Transformation of *Brassica napus* with the method of floral-dip

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Abstract

Two double-low rapeseed cultivars *westar* and *Zhong Shuang No.4* (*Brassica napus* L.)were used as recipient materials and the C-terminal fragment of *CRY1*, *CCT1*, fused to *GUS* (*GUS-CCT1*) was transferred with *Agrobacterium*-mediated floral-dip method. The transformation was performed at initial blossom stage. The effects of raceme position and surfactant *Silwet-77* on the transformation frequency were investigated. The results showed that the best branches for dipping treatment were the top inflorescence and the uppermost two first-order branches in the raceme. Three consecutive dippings were conducted on every another day within a 7 day period. The best concentration of *Silwet-77* was 0.05% (v/v) in the transformation buffer. The dipped racemes were bagged for self pollination and the seeds harvested were screened with Kanamycin in culture medium. The Kanamycin-resistant plants were further assayed by *GUS* reporter gene, and then by PCR test with a pair of specially designed primers based on the sequence of *CCT1*. The special DNA band from PCR was reclaimed, cloned and sequenced. The sequence of DNA was compared to *CCT1* on NCBI internet and the homogeneity of sequence was 100%. It was certified that the foreign gene *CCT1* was successfully transferred into the genome of the recipient rapeseed. The results showed that the *Agrobacterium*-mediated floral-dip method can be successfully applied to genetic transformation in rapeseed.

Key words: *Brassica napus* L, *CRY1*, Floral-dip, Transformation

Introduction

The *Agrobacterium*-mediated floral-dip transformation has been widely applied in transformation of target genes and studies on functional genomics in *Arabidopsis.* The floral-dip method for gene transformation is simple and efficient. Tissue culture is not necessary for it. The offspring of the transformed plants were genetically stable (Clough &Bent, 1998; Labra et al., 2004, Chang et al., 1994). The floral-dip method was originally derived from the *in planta* transformation in *Arabidopsis*. The *in planta* method was first adopted for transformation of germinating seeds with *Agrobacterium* suspension in *Arabidopsis* (Feldmann, 1987). The procedure was then mended, using vacuum infiltration with *Agrbacterium* suspension to transform the adult plants (Bechtold al., 1993). Later, this method was successfully applied in transformation of pakchoi (Qing et al., 2000) and *Medicago trumcatula* (Trieu et al., 2000). In 1998, the *'*floral-dip*'* method was developed for transformation of *Arabidopsis* (Clough&Bent, 1998). It was shown that the efficiency of this method was not lower than that of the vacuum infiltration. Studies have shown that the maternal reproductive organs or tissues were the primary target of *Agrobacterium*-mediated transformation with *in planta* and floral-dip method in *Arabidopsis* (Bechtold et al., 2000, 2003; Ye et al., 1999; Desfeux et al.2000; Bent, 2000).

Both rapeseed and *Arabidopsis* are cruciferous species. They have many common features which are convenient for floral-dip transformation. This study was aimed to investigate the possibility to apply the floral-dip method and the factors which may affect the efficiency of transformation in rapeseed.

Materials and Methods

Plant materials

Two cultivars of *B. napus*, *Westar* and *Zhong Shuang No.4*, conserved by Rapeseed Research Center, Sichuan Agricultural University, were used as recipient plants for the transformation. The materials were sown on the experimental farm of Sichuan Agricultural University in October, 2004. The transformation was carried out with *Agrobacterium*-mediated floral-dip method in March, 2005.

Bacterium strain and plasmid

The bacterium strain used in the present study is *Agrbacterium tumefaciens* strain *C58* carrying plasmid *pkyl 71* with *GUS-CCT1*. Both the bacterium and the reconstructed plasmid were provided by the Institute of Plant Physiology and Ecology, Shanghai Institute for Biological Sciences, CAS. The plasmid carries a *GUS* reporter gene and a selectable resistant marker gene *NPTII* which is resistant to kanamycin sulphate. The *CCT1* was the C terminal fragment of *CRY1* which encodes a blue light photoreceptor in *Arabidopsis* (Yang et al.,2000).

Preparation of bacterium suspension

The culture medium of *Agrobacterium tumefaciens* was LB (peptone 10g/L, NaCl 5g/L, autolyzed yeast extracyt 10g/L, pH7.0), supplemented with 50mgl^{-1} rifampicin and 100mgl^{-1} kanamycin sulphate. The bacteria were incubated at 28°C in dark

for 3d. Then a piece of agar with *Agrobacterium* was transferred into 30 ml of LB liquid medium and shaken at 28°C for 2 d. Afterwards 25ml of the bacterium suspension was transferred into 500ml of LB medium with 50mgl^{-1} kanamycin sulphate, and shaken for 24h until the OD600 value was 1.8 to 2.0. *Agrobacterium* was collected with centrifugation at 4000rpm for 15 min. The collected bacterium was then re-suspended with double volumes of transformation buffer medium (Yang et al., 2001).

Plant transformation

The transformation buffer was MS medium +(0.01mol/L) BAP+15%(m/v) sucrose+0.05%(v/v)*silwet-77* (Clough & Bent.,1998) with specific *Agrobacterium*. The inflorescences of rapeseed plants were prepared at initial blossom stage and submerged into the transformation solution for 5s. The treated inflorescences were immediately bagged with sulphate paper bags to keep moist. After 48h and 96h, the inflorescences were dipped again for the second and the third time, respectively. And the dipping treatments were done for 3 or 4 times within 7 days. Finally, these inflorescences were bagged until the end of flowering.

Screening for resistant plants

The optimum lethal concentration of Km (kanamycin) for rapeseed seedlings was screened at first with wild seeds of *Westar* and *Zhongshuang 4*. The transformed seeds were treated with the selected concentration of Km. The rapeseed seeds were sterilized with 75% (v/v) ethanol for 30s, followed by 0.1% (m/v) $HgCl_2$ for 10 minutes. The seeds were then rinsed in sterilized water for 3 or 4 times. The sterilized seeds were spread on a 1/4 MS medium with the selected concentration of Km. Two weeks later, the resistant seedlings were cut from hypocotyl and transferred onto the screening medium again for the second and the third times of screening for resistant seedlings. After 3 times of screening the finally resistant plants were transplanted in the field.

Molecular assay

The Km resistant plants were examined with *GUS* assay and PCR method. The *GUS* test was performed according to Jefferson *et. al*.(1989). For PCR test, total DNA was extracted from young leaves according to Lijian et al (1994). The volume of PCR reaction was 25μl, including 2.5μl 10×buffer, 2.0μl (25mM)$MgCl_2$, 2.0μl (25mM) dNTP, 1.0μl (0.02μM) primer, 2.0μl (200ng) DNA template and 1U DNA Polymerase. The PCR system was 94°C for 1min, 53.5°Cfor 2 min, 72°Cfor 2 min, 35 cycles, and finally 72°Cfor 8min. The special primers were designed with software Primer 5.0 based on the sequence of the C-terminal of *CRY1* gene from *Arabidopsis*. The sequences of the designed primers PCR were:

P1, 5′-GAGTTCCCAAGGGACATT-3′

P2, 3′-ACCATAACCTCCTCCTTC-5′

The sizes of DNA product from the PCR was approximately 413bp. The DNA product was recovered with1.2% agar gel electrophoresis. Then the objective band was cloned with PMT18-T in *E. coli* strain $DH_{5\alpha}$. Finally, the cloned DNA fragment was sequenced, and compared to that of *CRY1* gene on NCBI internet (blast).

Results

The influence of branch position on the transformation efficiency

In the raceme of plant, the average developmental stages of the flower buds on different branches are different. In the present study four branch positions were divided for the observation of treatment effects: i.e., the terminal (top) inflorescence, the first and the second (from top to bottom) first-order branches, the third and the forth first-order branches, the fifth and the sixth first-order branches. The different branch positions were treated at the same time at the initial blossom stage and the transformation frequencies were compared (Table 1). The results showed that the first and the second branches had the highest transformation frequency (1.34%), followed by the top inflorescence (1.00%). The transformation frequencies of the lower branches were remarkably lower. This suggested that the upper branches and the top inflorescence were the best branches for *Agrbacterium*-mediated floral-dip transformation in rapeseed.

Table 1 Transformation frequencies of different branch position

Branch position (from top to bottom)	Branches treated	Seeds harvested	Km resistant plants obtained	Transformation frequency (%)
Terminal inflorescence	14	3805	38	1.00
First and second branches	19	1268	17	1.34
Third and forth branches	31	5420	33	0.61
Fifth and sixth branches	12	2677	15	0.56
Total	76	13170	103	

The effects of surfactant SilwetL-77 *on the transformation efficiency*

The surfactant *silwetlL-77* was commonly used in *Agrbacterium*-mediated floral-dip transformation in *Arabidopsis*. Its main function was to reduce the surface tension of *Agrbacterium* suspension and to assist in the attachment of *Agrbacterium* to the surface of plants. In this experiment, we tested different concentrations of *silwetL-77* to find the best for use in the formal treatment. It was shown in Figure 1 that the best concentration of *silwetL-77* was 0.05% (v/v), which resulted in a remarkably higher transformation frequency than the other concentrations (Figure 1). The concentrations of 0.02% and 0.10%, however, also showed a marked effect on the transformation efficiency compared to the control.

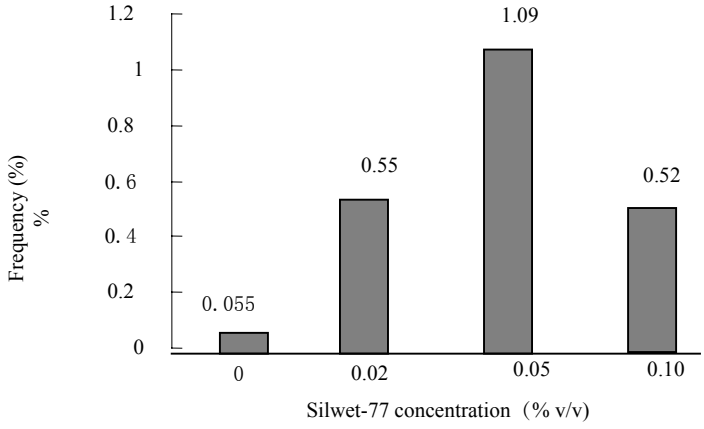
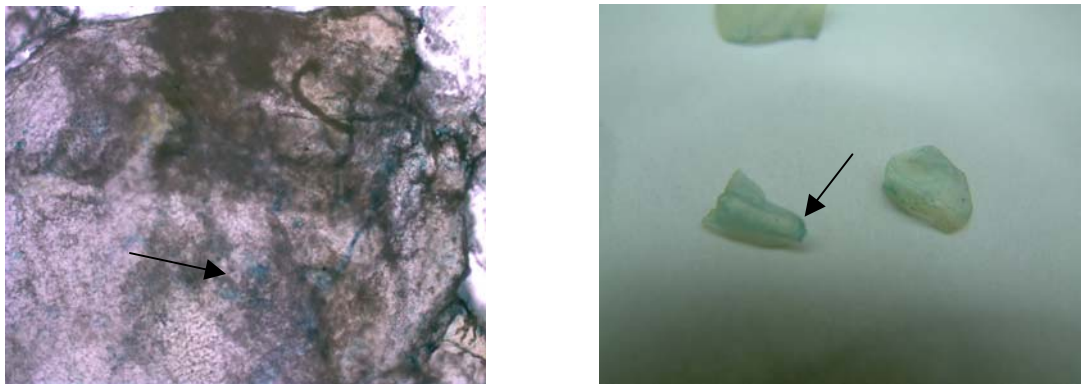


Figure1. Effects of different concentrations of Silwet-77 on transformation efficiency in *B.napus* based on Km resistant plants.

Assay of the transformed plants

Assay of the reporter gene The transient expression of *GUS* gene was first assayed with the young embryos on the treated plants of ***Westar*** (Figure 2, A). The adult plants of the T_2 generation were also assayed for the stable expression of *GUS* gene in young leaves (Figure 2, B). The results showed that the *Agrbacterium*-mediated floral-dip transformation was successful and the target genes (*GUS* and *CCT1*) was successfully integrated into the recipient genome.



A B

Fig.2 Assay of GUS expression in the transformed plants

A: The transient expression of *GUS* gene in the young embryo of *Westar*. The expression of *GUS* was shown in the young embryo one week after the floral-dip treatment. B: Stable expression of *GUS* gene in the leaves of the transformed plants. The expression of *GUS* was detected in the leaves of T_2 generation.

PCR amplification and blast of the C-terminal fragment of CRY1 DNA was extracted from the leaves of the transformed plants and also the wild plants (*Westar*). Special primers were designed based on the C-terminal sequence of *CRY1* Gene. PCR was performed together with DNA samples from the transformed plants, the wild plants (negative control) and the plasmid (*pkyl 71)* DNA (positive control, containing the C-terminal fragment of *CRY1*). A special band of DNA fragment was amplified from both the positive control and the transformed DNA(Figure 3). The special DNA band was then reclaimed and cloned, and sequenced. The sequence of the DNA fragment was 413bp. It was compared to the C-terminal fragment sequence of *CRY1* gene on NCBI internet(Blast). It was 100% homogeneous to the sequence of *CRY1* gene (data not shown). The results demonstrated that the sequence cloned from the transformed plants was identical to the C-terminal sequence of *CRY1* gene from *Arabidopsis*. So the C-terminal fragment of *CRY1* gene from *Arabidopsis* was successfully transferred into *Brassica napus.*

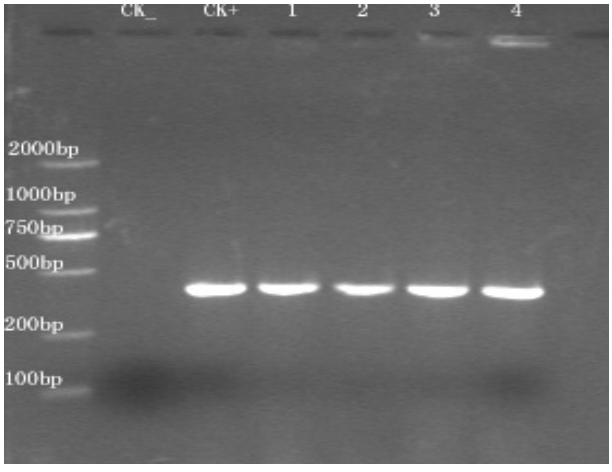


Figure 3 PCR analysis of the transformed plants

Note: CK-, negative control (wild plant); CK+, positive control (plasmid DNA); 1-4, transformed plants.

Discussion

The optimal position for transformation

The mechanism of floral-dip transformation has been studied in *Arabidopsis*, radish and other cruciferous plant. It was shown that the transformation by floral-dip occurred in the germ cells, especially the female gametophytes (Bechtold et al., 2000, 2003;Ye et al.,1999; Desfeux et al. 2000; Bent., 2000). Only in such conditions the target gene transformed can inherited to and be detected in the progeny. It is, therefore, considered that in the floral-dip transformation system the primary factor is to transfer target gene to the germ cells in order to obtain transformed plants (Clough & Bent, 1998). In the present study a big difference in transformation frequency was observed among different branch positions. The uppermost two branches and the top inflorescence showed the highest transformation frequencies. This indicated that at the initial blossom stage the upper branches were at a better development stage for transformation. In another word the optimal branches for floral-dip transformation at the initial blossom stage were the top inflorescence and the uppermost two first-order branches. The main reason might be that the upper branches had more germ cells at the same and the right developmental stage. Furthermore, the upper branches and the top inflorescence were more convenient for bagging. However, it is not known what exactly is the right development stage of the germ cells for floral-dip transformation. Further investigations are necessary to understand the optimal developmental stage for the germ cells in the floral-dip transformation system. Whereas the development stages of flower buds are always different even on the same branch, so we may improve the efficiency of floral-dip transformation through increasing the transformation times to overcome the differences of flower buds in development stages.

The effect of surfactant SilwetL-77 *on transformation efficiency*

Clough et. al. (1998) used surfactant *silwetL-77* to replace vacuum in infiltration transformation. They found that the transformation efficiency was not depressed by *silwetL-77*. So with a proper concentration of *silwet-77*, vacuum was no longer necessary. Curtis and Nam (2001) successfully transformed radish with floral-dip method. Two agents, *Tween 20* and *Plouronic F-68*, were compared to *silweLt-77*. It was found that the transformation efficiency of *silwetL-77* was better than *Tween 20* and *Plpuronic F-68*. In present experiment, we used *silwetL-77* for the transformation experiment, and found that the transformation frequencies of *silwetL-77* treatments were remarkably increased, compared to the blank control (Fig 1). The best concentration of *silwet-77* was showed to be 0.05% (v/v) which produced a much higher frequency of transformation than the other two, 0.02%and 0.1%. It was also found that in high concentration of *silwetL-77* (0.1%) the seed setting was obviously reduced. It is, therefore, suggested that 0.05% (v/v) of *silwetL-77*is sufficient for floral-dip transformation in *B. napus*.

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