Biolistic and *Agrobacterium*-Mediated Transient Expression of *Uid*A in Triticale Immature Embryos

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Abstract: The development of a protocol to transform a recalcitrant species like triticale (×*Triticosecale* Wittmack), based on biolistic or *Agrobacterium tumefaciens*, requires the identification and optimisation of the factors affecting DNA delivery. We have used immature embryos from the triticale doubled-haploid line 'ATOPE-22' in order to compare the transient expression of the *uidA* gene delivered by both biolistic and *Agrobacterium* systems. Particle bombardment experiments were made with gold micro-particles (1 μm diameter) coated with the plasmid pAHGUS, using Biolistic® PDS-1000/He (BioRad) equipment. The conditions that gave the maximun number of foci per immature embryo bombarded were 1100 psi of helium pressure and 6 cm of shooting distance. With *Agrobacterium*, we analysed the time required by the embryos in pre-culturing and the time of inoculation with the *Agrobacterium* strain AGL1 harbouring the plasmid combination pAL154 and pAL156 which was based on the pSoup/pGreen series. The times required to produce the maximum number of foci per immature embryo were 0.5 hours and 2–3 hours, respectively.

Keywords: *Agrobacterium*; triticale; GUS; biolistic

Genetic transformation of recalcitrant crops is a challenging process only achieved by a few laboratories worldwide. The development of a protocol to transform a recalcitrant species like triticale through particle bombardment or *Agrobacterium* transforming systems, needs the identification and optimisation of the factors affecting DNA delivery into tissue from which whole plants can be regenerated. It is only a decade since the recalcitrance of this crop to transformation was overcome and the first report of fertile adult transgenic triticale plants by biolistics were published (Zimny *et al*. 1995). However, research to make the *Agrobacterium*-based transformation method amenable to cereals has continued as the system is perceived to possess several advantages over other forms of direct transformation including the ability to transfer large segments of DNA with minimal rearrangement. The *Agrobacterium* based transformation system permits the precise insertion of fewer copies of inserted transgenes and lower cost than other methods (Amoah *et al*. 2001).

The most commonly used explant for wheat and triticale transformation is the immature scutellum, a specialised tissue that forms part of the seed embryo. It is amenable to both biolistic and *Agrobacterium*-mediated DNA delivery methods and can be readily induced to form embryogenic callus (Jones 2005). The aim of this study was to analyse the influence of pre-culture times, helium pressures, and shooting distances (biolistic method) and the duration of preculture and inoculation time (*Agrobacterium*-mediated transformation) in the transient expression of the gene *uidA* in triticale immature embryos.

MATERIAL AND METHODS

Plant material. 'ATOPE-22' is a doubled haploid line of triticale obtained by *in vitro* androgenesis from the intervarietal hybrid Torote × Presto fol-

Table 1. Mean and standard desviation (SD) of the three replications of each transitory expression experiments with the gene *uid*A in immature embryos of triticale

Pre-cultured (hours)	Helium pressure (psi)	Shooting distance (cm)	Foci/immature embryo (± sd)
0	1100	6	18.9 (2.4)
24	1100	6	8.9 (4.4)
48	1100	6	11.0 (2.0)
72	1100	6	5.6 (2.0)
0	1100	9	10.2 (3.0)
24	1100	9	2.8 (1.7)
48	1100	9	7.9 (3.7)
72	1100	9	2.9 (1.6)
0	1800	6	4.8 (1.2)
24	1800	6	4.9 (2.4)
48	1800	6	0.6 (0.1)
72	1800	6	1.2 (1.4)
0	1800	9	0.8 (0.7)
24	1800	9	2.0 (1.0)
48	1800	9	1.9 (1.5)
72	1800	9	1.9 (1.5)

lowing the protocol described by González *et al*. (1997). This line was used as mother plants for obtaining immature zygotic embryos.

Plasmids. pAHGUS (Figure 1) was used in the biolistic experiments (Rubio *et al.* 2004*)*. The binary vectors pAL154/156 (Figure 2) based on the pGreen/pSoup system (Hellens *et al*. 2000) were used in *Agrobacterium*-mediated transformation. The *uid*A or *gus* gene encoding β-glucuronidase, is driven by 35 S CaMV promoter in pAHGUS and by the maize Ubiquitin-1 promoter in pAL156.

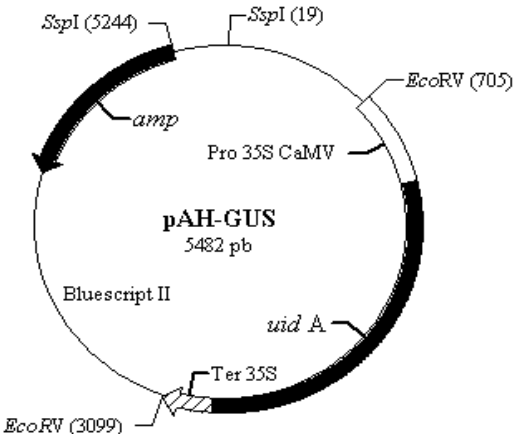

Fig. 1. pAHGUS plasmid used to in bombardment experiments

Particle bombardment. Preculture, osmotic treatment of immature embryos, preparation of DNA particles and DNA mix were done following the protocol described by Rubio *et al*. (2004). Plates containing the target tissue were placed 6 or 9 cm below the stopping mesh. Particle acceleration was accomplished using Biolistic® PDS-1000/He (BioRad) device at 1100 and 1800 psi. After bombardment, immature embryos were maintained on the osmotic protectant medium for 24 h and then transferred to MS medium (Murashige & Skoog, 1962) for a further 24 h.

Agrobacterium-*mediated transformation*. This was performed according to Wu *et al*. (2003), using *A. tumefaciens* strain AGL1 (Lazo *et al*. 1991) harbouring pAL154/156. The embryos were kept on induction medium containing Timentin for 2 days.

GUS assay. All of embryos were incubated at 37°C overnight in GUS substrate mixture as described by Jefferson (1987).

Statistical analyses. These were performed using the Statgraphics Plus (version 7.1) software package.

RESULTS AND DISCUSSION

After histochemical staining, the number of blue spots (foci) that every immature embryo showed

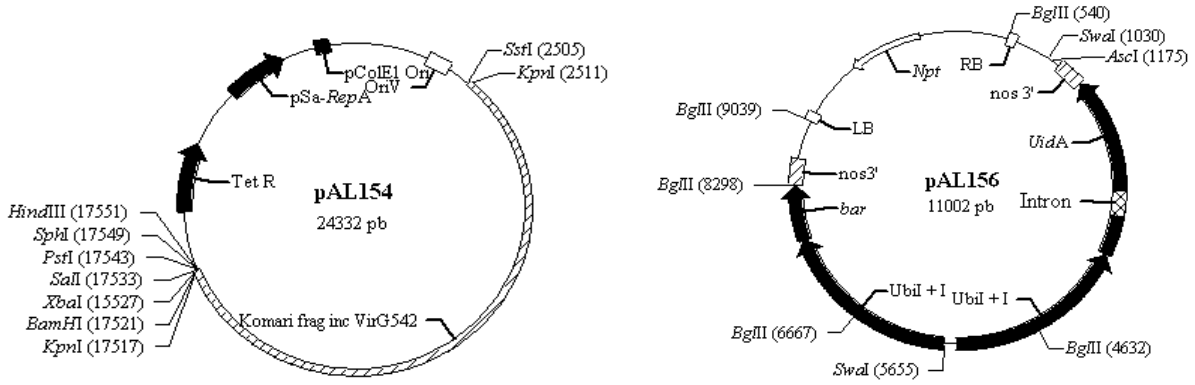

Fig. 2. pAL154/156 plasmids used to *A. tumefaciens* mediated transformation. DNA-T from pAL156 includes *bar* gene and *uid*A (GUS) gene with an intron within the open reading frame (to prevent expression in *Agrobacterium*). Both genes are driven by the maize Ubiquitin1 (Ubi1) promoter plus Ubi1 intron

was assessed and the number of foci per embryo calculated in each of the replications (three replications per experiment). A total of 90 immature zigotic embryos were used for every experimental condition. The mean of number of foci per embryo in every replication was transformed by the function arcsine $\sqrt{\alpha}$, $\alpha = x/100$ to normalise the distribution. With the normalised data, ANOVA was performed for each of the studied parameters (Table 3).

Rasco-Gaunt *et al.* (2001) published that in transformation experiments using immature embryos, it is normal that the latter undergo pre-culture for

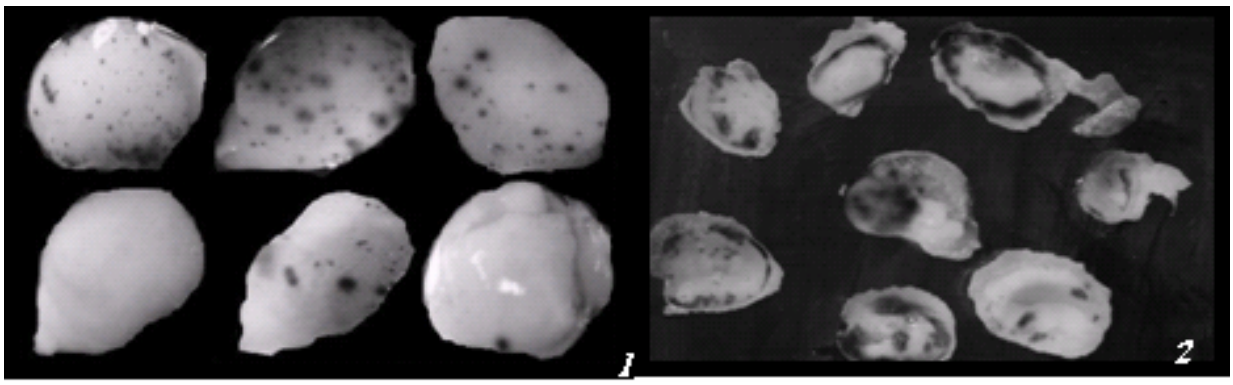

Figure 3. Immature embryos of line 'ATOPE-22' of triticale which were transformed with different conditions by particle bombardment (1) or *Agrobacterium*-mediated transformation (2)

Table 2. Mean and standard deviation (SD) of foci's number obtained from different inoculation and preculture times in triticale line 'ATOPE-22'

Preculture time	Inoculation time			
	1.5 h	2 h	3 h	Mean
0.5 h	14.13 (2.87)	17.22 (9.44)	14.80 (2.50)	15.38 (1.62)
1 h	0.17 (0.20)	7.75 (4.77)	11.75 (2.90)	6.56 (5.88)
3 h	1.60 (1.50)	3.87 (2.05)	7.73 (1.75)	4.40 (3.09)
24 h	0.10 (0.10)	1.10 (1.90)	0.40 (0.70)	0.20 (0.20)
Mean (SD)	4.00 (6.79)	7.48 (7.04)	8.67 (6.23)	7.00 (4.74)

Table 3. One way ANOVA for the variables analyzed

Variable	Source of variation	df	Sum of squares	Mean square	*F*-ratio	Significant level
Pre-culured	between groups	3	253.85	84.62	2.169	0.105
	within groups	44	1716.64	39.01		
	total	47	1970.48			
Shooting distance	between groups	1	159.76	159.76	4.059	0.049
	within groups	46	1810.72	39.36		
	total	47	1970.48			
Helium pressure	between groups	1	830.09	830.09	33.483	0.000
	within groups	46	1140.40	24.79		
	total	47	1970.48			
Preculture time	between groups	3	1933.52	644.50	18.64	0.000
	within groups	32	1106.54	34.58		
	total	35	3040.06			
Inoculation time	between groups	2	372.56	186.28	2.30	0.116
	within groups	33	2667.50	80.83		
	total	35	3040.06			

1 or 2 days. Then, in our particle bombardment experiments (Table 1, Figure 3), the immature embryos were pre-cultured in a MS medium containing 2 mg/l 2,4-D for 0, 24, 48 and 72 h, immediately before osmotic treatment and microbombardment. ANOVA indicates there were no differences between the different pre-treatments and the level of transitory expression of *uid*A in the immature embryos (Table 3). The helium pressure and the distance between the ejection point of the microparticles and the target cells influence the entry of DNA into the cells and the damage caused. In the present study, significant differences in the expression of *uidA* gene were observed with respect to the helium pressures settings (1100 and 1800 psi) (Table 3). With the lower pressure, the mean number of foci per embryo was 8.5 while for the second it was 2.3. Significant differences were also seen when the distance between the stopping screen and the immature embryos was 6 or 9 cm (Table 3). With the former distance, the number of foci for immature embryos was 7.0 with the latter it was 3.8. After different trials, the optimal conditions that gave the maximun number of foci per immature embryo bombarded was determined to be 1100 psi of helium pressure and 6 cm of shooting distance. This combination yielded 11.1 foci. The worst combination was 1800 psi and 9 cm. It yielded 1.65 foci per immature embryo.

In the *Agrobacterium* experiments, we analysed the time required by the embryos in pre-culturing and the time of inoculation with *A. tumefaciens* (Figure 3, Table 2). ANOVA indicated a significant difference between the different preculture times and the level of expression of *uid*A gene in immature embryos but there was no significant difference for different inoculation times (Table 3), although we could observe that there was an increasing of transitory expression of GUS when the inoculation time was greater, as was reported by Wu *et al*. (2003). The conditions to produce the maximum number of foci per immature embryo were 0.5 h for the preculture time and 2 to 3 h for the inoculation time of *Agrobacterium*-mediated transformation.

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