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ORIGINAL RESEARCH ARTICLE

The progress of transgenic cucumber mediated by Agrobacterium tumefaciens

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ABSTRACT

Cucumis sativus is an important vegetable crop in the world. Agrobacterium mediated transgenic technology is an important means to study plant gene function and variety improvement. In order to further accelerate the transgenic research and breeding process of cucumber, aiming at the Agrobacterium mediated genetic transformation method of cucumber, this paper expounds the research progress and existing problems of Agrobacterium mediated transgenic cucumber from the aspects of influencing factors of cucumber regeneration ability, genetic transformation conditions and various added substances in the process, and prospects the future of improving the efficiency of cucumber genetic transformation and the application of safety screening markers, in order to provide reference for cucumber stress resistance breeding and fruit quality improvement.

Keywords: Cucumber; Transgenic; Regeneration Ability; Genetic Transformation Efficiency

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1. Introduction

Cucumis sativus is one of the most important vegetable crops in the world, and its gene function research and genetic breeding have been widely concerned. Agrobacterium mediated genetic transformation technology is a natural plant transgenic system. With the help of Agrobacterium, to infect injured plants, the modified T-DNA region is inserted into the plant genome, and then the transgenic plants are obtained through tissue culture technology. It has the advantages of good stability, low cost, simple technology and low equipment requirements. It has gradually become an important means of cucumber gene research and variety improvement[1].

Agrobacterium Tumefaciens mediated cucumber transgenic technology is the most mature and widely used genetic transformation method. The key to this technology is to create suitable genetic transformation conditions for cucumber by relying on the in vitro regeneration system of cucumber, supplemented by appropriate additives, which generally includes the acquisition of sterile explants, pre-culture, Agrobacterium infection and transformation, co-culture of Agrobacterium and explants, induction and screening of resistant buds, elongation and rooting of resistant buds, seedling refining and transplanting. Since the introduction of tissue culture and genetic transformation technology of cucumber[2,3], through continuous improvement and optimization by follow-up researchers, fruitful achievements have been made in the genetic transformation of genes such as resistance to biological stress[4,5], resistance to abiotic stress[6,7], fruit quality improvement[8,9], growth

and development[10,11]. However, due to different genotypes, explant types and other factors, the regeneration ability of cucumber is still quite different. The research on the conditions of genetic transformation is still not comprehensive. The research on the principle and function of various additives in the process of cucumber genetic transformation is still not thorough. As a result, browning, pollution, glass seedlings, old and young seedlings, low efficiency of genetic transformation, gene expression and genetic instability still plague researchers. Based on the ability of cucumber regeneration in vitro, genetic transformation conditions and various additives in the process, this paper analyzes and summarizes the current progress and achievements, and provides a reference for the in-depth research and future development of Agrobacterium Tumefaciens mediated cucumber transgenic technology.

2. Factors affecting regeneration ability of cucumbers

High regeneration ability is the basis of successful genetic transformation. In vitro regeneration of cucumber mainly includes two ways: Obtaining adventitious buds directly from explants and inducing callus first and then inducing adventitious buds. These two methods are basically same in culture steps. The difference is that the method of directly inducing adventitious buds has the characteristics of short regeneration cycle and high regeneration rate. And, the method of inducing callus and then inducing adventitious buds is more conducive to the positive screening of subsequent genetic transformation[4]. The main factors affecting the regeneration ability of cucumber are as follows.

2.1 Variety genotype

The regeneration ability of different cucumber varieties was greatly different. Todd *et al*.[3] conducted in vitro regeneration experiments on 85 cucumber varieties, but only 28 varieties can regenerate buds from cotyledons, which proves that the regeneration ability of different cucumber genotypes is quite different. Moreover, even cucumber varieties of different genotypes that are easy to regenerate also have significant differences in their regeneration ability[12]. For example, studies have found that the regeneration frequency of cucumber variety 'Jilin Dry Melon' can reach 97%, while that of cucumber variety '9910 GT' is only 67%[13]. At the same time, it is pointed out that under the same culture conditions, 'Jilin Dry Melon' is significantly faster than the other five varieties in terms of the average number of regenerated buds of explants, seedling emergence, growth and differentiation. At present, many studies have obtained similar conclusions[14–16]. Wang *et al*.[17] found that the gene Csa1G642540 (DnaJ, which is involved in responsing to abscisic acid regulation, meristem regeneration and plasma membrane H^+-ATP activity regulation, etc.) plays an important role in cucumber regeneration in vitro through QTL mapping and SNP molecular marker technology. It is the first time to confirm the difference of regeneration ability of different cucumber varieties in genetic mechanism. Suitable genotypes have strong regeneration ability, rapid and consistent growth and differentiation, and strong adaptability to adversity, which can effectively reduce the pollution and accumulation of harmful substances in the tissue culture micro-environment of regenerated plants.

2.2 Explant selection

The regenerated shoots of cucumber need to be differentiated from the cut explants. In this study, the in vitro regeneration system of cucumber was optimized according to the seedling age, seedling state and different explant types.

In the study, the best seedling age of cucumber is usually 1–4 days[18–20], and 5–7 days is also selected as the best seedling age[21]. In the seedling state, cotyledons that are not fully folded[6,22], cotyledons that are completely upright[21] and cotyledons of short seedling age that are not cultured under light[10,18] are selected. Seedling age is not only the main factor that affects the yield, vitality, division rate, plant hormone level, nutrient content, etc. of protoplasts, but also determines the seedling state of cucumber tissue culture seedlings. In the study, the differences in seed vitality or seed soaking methods may lead to different germination rates of cucumber seeds, and thus affect the seedling age of

cucumber tissue culture seedlings to achieve the best regeneration state[23], it is more reasonable to take seedling state as the main reference factor for optimal regeneration efficiency.

The types of explants mainly include cotyledon, cotyledon node, hypocotyl, epicotyl, etc., of which cotyledon[6,24] and cotyledon node[25–27] are the most used. The regeneration frequency can reach 96.7%[9] and 100%[28] respectively, while the regeneration frequency of hypocotyl is only 21%[26], which is significantly lower than cotyledon and cotyledon node. The reason for this difference may be mainly affected by genotype. In addition, the treatment method of explants and other factors such as culture environment also affect the regeneration ability of different explants. Using cotyledon, cotyledon node and hypocotyl as explants, it has been reported that transgenic plants have been successfully obtained. However, studies have confirmed that the transgenic efficiency of cotyledon and cotyledon node is higher than that of hypocotyl[29]. In general, the most suitable explant type for in vitro regeneration of cucumber is cotyledon node or cotyledon[30,31].

3. Agrobacterium mediated transformation of cucumber

3.1 Pre-culture conditions

It is generally believed that pre-culture can adjust the level of endogenous hormones and physiological state of plant cells in explants, reduce the stress of adversity on young explants, and promote cell division and integration of foreign genes[32]. And pre-culture is generally carried out in the way of dark culture, which has a significant impact on the regulation of peroxidase activity growth[33]. According to the sprouting rate of resistant buds[21] and the GUS transient expression rate[34] after different pre-culture days, it is found that different pre-culture time has a significant impact on genetic transformation efficiency[32], and 1–2 days are the pre-culture days selected in most studies[10,35]. The analysis shows that the explants can not reach the best physiological state within a too short pre-culture time, and a too long pre-culture time will make the explant wound heal, the effect of Agrobacterium infection will be greatly reduced, which will affect the efficiency of genetic transformation.

3.2 Infection conditions

It is an important step in the process of cucumber genetic transformation to infect injured explants with prefabricated infection solution[36]. Agrobacterium Tumefaciens GV3101[8], LBA4404[37], EHA105[38] and other strains have been reported to be used in the study of cucumber genetic transformation, and transgenic plants have been successfully obtained. After culturing the Agrobacterium Tumefaciens containing the target plasmid to the OD_{600} value of 0.6–0.8, the suspension and dilution of the Agrobacterium Tumefaciens to the OD_{600} value of 0.2–0.3. The study shows that the OD_{600} value of the culture medium is between 0.6 and 0.8, and the Agrobacterium Tumefaciens is in the logarithmic growth stage. At this time, the activity of Agrobacterium Tumefaciens is the highest, the OD_{600} value after dilution is between 0.2 and 0.3, which can avoid the explant corruption and death caused by excessive concentration of Agrobacterium[6–8]. The infection mode of explants is mainly immersion infection[4,10]. There are also reported methods to improve the infection depth by using vacuum negative pressure and other equipment[39,40]. Yang Li[39] used green fluorescent protein to monitor the genetic transformation process under different infection conditions. It was found that the regeneration site was not consistent with the infection site, and the infection rate was only 30%. After changing to vacuum negative pressure infection, the infection rate increased to 90%. Although the infection time is limited by the infection mode, it is mostly between 10–30 min[14,41]. The reports that the infection time is less than 10 min[32] or more than 30 min[42] are rare.

3.3 Co-culture conditions

The co-culture stage is set after the explants are infected, which is an important stage for the integration of target genes into plant chromosomes. The length of co-culture time needs to be optimized according to the differences of explants' tolerance,

infection solution concentration and co-culture operation mode. Many studies choose the best co-culture time within 2–4 days based on the healing rate of resistant buds or GUS positive expression[34,39]. According to the study on co-culture temperature, we can not only consider the optimal temperature for cucumber explant differentiation, agrobacterium growth or T-DNA transfer, but also should consider the interaction between co-culture temperature and co-culture time. When co-culture for 2 days, 26 ℃ is more conducive to genetic transformation[42]. As for the influence of co-culture medium pH on genetic transformation efficiency, it was found that the co-culture medium pH between 5.0 and 5.2 was more conducive to the improvement of resistant bud rate[38]. It was believed that the additive Acetosyringone (AS) had better effect in acidic environment, and it was also believed that lower pH was conducive to the activation, transfer and expression of Agrobacterium Virgene. With the improvement of co culture time, temperature and medium pH, it is of great significance to improve the efficiency of Agrobacterium Tumefaciens mediated transformation of cucumber.

In the study, the research group first used 1/2MS liquid medium to elute the explants after co-culture for 3 times, and then dried the explants and transferred them to the screening medium. It was found that the growth of Agrobacterium and the use of antibacterial antibiotics in the screening culture stage could be greatly reduced. Compared with the untreated group, the resistant bud rate increased to 67%. This method of eluting Agrobacterium can significantly promote the normal growth of explants. The next step will be to improve the elution time. The factors such as the amount of antibiotics added and the effect on the efficiency of genetic transformation were further studied.

4. Application of additive in transgenic cucumber

4.1 Plant hormones

It was found that the endogenous hormone content of the cut explants changed dramatically at different regeneration stages, and the content of Abscisic acid (ABA) increased significantly at the callus induction stage. The content of ABA/ Indole-3-acetic acid (IAA) and ABA/ Gibberellin (GA) were also related to the callus induction stage[43], which indicates that the explants were in various processes of in vitro regeneration, the contents of various plant hormones play an important role in regulation. Therefore, researchers used the method of adding different concentrations of plant hormone combinations at different stages of cucumber regeneration in vitro to screen the optimal hormone combinations[40]. The two widely used plant hormones are 6-Benzylaminoadenine (6-BA) and ABA (**Table 1**).

Table 1. Application of plant hormone and $AgNO_3$ in the explant bud induction stage of different varieties of cucumber

Variety	Explant type	Medium formula	Regeneration frequency (%)
Xintai mici[4]	Cotyledonary nodes	MS + 0.5 mg/L 6-BA + 1.0 mg/L ABA	82.7
Jinyan No.4[7]	Cotyledonary nodes	MS + 3.0 mg/L 6-BA + 1.5 mg/L ABA + 1.5 mg/L $AgNO_3$	90.0
9930[9]	Cotyledon	MS + 1.5 mg/L 6-BA + 1.0 mg/L ABA	96.7
Jinyou No.1[11]	Cotyledon	MS + 2.0 mg/L 6-BA + 2.0 mg/L $AgNO_3$	86.1
Jinyou No.1[18]	Cotyledonary nodes	MS + 2.0 mg/L 6-BA + 2.0 mg/L $AgNO_3$	90.7
9930[19]	Cotyledonary nodes	MS + 2.0 mg/L 6-BA + 1.0 mg/L ABA + 2.0 mg/L $AgNO_3$	90.2
S06[20]	Cotyledon	MS + 1.5 mg/L 6-BA + 0.5 mg/L ABA + 2.0 mg/L $AgNO_3$	91.2
Changchun mici[22]	Cotyledonary nodes	MS + 1.5 mg/L 6-BA + 0.5 mg/L ABA + 2.0 mg/L $AgNO_3$	90.0
S52[23]	Cotyledonary nodes	MS + 1.5 mg/L 6-BA + 0.5 mg/L ABA + 2.0 mg/L $AgNO_3$	100.0
Gl[34]	Cotyledonary nodes	MS + 1.5 mg/L 6-BA + 1.0 mg/L ABA + 2.0 mg/L $AgNO_3$	100.0

There are also reports of using GA, IAA, Indole butyric acid, Naphthylacetic acid, etc. The concentration range of 6-BA is mostly in 0.5–2.0 mg/L, and the concentration range of ABA is 0–2.0 mg/L. According to the different cucumber genotypes, explant types and regeneration stages in vitro, the best hormone combinations for in vitro regeneration of cucumber strains such as Jin You series[11,18], Jin Yan series[7,24], Xin Tai Mi Ci[4,37], 9930[17,19] have been studied and improved, and the highest regeneration frequency of 100% has been obtained (**Table 1**)[19]. In the process of inducing the explants of 'Xin Tai Mi Ci' to directly differentiate into buds, our research group found that the combination of

0.5 mg/L 6-BA and 1.0 mg/L ABA had the best effect.

After the induction of adventitious buds, the endogenous hormones in the explants gradually tend to be stable. In the study, it is often used to reduce the concentration of added plant hormones, to change or to reduce the types of hormones used, so as to promote the elongation of regenerated buds[20,22], and the cycle of this step is about 7–15 days. When the regenerated seedlings extend to about 2 cm, rooting can be induced. In some studies, a small amount of plant hormones were added to promote the rooting of regenerated buds[10,15], but it is generally believed that rooting can be induced without adding plant hormones[16,18], which may be related to the fact that cucumber regenerated buds are relatively easy to induce roots[4].

4.2 $AgNO_3$

$AgNO_3$ is widely used as an additive in the regeneration of cucumbers in vitro. The research shows that Ag^+ plays a significant role in promoting explant differentiation, growth and preventing browning, which is positively related to the inhibition of ethylene synthesis by Ag^+ and the inhibition of explant regeneration[44]. It is generally considered that adding 0.5–2.0 mg/L $AgNO_3$ can achieve the best effect, and excessive use will have a negative effect[13,31], however, in some studies, higher regeneration frequency can still be obtained without $AgNO_3$ (**Table 1**) and good growth state of regenerated buds can be maintained[5], which may be due to differences in genotype and explants, resulting in different stress resistance of explants. In the study, the optimal concentration of $AgNO_3$ needs to be further screened.

4.3 As and antioxidants

Many studies have added a certain concentration of AS[18,19] in the pre-culture[23], infection and co-culture stages of genetic transformation, and found that as has a significant effect on improving the differentiation rate of resistant buds. This is because as and other phenolic substances can activate the Vir region of Agrobacterium, promote the introduction of foreign genes into the plant genome, and then improve the efficiency of genetic transformation[4,35]. The lowest concentration of as in co-culture medium is 20 μmol/L[19], and the maximum is 150 μmol/L[32]. The concentration of as in the infection solution is 100 μmol/L[18,42], although there are differences in the addition stage and concentration, it shows that phenols can promote the genetic transformation of cucumber. There are also studies to improve the efficiency of genetic transformation by inhibiting the oxidation of phenolic substances, i.e. adding antioxidants, such as α-Octyl sulfate[16], L-Cytine, Dithiothreitol, $Na_2S_2O_3$[35] are common and have good effects[16], but they are not widely used in genetic transformation of cucumber. Whether as is directly added or antioxidant is added, the efficiency of genetic transformation is improved by increasing the content of phenolic substances. However, such substances are often dissolved in toxic organic solvents, or they will have a certain toxic effect at high concentrations, and effectively inhibit the growth of Agrobacterium together with the infected liquid. classes[41], co-culture medium pH[38], co-culture temperature[42]. However, the specific addition stage and concentration need to be further discussed.

4.4 Antibiotics

In the screening and culture stage of cucumber genetic transformation system, screening antibiotics and antibacterial antibiotics are often used together. Kanamycin (Kan)[16,40], Hygromycin[10,32], Glyphosate[25] are the most used screening markers. It is generally believed that the screening antibiotic tolerance pressure of cucumber explants is closely related to genotype[19,28], and culture stage[20,26]. For example, the cucumber ‘Chuan Lv 2’ can not completely inhibit budding at the concentration of 140 mg/L Kan, while the ‘Bai Si Tiao’ and ‘Er Han Zi’ can completely inhibit sprouting at the concentration of 40 mg/L and 60 mg/L[42]. The critical Kan concentration of cucumber ‘S06’ in the bud induction stage is 140 mg/L, and 100 mg/L Kan in the rooting stage can completely inhibit rooting[20]. The concentration of screening antibiotics should not be too high or too low. Too high concentration is easy to kill young plants or untransformed cells. Too low concentration will lead to too high false positive

rate and greatly increase the workload of positive identification in the later stage. In recent years, researchers prefer to set gradient concentration for positive screening[26], that is, low concentration of screening antibiotics is used during bud induction, and higher screening concentration is used at the stage of strong bud and rooting. This dynamic screening based on the change of genotype and culture stage can effectively solve the problems caused by too low or too high screening concentration.

Different from screening antibiotics, which have obvious inhibitory effect on explant differentiation, we often use antibiotics that do little damage to plants and have certain inhibitory effect on Agrobacterium. Commonly used antibacterial antibiotics include Carbenicillin (Carb), Cefotaxime sodium (Cef), Timentin (TM), Ampicillin etc.[32,36] Although these antibiotics can effectively inhibit the growth of Agrobacterium Tumefaciens, they are often used in high concentrations often in 300–500 mg/L. And it also has certain influence on the differentiation and growth of explants.

It was found in some reports that CEF is more conducive to callus induction and budding than Carb[26,32]. TM is a new antibiotic that inhibits Agrobacterium Tumefaciens, and its biggest feature is the strong persistence of bacteriostasis, which can effectively inhibit bacteria within 70 days[45], but the use cost is much higher than CEF. Therefore, in the process of cucumber genetic transformation, the most appropriate bacteriostasis can be selected according to different culture stages and cycles antibiotic. For example, in the differentiation and induction stage, it is necessary to select the species that have less influence on explants and callus induction, and in the long growth cycle stages such as strong buds or rooting, it is necessary to select the species that have significant and lasting antibacterial effects. In addition, the use concentration can be appropriately increased or decreased according to the pollution situation, so as to minimize the impact of Agrobacterium and antibiotics on the explants during genetic transformation.

Based on the previous research results[46], the research group applied activated carbon as an exogenous additive in the process of cucumber genetic transformation. It was found that the medium with activated carbon could remain colorless and transparent for a long time, while the medium without activated carbon would gradually turn yellow in about a week. It was also found that activated carbon had a significant effect on inhibiting browning and promoting rooting at a mass concentration of 0.5%, however, the specific addition stage and the adsorption of the original substances in the culture medium need to be further studied.

5. Discussion and Outlook

At present, cucumber is recognized as a species that is difficult to carry out transgenic. It has been reported that after detection by PCR, Southern blotting, qPCR and other methods, the highest genetic transformation efficiency can reach to 29%[47], while the lowest is only 0.1%[48], mostly between 1%–10%, and cannot be stably inherited to the next generation. Gene loss or silencing often occurs[5]. The inconsistency between the infection site of Agrobacterium and the regeneration site of explants is an important reason for the low efficiency of genetic transformation of cucumber[48]. The culture time, temperature, pH value of culture medium, infection mode, strain and carrier type at each stage are the main factors affecting the depth of infection site. However, in the existing studies, these parameters are different and lack of sufficient details. In the basic culture medium, curing agent[16], light environment and gas environment of tissue culture, there are few studies on the domestication of regenerated plants. The serious pollution of Agrobacterium and the death of transgenic plants in the domestication stage are also other important reasons for the low efficiency of cucumber genetic transformation. At present, various antibiotics are still the main screening markers in the cucumber transgenic system. Although safety screening markers such as Mannose[49] have been reported to be used in the cucumber transgenic process, their development is relatively slow[50], and there is still a great research development space compared with the traditional antibiotic screening markers.

With the continuous development of molecular biotechnology and the continuous optimization of cucumber transgenic technology, researchers have actively explored the cucumber transgenic technology mediated by Agrobacterium Tumefaciens. Ultrasound, vacuum pump and other tools are more and more widely used[41]. In vivo transfection[50], new nano particle mediated[51] and other methods have gradually entered the researchers' attention. It can be predicted that the in vitro regeneration and genetic transformation system of cucumber will be more and more perfect. CRISPR/cas9 technology[48] and safety screening markers will be more and more applied to the research of antibiotic free cucumber genetic transformation. Some problems that are difficult to break through at present will be solved with the deepening of research.

Conflict of interest

The authors declare that they have no conflict of interest.

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