the leaves caused by prolonged exposure to air. During the culture process, if the callus is found to stop growing or turn black and die, it may be due to too long culture time on the original medium, resulting in insufficient nutrition or the accumulation of toxic metabolites. At this time, a new medium must be replaced and the leaves transferred to the new medium to ensure their normal growth. In addition, in order to maintain the long-term growth and proliferation of the leaves, it is necessary to change the medium at about 4 weeks of each subculture and re-inoculate the leaves on a fresh medium. This avoids growth stagnation or death due to the aging of the medium or the accumulation of toxic metabolites. At the same time, the new medium can also provide enough nutrients to support the continuous growth and proliferation of leaves. In general, through aseptic operation, regular change of medium, environmental control, and other measures, lettuce leaves can be successfully cultured in vitro to achieve long-term growth and proliferation. This is a delicate and rigorous process that requires researchers to have a wealth of experience and strict operating procedures to achieve successful experimental results.

2.4 A Stable Transformation Step

Delayed culture: in vitro leaves were transferred to a delayed culture with only 250 mg/L Cef and incubated in the dark for 3 d to observe the tolerance of leaves to Agrobacterium. Screening: in vitro leaves were transferred into screening medium containing 250 mg/L Cef and 25-35 mg/L Kan for 2 weeks in the dark to observe leaf budding and incubated in light for 2 weeks later. Selection: After light culture, the color of the regenerated bud turned green, cut from the bud to 1-2 cm, and transferred into the secondary medium (containing 250 mg/L Cef and 30 mg/L Kan) to observe the growth of the regenerated bud. No bleaching after 4 generations of screening and stable growth can be verified. Rooting culture: the tissue culture seedlings of about 5 cm without disease that grew normally for 30 d after screening were selected and put in the rooting medium.

2.5 Activation Steps of Agrobacterium Solution

Activation: The preserved agrobacterium solution was removed from the -80 °C refrigerator and placed on the ice immediately. After melting on the ice, appropriate amount of the bacterial solution was dipped in the inoculating ring to mark the resistant LB medium. After the plate was slightly dried, it was sealed with a sealing film and placed in a constant temperature incubator at 28 °C for culture.

Bacteria picking: Select a good growing and pollution-free agrobacterium plate, select a single colony from the plate with a sterilized gun head, and place it in a 1.5 ml centrifuge tube containing resistant LB liquid medium, and culture it in a shaking table at 28 °C for 200 r/min. The bacteria concentration was about 1.0, and the bacteria solution was changed into LB medium containing corresponding antibiotics, and the culture was shaken to the logarithmic growth stage. The bacterial solution was centrifuged on the centrifuge at 5000 r/min for 10 min, and the bacterial bodies were collected, and the bacterial cells were suspended with 1/2 ms liquid.

2.5 Agrobacterium Transformation of Expression Vector

First, 2-5 μL expression vector plasmid DNA was transformed into 50-100 μL Agrobacterium receptive cells, resting on ice for 30 min, then placed in liquid nitrogen for 2 min, and then in 37 °C hot water bath for 5 min, immediately placed on ice for 5 min. Add 400 uL of non-resistant liquid LB medium into the bacterial solution and shake it in a shaker at 28 °C for 3 hour under dark conditions. (Therefore, only in this step is the liquid LB non-resistant, and in the following steps, the LB is either resistant liquid LB or LB solid medium.) The shaken bacterial solution was absorbed 20-50 μL with the tip of a gun, coated on LB solid medium with corresponding antibiotics, and placed in inverted culture at 28 °C in the dark. The frozen Agrobacterium with transformed expression vector was removed from the refrigerator 4 day in advance, striated and activated on LB solid medium with corresponding antibiotics, and cultured inversely under dark conditions at 28 °C. The whole process was generally 2 day. Small shaking: After 2 day, colonies grew in the medium, single colonies were picked out, placed in 700 μL liquid LB with corresponding antibiotics at 28 °C, shaken in the dark for 3 hour, and then identified by transformation PCR. After identification, the correct bacterial solution continues to shake until the bacterial solution is completely orange. Big shake: Pour all the bacteria liquid that has been shaken into orange yellow into 500 ml conical bottle, add liquid LB with corresponding antibiotics at a ratio of 1:100, and shake at 28 °C in the dark for about 6 hour, until the bacteria liquid has completely turned orange. The orange-yellow bacterial solution was centrifuged at 8000 rpm for 5 min to collect the bacterial solution. The 1/2 ms culture solution was used AS the regulator to add 200 μM of AS and MES and $MgCl_2$ with a final concentration of 10 μl, and the concentration of the bacterial solution was adjusted to OD_{600} of about 0.1-0.3. The prepared bacterial solution was used to infect the explants, and the activity of the bacteria was the best within 1 hour.

3. Results

3.1 Disinfection of Lettuce Seeds

In order to improve the efficiency of genetic transformation, normal and full lettuce seeds were selected in the early stage, and the contamination rates of 4 different disinfection concentrations were calculated, including 5% NaClO solution for 5 min, 10 min, and 15 min. The sterile rates were 52.2%, 97%, and 99%, respectively (Table 1). The results showed that with the increase of NaClO solution concentration, the contamination rate decreased gradually, the disinfection time increased, and the sterility rate increased correspondingly. The seeds grew normally after 10 min of disinfection, and the contamination rate was less (Figures 1 and 2). However, when the disinfection time is 15 min, although the contamination rate is very low, some seeds are not easy to germinate. This may be because the disinfection time is too long, which has damaged the internal tissue of the seeds, resulting in poor differentiation. The structure and function of lettuce seeds are very complex, and the disinfection process not only needs to kill the bacteria and viruses on the surface but also needs to reduce the damage to the seeds as much

as possible under the premise of ensuring the health of the seeds. Disinfectants such as NaClO can kill germs and viruses, but they can also have a negative effect on the seeds. This is because NaClO, as an oxidizing agent, can damage the cell membrane and cell wall of the seed, which in turn affects the germination and growth of the seed. In addition, too long disinfection time may also destroy the internal structure and function of the seeds, leading to poor differentiation. In this case, although the surface of the seed appears normal, the inside may have been damaged and not germinated properly. Therefore, when selecting the disinfection time and concentration, it is necessary to consider the disinfection effect and the damage degree to the seeds in order to find the best balance point. In short, disinfection is an important step in the process of improving the efficiency of genetic transformation. The correct choice of disinfectant and disinfection time can minimize the contamination of germs and viruses while ensuring the healthy growth of seeds.

Table 1. Effects of different disinfection concentrations on lettuce seeds

Time	Sterility rate %	Survival rate %	Seed status
5 min	54.2 b	67.5 b	The seeds germinate normally, but are heavily contaminated
10 min	97.5 a	99.8 a	Seeds germinate normally, no pollution
15 min	99.1 a	95.2 a	Individual seed color light color, no germination, no pollution

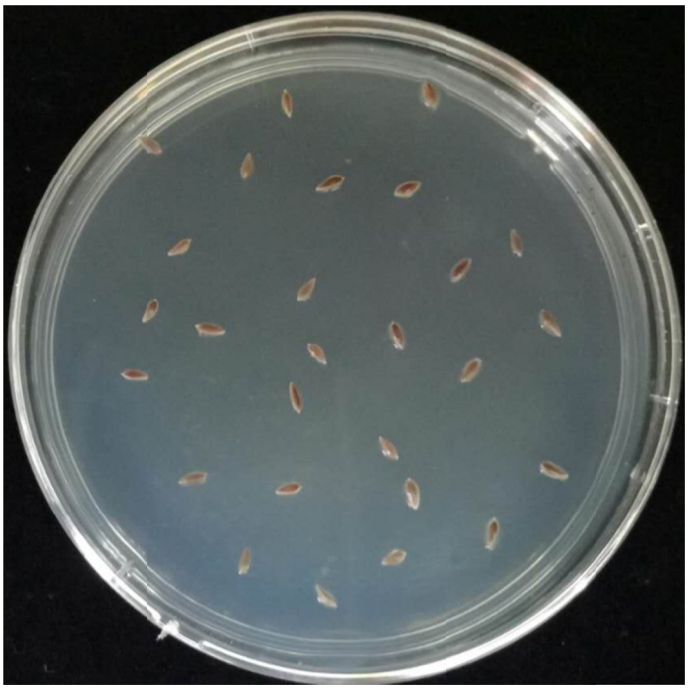

Figure 1. Sterile seeds without contamination

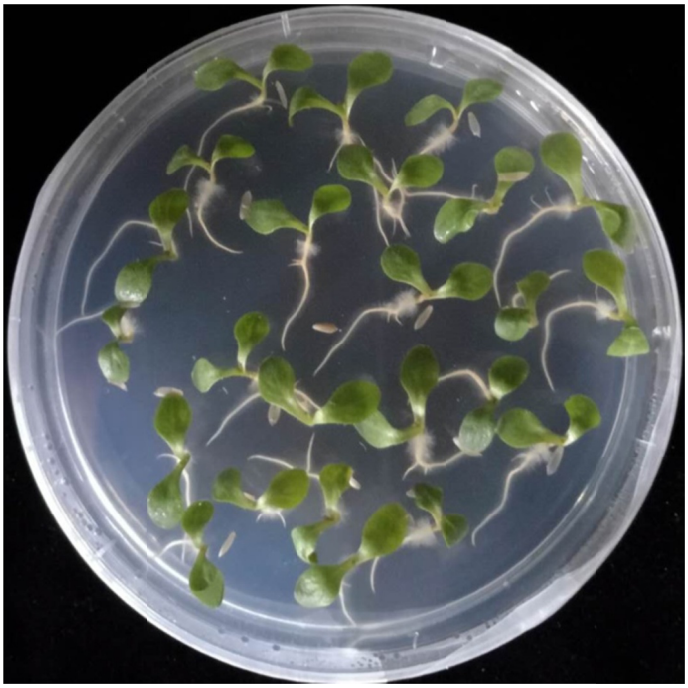

Figure 2. The seeds germinate normally after disinfection

3.2 Screening of the Regenerated Culture Media

In the process of plant regeneration, the concentration and proportion of hormones have a crucial effect on the regeneration efficiency. In this study, the concentration of 6-benzyladenine (6-BA) and naphthalene acetic acid (NAA) was systematically adjusted to find the best combination of hormones to improve the regeneration efficiency of lettuce cotyledon. Nine concentration gradients were designed. The concentrations of 6-BA ranged from 0.05 to 0.15 mg L^{-1}, and the concentrations of NAA ranged from 0.04 to 0.06 mg L^{-1}, with three replicates set for each concentration combination. The experimental results showed that when the concentration of 6-BA was 0.1 mg L^{-1} and the concentration of NAA was 0.5 mg L^{-1}, the regeneration rate of adventitious buds reached 97.9%, which was the best leaf regeneration hormone concentration (Table 2). This result provides a basis for further improving the efficiency of genetic transformation. When the concentration of 6-BA was maintained at 0.1 mg L^{-1}, and the concentration of NAA was 0.4 mg L^{-1} and 0.5 mg L^{-1}, the regeneration rate of advadvent buds also reached more than 80%. This suggests that moderate changes in NAA concentration do not have much effect on bud regeneration. However, when the concentration of 6-BA was 0.05 mg L^{-1} and 0.15 mg L^{-1}, respectively, the regeneration rate of advisable buds was lower than 50%. This indicates that the concentration of 6-BA plays a leading role in leaf regeneration. In addition, the regeneration rate performed best when the concentration of 6-BA was in the range of 0.1 mg L^{-1}. This suggests that either too high or too low 6-BA concentration has a negative effect on leaf regeneration. Based on these experimental results, 6-BA of 0.1 mg L^{-1} and NAA of 0.5 mg L^{-1} were selected as leaf regeneration culture hormones in subsequent experiments (Figures 3 and 4). These optimized hormone concentrations can provide a better basis for further genetic transformation experiments, thereby improving the efficiency and stability of genetic transformation. The study also highlights the importance of precise control of hormone concentrations for plant regeneration. Different plant species and different physiological states may have different sensitivity to hormones. Therefore, it is of great significance to find out the optimal combination of hormone concentration through experiments for subsequent experiments.

Table 2. Effect of hormone concentration on leaf regeneration

6-BA Concentration (mg/L)	NAA concentration (mg/L)	Number of explants cultured	The rate of bud regeneration%
0.05	0.04	30	21.3 c
0.1	0.04	30	86.2 b
0.15	0.04	30	44.5 c
0.05	0.05	30	28.9 c
0.1	0.05	30	97.9 a
0.15	0.05	30	45.2 c
0.05	0.06	30	38c.7
0.1	0.06	30	81 b.3
0.15	0.06	30	30.3 c

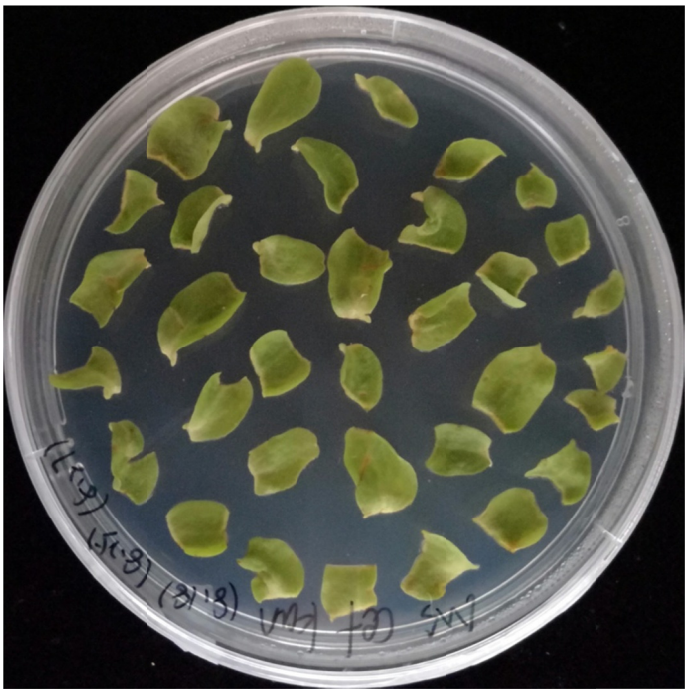

Figure 3. Lettuce leaf regeneration rate is high

Figure 4. Lettuce leaf regeneration rate is high

3.3 Agrobacterium Coculture

In the absence of antibiotics, the results of co-culture of Agrobacterium with leaves showed that on day 1 of co-culture, a low-density Agrobacterium suspension (OD_{600} = 0.2) was introduced into the leaf environment. This concentration of agrobacterium had no significant negative effects on the leaves, and the leaves grew normally without any signs of disease or infection. However, at this stage, the conversion efficiency is relatively low due to the small number of agrobacterium (Table 3). On the second day of co-culture, Agrobacterium began to make deeper contact with the leaves, and the transformation process began to accelerate. At this stage, although the growth condition of the leaves is still normal, some changes in the color and texture of the leaves can be observed. These changes are due to the fact that Agrobacterium began to produce a piece of DNA called T-DNA, which is able to transfer certain genes from the bacteria into plant cells. This transfer process is called transformation. On day 3, the conversion efficiency reached its maximum. This is because during the first two days of co-culture, T-DNA successfully introduced some specific genes into the leaf cells. The introduction of these genes promoted the growth and division of leaf cells, thus improving the conversion efficiency. At the same time, the normal growth of the leaves at this stage also reflects that these imported genes have no obvious side effects on the leaves. However, when the co-culture period lasted until day 4, or even longer, the situation began to deteriorate. As the number of agrobacterium continues to increase in the absence of antibiotics, a large number of leaves are infected with agrobacterium. These infected leaves do not grow normally, and some even have severe lesions and necrosis. Moreover, even if antibiotics were added to subsequent cultures to inhibit the growth of Agrobacterium, the leaves could not return to their normal growth state. Therefore, when the co-culture time is 3 days, the best conversion efficiency is the highest. In addition, this study also provides a basis for further exploration of Agrobacterium transformation mechanism.

Table 3. Agrobacterium coculture state

OD_{600}	Time of coculture/day	Leaf state
0.2	1	The conversion efficiency is relatively low due to the small number of agrobacterium
0.2	2	The growth condition of the leaves is still normal, some changes in the color and texture of the leaves can be observed
0.2	3	Leaf growth is good, agrobacterium contamination is less, the conversion rate is high
0.2	4	Agrobacterium continues to increase in the absence of antibiotics, a large number of leaves are infected with agrobacterium

3.3 Effects of the Infection Concentration and Time on the Leaves

Because the concentration of the bacteria fluid and the time of infection are important influencing factors in the infection process. To ensure the transformation effect and the stability of the test conditions, the infection concentration and the infection time were screened. The gradient test for the infection concentration of the leaves, OD_{600} values were 0.1-0.3, the infection time was 10-20 min, and 3 concentration gradients were set to total 9 test groups. Test results show that when the OD_{600} value was 0.2 and the infection time was 15 min, the infected leaves were in good condition, controllable Agrobacterium, good leaf growth status and high rate of resistant plants, which was the best choice for infection concentration. And when OD_{600} at the value of 0.4, Agrobacterium is easy to be screened during leaf growth, resulting in very low transformation efficiency; when OD_{600} at the value of 0.6, the concentration of the infected bacterial solution was too high, which was prone to agrobacterium contamination, leading to the loss of activity of the leaves. Therefore, the highest at the OD_{600} value of 0.5. Therefore, an OD_{600} value of 0.6 was selected as the infection concentration in the subsequent test. However, when the infection time is 10 min, the infection time is too short, although there is no Agrobacterium contamination, Agrobacterium is not easy to enter cells, resulting in high false positives and low conversion rate; when the infection time is 20 min, the infection time is too long, leading to the blackening of large areas of Agrobacterium contaminated leaves, without the ability to differentiate buds, and reducing the transformation efficiency. Therefore, the infection concentration of 0.2 was selected with an infection time of 10 min as the infection condition for the leaves (Table 4).

Table 4. Effect of infection fluid concentration and time on leaves

OD_{600}	Time of infection/min	Leaf state
0.1	10	The leaves were in good growth condition without Agrobacterium contamination
0.1	15	The leaves are in good growth condition
0.1	20	Leaf growth was good with a small amount of Agrobacterium contamination
0.2	10	The leaves were in good growth condition without Agrobacterium contamination
0.2	15	Leaf growth was good with a small amount of Agrobacterium contamination
0.2	20	The leaves were yellow and polluted by agrobacterium
0.3	10	Leaves withered yellow
0.3	15	Large-scale contamination of Agrobacterium sp
0.3	20	Black leaves and extensive contamination of Agrobacterium sp

3.4 Kana Concentration

Given the obvious phenotype of Kan antibiotic screening plants, it is widely used to screen plant resistant plants, but Kan tolerance varies greatly among different tissues in different environments. According to the resistance of the vector, Kan was selected for the screening of callus genetic transformation in this experiment. Kan concentration gradients were set with concentrations between 25 and 35 mg/L, and WT and transgenic plants were placed in MS medium containing different Kan concentrations with six concentration gradients for a total of six test groups (Figures 5 and 6). The results showed that the Kan concentration was 30 mg/L, and the leaves remained pale green, which was the most suitable Kan concentration for screening. At the Kan concentration of 25 mg/L, fast growth, but low conversion and more false positive plants, because the Kan concentration was too low. When the Kan concentration was 35 mg/L, the growth was slow and the leaves underwent massive etiolation because the Kan concentration is too high to exceed the tolerance of the plant, which in turn leads to etiolation. However, after adding Kan to WT plants, the plants grew slowly, and when the Kan concentration

was above 25 mg/L, the plant bleaching appeared and inhibited the growth of WT plants. This indicated that the Kan concentration was most appropriate at 30 mg/L, and the resistant plants were selected while excluding false positive plants (Table 5).

Table 5. Effect of Kana concentration on plant screening

Kan (mg/L)	WT plant status	Transgenic plant status
25	Do not grow, a large number of plant leaves turn white	Grow faster
30	No growth, a large number of leaves and roots are white	Normal growth
35	No growth, plant leaves and roots white off	Slow growth, a lot of leaf yellowing

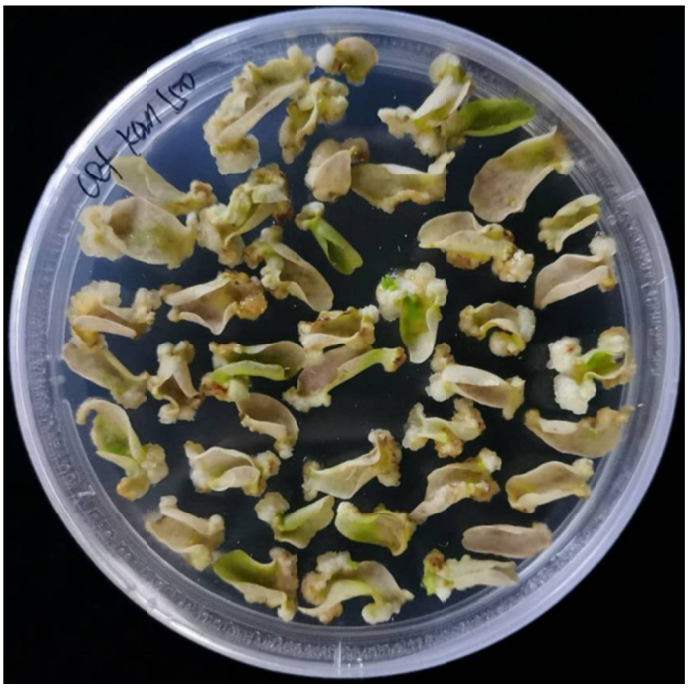

Figure 5. Kana screening

Figure 6. Kana screening

3.5 Rooting of Transgenic Plants

Since rooting is a characteristic of complete plant development, tissue culture seedlings can no longer rely on the medium after rooting, and the roots absorb external nutrients to supply the plants. Meanwhile, the quality of the roots also affects the survival rate of transplanting. To ensure the root of the plant and the experimental conditions are good, the secondary medium is screened first. The gradient test of hormone concentration in the medium was carried out, and six experimental groups were set up, including NAA alone and NAA combined with 6-BA, NAA 0.05-0.15 mg L^{-1}, and 6-BA 0.2 mg L^{-1}. The results showed that when NAA 0.1 mg L^{-1} was added alone, the roots were robust, the taproots were numerous, the plants were robust, and the rooting rate reached 95.9%, which was a suitable medium for rooting (Table 6). When mixed with NAA 0.1 mg L^{-1} and 6-BA 0.2 mg L^{-1}, the roots were short, fibrous roots were abundant and the plants were small. When only a small amount of NAA was added to the medium, for example, the combination of NAA 0.05 mg L^{-1} and 6-BA 0.2 mg L^{-1} could not promote rooting. When only a large amount of NAA was added to the medium, the combination of NAA 0.15 mg L^{-1} and NAA 0.2 mg L^{-1} could promote rooting, but the rooting rate was 48.9% and 46.2%, respectively. It can be seen that NAA is the main factor of rooting, and the addition of 6-BA can make the root system strong and is the auxiliary factor of rooting. However, when the concentration of NAA is too high, it causes yellow leaves, which is not conducive to plant growth. Therefore, NAA 0.1 mg L^{-1} and 6-BA 0.2 mg L^{-1} were selected as the medium hormone concentrations of transgenic plants in subsequent experiments (Figures 7 and 8).

Table 6. Rooting of transformed screening

Hormone concentration	Rooting rate/%	Rooting situation
NAA 0.05 mg/L	0.00	The plant has no roots and does not grow
NAA 0.1 mg/L	95.9 a	The roots are long, short and strong
NAA 0.15 mg/L	48.9 b	Low root system
NAA 0.05 mg/L +6-BA 0.2 mg/L	0.00	The plant has no roots and grows slowly
NAA 0.1 mg/L +6-BA 0.2 mg/L	88.3 a	Fewer taproots, more fibrous roots, small plants
NAA 0.15 mg/L +6-BA 0.2 mg/L	46.2 b	Taproot less, leaves yellow

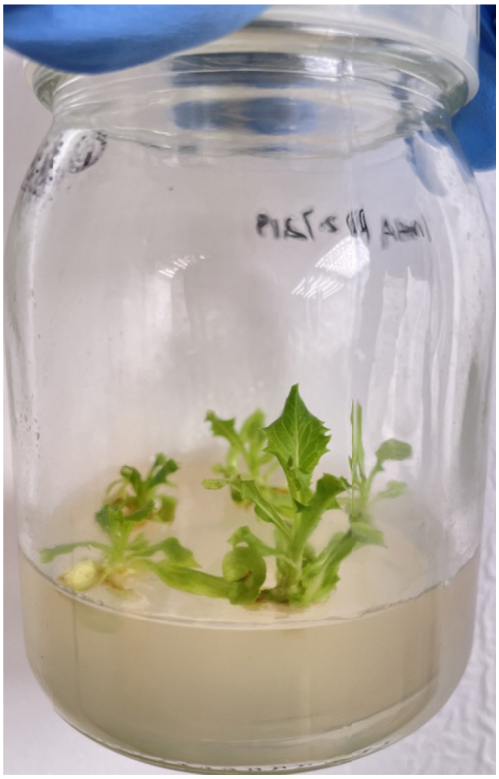

Figure 7. Normal subgeneration of buds

Figure 8. Lettuce plant root

3.6 Plants Growth Acclimation

After the plants take root in the medium, they need to be acclimated before they can be transplanted to natural conditions for growth. To improve the transplanting survival rate of tissue culture seedlings, acclimation is necessary. First, we should select robust rooting tissue seedlings; The tissue seedlings were transferred to a greenhouse, where they were domesticated. In the process of domestication, it is necessary to control the temperature and humidity in the greenhouse and give appropriate lighting to make the tissue culture seedlings gradually adapt to the external environment. After a certain period of domestication, the utilization of photosynthesis in the leaves of tissue culture seedlings was gradually improved, at the same time, the resistance was also gradually improved, and the ability to adapt to changes in the external environment was gradually enhanced. At this time, the tissue culture bottle cap can be opened to let the tissue culture seedlings contact the external environment to further improve the resistance to the environment. After a period of contact, tissue culture seedlings can be transplanted into the seedling substrate. After fully watering, the tissue culture seedlings are placed in the greenhouse, and continue to control the temperature and humidity. When the plants are about 10 cm tall, they can be transplanted into natural soil for cultivation (Figure 9). When cultivated in natural soil, regular watering, fertilization, and management are required to ensure normal growth and development of the plant.

Table 9. Lettuce plant root

4. Discussion

The establishment of an efficient transformation system is the key step to realizing vegetable transgenic, and also an important way to improve the efficiency of transgenic. Through Agrobacterium infection, stable transgenic strains can be obtained, which provides an important basis for follow-up research (De et al., 2021). There are many effects of genetic transformation, among which plant growth regulators are one of the important factors affecting leaf regeneration in vitro (Xiang et al., 2022). Plant growth regulators can affect plant cell division and differentiation, and then affect the process of leaf regeneration in vitro. In the process of transgene, the type and concentration of plant growth regulators need to be strictly controlled to obtain the best conversion effect. Cytokinin is a necessary substance for leaf differentiation to produce Adventist buds. Its main function is to cause cell division, induce bud formation, and promote bud growth. Phytocytokinin 6-BA is an important hormone that regulates cell growth during plant growth and development (Qi et al., 2023). NAA is a broad-spectrum plant growth regulator that promotes cell division. The combination of the two can induce adventitious buds at appropriate concentrations (Kazeroonian et al., 2018). The combination of 6-BA and NAA was established, and the experimental results showed that auxin binding could achieve a higher regeneration rate. In our test results, it was found that 6-BA of 0.1 mg L^{-1} and NAA of 0.5 mg L^{-1} were the optimal hormones for leaf regeneration culture (Figure 2). In short, the establishment of an efficient transformation system is one of the important steps to achieve vegetable transgenic. In the conversion process, it is necessary to strictly control various factors in order to obtain the best conversion effect.

During the infection process, the appropriate amount of acetylsyronone can promote the infection effect (Gasparis et al., 2017). In the configuration of Agrobacterium infection solution, 200 μM/L acetylsyringone significantly promoted the transformation of ‘Wang Lin’ callus, and the induction rate of resistant callus was significantly increased. In the green sleeve apple study, the amount of acetylsyringone during infection was 100 μM/L (Chauhan et al., 2021). If the leaf culture time is short, the cell differentiation ability is weak, and the resistance is poor after Agrobacterium infection is infected, leading to the death of Agrobacterium contamination; and the leaf preculture time is long, the cell aging, not easy to grow new callus, resulting to the decline of differentiation ability. Therefore, in the experiment, the leaf culture time should be selected, the time should not

be too long or too short. In the study of cotton embryo callus, 1-2 was the best culture time, and the infection effect of Agrobacterium was the best (Zhao et al., 2022).

OD of Agrobacterium fluid concentration during transformation of Agrobacterium600Values and infection time affect transformation efficiency. The concentration of the bacterial liquid is over or below the preset value, and the infection time is too long or too short will have a great impact on the transformation efficiency (Luo et al., 2023). If the concentration of bacterial fluid exceeds the preset value or the infection time is too long, it will poison the plant and even cause the plant death; if the concentration of bacterial fluid is lower than the preset value or the infection time is too short, the Agrobacterium only stays on the surface of the plant and cannot infect the cells, and causes the contamination of Agrobacterium to the plant, and the conversion efficiency will be reduced. The suitable OD_{600} value and infection time varied for different plant materials (Duan et al., 2022). This study found that, when the OD_{600} value was 0.2, and the infection time was 15 min. And when OD_{600} at 0.5, no matter the infection time was 10 min or 20 min, resistant plants could not be obtained. Moreover, due to the high concentration of bacterial fluid, Cef could not effectively inhibit the growth of Agrobacterium in the sterilization stage, which makes the growth of Agrobacterium in the later stage vigorous, and the plants were poisoned and did not have the differentiation ability, thus greatly inhibiting the transformation efficiency.

Coculture is a key step in plant transformation, the purpose of which is to allow agrobacterium and explants to co-culture for some time without antibiotics to promote agrobacterium transformation into plant cells (Zhang et al., 2019). The time of coculture needs to be precisely controlled, because too short a time may prevent agrobacterium from fully reaching and entering the explants, resulting in reduced conversion efficiency; However, too long a time may lead to excessive growth of agrobacterium, covering the surface of the explants, and adversely affecting subsequent growth and screening (Song et al., 2020). The study results of coculture time are mainly based on experimental data and experience. Studies have found that the most appropriate co-culture time is usually 2 to 3 days. During this period, Agrobacterium can fully contact and enter the explants to achieve effective transformation. If the coculture time is too short, such as 1 day or less, Agrobacterium may not be able to make adequate contact with the explants, resulting in reduced conversion efficiency (Fu et al., 2023). Therefore, by controlling the co-culture time, the transformation efficiency of Agrobacterium can be improved, and the pollution and screening pressure can be reduced, to achieve efficient plant gene transformation.

Lettuce rooting is a more important step, affecting the survival rate of the plant. When NAA was added 0.2 mg/L alone, the roots were robust, the taproots were numerous, the plants were robust, and the rooting rates reached 90% and 98.3%, respectively, which was a suitable medium for rooting. The ratio of auxin and cytokinin can affect the rooting of plants, and the survival rate of transplanting can be improved by strong root system. Since plants need to be transplanted under natural conditions for growth after taking root in the medium, then physiological experiments can be carried out in the later stage. In order to improve the survival rate of transplanting tissue culture seedlings, domestication is necessary. Firstly, healthy rooting tissue seedlings were selected and transplanted into the greenhouse to improve the utilization of photosynthesis by leaves after domestication. After 3 days, the tissue culture bottle cap is opened, so that the tissue culture seedlings contact the external environment, improve the resistance to the environment; 2 days later, the tissue culture seedlings were transplanted into the seedling substrate, watered fully, placed in the greenhouse, and then transplanted into natural soil for culture when the plant grew to about 50 cm.

5. Conclusion

In this study, we optimized the stable genetic transformation system of lettuce, studied the factors affecting the genetic transformation of lettuce, and obtained transgenic plants, and the conversion rate increased by 2%. At present, the most common method is the transgenic lines, but the transformation efficiency is improved only by constructing high-quality system, and the transformation efficiency is improved by constructing safe, new and efficient expression vector. In general, the development of new methods suitable for lettuce and the efficiency of agrobacterium-mediated transformation are still the focus of future lettuce breeding work.

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