

Gene transformation in *Brassica* sp. using particle bombardment technique.

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ABSTRACT

Gene transformation in Brassica sp. was studied using particle bombardment. Cotyledon and hypocotyles of different species of Brassica have been used as target explants. Transient expression of uidA gene has been obtained when either been constructed with CaMV35S or Actin promoters. The highest expression was recorded between 10 to 15 hours after bombardment. Plasmids pBI121, pBI221 and pDM803 were used to carry uidA gene. Further transformation events should be carried out to obtain highest transformation efficiency.

Key words: gene transformation, bombardment, *Agrobacterium*

INTRODUCTION

Brassica is one of the most agronomically important crops in the world. Oil produced by this crop makes 10% of total edible oil consumption in the world. However, productivity of oil seed crops is severely hampered by the infection of different kinds of diseases such as Alternaria blight [*Alternaria brassicae* (Berk) Sacc., *A. alternata* (Fries) Keissler]. The loss in productivity of rapeseed and mustard due to Alternaria blight ranges from 30-70% depending on severity of the infection. Efforts have been done by conventional breeding methods to develop Brassica varieties which could resist damage due to these fungi. However, there is no variety yet, which carries resistance against this disease at a satisfactory level. Chemical method, on the other hand, can prevent from spreading of the disease to a certain extent but it is very expensive and is eco-unfriendly. With the advent of modern plant biotechnology methods, these limitations can be circumvented. Biotechnology methods offer new ideas and techniques to improve crop productivity and nutrient values. Genetic structure of the crops can also be engineered to improve their resistance to insects, diseases and abiotic factors.

During the past ten years, with the fast growth of recombination DNA technology, several genes have been identified, isolated and modified to study their function and regulation. Such modified genes are then

introduced into the defined recipients for obtaining expected plants. Recently, fertile transgenic plants of *Brassica* sp. carried genes of interest were produced by electroporation of protoplast (Bergman and Glimelius 1993), *Agrobacterium* (Narasimhutu 1992) and biolistic transformation (Chen and Beverdorf 1994). The present study was undertaken to investigate some preliminary parameters which could provide some basic ideas for the long term strategy leading to complete success in obtaining fertile transgenic plants of *Brassica* species resistant to *Alternaria brassicae*.

OBJECTIVE

To find out several suitable factors in transformation of Brassica tissues using microprojectile bombardment.

MATERIALS AND METHODS

i) Source of explants:

Cotyledons and hypocotyls of *Brassica campestris* cv. PT303., *B. cv. Divya*, *B. oleracea* were used as target explants

ii) Source of Bacterial strains:

XL1 Blue MRF strain was purchased from Clontech Co. with *uid A*, *lac* and ampicillin resistant genes for selection. This 2.88 kb XL1 Blue MRF strain also containing pUC18 polylinker cloning sites. The *uidA* gene was driven by *CaMV35S* and *Actin* promoters. SOB and SOC media were used to grow bacterium for DNA extraction.

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All chemicals were analytical grade purchased from Hi-media, Qualigene, Strategene, Sigma and Japan.

The biolistic apparatus (PDS-1000/Helium device, BIORAD, USA).

Steps for coating gold particles:

An aliquot of gold particle suspensions (60mg/ml) was added into a sterile eppendorf tube.

A 1ml of 70% ethanol was added into the tube, vortexed vigorously and kept at room temperature for 50min.

Particles were pelleted by centrifugation at 13000rpm for 2 min. The supernatant was removed and 1ml of sterile distilled water was added.

Centrifugation was done at 13000rpm for 2min to remove supernatant. The above step was repeated once and the pellet was collected.

Add 1ml of 50% sterile glycerol solution and vortex vigorously on cyclomixer to keep particle suspended.

Add the following items in the order 10 μ l DNA (0.1 μ g/ μ l), 50 μ l of 2.5M CaCl₂, 20 μ l of 0.1M fresh spermidine. Gently mixed and incubated on ice for 10min.

The coated particles were pelleted by centrifugation at 13000 rpm for 5 seconds. Supernatant was carefully removed.

The particles were washed with 140 μ l of 70% ethanol then with 140 μ l of 100% ethanol.

The pellet was then suspended in 50 μ l of 100% ethanol. It was ready for loading on macrocarrier disks.

Spreading of DNA coated particles on macrocarrier disks:

DNA coated particles was withdrawn from the suspension stock and spreaded on macrocarrier disks, dried in a desiccator and fixed to the macrocarrier holder for bombardment.

Preparation of explant targets

Cotyledon and hypocotyle segments were precultured in MS medium containing 0.6% agar and NAA (0.5mg/l) + BAP (1.0mg/l) for 2 days in dark before bombardment. Explants were arranged in a 2cm diameter circle. Samples plates were placed at different distance in the device. The bombarded plates were incubated in dark and taken for transient expression assay. A bombardment without DNA (only gold particles) represented negative control plates. Gus expression was examined at every 5 hour intervals by staining randomly bombarded tissues with X-Gluc.

RESULTS AND DISCUSSIONS

1. Effects of types of plant tissue on transient expression

Results in table 1 showed that *Brassica oleracea* exhibited the highest level of GUS activity in all samples taken for histochemical analysis. Numbers of blue spots developed on the tissues were able to count due to very dense aggregates of particles hit on a small area of tissues. Hypocotyls of cv. Divya exhibited comparatively higher GUS activity than hypocotyls of cv. PT303. Cotyledons from these cultivars, in contrast, did not show remarkable GUS expression, hardly few cotyledons were transformed. The transformed cells exhibited transiently GUS activity at low level in the first 5 hours after bombardment, and gradually increased in the next 5 hours before reaching the highest levels at 15 hours and got plateau at 20 hours in all tissues. The staining pattern of these tissues suggested that cauliflower possessed a suitable regulation system which could be in *Brassica* cotyledons. In addition, a "pachy" distribution and dark blue color of GUS expression on cauliflower tissues made it difficult to quantitate efficiency of transformation events. This also suggested that a further refinement in coating and spreading of DNA bearing particles should be improved to obtain an even distribution of particles and to minimize damage to the tissue. In this regard, attempts have been made to study effects of distance between the stopping plate and target cells (data not shown). It is observed that the highest number of blue spots was at the middle distance (7cm) tested followed by the shortest distance (3cm). A negative correlation between transformation efficiency and increased distance of target cells was observed at 10.5cm. The results are consistent with earlier reports by Oard et al. (1990) that they could not detect GUS expression at 10.5cm whereas a highest activity was recorded at 7cm distance on bombardment of rice calli. Typically, very few numbers of blue spots were observed in the central zone of the tissue while expression was higher in the area between 1-2 cm zone. The result was contrary to the observation by Klein et al. (1988) in which the central zone from 4-9cm² in tobacco leaf targets were hit by a large numbers of DNA-coated particles. Beyond this area only few blue cells were seen. The difference could be attributed to the selection of different devices. While the bombarded tissues with

their own apparatus, we used PDS/1000-He device made by BIORAD, USA.

Table 1: Transient expression of GUS gene on various *Brassica* tissues by microprojectile bombardement

Tissues used	Expression of GUS gene at time intervals				
	5h	10h	15h	20h	24h
<i>B. camp cv. PT303</i>					
Hypocotyls	+	+	++	++	++
Cotyledons	-	-	+	+	+
<i>B. juncea cv. Divya</i>					
Hypocotyls	+	++	+++	+++	+++
Cotyledons	-	+	++	++	++
<i>B. oleracea</i>					
Hypocotyls	+	+++	+++++	+++++	+++++
Control	-	-	-	-	-

Means of two repeats
Control: shot w/o DNA
pBI 121: 10 µg/ 60mg Au particle

2. Effects of different promoters on transient expression of GUS gene

The *actin* promoter derived from rice was widely employed in monocot transformation. Therefore, it is interesting to study the relative effectiveness of this promoter in promoting GUS expression in dicot family as compared to *CaMV35S* promoter.

Tissues of cv.PT303 and cv. Divya as well as cauliflower were selected as targets and placed at 7cm distance from the stopping plate in the device. Helium pressure was at 1100 psi. Results in the table 2 showed that there was no distinct difference between these two promoters in expressing of *uid A* gene in all three *Brassica* species. Cauliflower again exhibited maximal expression as compared to other two cv.PT303 and cv. Divya. The highest expression of *uid A* gene was observed at 16 hours and got plateau at 24 hours in all cases. Frequency of GUS expression was low in cotyledons of both cv. PT303 and cv. Divya whereas higher GUS activity was recorded in hypocotyls regardless of their respected promoters. The observation under microscope revealed that all particles have penetrated into phloem of the hypocotyl tissues. Therefore, blue color has diffused to larger distance along the length of segments. As many as 100% of the length of cotyledons and hypocotyls have developed blue color indicated that a higher number of cells has been transformed and expressed. GUS activity driven by the two different promoters remained same in all *Brassica* species tested. Thus, it is possible to use *actin* promoter in transformation of dicot plants. Different expressions observed on different tissues may be due to the regulatory systems which may inhibit the full potential of promoters. For

example, Twell et al. (1989) reported that LAT51 promoter derived from tomato could direct GUS expression better in tomato pollen than did the *CaMV35S* (fused with pBI221). Otherwise, bombardement of pBI221 on tomato anther and petal showed highly transient expression of *uid A* gene, whereas the same could not detect if pLAT52 was used. Variation of fidelity in GUS expression could be explained in term of tissue specific and regulatory factors which regulate the whole plant. The satisfactory activity of any promoter is, therefore, greatly dependent on such factors (Boston et al. 1987). Regarding to the high intensity of blue spots which covered whole tissue surface, it is felt that, beside essential refinements in coating and spreading of DNA-coated particles, the concentration of gold particle should also be reduced. An ideal bombardement should be designed in such a way that particle can enter cells but still preserve their integrity for subsequent stable recovery. High intensity of blue color in these experiments suggested that a very high velocity impact of dense particles has hit on a small area of the tissue. As a result. A large damage for the tissue without inducing satisfactory transformation occurred. Therefore, an optimal protocol for such parameters is very essential for getting high number of cell to be transformed rather than more number of copies entering one cell.

Thus transient expression obtained by microprojectile bombardement in this and previous experiments has indicated that *uid A* gene can be used as a reporter gene in transformation of *Brassica campestris* cv.PT303, *B. juncea* cv. Divya and *B. oleracea*. The presence of transformants can be detected by histochemical analysis of GUS

expression. Furthermore, an equally efficient promoter can also be employed to promote potential of the *actin* promoter in direction of GUS expression in dicot plants, particularly in the expression of *uid A* gene as compared to *Brassica campestris*, *B. juncea*, and *B. oleracea*. *CaMV35S* promoter suggested that the *actin*

Table 2: Effects of different promoters on GUS expression in different *Brassica* tissue through microprojectile bombardment

Tissues used	Expression of GUS gene at time intervals					
	8h		10h		24h	
	35S	Act	35S	Act	35S	Act
<i>B. campestris</i> cv. PT303						
Hypocotyls	+	+	++	++	++	++
Cotyledon	+	+	+	+	+	+
<i>B. juncea</i> cv. Divya						
Hypocotyls	+	++	++	++	+++	+++
Cotyledon	+	+	+	+	+	+
<i>B. oleracea</i>						
Hypocotyls	++	++	+++++	+++++	+++++	+++++
Control	-	-	-	-	-	-

Mean of two repeats; Control: shot w/o DNA ; pBI121: 10 μ g/ 60mg Au particle; 35S: *CaMV35S* promoter; Act: *actin* promoter

Though we obtained the highest transient expression between 10 to 15 hours after bombardment of *uidA* gene which has been constructed with either *CaMV35S* (in pBI121/pBI221) or with Actin promoters (in pDM803), an optimal condition for DNA, particle concentration and stable transformation events need to be further studied.



Fig 1: Micro projectile bombardment facility (BIORAD)

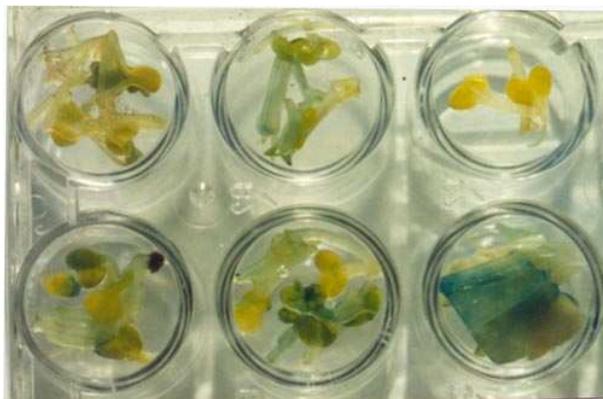


Fig 2: GUS expression

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SUMMARY IN VIETNAMESE

Nghiên cứu chuyển nạp gen vào mô cải dầu bằng kỹ thuật bắn gene

Cotyledon và hypocotyls của cải dầu được sử dụng làm vật liệu thí nghiệm. Gene UidA được mang bởi plasmid pBI121, pBI221 hoặc pDM803 và dưới sự điều khiển của promoter CaMV35S hoặc Actin đều được thể hiện cao nhất trong mô cải dầu ở 10 đến 15 giờ sau khi chuyển nạp. Promoter Actin không những hoạt động tốt trong cây 1 lá mầm mà còn có cả loại cây 2 lá mầm. Tuy nhiên cần nghiên cứu thêm 1 số nhân tố khác để đạt được hiệu quả cao nhất trong việc chuyển nạp gen.
