Indian J. Genet., **61**(4): 298-300 (2001)

Studies on factors involved in transfer of foreign gene by pipetting *Agrobacterium* into spikelets of wheat

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(Received: September 1998; Accepted: August 2001)

Abstract

Studies were conducted on wheat cv. UP 2338 for various factors involved in gene transfer by pipetting *Agrobacterium* suspension into florets using indirect pollen system. Pollen grains showed germination after 20 min of pollination that was considered as an optimum time for *Agrobacterium* inoculation. After 20 min of pollination 5µl of *Agrobacterium* (LBA4404 containing binary vector pBI121) suspension was pipetted into the florets. Gram negative staining for *Agrobacterium* showed presence of pink colour after 20 min of *Agrobacterium* inoculation and at later stages some parts of pistil and pollen took pink colour. The seeds obtained after *Agrobacterium* inoculation into the florets showed 5.5% putative transformation frequency when screened against 500 mg/l kanamycin. Based on phenotypic observations it can be concluded that it is possible to transfer foreign gene via pollen through *Agrobacterium*.

Key words : *Agrobacterium*, pollen, transformation, *Triticum aestivum*

Introduction

Plant cell biology and molecular biology have a major impact on agriculture by supplementing the present activities of breeding process. By using various vector mediated and direct DNA delivery methods transgenics have been developed in different crop species. Pollen mediated transformation has been discussed for many years as an alternative gene transfer procedure which is not restricted by any host range or tissue culture limitations. The unique biology of pollen appears to make it ideally suited as a target cell for transformation. But, pollen preparations usually show high DNase activity. Therefore, it seemed better to use *Agrobacterium* pollen mediated transformation [1]. The present investigation was carried out to study the various factors like pollen and pollen tube development for determining the best time of *Agrobacterium* inoculation for pollen mediated gene transfer and phenotypic selection of putative transformed seeds.

Materials and methods

The wheat variety UP 2338 was used in the present study. *Agrobacterium* strain LBA4404 with binary vector pBI121 was used which contained *gus* reporter gene under the control of 35s promoter and nptII selectable marker gene under the nopaline synthase (NOS) promoter. The strain LBA4404 obtained in the form of either stab culture and/or glycerol stock were streaked on YEP medium plates (Yeast extract - 10 g/l, peptone - 10 g/l, Sodium chloride - 5 g/l, pH 7.0) which was further supplemented with antibiotics (50 mg/l kanamycin + 50 mg/l streptomycin + 50 mg/l rifampicin). The plates were sealed with parafilm and incubated at 28°C for 2 days in dark. The *Agrobacterium* suspension was prepared by picking single colony from the YEP medium plate and inoculated in 5 ml of liquid YEP medium containing antibiotics. Cultures were incubated for a day on a shaker with 100 rpm. The O.D. of the culture was measured at A-600 nm. The culture was then centrifuged at 2500 rpm for 10 min. The supernatant medium containing antibiotics was discarded and the bacterial pellet was resuspended in YEP liquid medium without antibiotics and maintaining an O.D. of 0.1.

Pollen Germination Studies - For the study of pollen germination stages, the pollinated spikes with the pollen of same variety were collected at intervals of 5, 10, 20, 30 min and 1, 2, and 8 h. These spikes were fixed in 1:2 lactoalcohol for 24 h and preserved in 70% alcohol. These were stained with cotton blue for 10-15 sec and destained with 1:1:1 mixture of 40% lactic acid : orthophosporic acid and distilled water and mounted in pure lactic acid.

Agrobacterium Staining - Florets after 20 min of pollination were inoculated with 5µl *Agrobacterium* suspension by pipetting while control experiments were conducted by pipetting 5µl medium into the florets. The *Agrobacterium* inoculated florets were collected at

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different time intervals of 20 min and 1, 2 and 8 h. for *Agrobacterium* staining. Gram negative staining method was used. Whole pistils were stained with crystal violet for 30 sec, rinsed with distilled water and decolourized with 95% alcohol for 10-20 sec. After proper time interval counter staining with safranin for 20-30 sec was done which was followed by staining with cotton blue. The presence of pink colour indicates Gram negative bacteria.

Pollen mediated transformation - 5µl of *Agrobacterium* suspension was pipetted into all the florets after 20 min of pollination while controls were inoculated with *Agrobacterium* YEP medium. The spikes were then covered with butter paper bags.

Screening of grains - Seeds obtained after treatment with *Agrobacterium* or medium were surface sterilized with 40% commercial sodium hypochlorite solution for 20 min followed by 5 rinses with sterile distilled water. Grains were initially allowed to swell for 2 h in distilled water and then placed in sterile petridishes containing sterilized distilled water for 2 days. *Agrobacterium* treated seeds were placed in Elisa plates (6 × 4 wells) with 0.5 ml of WH medium of Wagner and Hess [1] containing 500 mg/l kanamycin for 4 days. Control seeds obtained after YEP medium inoculation were put in the same way in WH medium alone and also WH medium with 500 mg/l kanamycin. Uninoculated control seeds were put in the same way in water and water containing 500 mg/l kanamycin. After 4 days, surviving green plantlets were transferred to tubes containing same medium in which they were grown in Elisa plates.

Results and discussion

Pollen germination - For pollen based transformation studies it is important to determine the best time for *Agrobacterium* inoculation so that foreign gene can be transferred via pollen as a vector. To determine at what time pollen starts germinating, pollinated spikes were collected at different time intervals. Maximum pollen fall was recorded after 5 min and the lowest after 30 min of pollination. Pollen grains showed some swelling after 10 min of pollination. After 20 min of pollination, pollen grains showed germination with an average germination of 45.4%. The emergence of pollen tubes in the hairy stigma and the bifurcated style was seen after 30 min of pollination. There was a constant increase in pollen tube growth after 1, 2 and 8 h of pollination. Germination of pollen grains in wheat reported to start after 5 min of pollination [2]. However, our studies indicate pollen tube emerges from thick pollen wall after 20 min of pollination.

Agrobacterium staining - Gram negative staining for *Agrobacterium* was followed at different time intervals after 5 µl of *Agrobacterium* has been inoculated into the florets after 20 min of pollination. Pistils were first stained with Gram negative staining followed by staining with cotton blue for pollen. The parts of the pistil that took pink colour indicate the presence of Gram negative bacteria. After 20 mm. stage pink colour was seen on the edges of pollen, which indicate attachment of *Agrobacterium*. At a later stage of 1h some parts of stigma and pollen took pink colour, while after 2h the whole stigma with pollen tube became pink which indicate the presence of *Agrobacterium*.

Table 1. Screening of wheat seeds with kanamycin obtained after *Agrobacterium* or medium inoculation into the florets

Treatment	No. of seeds	Screening	WH + 500 mg/l kan	WH medium	Water + 500 mg/l kan	Water
A.t (pBI121) inoculated	475	1st	129 (27.1)	-	-	-
		2nd	26 (5.5)			
A.t medium inoculated	100	1st	10 (10)	-	-	-
		2nd	1(1)			
	100	1st	-	84 (84)	-	-
		2nd		84 (84)		
Untreated normal seeds	100	1st	-	-	9 9)	-
		2nd			1 (1)	
	100	1st	-	-	-	87 (87)
		2nd				87 (87)

A.t Agrobacterium tumefaciens; WH - Wagner and Hess medium; kan - kanamycin, values in parentheses indicate percentage

Screening of grains - The seeds obtained after inoculation of florets with either *Agrobacterium* or medium were screened against kanamycin antibiotic (Table 1). Seeds obtained after *Agrobacterium* inoculation when screened against 500 mg/l kanamycin showed 27.1% survival after 1st screening of 4 days and 5.5% plants survived after second screening. Seeds obtained after *Agrobacterium* medium inoculation when put on kanamycin containing medium showed 1% survived after 2nd screening. Only one plant, which survived, showed very restricted growth. Seeds germinated on WH medium alone showed normal growth. Uninoculated normal seeds when grown in

water and water with kanamycin showed 87 and 1% survival respectively. This screening was based on phenotypic appearance of plants. De la Pena [3] injected DNA into the young floret tillers of rye and succeeded in getting single plant resistant to kanamycin. However, this could not be repeated. It was reported that DNA is inactivated by nuclease action before it enters the pollen. In a modified approach Hess *et al.* [1] used *Agrobacterium* to transfer T-DNA via pollen as a vector to overcome the nuclease action on DNA and reported 1.04% transformation frequency in wheat. Transgenic wheat plants were obtained with pollen tube pathway method when a drop of 100 mg/l plasmid (pBI121) DNA solution was delivered to the end of the styles [4]. Based on phenotypic observations it can be concluded that it is possible to transfer foreign gene via pollen through *Agrobacterium.*

References

1. **Hess D., Dressler K. and Nimmrichter R.** 1990. Transformation experiments by pipetting *Agrobacterium* into the spikelets of wheat (*Triticum aestivum* L.). Plant Sci., **72**: 233-244.

2. **Watanabe Y.** 1955. Studies on the cytological instabilities of common wheat. 1. The meiotic abnormalities of dwarf wheat, Norin 10, with special reference to the appearance of tall plants. Jap. J. Breed., **4**: 76-76.

3. **De la Pena A., Lorz H. and Schell J.** 1987. Transgenic plants obtained by injecting DNA into young floral tillers. Nature, **325**: 274-276.

4. **Zeng I. Z., Wang D. T., Wu Y. Q., Zhang J., Zhou W. J., Zhu X. P. and Xu N. Z.** 1994. Transgenic wheat plants obtained with pollen tube pathway method. Science in China Series B, Chemistry, Life Sciences and Earth Sciences, **37**: 319-325.