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BRIEF COMMUNICATION

Exploration on the vacuum infiltration transformation of pakchoi

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Abstract

Agrobacterium tumefaciens mediated vacuum infiltration transformation *in planta* has been established in pakchoi, a kind of Chinese cabbage, but the transformation frequency in harvested seeds has varied in the range of 0.5 to 3.0×10^{-4} over several years and is much lower than the transformation frequency in *Arabidopsis thaliana*. To understand that, the distribution and vitality changes of *A. tumefaciens* in plant tissues were examined. Results revealed that there was a majority of *A. tumefaciens* in the flower compared with that in the stem and in the leaf at all times after infiltration. As fact of transformants in the upper part of the treated plant (T_0) stalk and fact of the survival of *A. tumefaciens* in the plant were proved, possibilities of optimizing the transformation conditions to increase the transformation frequency in pakchoi was discussed.

Additional key words: *Agrobacterium tumefaciens*, *Arabidopsis, Brassica rapa* ssp. *chinensis.*

The genetic improvement of plant by *A. tumefaciens* has become a common technique in the last decades. The standard tissue culture approach is that the explants were co-cultured with *A. tumefaciens* under *in vitro* conditions and transformants were identified from regenerated plants (Yang *et al*. 2007, Guo *et al*. 2007, Nandakumar *et al*. 2007). This is genotype-dependent due to *in vitro* culture, especially for those recalcitrant species such as Chinese cabbage (Narasimhulu *et al*. 1988, Zhang *et al*. 2000). There has been a continuous effort to find alternative methods for plant transformation. *In planta* transformation was first introduced in *Arabidopsis* using germinating seeds (Feldmann *et al*. 1987). Later a variety of *in planta* methods have been developed in *Arabidopsis* (Bechtold *et al*. 1993, Chang *et al*. 1994, Katavic *et al*. 1994, Clough *et al*. 1998, Richardson *et al*. 1998). Then the transformation frequency increased from original about 0.3 % to 0.4 - 3 %. These simple, transformation techniques have been utilized intensively in reverse genetic experiments, positional cloning, and the insertional mutagenesis (Azpiroz-Leehan *et al*. 1997, Krysan *et al*. 1999, Clough *et al*. 2000). A number of laboratories have tried to use *in planta* transformation on other plant species. Although many failed, it has succeeded in pakchoi (Liu *et al.* 1998), *Medicago truncatula* (Trieu *et al*, 2000) and radish (Curtis *et al.* 2001). The transformation frequency in radish is similar with *Arabidoposis*, but it differs greatly for pakchoi (Liu *et al*. 1998) and *Medicago truncatula* (Trieu *et al*. 2000). To clarify the biological mechanism of *in planta* transformation, research on the site and timing of transformation has been done by three independent research groups (Ye *et al*. 1999, Desfeux *et al*. 2000, Bechtold *et al*. 2000). All the results in *Arabidopsis* showed that the ovule is the target of transformation. The striking correlation between the closure time of the ovary and the siliques that gave transformants was specifically addressed (Desfeux *et al*. 2000).

Chinese cabbage (*Brassica rapa*) is an important vegetable crop. Its *in vitro* transformation is not easy due to the genotype dependent shoot regeneration capacity and the sensitivity of explants to *Agrobacterium* (Zhang *et al*. 2000). Establishing a simple and high frequency

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transformation technique will greatly benefit research in genetic modification and the genome of this important economical crop.

The plant material used was *Brassica rapa* ssp. *chinensis* var. *utilis*, cv. 49 Caixin, a very early-maturing cultivar of pakchoi originating from southern China. This flowering plant is 30 - 40 cm high and is easy to use in a vacuum infiltration chamber. Plants with non-elongated inflorescences and younger flower buds or plants with elongated inflorescences and a few opened flowers were used for transformation. The *Agrobacterium tumefaciens* strain C58C1(pMP90) (Koncz *et al*. 1986) contained the binary vector pBBBast-pin II or pBBBast-gus-intron where the phosphinothricin resistance gene *Bar* and proteinase inhibitor gene *Pin* II or the intron-containing *Gus* gene all were under the control of CaMV 35S promoter. The constructed *Agrobacterium* strains were resistant to 100 mg dm^{-3} kanamycin, 50 mg dm^{-3} rifampicin and 100 mg dm^{-3} gentamycin. Vacuum infiltration transformation was done following the procedure formerly reported (Cao *et al*. 2000). In the *Gus* gene expression experiment, the plasmid pBBBasta-gus-intron was used, otherwise the plasmid pBBBasta-pin II was used. At harvest time, the flower stalk was divided into two parts: one third of the inflorescences from the bottom are referred to as the lower part and the remaining two thirds are referred to as the upper part and includes all lateral branches. Siliques from the two parts were collected in bulk separately. Transformants were selected by spraying *Basta* solution 3 times (0.75 g dm^{-3} phosphinotricin) on the seedlings every week in one week intervals.

Relative quantity and viability changes of *A. tumefaciens* in different plant organs were studied by counting the clone formation units (CFU) *in vitro*. The stem, leaf, or complete inflorescence (1 g) were sampled and washed 5 times with autoclaved water then ground with a mortar with 3 cm^3 0.8 % (m/v) NaCl solution. In an attempt to minimize the possible different physiological conditions in the plant tissues, at least 3 parts of each plant were used for sample preparation. The extracted sap was diluted to 10^{-7} with 0.8 % (m/v) NaCl solution, and then inoculated on medium at 0.2 cm^3 per dish with 3 repeats. The culture medium was special for *A. tumefaciens* growth (Fang 1998), and contained 100 mg dm^{-3} kanamycin. The culture was grown at 28 °C. The clone formation units (CFU) were counted after 4 d of culture using a stereomicroscope. To explore the viability changes of *A. tumefaciens* in the plant, the stem, leaf, and inflorescence were sampled 2, 6, 9, 14, and 19 d after infiltration treatment. Clone identity was checked by polymerase chain reaction (PCR). Plasmid DNA was extracted from a randomly chosen *A. tumefaciens* clone for the PCR reaction as described by Sambrook *et al*. (1989). The primers used for *bar* gene amplification were: 5′-AAC TTC CGT ACC GAG CCG CA-3′ and 5′-ATG CCA GTT CCC GTG CTT GA-3′.

The transformation frequency in the harvested seeds was 0.1 ~ 0.3 ‰ over several years (Table 1). It is much lower than the frequency in *Arabidopsis* transformation which is about 0.4 to 6.0 % (Bechtold *et al*. 1993, Ye *et al*. 1999). Regarding the transformants produced per treated plant (referred to as the transformation efficiency of plants), the data varied a lot in different experiments (Table 1).

In the spring, the plants were sensitive to low temperature and flowered easily. The vegetative growth period was short and resulted in a relatively small plant whose growth recovery after infiltration was not vigorous when followed by a higher temperature environment. In the autumn, the vegetative growth period was longer, which resulted in a bigger plant. The growth recovery was better when followed by a cool temperature environment, and more seeds were collected. The transformation efficiency of plant was ten times higher in the autumn than in the spring. This suggests that good recovery and good seed setting of the treated plants provides a significant advantage for transgenic seeds production. Our observations also revealed that transformation in pakchoi was not consistent: some T_0 plants produced no transformants, whereas others were transformed at a relatively high frequency.

To clarify the distribution of transformed seeds on the T_0 plant stalk, seeds were collected in bulk from the upper and lower part of the stalk separately and screened by *Basta* resistance. Results show that transformants could come from both the upper parts and the lower parts of the infiltrated plants. More transformants were found from

Table 1 Transformation frequency in three experiments in spring and four experiments in autumn of vacuum infiltrated pakchoi. Transformation frequency of seeds was designated as the number of *Basta* resistant T_1 plants *versus* the total number of seeds assayed. Transformation efficiency of plants was designated as the number of *Basta* resistant T_1 plants *versus* the number of surviving T_0 plants after infiltration.

	Spring			Autumn			
	1	2	3	1	2	3	4
Number of T_0 plants	29	44	57	16	16	11	30
Number of survived T_0 plants	16	35	49	15	15	10	29
Number of seeds per plant	350	1043	388	2902	2741	6098	2010
Transformation frequency of seeds [$\times 10^{-4}$ %]	1.79	0.55	2.10	1.84	2.67	1.31	2.23
Transformation efficiency of plants [%]	3.45	4.54	7.02	53.33	73.33	80.00	44.83

Table 2. Distribution of transgenic seeds on the T_0 plants.

Experiment	Upper stalk number of seeds assayed	number of resistant seedlings	transformation frequency	Lower stalk number of seeds assayed	number of resistant seedlings	transformation frequency
1	15748	11	7×10^{-4}	769	3	3.9×10^{-3}
2	31282	10	3×10^{-4}	3993	9	2.3×10^{-3}
3	27890	11	4×10^{-4}	1329	3	2.3×10^{-3}
4	35632	12	3×10^{-4}	4360	9	2.1×10^{-3}
Average	27638	11	4×10^{-4}	2613	6	2.3×10^{-3}

seeds harvested from the upper parts, but the transformation frequency in the lower parts was about 4 times higher than that in the upper parts of the treated plants (Table 2). Seeds in the lower part came from flower buds that had already matured at the infiltration time, while seeds in the upper part came from newly developed flower buds that were small at infiltration time. Since pakchoi contains indeterminate inflorescences, it provides an indeterminate number of target organs for transformation. Our experiments also showed that *Basta* resistant seedlings could come from T_0 plants with unelongated inflorescences and small flower buds, as well as from T_0 plants with elongated inflorescences and a few opened flowers. The transformation frequency in both cases was about 0.08 %. Although transformants could be found in infiltrated T_0 plants with just emerged inflorescences, the transformation frequency was about 0.04 %. The results indicate that: *1*) *A. tumefaciens* mediated transformation in pakchoi could happen in a long period of plant development after infiltration; *2*) the transformation events happened more frequently in the short period directly after infiltration than in the longer period of plant growth after infiltration.

Distribution and vitality changes of *A. tumefaciens* in infiltrated pakchoi plants were examined. Histochemical assays of paraffin sections revealed that *A. tumefaciens* did not distribute equally in different organs and tissues of the treated plant. There were more green particles (stained *A. tumefaciens*) in flowers than in the stem and leaf. The quantity of *A. tumefaciens* in different organs was surveyed by counting the clones formed (referred as CFU) on the *A. tumefaciens* culture plate inoculated with the extracted sap from different organs. The formed clones were identified by PCR of plasmid DNA by the sequence of the *Bar* gene. Amplification results show that all of the 30 separate clones randomly chosen had the specific 400 bp *Bar* gene fragment. Combining the kanamycin selection medium, we have concluded that the clones formed on the medium were the engineered *A. tumefaciens* used in the infiltration transformation.

The CFU of flower samples 2 d post infiltration were several-fold higher than that of the stem or the leaf (Table 3). This result suggested that *A. tumefaciens* infiltrated easier into the flower tissues compared with the stem and leaf. The quantity of living *A. tumefaciens* and their distribution is important to the transformation event. More live *A. tumefaciens* in flower of pakchoi after infiltration could be due to easier entrance to the flower than the stem and leaf, or the micro-environment in flower is better for *A. umefaciens* fitness.

Table 3. The clone formation units (CFU) in different organs of pakchoi plants and different days post *A. tumefaciens* infiltration. DPI stands for days post infiltration. Each value represents the mean of three independent experiments.

DPI	CFU [$\times 10^3$ g^{-1}(f.m.)] stem	leaf	flower
2	150.40	1780	11070
6	20.15	622.1	626.2
9	9.80	2.3	958.2
14	0.18	0	65.4
19	0	0	0.326

Further assays were done to show changes in *A. tumefaciens* vitality in the plant in a period of time post infiltration. Results showed that the live *Agrobacterium* in plants were significantly decreased 6 d post infiltration, and were sharply decreased 14 d post infiltration. In spite of variable datum, the tendency was that *A. tumefaciens* in plants decreases with time in the organs examined. There were always more CFU in flower samples than in stem or leaf samples (Table 3). The lack of order in the datum reflects the variation among different samples. Chung *et al*. (2000) reported that no matter which transformation method was used in *Arabidopsis*, the transformation ratio was the highest in the seeds collected within 3 - 4 weeks post treatment and dropped significantly after five weeks. This work also showed that the transformation rate was increased two-fold when the floral-dip treatment was increased from 5 s to 5 min and increased about 2.5-fold when floral sprays were increased from one to three times. These results suggest that a method for keeping or increasing the quantity and vigor of *A. tumefaciens* in the plant would be an efficient means for increasing transformation frequency. A large quantity of live *A. tumefaciens* during a relatively long period post infiltration would result in more chances for *A. tumefaciens* to come in contact with the target plant cell.

The goal of this study was to explore vacuum infiltration transformation in pakchoi, and to compare the

results in pakchoi and in *Arabidopsis* to clarify the general features and the differences among different plants. Our work in pakchoi confirms the observations in *Arabidopsis* that *A. tumefaciens* mediated transformation could happen in a relatively long period during plant development and the transformants distributed discontinuously on the plants. Furthermore, detailed data in pakchoi about *A. tumefaciens* distribution and the vitality changes in infiltrated plants showed that flower buds had a higher quantity of *A. tumefaciens* than leaves and stems after infiltration, and it showed a relatively higher number of living *A. tumefaciens* even after 9 d of treatment.

As both *Arabidopsis* and pakchoi have an indeterminate inflorescence, there will always be some flower buds whose pistils are at the right developmental stage for transformation. So if it is ensured that there is a significant quantity of living *A. tumefaciens* around these tissues, there should be a consistently higher transformation frequency. In pakchoi, transformation frequency could rise to 0.39 % if seeds from the completely non-transformed plants were not included (Table 2). This frequency is close to that seen in *Arabidopsis*. Therefore, it appears that favorable conditions may increase the *A. tumefaciens* induced transformation frequency in pakchoi.

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