

Development of a method for *in planta* transformation in *Brassica napus* L.

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Abstract

Plant transformation is known to be an important research tool for cultivar improvement and in plant biology. The transfer of foreign genes in many species including of *Brassica* family has been performed mainly by tissue culture method, that is reported to produce the high percentage of escaper regardless of cultivars though labor-intensive. But *in planta* transformation, especially floral dipping method, is used commonly in *Arabidopsis* with relative high efficiency and simple procedures. Its application is now extending into other crop such as packchoi, radish, and the legume *Medicago truncatula* by its advantages. Here we report that transformants can be successfully obtained in *B. napus* using *in planta* transformation methods with similar transformation efficiency as in *Arabidopsis*.

Introduction

The capacity to introduce and express many foreign genes in plants, first described for tobacco in 1984, has been extended to over 120 species in at least 35 families (1). This method is revealed to be a powerful experimental tool, allowing direct testing of some hypotheses in plant biology that have been exceedingly difficult to resolve using other biochemical approaches.

In tissue culture systems for plant transformation, what is most important is a large number of regenerable cells that accessible to the gene transfer treatment, and that will retain the capacity for regeneration for the duration of the necessary target preparation, cell proliferation, and selection treatments. But the somaclonal variation and high percentage of escapers are now more a barrier of gene transfer systems, although is considered a potentially useful source of genetic variation for plant improvement. Particle bombardment method using meristematic tissue of excised embryonic axes, shoot proliferation to yield some lines with transformation of germline cells have the limiting factors, that is, the ability to mechanically preparation of the explants, transfer genes into regenerable cells, and select of screen for transformation at an efficiency sufficient for practical use in cultivar improvement .

The floral dipping method, the simplest transformation system in non-tissue culture approaches, is known to be a submergence of developing floral tissue into a solution containing agrobacterium, improved from vacuum infiltration methods using *Arabidopsis* (2, 3, 4). Recently the successful transformation of relative non-small-sized plants such as Packchoi, the radish (*Raphanus sativus* L.) and the legume *Medicago truncatula* is reported (4, 5, 6), although some aspects such as small plant size, rapid generation time, and high seed yield per plant are prerequisites for this methods. Here, we report the possibility that the simply modified floral dipping method can be used successfully in the transformation of rapeseed plant

Material and Methods

Mokpo-CGMS and Mokpo 64 plants (*Brassica napus* L. Mokpo 64) which were exhibited a range of floral development and three *agrobacterium tumefaciens* strain carrying the binary vector pCAMBIA 3301 carrying bar and gus gene were used in transformation studies. The protocol for floral dipping transformation described by Clough and Bent (1998) was used with minor modification. Agrobacteria cell were resuspended in filtration media contained 1X B5 salt, 5% sucrose, 0.02-0.05% Silwet L-77. The flowers of Mokpo 64 plants to have been

fertilized were eliminated, and agrobacteria resuspended in filtration media were sprayed on the whole plants several times at intervals of 3-4 days. The transformants were selected in subsequent generation by basta (~0.01%). The other method for *in planta* transformation, was used the immature pollen grains in order to transfer the agrobacteria into receive plants. Mokpo 64 pollen grain were harvest, and then suspended into distilled water under sterile condition, passing the suspended pollen solution through a 59 µm nylon mesh, and washing the pollen suspension twice with distilled water. Finally, the pollen was resuspended into infiltration media containing agrobacteria, and then incubated for 12 hr at room temperature in dark with gentle shaking. After 12 hr, the pollen grain was used to fertilize the Mokpo-CGMS plants. The transformants were selected in subsequent generation by basta.

Results and Discussion

Transformation by modified floral-dipping method

The application of *in planta* transformation methods now extend to other crops due to the simple procedures, time saving and its reproducibility. The small size plants are submerged into infiltration media containing agrobacteria in floral dipping method, which is focused on the flower organ. Here, we changed the dipping procedure to the spray procedure because of the large size of rapeseed plant. To supply agrobacteria enough, the infiltration solution containing agrobacteria was sprayed into whole plant several times with some intervals. Three agrobacteria strains were chosen for this transformation study, which were used in tissue culture transformation in other important crops, and *in planta* transformation in arabidopsis. The seed production from 40 plants respectively was decreased by increase of agrobacteria virulence (Table. 1). In case of agrobacteria II commonly used in arabidopsis *in planta* transformation, the number of basta resistant plant was increased although the seed production was the same level as in case of agrobacteria III. This result is supposed that there is the preference of *A. tumefaciens* in transformation of *B. napus*. A recent literature survey of *A. tumefaciens* vectors used of transformation of *B. napus* reveals that octopine strains are generally reported to be ineffective in this process compared to nopaline strains (7).

Table.1 Transformation efficiency by modified floral dipping method.

Agrobacteria	Seed production (T1 seed, g)	The number of Basta resistant plants / 1600 T1 plant	Efficiency (%)
I	649.5	2	0.1
II	462.2	9	0.6
III	431.4	2	0.1

The Basta resistant plants were selected by ~0.01% Basta solution among T1 plants.

Transformation using pollen

Because the floral dipping method was developed on basis of the sexual organ, we focused more on the usage of pollen grain than any other organ to reduce the number of plant to be transformed. Previously reported pollen transformation requires the pollen containing the foreign DNA to be placed onto a receptive stigma of a flower, and the pollen will translocate the DNA to the ovule upon fertilization. There is currently no evidence about nuclear incorporation of the foreign DNA by pollen nuclei or translocation of foreign DNA as part of the cytoplasmic constituents of the germinating pollen grain, but transformations are rescued. On basis of that, we used this pollen as a mediator to transfer agrobacteria containing foreign DNA to receptive flower. The agrobacteria treatment of pollen collected from Mok-po 64 plants were treated with three different methods. First, the pollen or anther collected from Mok-po 64 applied to the stigmas of Mokpo-CGMS flowers, and then these stigma were inverted into agrobacteria II infiltration solution contained binary vector carrying bar and gus gene for a few seconds. Second, some drops of the agrobacteria infiltration

solution were applied on the stigma of Mokpo-CGMS flowers. After 3 hr, the drop had dried on the stigma, the stigma were crossed the pollen from Mok-po 64 plants. Third, pollen was collected from Mok-po 64 flowers, washed with distilled water under sterilization condition, and then incubated agrobacteria infiltration media for 12 hr at room temp. in dark. After the incubation, the stigmas of Mokpo-CGMS flowers were submerged into this solution for 2-3 seconds. In case of first and second methods, the subsequent seed or basta resistant plant was not acquired. Basta resistant T1 plants were selected only in case of agrobacteria and pollen co-culture, not showing the gus staining in pollen (Table. 2). Transformation by pollen incubated with the agrobacteria showing O.D₆₀₀ of above 1.0 was less effective, though seed production was increased compared to agrobacteria showing O.D₆₀₀ of below 1.0. These results are supposed that pollen can translocate the agrobacterium containing foreign DNA into the ovule.

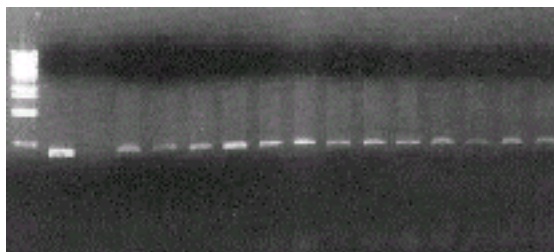
Table.2 Transformation efficiency by transformation using the pollen grain

Agrobacteria	O.D ₆₀₀	Basta resistant plant / total seeds	Efficiency (%)
II	> 1.0	3/231	1.3
	< 1.0	2/365	0.55

PCR analysis of Bar gene

In PCR analysis using primer for bar gene, DNA fragments of expected size of about 600 bp in length were amplified from the total genomic DNA for the putative transgenic plants (Fig.1). These fragments were not detected in genomic DNA from wild type. The preliminary Southern blot analysis of these putative transgenic plants showed the 1 or 2 copy bar gene integration (data not shown).

Fig.1 PCR of putative transgenic plants (T1), amplified about 600 bp Bar gene.



Lane 1: size marker (lambda DNA digested by Bst EII enzyme)

Lane 2 : pCAMBIA 3301 (positive control).

Lane 3: wild type (negative control)

Lane 4 ~ Lane11: DNA from the leaves of 8 T1 generation plants transformed by modified floral-dipping method

Laen 12 ~ Lane 16 : DNA from the leaves of 5 T1 generation plants transformed using pollen.

Now these putative transgenic plants are T1 generation, so will be assayed the incorporation and inheritance of T-DNA into genomic DNA, GUS staining and Mendelian segregation on T2 generation.

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