JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1387-1395

Agrobacterium-Mediated Genetic Transformation and Regeneration of Low-Toxicity Variety of Castor (*Ricinus communis* L.)

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(Received: 09 January 2014; accepted: 18 March 2014)

Low-toxicity transgenic castor (*Ricinus communis)* plants were produced through *Agrobacterium*-mediated genetic transformation. Ricin, which is highly toxic, affects the application of castor. In this research, a 351-bp fragment located at ricin A chain gene (RTA) was selected to establish an RNA interference construct that was introduced into epicotyl of castor, thus transformants were obtained. Through semi-quantitative analysis on RTA, it was confirmed that the RTA expression quantity of the transformants was apparently decreased, demonstrating the interference efficiency of the transformation vectors and their function of reducing the expression of ricin at the RNA level. An improved protocol for the transformation and regeneration of less toxic caster has been developed for the first time.

Key words: Castor, ricin, *Agrobacterium tumefaciens*, RNAi

Castor is a very important industrial oil crop whose beans have a high oil content of 46%-60%. Castor oil does not solidify at -18°C, and does not degenerate or combust at a temperature up to 500-600°C. With these advantageous properties, thousands of chemical derivatives made from castor oil are widely applied in areas such as national defense, machinery, chemical industry, pharmacy, and agriculture, etc.[1]. Castor oil is considered an excellent alternative to petroleum as well as a renewable energy, and the demand for castor is rising year by year.

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However, due to several reasons, castor production is limited. One important reason is that castor contains ricin, which is composed of two peptide chains, Chain A (Ricinchain A, RTA) and Chain B (Ricinchain B, RTB)[2]. RTA encodes a glycosidase, and with the assistance of Chain B (RTB), it can pass through cell membrane and destroy 60s ribosomal subunits, thus inhibiting protein synthesis and causing cell death[3-4]. Ricin has a deadly toxicity—it is 6000 times more toxic than that of an equivalent weight of cyanide and 1g of ricin can kill tens of thousands of people[5]. Thus the planting of castor is restricted in some countries and the development of castor industry is affected. To solve this problem, developing low-toxicity or non-toxic castor varieties has become one of the key research topics of castor breeding.

With the development of biotechnology, transgenic technology has become one of the most efficient methods for plant breeding, and molecular breeding technology is a good choice for cultivating castor varieties. Although having an early start[6-7], due to the growth feature of castor, the molecular breeding research of castor progresses slowly and has not obtained preliminary achievements until recent years. Sujatha and Reddy[8] first reported a relatively stable regeneration system in 1998. They used the hypocotyls and stem tips of germinating seeds as explants, and under the effect of TDZ and BA, acquired a lot of shoots. On this basis, in 2005 and 2008 respectively, Sujatha *et al*.[9] and Sailaja *et al*.,[10] successfully constructed the transformation systems mediated by *Agrobacterium* and gene gun, and obtained transformed castor plants, but with extremely low transformation rate. Malathi *et al*.,[11] successfully cultivated an anti-inchworm castor variety with an *Agrobacterium*-mediated transformation system in 2006. Ahn *et al*. constructed a regeneration system with cotyledon[12] and Hypocotyl[13] as the explants, which greatly improved the regeneration efficiency. In 2009, Sujatha *et al*.[14] expressed the cry1EC gene in castor with the transgenic technology, and obtained an insect-resistant castor transformation variety. These studies have positively accelerated the process of genetic transformation of castor and promoted the development of castor industry.

RNAi is a mechanism to interfere with the translation of target genes by degrading their mRNAs through creating dsRNA (double-stranded RNA)[15-16]. RNAi technology is now widely applied in the molecular breeding of crops[17-18].

In this study, castor transformants with low RTA expression were obtained through the design of RNAi sequence which targeted the RTA encoding gene and was transferred into castor cells by *Agrobacterium tumefaciens* mediated method. Also, the castor transformation system of explants selection and co-culture phase were further optimized.

MATERIALS AND METHODS

Plant material and explant preparation

The selected castor seeds were Variety NO. 2 provided by China Shandong Jiaxiang Castor Seed Industry Technology Co., Ltd. After being shelled, the seeds were immerged into 0.1% $KMnO_4$ solution for 10 min for sterilization before being cleaned with sterile water for 2-3 times. The sterilized seed endosperms were then carefully pushed aside to detach the intact embryos, which were then inoculated in the germination medium (MS medium[19], pH 5.7, 30g/L sucrose, supplemented with 0.2 mg/L BA). The embryos were cultured for five days in darkness at 26 °C. When the seedlings grew to 7—10 mm and their embryo tips and hypocotyls could be clearly identified, the cotyledons were removed and the epicotyls were cut out as explants and collected for infection test after being cultured on the recovery medium (MS supplemented with 0.2 mg/L BA) for three days.

Construction of recombinant vectors and preparation of transformed *Agrobacterium tumefaciens*

EHA105 (*Agrobacterium tumefaciens*), pBI-121 vector and pHANNIBAL vector used in the experiment were preserved in our laboratory; the endonuclease and ligase were purchased from Takara Co., LTD. According to the sequence of chain A of ricin published by Genabank (Accession: DQ661048.1), and through adding the corresponding restriction enzyme cutting site, primers were designed as follows: RTA-F (forward) and RTA-R (reverse), anti-RTA-F (forward) and anti-RTA-R (reverse), as detailed in Table 1. All primers were inspected in GenBank to avoid other homologous sequences. Using the DNA of castor tissues as the template, both the sense and anti sense strands of RTA gene for ricin's Chain A were amplified, and then after digestion, ligated to pHANNIBAL to constitute the RNAi parts (containing the RTA, the intron and the anti-RTA), thus pHAN-RTA vectors were obtained. RNAi parts were then restricted by endonuclease, ligated to the pBI-121 to construct recombinant vectors pBI-RTA-RNAi, and transferred into EHA105 by the method of heat shock to make engineering bacteria. The structures of the vectors are shown in Fig. 1.

The infection of *Agrobacterium tumefaciens* and cocultivation

Agrobacterium tumefaciens was inoculated to 50 ml of activation medium (LB supplemented with Kan 25 mg/L, Rif 50 mg/L), and

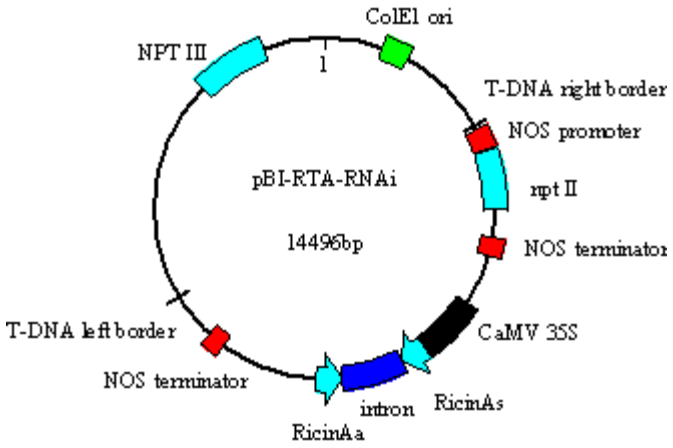


Fig. 1. The Gene Map of the vector pBI-RTA-RNAi

was fully activated under the conditions of 180 r/min, 28°C and darkness. After the activated bacteria liquid was decentered, bacteria were collected and re-suspended in 100 ml of infection solution (sterile water supplemented with AS 20 mg/L, DTT 152.4 mg/L). Then the prepared castor epicotyl explants were immersed in the infection solution, and after 30 min of infection under the conditions of 28°C, 80r/min and darkness, the explants were removed and the extra liquid on the explants was removed with sterilized filter paper. The explants were then inoculated into co-culture medium (MS, pH 5.7, 30 g/L sucrose, supplemented with BA 0.5 mg/L, IBA 0.5 mg/L, AS 20 mg/L, $Na_2S_2O_3$124 mg/L+ DTT 152.4 mg/L, after the culture medium solidified, a piece of sterilized filter paper was placed on top), and were cultured in darkness at 25 °C. By using the above-mentioned procedure, the gradient test was performed by varying the concentration of the infection liquid and the co-culture duration, as illustrated in Table 2.

Regeneration and selection

The co-cultured explants were placed in a sterilized triangle bottle, rinsed with sterile water 3-5 times before being inoculated into the recovery medium (MS supplemented with BA 0.35 mg/L, Cef 300 mg/L) and cultured for five days with a photoperiod of 16/8 at 25°C. Then they were transferred into the shoot induction screening medium (MS supplemented with BA 0.35 mg/L, IBA 0.25 mg/L, Kan 250 mg/L, Cef 300 mg/L), which was replaced once every seven days to induce and screen for resistant shoots. When shoots grew to the length of 2-3 cm (the term is generally four to eight weeks with large individual variabilities), the regenerated shoots were carefully excised from the base with a sterilized scissor, and transferred to the rooting medium (1/2MS medium supplemented with 0.2 mg/L IBA) to induce rooting. Seven to fourteen days later, when a large number of adventitious roots came out of the resistant shoots, the regeneration plants were taken out, and the culture medium clinging on the roots was washed off with autoclaved water, the plants were transferred to pots containing wet soil (the portion of nutrient soil and vermiculite was 1:2). The pots were covered with transparent polythene to keep moisture. The regenerated plants were strengthened in the greenhouse for 5-7 d, after that polythene film was removed, and the plants grew in a greenhouse until mature.

Screening transgenic plants by PCR

DNA was isolated from the leaves of putative transformants by the CTAB method [20]. Vector pBI-RTA-RNAi was used as the positive control, while genomic DNA from untransformed plants was the negative control. Using the primers targeting plasmid's PBI-121 NPT (c-PBI-F (forward) and c-PBI-R (reverse), shown in Table 1), PCR amplification was performed under the following thermal cycling: one cycle at 94°C for 5 min, then subjected to 30 cycles of ampliûcation (94°C for 30 s, 57°C for 1 min, 72°C for 40 s), followed by a ûnal elongation step at 72°C for 10 min. Ampliûed products were electrophoresed on a 1.5% agarose gel with EB, and visualized under ultraviolet (UV) light. Then the transformation efficiency was calculated.

Table 1. Primers used in this study

Name	Sequence of primers (upstream) (downstream)	Annealing temperature (°C)	Product size (bp)
RTA	F:CCGCTCGAGAACAGAGTTGGTTTGCCTAT R:CGGGGTACCAGTTGGAAGCTGAGTGCC	56	369
anti-RTA	F:GCTCTAGAAACAGAGTTGGTTTGCCTAT R:CCCAAGCTTAGTGGAAGCTGAGTGCC	56	368
c-PBI	F:GCGGTTTTTCGCTTCTTGGT R:AGTATTTGGGCAAGGGGTCG	57	520
antin	F:TGATGATGCTCCCAGGGC R:GTGAGAAGCACAGGATGC	57	249
c-RTA	F:GCCTTTGGTGGTAATTATG R:GGTGCGCATCTATACACC	57	431

Analysis of RTA gene expression

200 mg of fresh leaves were taken from all seven positive transformed plants as the test samples, and the WT castors as the control, smashed by liquid nitrogen and RNA was extracted using Plant RNA kit from OMEGA. The extracted RNA was verified by measuring 260 nm absorption for purity. The integrity of the RNA was also examined through 1.5% agarose electrophoresis. 1 ug of RNA was then reverse-transcribed into cDNA (using cDNA Syhthesis Kit from Takara) and diluted using 75 ul of DNase-free water. Using this cDNA as the template, semi-quantitative gene expression analysis was analyzed.

Primers A-F and A-R were designed using castor Actin gene (GeneBank:AY360221.1) as the internal control, then RT-qPCR (Real-time quantitative PCR) amplification was performed using primers RTA-F and RTA-R. The QT-qPCR reaction solution was in the volume of 20 ¼L, containing 1¼L of cDNA, 1¼L of forward and reverse primers, 10¼L of Q-PCR Mix(with SYBR Green I; from Takara), and 7¼L of sterile ddH_2O. RT-qPCR was performed in an iQ5 thermocycler (Bio-Rad), and the reaction was done under the following cycle: 10 min of pre-denaturation at 94 °C; then 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, and elongating at 72°C for 30 s; followed by a ûnal elongation step at 72 °C for 10 min. Products were then run on a 1.5% agarose gel containing EB. The expressions in transgenic plants were then normalized using the WT gene intensity. Each group was repeated 3 times.

Numerical data are presented as mean±SEM. The difference between means was analyzed using one-way ANOVA. All statistical analyses were performed by using SPSS 17.0 software (Chicago, IL, USA). $P<0.05$ was considered signiûcant.

RESULTS

Regeneration System

Several improvements to the transformation method were made in this research. The explants were derived from embryo germination instead of seed germination, and the epicotyls were cut and used as infected explants. This improvement could avoid both variabilities in seed germination progress due to individual variabilities, and effectively reduce pollution. In the transformation process, the shooting induction phase after the co-culture was the critical phase for transformation efficiency, and with the application of this method, the shooting rate (number of shooting explants / total number of explants) could reach 50% (Table 2). Single explant could send forth 3-5 stretchable shoots (herein only stretchable shoots that were used for rooting were calculated and included in the statistic; after shooting induction, some explants could send forth 20 or more shoot points, but these shoot points could not grow into complete plants, therefore they were not included in the statistic).

Castor's clustering shoots could root easily, using of the method reported in this experiment, adventitious shoots could send forth strong roots in one to two weeks, and after seedling strengthening, their survival rate in transplanting could reach 100%. The processes of transformation and regeneration are shown in Fig. 2.

a. Embryos dissected from mature seeds were placed on MS medium. b. Explants 5 d after culture initiation. c. Hypocotyl explants after 3 d of culture initiation. d. Hypocotyl explants of the co-culture stage. e. Adventitious shoots in screening medium. f. Shoot elongation on screening medium. g. Regenerated shoots developed root system in one to two weeks. h. Complete transgenic plant. I. Plantlets were successfully acclimatized in the soil.

Fig. 2. *Agrobacterium tumefaciens*-mediated transformation of castor

Table 2. Influence of different infection stages and co-culture time on the transformation system

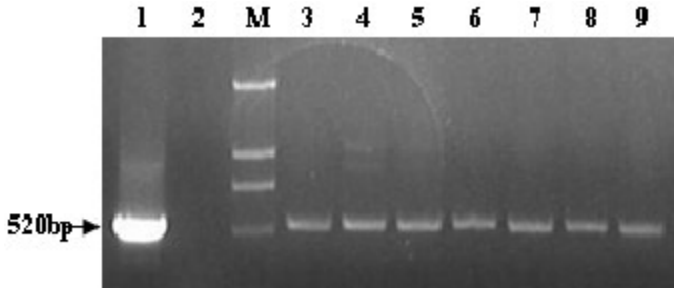
No.	*Agrobacterium* concentration	Co-culti vation period (days)	Total no.of explants	Shoot survival frequency in Resto-ration(%)	No. of Shoot or screening 7 days	No. of shoot for screening 14 days	No. of shoot for screening more than 21 days	No. of Regen--erated plants	No. of PCR positive plants
Q-1	OD600=0.6	3	285	55.2	132	92	35	5	0
Q-2	OD600=0.6	5	272	57.5	141	95	42	4	2
Q-3	OD600=0.6	7	288	38.2	102	73	11	0	0
Q-4	OD600=0.8	3	295	64.6	174	112	48	4	1
Q-5	OD600=0.8	5	283	53.8	113	101	55	6	2
Q-6	OD600=0.8	7	272	35.1	85	42	5	3	0
Q-7	OD600=1.0	3	253	62.8	114	75	31	5	1
Q-8	OD600=1.0	5	283	49.8	115	78	21	3	1
Q-9	OD600=1.0	7	275	28.8	64	25	3	0	0

Optimization of the infection and co-culture phase

Gradient test was performed to the concentration of infection bacteria liquid and co-culture duration in the infection and co-culture phase — the key phase of the infection to optimize the configuration. From the results shown in Table 2, it can be seen that the concentration of infection bacteria liquid had a minor influence on shooting and transformation efficiency, while differences in co-culture duration could to some extent affect shooting and the late transformation efficiency. The longer the co-culture was (especially when the duration was more than five days), the lower the shooting rate was; yet the number of resistant

shoots slightly increased. Finally five days was chosen as the culture duration.

The test confirmed that following co-culture, the addition of a recovery culture phase without kan favored the induction of clustering shoots. 30 regeneration plants were obtained through screening with kan. And through PCR detection, seven positive plants were finally acquired. The result of PCR test is shown in Fig. 3. The best transformation condition was Q-5 (Table 2).

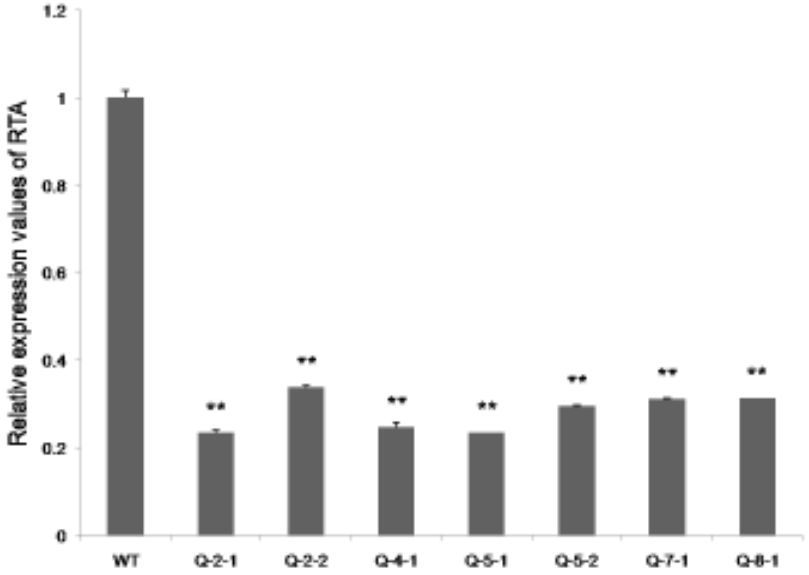


1: PCR detection of PBI121; 2: PCR detection of Wild castor; M: DL2000 DNAMarker; 3-9:Transformation plant of Q-2-1,Q-2-2,Q-4-1,Q-5-1,Q-5-2,Q-7-1,Q-8-1.

Fig. 3. PCR analysis of transgenic plants for the presence of *npt* gene

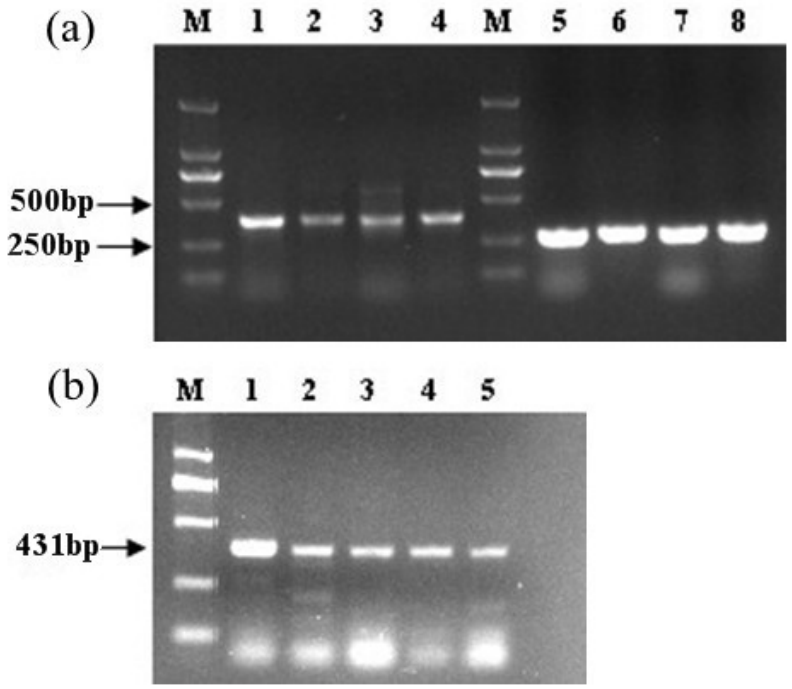
Analysis of RTA gene expression

RTA semi-quantitative analysis was conducted on the seven positive plants, and the average value was removed after three repetitive tests to analyze the interference trend of RNAi. Results demonstrated that the RTA expression levels of seven transformed castors were relatively lower than those of the untransformed of RTA to various degrees (Fig. 4), the effects of interference on Q-2-1and Q-5-1 were the best. The result of agarose electrophoresis also showed that RTA was decreased apparently (Fig. 5). All above proved the effectiveness of the designed interference vectors, and further proved the success of the transformation.



Q-2-1, Q-2-2, Q-4-1, Q-5-1, Q-5-2, Q-7-1, Q-8-1 were 7 transgenic plants. Data represent averages of three experiments; error bars show standard deviations. *Asterisk* indicates that difference is signiûcant at P<0.05 compared with untransformed control (WT).

Fig. 4. RTA relative expression analysis of 7 transgenic plants



(a) M: DL2000 DNAMarker; 1: The RTA PCR result of WT; 2-4: The RTA PCR result of Q-2-1,Q-2-2,Q-4-1; 5: The actin PCR result of WT; 6-8: The actin PCR result of Q-2-1,Q-2-2,Q-4-1.(b) M: DL2000 DNAMarker; 1: The RTA PCR result of Non-transformed plant; 2-4: The RTA PCR result of Q-5-1,Q-5-2,Q-7-1,Q-8-1.

Fig. 5. RT-PCR analysis of the RTA

DISCUSSION

Transformation system

Experiments showed that overlong co-culture could cause reduced explant germination rate as well as increased rate of explant browning and necrosis, while the concentration of infection bacteria liquid had a minor effect on the shooting and transformation rate, as in accordance with the predication. Although the concentration of infection bacteria liquid varied, the growth of plant cells was not largely impacted since the duration of infection was relatively short and the excess fluid was removed after the infection. The co-culture step is actually the major phase of *Agrobacterium tumefaciens* infecting plant cells and co-culture condition is more favorable for the growth of *Agrobacterium tumefaciens*, therefore the longer the co-culture duration is, the greater the theoretical transformation chances will be. *Agrobacterium* infection is inherently harmful to plant cells, a greater infection intensity would lead to more explant deaths, therefore it has a great influence on the shooting efficiency of explants, and might even lead to the necrosis of the entire explants. Thus the identification of appropriate co-culture duration is very important; it can not only ensure the full reaction between *Agrobacterium* and explants but also prevent excessive damage.

In addition to optimization of co-culture duration, the right amounts of AS, DTT and $Na_2S_2O_3$ were stilled added in the infection and co-culture stage. AS is a kind of phenolic compound that has been proved can effectively activate the vir region of *Agrobacterium* to promote the transfer of T-DNA[21]. DTT and $Na_2S_2O_3$, both having reduction functions, were added in the co-culture stage, and to some extent prevented the plant cells from dying of excessive oxidation caused by the infection of *Agrobacterium tumefaciens*. Adding filter paper on the co-culture medium could avoid the over-proliferation caused by direct contact between the bacteria and the medium as well as make it easier to remove *Agrobacterium* after the co-culture[22]. Eventually, five days was determined as the optimal culturing time in this experiment; the duration is in accordance with the experimental result of Sujatha *et al*.[9], but the best co-culture

time was different in our transformation on other varieties. The reason is still unclear—it might be due to the differences in castor genotypes, screening methods or culture media.

Vitrification phenomenon

In the process of regeneration, some of the explants were severely vitrified. Vitrified cells looked translucent or were soaked with loose and fragile textures, and their growth was deteriorated to an almost halt state[23]. As a widespread phenomenon in plant tissue culturing process, vitrification has a lot of causes, and different dominant factors exist in different plants and experiments, therefore there are mixed opinions on the formation mechanism of vitrification phenomenon. Studies have shown that vitrification is mainly related to culturing temperature, lighting time, ventilation and relative humidity in the culturing environment[24], and according to Zvi *et al*.[25], the state of moisture in media is also an important cause for vitrification. Other studies[26-27] have indicated that vitrification of cell tissues is due to the disorders of internal physiology, and that factors like cytokinin concentration and culture temperature have close links with the generation of vitrification.

Vitrification in this system may be caused by the water vapor condensation due to temperature difference, and lowering the rate of it through optimization can further improve the regeneration rate and transformation rate, which is also one of the key aspects in our future research.

Regeneration and transfer of plants to soil

Fortunately, the phenomenon of hard rooting for adventitious shoots, which is common in wheat and soybeans, did not appear in the castor tissue culturing. After the formation of resistant shoots, thick and strong roots came out extremely easily. The transformation of castor has been previously facing a lot of problems especially in the process of transferring the regenerated plants in soils. It was found that the survival of the transferred plants could be significantly impacted by the ratio of soil, humidity and the adaptation of regenerated plants to the external environment. The application of the seedling strengthening transplant method introduced in this article can effectively improve the survival of transplants, and provide a guarantee for the follow-up test.

Efficiency of RNAi interference

Many studies reported the interrelation between the interference efficiency of RNAi and factors including the size of introns in the interference structure and the structures of the interference fragments[28]. In this research, the same RNAi plasmid was transformed, but diversified interference effects were acquired. These problems have also been encountered in other studies, yet the cause is not clear-it could be the difference in expression level, difference in insertion sites, or differences in siRNA levels[29-30]. The overall working mechanism of RNAi is still a mystery, and gene expression itself is a very complex process regulated by many mechanisms. Scientists need to make more efforts to gain a more effective application of this tool.

Cultivation of low-toxicity castor variety

In this research, seven castors with reduced RTA expression were obtained through transformation, and the amount of ricin synthesis was decreased at the molecular level. According to literature review this is the first report of the regeneration of low-toxicity castor variety by genetic transformation, providing a new possibility to cultivate low-toxicity castor variety commercially and to promote the castor oil industry.

ACKNOWLEDGEMENTS

The authors thank Zheng Hu for the help about vector construction, and Jingfang Wei and Grace Chen for their excellent technical help.

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