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Establishment of Highly Efficient Genetic Transformation System of Sugarcane Callus Mediated by *Agrobacterium*

Dong-Liang HUANG[1*], Cui-Xian QIN[1*], Zhong-Liang CHEN[1], Yi-Yun GUI[1], Miao WANG[1], Jian-Hui ZHOU[2], Qing LIAO[2], Li-Tao YANG[1,2] Yang-Rui LI[1, 2]**

[1]Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Sugarcane Research Center, Chinese Academy of Agricultural Sciences, Guangxi Key Laboratory of Sugarcane Genetic Improvement, Key Laboratory of Sugarcane Biotechnology and Genetic Improvement (Guangxi), Ministry of Agriculture, P. R. China, Nanning 530007, China
[2] Guangxi Academy of Agricultural Sciences, Nanning 530007, China
*Authors contributed equally to this work.
**Corresponding Author's Email: liyr@gxaas.net

***Abstract* – To date, it is difficult to transform sugarcane using *Agrobacterium* because sugarcane belongs to Monocotyledonae, which are not native host of *Agrobacterium*. To establish a highly efficient sugarcane transformation system, the optimal concentration of antibiotic (Km) was screened for subculture and differentiation of sugarcane calli, as well as seedling rooting, respectively. The factors that influenced sugarcane transformation, such as pre-culture time, infection time and co-culture time were also studied. The results showed that the optimal concentrations of antibiotic (Km) for subculture, differentiation and seedling rooting were 300 mg/L, 40 mg/L and 20 mg/L, respectively. The optimal pre-culture time, infection time and co-culture time were 4 days, 30 min and 4 days, respectively. PCR detection showed that 61.6% of resistant plants were transgenic. This work lays a solid foundation for further study on gene function and transgenic research of sugarcane.**

***Keywords* – Sugarcane, Genetic Transformation, High Efficient, Callus, *Agrobacterium*.**

I. INTRODUCTION

Sugarcane (*Saccharumofficinarum* L.) is an important sugar and energy crop that is significant to the economic development of China [1]. Sugarcane breeding is the core technology of cane sugar production. Breeding new varieties are the most cost-effective ways to improve cane sugar production. At present, new sugarcane varieties are primarily developed through cross breeding. Since sugarcane is highly heterozygous, aneuploid, and polyploid as well as of complex genetic background, conventional sexual hybridization requires long cycles to achieve variety improvement and the efficiency is very low, generally it takes 10–12 years to develop a new variety [2] [3]. The efficiency of sugarcane breeding can be greatly increased by use of transgenic technologies. Among the commonly used transgenic techniques, including *Agrobacterium*-mediated transformation, particle bombardment technology, and pollen tube pathway-mediated transformation [4], the *Agrobacterium*-mediated transformation is easy to operate, highly efficient, fast, and currently, it is the main method used in plant genetic transformation [5]. More than 80% of transgenic plants have been obtained via *Agrobacterium*-mediated transformation.

Sugarcane belongs to family Gramineae, class Monocotyledonae. Since monocots are generally not native hosts of *Agrobacterium*, transforming Gramineae using *Agrobacterium* has always been difficult [6]. However, thanks to the advances made in transformation of Gramineae, such as rice mediated by *Agrobacterium*, significant progress was made in transformation of sugarcane mediated by *Agrobacterium*. Arencibia et al. [7] established a preliminary *Agrobacterium*-mediated transformation system of sugarcane. Enriquez-Obregón et al. [8] produced herbicide-resistant sugarcane plants via *Agrobacterium*-mediated transformation of sugarcane callus. Elliott et al. [9] generated transgenic sugarcane plants that expressed the green fluorescent pigment (GFP) by transforming sugarcane calli with the strain AGLO containing the GFP genes. Some domestic researchers also obtained transgenic sugarcane using *Agrobacterium*-mediated methods [10] [11][12] [13]. Nevertheless, the low transformation efficiency of *Agrobacterium*-mediated transformation of sugarcane remains a major hindranceof this technique. Therefore, optimization of the *Agrobacterium*-mediated genetic transformation system of sugarcane callus and improvement of the transformation efficiency of sugarcane are essential to sugarcane transgenic breeding. In this study, antibiotic concentrations (Km) were screened to determine the optimal concentrations for subculture and differentiation of sugarcane calli, as well as seedling rooting. In addition, factors affecting genetic transformation, such as preculture time, infection time, and co-culture time during *Agrobacterium* infection process were optimized, to establish a high-efficiency sugarcane transformation system.

II. MATERIALS AND METHODS

A. Plant Materials and Growth Conditions

Sugarcane (*Saccharumofficinarum* L.) cultivar ROC22 was currently the predominant cultivar in Guangxi, China. Plants of sugarcane were grown in our experimental field under normal conditions.

B. Bacterial Strains and Plasmids

Agrobacterium tumefaciens strain EHA105, stored in our lab, and plant expression vector pRI101-ON, purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd.

C. Media

All chemicals were purchased from Shanghai Sangon Biological Engineering co., LTD (China).

Callus induction and subculture medium (MS1): MS + 3 mg/L 2,4-D + 3% sucrose + 0.8% agar (pH 5.8);

Differentiation medium (MS2): MS + 0.1 mg/L NAA + 2 mg/L6-BA + 3% sucrose + 0.8% agar (pH 5.8);

Rooting medium (MS3): MS + 4 mg/L NAA+3% sucrose + 0.8% sucrose agar (pH 5.8);

Co-culture medium (MS4): MS + 2mg/L 2,4-D + 100 µmol/L AS + 3% sucrose + 0.8% agar (pH 5.8);

Subculture screening medium: MS1 + 300 mg/L Cef + 300 mg/L Km (pH 5.8);

Differentiation screening medium: MS2 + 300 mg/L Cef + 40 mg/L Km (pH 5.8);

Rooting screening medium: MS3 + 300 mg/L Cef + 20 mg/L Km (pH 5.8).

D. Sugarcane Callus Induction

Shoot tips were obtained from healthy sugarcane plants and their surface was disinfected with 70% ethanol. Young leaf tissues about 10 cm from the apical meristem were used as explants, which were then cut into 0.2 mm thin slices and cultured in the callus induction medium at 26 °C–28 °C in dark for 15–20 days to form embryonic calli.

E. Optimal Antibiotic Concentration Screening

Antibiotic concentrations of 0, 20, 40, 50, 100, 200, 300 and 400 mg/L were screened for concentrations (Km) that are most suitable for subculture and differentiation of calli as well as seedling rooting (20 calli treated in each group, 3 repeats per group). The induction rate, differentiation rate, and rooting rate were calculated respectively.

F. Optimization of Agrobacterium Infection Conditions

Loose embryonic calli were selected and broken into small pieces, about 2 mm each, which were then transferred to Petri dishes. A liquid medium of *Agrobacterium*, activated using 200 µmol/L AS for 2 h, was added for inducing infection. Mild shaking was applied during the process. Next, the calli were wiped dry with sterile filter papers before being transferred to the co-culture medium containing 100 µmol/L AS and no antibiotics. They were later transferred to the shoot induction medium containing antibiotics. After the development of adventitious buds, the calli were transferred to the shoot growth medium, and then, when the shoot grew to a length of 2–3 cm, they were transferred into the rooting medium containing an antibiotic, where they developed roots and grew into full plants.

In this experiment, the effects of different pre-culture times (0, 1, 2, 3, 4 and 5 days), infection times (10, 15, 20, 25, 30, 35 min), and co-culture times (0, 1, 2, 3, 4 and 5 days) on transformation efficiency were observed by treating 300 calli in each group.

G. PCR Molecular Detection of Transgenic Plants

A pair of primers, NPTII-F (5'-ctattcggctatgactgggcaca-3') and NPTII-R (5'-cctgatgctcttcgtccagatca-3'), targeting the NPTII gene, were designed based on the plasmid pRI 101-ON sequence. PCR assays were performed with primers, which targeted the *NPTII* gene, and genomic DNA of regenerated resistant plants and untransformed plants (control) as templates. Target fragment length was 436 bp. PCR products were analyzed on 1.2% agarose gel.

III. Results

A. Screening for Antibiotic Concentration (Km) Optimal for Callus Induction

In the callus subculture phase, sugarcane cultivar ROC22 calli with uniform growth were selected and inoculated into subculture medium with different antibiotic concentrations (Km). After 20 days, it was observed that in the culture medium at concentration 0 mg/L all the calli grew dry, dense, and yellowish new calli; at 20 mg/L, more than 70% of the calli grew new calli; at 40–50 mg/L, the callus induction rate was below 30%; at 100–200 mg/L, the rate dropped below 10%; and at 300 mg/L, all calli died before developing any new callus (Fig. 1). The results showed that the screening criteria were reached at a concentration of 300 mg/L. Hence, the optimal antibiotic concentration (Km) for induction was determined to be 300 mg/L.

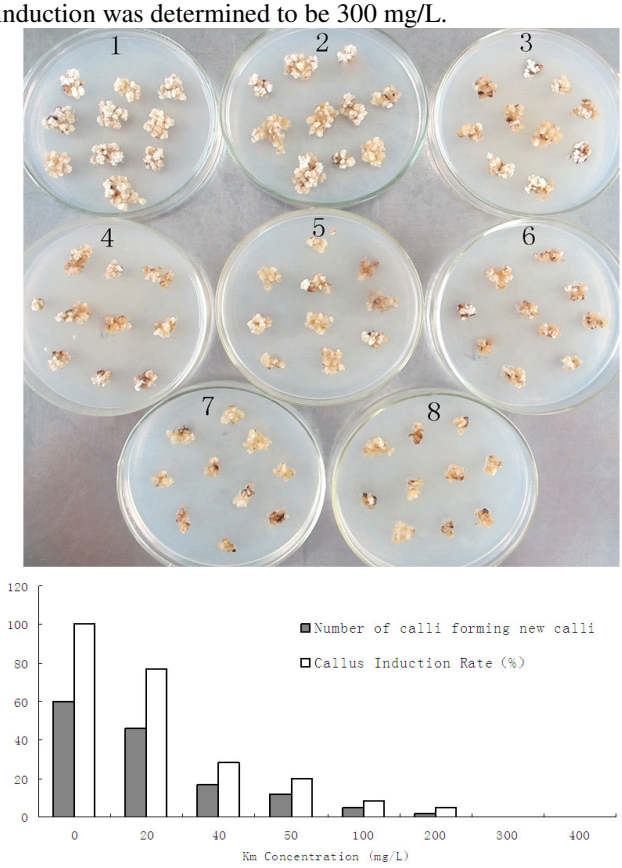

Fig.1. Effects of Km concentration on subcultures of sugarcane calli.1: 0 mg/L; 2: 20 mg/L; 3: 40 mg/L; 4: 50 mg/L; 5: 100 mg/L; 6: 200 mg/L; 7: 300 mg/L; 8: 400 mg/L

B. Screening for Antibiotic Concentration (Km) Optimal for Callus Differentiation

In the callus differentiation phase, the uniformly grown embryonic calli were inoculated into the differentiation medium with different antibiotic concentrations (Km).

After 20 days, it was observed that, calli grown in culture medium at a concentration of 0 mg/L differentiated normally and developed green, strong, and healthy seedlings; at 20 mg/L, only 2 calli developed into seedlings, one healthy and the other albino that was unable to grow normally, while the rest of the calli died; at 40–400 mg/L, all calli died (Fig. 2). The results showed that, as the Km concentration increased, the growth of calli was severely impeded. At the concentration of 40 mg/L, the growth of callus was completely suppressed, which matched the screening criteria. Hence, the optimal antibiotic concentration (Km) for differentiation was determined to be 40 mg/L.

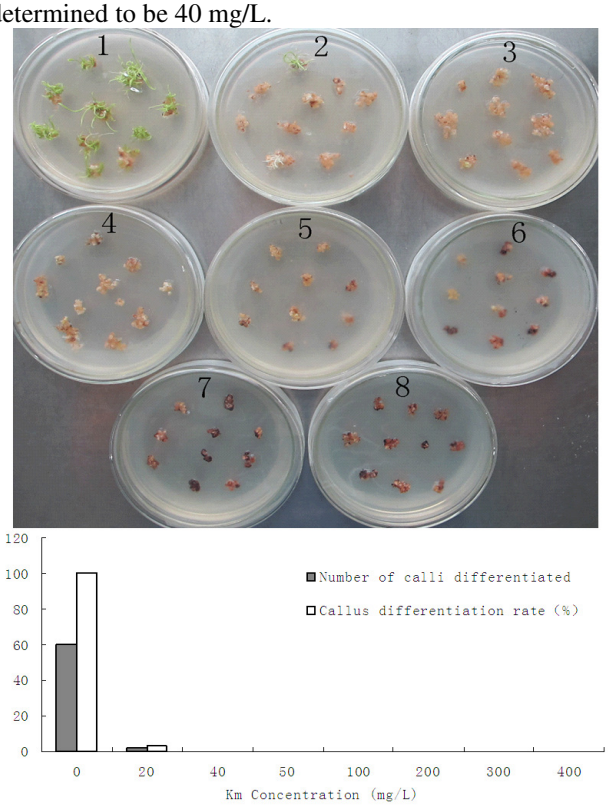

Fig.2. Effects of Km concentration in the medium on the differentiation of sugarcane calli.1: 0 mg/L; 2: 20 mg/L; 3: 40 mg/L; 4: 50 mg/L; 5: 100 mg/L; 6: 200 mg/L; 7: 300 mg/L; 8: 400 mg/L

C. Screening for Antibiotic Concentration (Km) Optimal for Seedling Rooting

The uniformly grown seedlings were inoculated into the rooting medium with different concentrations (Km). After 20 days, it was observed that, in the culture medium at a concentration of 0 mg/L, all the seedlings normally grew abundant, thick, and dense roots and differentiated into tender green seedlings with many tillers; at 20 mg/L, 65% of the seedlings grew roots, but the seedlings were unhealthy albinos that were unable to grow; at 40–50 mg/L, although all seedlings grew roots, the newly produced seedlings were all albinos; at 100–400 mg/L, there was no root formation (Fig. 3). The results showed that, when the Km concentration was 20 mg/L, 65% of the seedlings grew roots but none of them was healthy. As a result, the optimal antibiotic concentration (Km) for rooting was 20 mg/L.

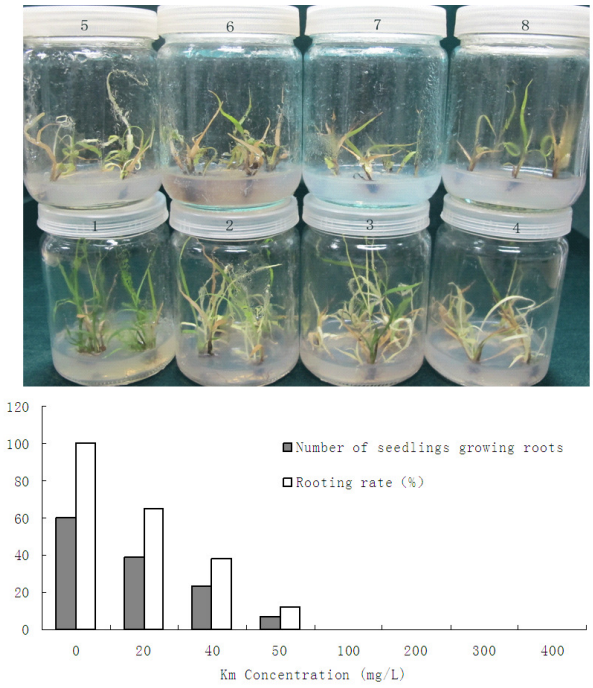

Fig.3. Effects of Km concentration on rooting of sugarcane seedlings.1: 0 mg/L; 2: 20 mg/L; 3: 40 mg/L; 4: 50 mg/L; 5: 100 mg/L; 6: 200 mg/L; 7: 300 mg/L; 8: 400 mg/L

D. Effects of Preculture Duration on Transformation Efficiency

Preculture can help explants adapt to the culture environment faster before entering the germination stage. It also improves transient expression and transformation rate of exogenous genes by promoting cell division. This study indicated that preculturing for 3–4 days facilitated transformation: as many as 61–65 buds developed were resistant, representing 20.33%–21.67% of the samples, significantly more than the number of resistant bud production in the control group (0 day of preculturing).

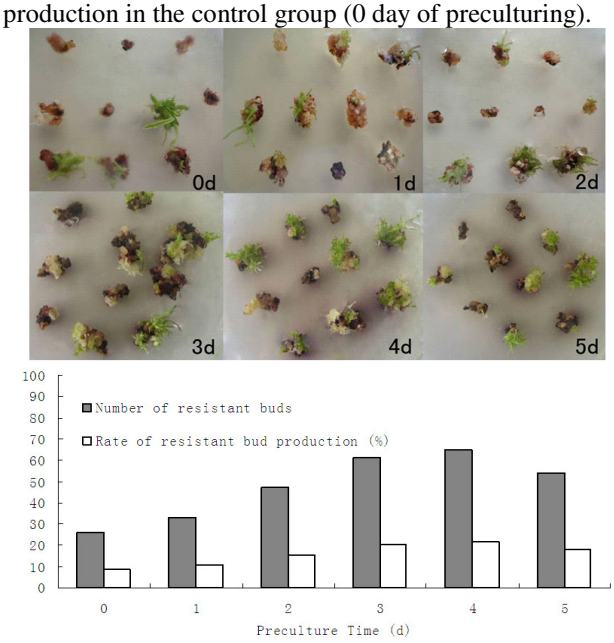

Fig.4. Effect of pre-culture time on sugarcane transformation.

On the other hand, the rate of resistant bud production declined as the preculture duration increased. The rate dropped to 18% (Fig. 4) if precultured for 5 days. This is because extended preculture time induces the formation of latent buds in explants, which causes a decrease in the transformation rate and an increase in the false positive rate. Therefore, the optimal preculture duration was 4 days.

E. Effect of Infection Time on Transformation Efficiency

In the series of infection time in the time gradient, 30-min infection resulted in the highest transformation efficiency reaching 27.33% (Fig. 5). If the infection time was too short, *Agrobacterium* could not fully adhere; if too long, the overgrowth of *Agrobacterium* caused difficulty in suppressing *Agrobacterium* or browning of explants and led to subsequent death due to strong toxicity. The infection time that led to the highest resistant bud production rate was considered the most suitable duration for infection, which was found to be 30 min.

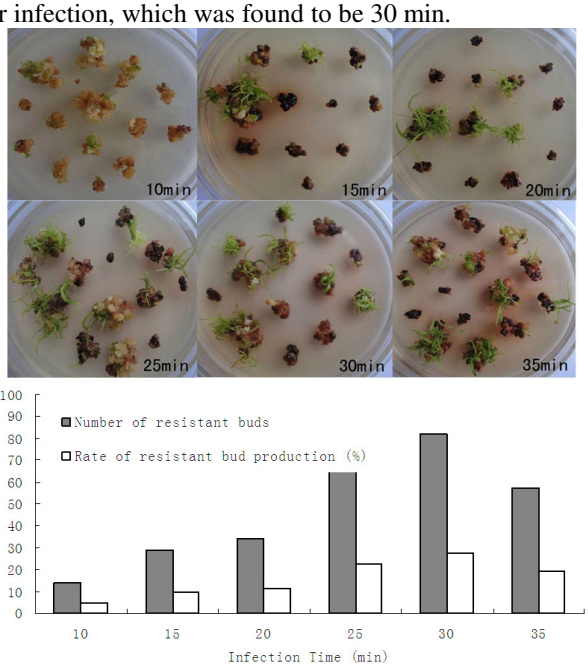

Fig.5. Effect of infection time on sugarcane transformation.

F. Effect of Co-culture Time on Transformation Efficiency

Co-culture is an important phase of the transformation process, after the completion of *Agrobacterium* attachment and T-DNA transfer and integration. Therefore, appropriate co-culture time can improve the transformation efficiency. This study showed that co-culturing for 0–4 days improved the transformation efficiency as the co-culture time increased. When the co-culture time was 4 days, the transformation rate reached 24.67%, which, however, declined to 7.67% at the co-culture time of 5 days (Fig. 6). This was caused by the overgrowth of *Agrobacterium* due to the long co-culture time, which in turn contributed to the death of plant cells caused by toxicity. Thus, the most appropriate co-culture time was 4 days.

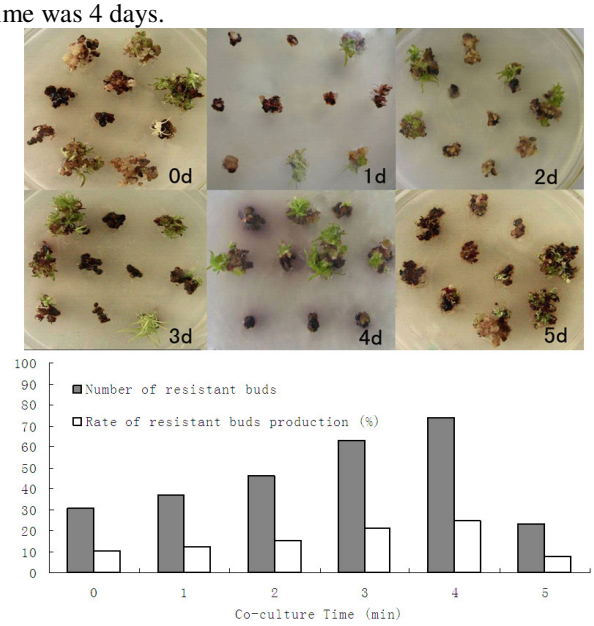

Fig.6. Effect of co-culture time on sugarcane transformation.

G. Agrobacterium-mediated Genetic Sugarcane Transformation

This study tested a series of optimization factors for *Agrobacterium*-mediated genetic transformation of sugarcane in order to determine the optimal transformation conditions. Under optimal transformation conditions, a total of 364 calli were transformed, which were later screened against the antibiotic Km in a series of subculture, differentiation, and rooting processes (Fig. 7). At the end of the tests, 89 resistant plants were obtained and the transformation rate was 24.45%.

Fig.7. Regeneration process of transgenic sugarcane.

H. Examination of Resistant Plants

Genomic DNA was extracted from 60 resistant plants, divided into 3 sets and amplified by PCR using primers targeting the NPT II gene. The results showed that 400–500 bp fragments were produced in the PCR amplification of DNA extracted from 37 resistant plants. This length was consistent with the estimated length of 436 bp and the target fragment was not present in the untransformed plants (Fig. 8). The positive rate was 61.6%, which confirmed the integration of the exogenous genes into the genome of transgenic sugarcane.

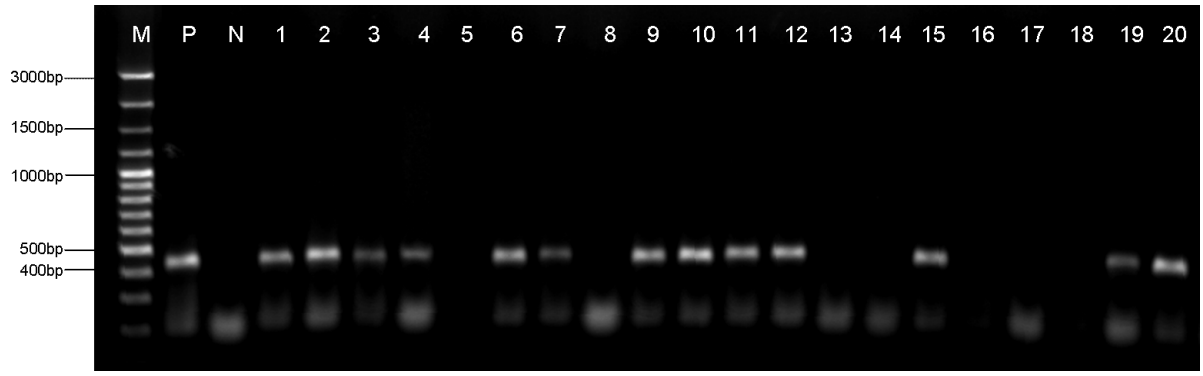

Fig.8. PCR detection of resistance plants.M: 100bp Ladder Plus (Dongsheng); P: Positive control (pRI101-ON); N: Negative control (Untransformed plants); 1-20: Resistance plants

IV. Discussion

Selective pressure on explants has a great impact on transformation efficiency. If the selective pressure is too low, the false positive rate will be high, and this will lead to higher requirement of manpower and resources to detect errors in later stages. If the selective pressure is too high, growth and differentiation of the resistant materials will be affected. Through a Km concentration gradient, Yu et al. [14] selected a Km concentration of 10 mg/L for the screening of sugarcane seedlings, and 10mg/L for rooting screening based on screening experiments with different Km concentrations. Our experiments indicated that the optimal antibiotic concentrations for subculture and differentiation of sugarcane callus and rooting were 300 mg/L, 40 mg/L, and 20 mg/L, respectively. These results also indicated that there are great gaps between the Km tolerance of explants at different growth stages. When Km is used as a selective agent, sensitivity testing is required at different phases of development—subculture and differentiation of calli, and rooting—to lower false positives rate, ensure the survival of true transgenic plants, and enhance transformation efficiency.

There are many factors affecting *Agrobacterium*-mediated genetic transformation, such as preculture time, bacterial concentration, and infection time. Many studies have focused on this aspect in different crops. He et al. [15] demonstrated that the optimal durations for *Agrobacterium*-mediated transformation of wheat include 1–2 days for preculture, 3 days for co-culture, and 45 min for infection at a bacterial concentration of OD600 = 0.6. Luo et al. [16] used sugarcane cultivar ROC10 as the receptor in the transformation experiments and, through the optimization of a series of conditions affecting the *Agrobacterium*-mediated genetic transformation, determined that the optimal infection time was 30 min and co-culture time was 4 days. Sun et al. [17] studied factors affecting genetic transformation in peanuts and determined the optimal preculture time to be 1–2 days and co-culture time as 3 days. Sun et al. [18] studied *Agrobacterium*-mediated genetic transformation in maize and determined that the *Agrobacterium* concentration of OD600 = 0.6, infection time of 15 min, and co-culture time of 3 days are the optimal conditions for genetic transformation. To establish a complete *Agrobacterium*-mediated genetic transformation system in sugarcane, this study used embryonic calli of sugarcane cultivar ROC22 as explants, and explored the effects of preculture time, infection time, and co-culture time on the genetic transformation system. The results indicated that appropriate preculture treatment of explants before transformation could increase the transformation rate; furthermore, preculture may improve transient expression and transformation rate of exogenous genes by promoting cell division [19] [20]. The optimal preculture time was 4 days, and 65 resistant buds were produced and the resistant bud production rate was 21.67% in this case. The effect of infection time was also apparent. If the infection time was too long, the calli will be softened and rotted due to the toxicity of *Agrobacterium* and oxygen deprivation, and it was difficult to inhibit bacterial growth after co-culturing. If the infection time was too short, *Agrobacterium* was unable to fully attach to the surface, resulting in a low transformation rate [19]. In this study, the optimal infection time was 30 min, with a transformation rate of 27.33%. After co-culturing for 3–4 days, the resistant bud production rate was >20%, while 5 days of co-culturing resulted in the rate drop to <7.67%. This indicated that, without co-culturing or without sufficient co-culturing, the transformation rate will be very low. As the co-culture time increased, the transformation rate increased as well, but if the co-culture time was too long, *Agrobacterium* overgrew would cause the death of plant cells due to toxicity, and eventually result in lower transformation rate. PCR results showed that 61.6% of resistant sugarcane plants obtained from optimized transformation systems

could be amplified to produce the expected segment, which indicated that the exogenous genes had already been integrated into the sugarcane genome. This study establishes a solid foundation for further research on the verification of sugarcane gene functions and genetic transformation.

V. Conclusion

A high efficiency genetic transformation system of sugarcane callus mediated by *grobacterium* was established by screening the optimal concentration of antibiotic (Km) for subculture and differentiation of sugarcane calli, and seedling rooting, as well as studying the factors that influenced sugarcane transformation. This work will facilitate further study on gene function and transgenic research of sugarcane.

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