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Agrobacterium-mediated transformation and plant regeneration of *Triticum aestivum* L.

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Abstract

The use of two *Agrobacterium tumefaciens* strains for transformation of *Triticum aestivum* L. cv. Vesna was studied. Immature embryos, isolated 15 d after pollination, were co-cultivated with the super-binary LBA4404/pTOK233 and the binary AGL1/pDM805 vectors. While the transient GUS-intron expression was high (69.9 and 80.0 %), the number of plants regenerated on selective media containing hygromycin or phosphinotricin did not exceed 0.4 and 0.13 %, respectively. Nevertheless, the regenerated plants were fertile and produced seeds. The T_0 plants, as well as the T_1 seedlings, displayed the activity in the β-glucuronidase histochemical assay and a positive signal in PCR analysis for the presence of *uidA* gene sequences in their genomes. The data suggest that the transformation of wheat cv. Vesna with both *Agrobacterium* strains is feasible.

Additional key words: *bar* gene, GUS expression, *hpt* gene, immature embryos, regeneration, transgenic plants.

Introduction

The success in plant genetic transformation depends on efficient *in vitro* regeneration system and on reliable procedure for gene transfer. Cereals have generally been considered as most recalcitrant species in both respects (Vasil 1994). Important advances have been achieved using the microprojectile bombardment of embryogenic tissues, and first fertile wheat transgenic plants were obtained by Vasil *et al.* (1992). In the same time the attention of several authors was turned to the use of *Agrobacterium* for gene transfer in monocotyledonous crops, which resulted in successful transformation of rice (Chan *et al.* 1993, Hiei *et al.* 1994), maize (Ishida *et al.* 1996), barley (Tingay *et al.* 1997, Wu *et al*. 1998), and sugarcane (Enríquez-Obregón *et al.* 1998). Cheng *et al.* (1997), Xia *et al.* (1999), Khanna and Daggard (2003), and Wu *et al.* (2003) were also successful in producing transgenic wheat plants of different cultivars, using *Agrobacterium* as a vector. To apply this technique in wheat breeding, it is important to use wheat lines or cultivars that are suitable for cultivation in particular regions in different countries. We report here the genetic transformation of a novel spring wheat cultivar Vesna selected for its valuable agronomic traits in the Center at Zaječar. Based on the results of previous study (Mitić *et al.* 1999), Vesna was chosen among several domestic cultivars for its high *in vitro* regeneration capacity.

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Abbreviations: GUS - β-glucuronidase; MS medium - Murashige and Skoog (1962) medium; MS-co - cocultivation medium; MSL-inf - infection medium; MS-reg - regeneration medium; MS-root - rooting medium; MS-sel - selection medium; PCR - polymerase chain reaction.
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Materials and methods

Plants and tissue culture: Immature seeds of spring wheat (*Triticum aestivum* L. cv. Vesna) were collected 15 d after pollination from plants grown in the experimental field. The plants were self-pollinated by hand and protected afterwards by paper bags till harvesting. Immature seeds were surface sterilized and the embryos, about 2 mm long, were isolated aseptically under a stereomicroscope, and used as target explants for transformation. The media used during inoculation and culture of embryos are listed in Table 1.

Table 1. Supplements [mg dm^{-3}] to the media used for culture and transformation of wheat embryos; all media contained MS salts and vitamins, 4.5 g dm^{-3} agar except MSL-inf, 30 g dm^{-3} sucrose, 100 mg dm^{-3} inositol; pH 5.8.

Substance	MSL-inf	MS-co	MS-sel	MS-reg	½ MS-root
Acetosyringone	20.0	20.0	0	0	0
2,4-D	2.0	2.0	2.0	0.5	0
Cefotaxime	0	0	300.0	300.0	300.0
Hygromycin	0	0	25.0	25.0	25.0
PPT	0	0	1.0	1.0	1.0

***Agrobacterium* strains:** Two *A. tumefaciens* vectors were used: *a*) LBA4404, harbouring the super-binary plasmid construct pTOK233 (Hiei *et al.* 1994) with the kanamycin-resistance gene (*nptII*), the hygromycin-resistance gene (*hpt*), and the β-glucuronidase (GUS-intron, *uidA*) gene (Fig. 1*A*), and *b*) AGL1, carrying the binary plasmid pDM805 (Tingay *et al.* 1997), containing phosphinothricin (PPT) acetyl transferase (*bar*) gene, and the β-glucuronidase (GUS-intron, *uidA*) gene (Fig. 1*B*). The LBA4404/ pTOK233 was maintained on agar solidified AB medium (Chilton *et al*. 1974), supplemented with 50 mg dm^{-3} hygromycin. The AGL1/pDM805 was cultured on MG/L medium (Garfinkel and Nester 1980), containing 20 mg dm^{-3} rifampicin and 5 mg dm^{-3} tetracycline. Prior to inoculation, samples of both strains were grown at 27 °C for 48 h. A single bacterial colony was transferred to suspension, were centrifuged and the bacterial pellet

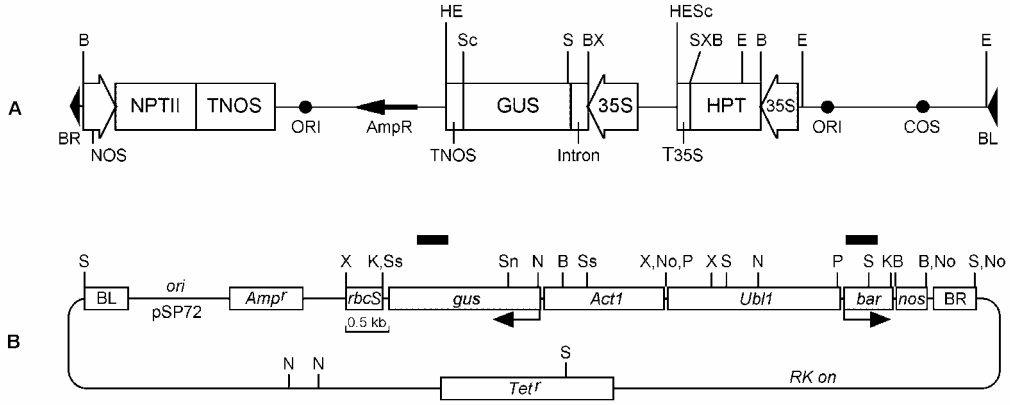

Fig. 1. T-DNA regions of (*A*) the super-binary vector pTOK233 (Hiei *et al.* 1994) and (*B*) the binary vector pDM805 (Tingay *et al.* 1997).

resuspended in 10 cm^3 of MSL-inf medium. 100 μM of acetosyringone was added to the suspension, 1 h before infection of immature embryos.

Co-cultivation and selection: Immature embryos were immersed in bacterial suspension for 15 - 30 min, while the control embryos were soaked in bacteria-free MSL-inf medium. After infection, the explants were blotted dry with sterile filter paper and placed on the MS-co medium, at 27 ºC for 3 d in the dark. Then the explants were transferred to MS-sel media and incubated at 25 ± 2 °C, in white fluorescent light, with irradiance of 47 μmol m^{-2} s^{-1}, and the 16-h photoperiod. After 6 weeks of culture the survived calli were transferred to MS-reg medium. Regenerated plantlets were cultured on ½ MS-root medium.

β-Glucuronidase assays: β-Glucuronidase enzyme activity (GUS-assay) was determined histochemically in immature embryos and leaf and root segments of regenerated plants, after overnight incubation at 37 °C, using X-gluc as a substrate (Jefferson *et al.* 1987). Green leaves were soaked in 96 % ethanol for removing chlorophyll. The presence of blue sectors was examined under a stereomicroscope.

PCR analysis: The genomic DNA was isolated from leaves of the putative transformants using CTAB extraction method (Xiaomei *et al.* 1994). The PCR analysis was performed using the primers GUS 392 (5'-TAGCGGGACTTTGCAAGTG-3') and GUS 22 (5'-GTTTTTGCAGCAGAAAAGCC-3'), which ampli-

fied a 366 bp fragment of the *uidA* coding region. PCR reaction was carried out at 95 °C for 4 min, followed by 30 cycles of 30 s each, at 94 °C, at 60 °C, and at 72 °C, respectively, and finally at 72 °C for 5 min, in a *Techne* (Cambridge, UK) thermocycler.

Results

After 3 d of co-cultivation, the immature embryos infected with both LBA4404/pTOK233 and AGL1/pDM805 displayed a high transient GUS expression, 69.9 and 80.0 %, respectively. The blue sectors were mainly present in the scutellum tissue (Fig. 2), but blue coloured embryo axis and root meristem

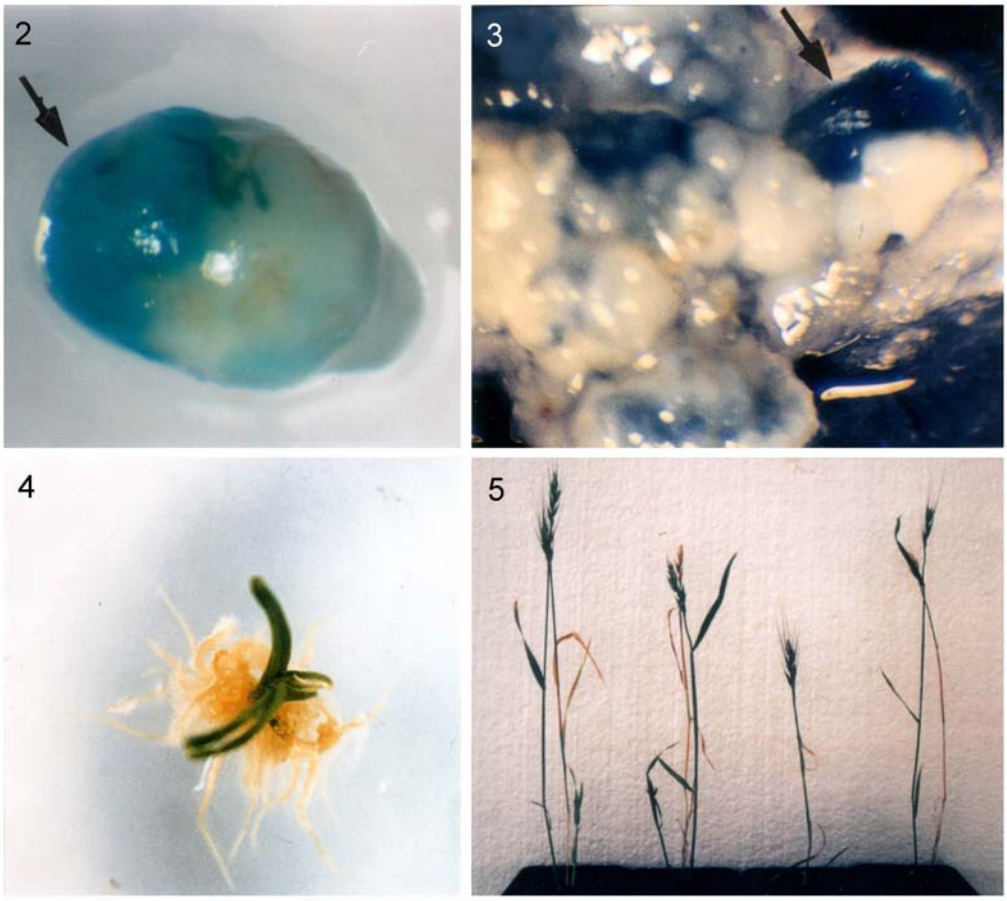

Fig. 2. Transient GUS expression in the scutellum (*arrow*) of an immature embryo, 3 d after co-cultivation with LBA4404/pTOK233.
Fig. 3. A transformed callus and developing shoot of wheat, exhibiting GUS activity (*arrow*), on the selection medium with 25 mg dm^{-3} of hygromycin.
Fig. 4. A transformed plantlet, survived on MS-reg medium, with 25 mg dm^{-3} of hygromycin.
Fig. 5. T_0 fertile plants transformed with LBA4404/pTOK233.

Table 2. Transformation of immature embryos and plant regeneration of wheat cv. Vesna.

Vector	No. of infected embryos	Surviving calli 2 wk	4 wk	6 wk	Regenerating calli	Regenerated plants
LBA4404/pTOK233	1209	149	92	32 (2.6 %)	7 (0.6 %)	5 (0.41 %)
Control	50	9	0	-		
AGL1/pDM805	796	113	71	17 (2.1 %)	5 (0.6 %)	1 (0.13 %)
Control	50	12	0	-		

Table 3. Inheritance of *uidA* gene in T_1 progeny.

Vector	T_0 plant	Number of seeds [plant^{-1}]	Germinated seeds	T_1 seedlings	GUS^+ T_1 plants histochemical	PCR
pTOK233	TA	0	-	-	-	-
	TB	2	2	2	1	1
	TC	9	5	3	1	-
	TD	10	7	4	2	2
	TE	7	4	3	-	-
pDM805	DA	6	3	2	1	1

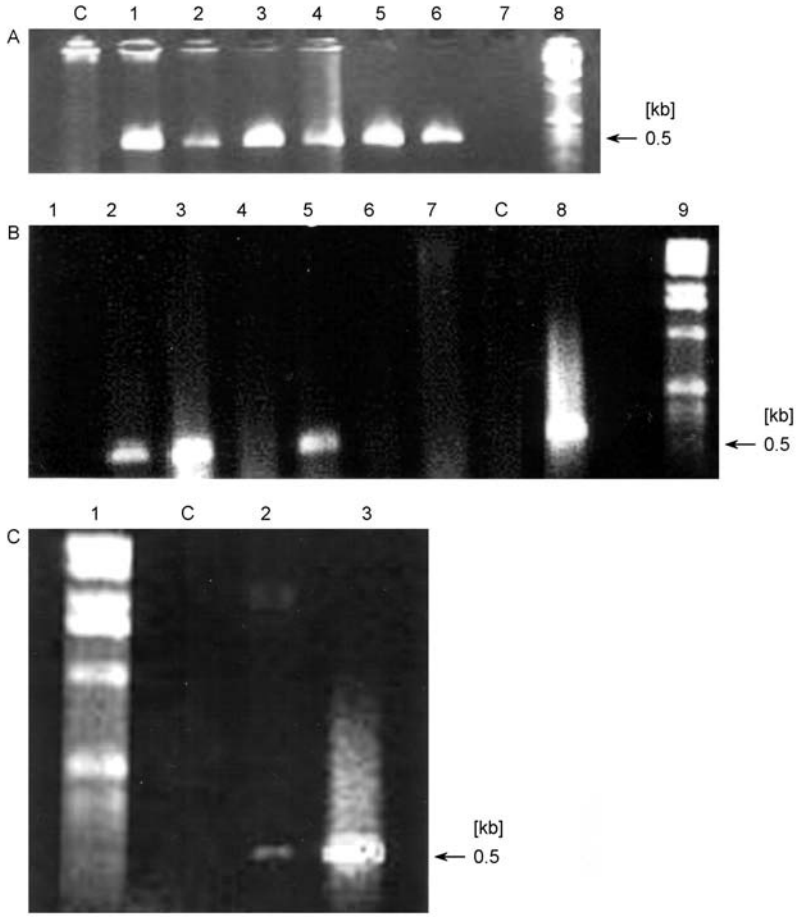

Fig. 6. PCR analyses of T_0 plants (*A*) transformed with LBA4404/pTOK233 and AGL1/pDM805 and their T_1 progeny (*B*,*C*). *A*: *lane C* - DNA sample from nontransformed control plant; *lanes 1, 2 and 3* - DNA samples of the primary transformants TB, TC and TD transformed with LBA4404/pTOK233; *lane 4* - DNA sample from putative primary transformant DA transformed with AGL1/pDM805; *lane 5* - pTOK233 as a positive control; *lane 6* - pDM805 as a positive control; *lane 7* - blank; *lane 8* - DNA ladder 1 kb. *B*: *lane 1* - blank; *lanes 2-7* - DNA samples from T_1 progeny transformed with LBA4404/pTOK233 (plants TB_1, TD_1, TD_2, TE_1, TE_2); *lane C* - DNA sample from nontransformed plant; *lane 8* - pTOK233 as a positive control; *lane 9* - DNA ladder. *C*: *lane 1* - DNA ladder; *lane C* - DNA sample of nontransformed control plant; *lane 2* - DNA sample from T_1 progeny plant (DA_1) transformed with AGL1/pDM805; *lane 3* - pDM805 as a positive control.

were also observed. All calli developing on the control embryos died after the first cycle on MS-sel medium (Table 2). During the continuous selection on hygromycin- or PPT-containing media, the number of surviving calli, derived from infected embryos, constantly decreased. Finally, 32 (2.6 %) calli, derived from 1209 embryos initially co-cultivated with LBA4404/pTOK233, and 17 (2.15 %) calli derived from 796 embryos infected with AGL1/pDM805 survived selection. They were transferred to MS-reg media and only seven (0.6 %) of

hygromycin resistant and five (0.6 %) of PPT-resistant calli regenerated green shoots after about four weeks. These shoots were intensively blue coloured in the GUS histochemical assay (Fig. 3). Five plants resistant to hygromycin (Fig. 4) and one resistant to PPT were obtained from surviving regenerative calli. The transformation frequency was 0.41 and 0.13 % for LBA4404/pTOK233 and AGL1/pDM805, respectively. All regenerated plants were rooted on MS-root medium. Some of these plants were further multiplied by tillering, producing 2 - 3 new plants. Seven rooted hygromycin-resistant plants, originated from four primary transformants, and one PPT-resistant plant were transferred to soil and grown for three months in the greenhouse, where all except one produced seeds (Table 3). Due to unsatisfactory conditions in the greenhouse during the winter, the transformed plants had shorter stems and spikes, and produced a rather low number of T_1 seeds (2 - 10 seeds per plant) (Fig. 5). Histochemical assays of GUS activity were carried out on expanded leaves and roots of T_0 plants. Segments of leaves and the pericycle zone of roots were stained blue, indicating the expression of the *uidA* gene. No colouration was visible in control plants. The PCR analysis of DNA extracted from leaves of three T_0 hygromycin-resistant plants indicated the presence of *uidA* sequences from the super-binary vector LBA4404/pTOK233 (Fig. 6*A*, *lanes 1-3*), and the presence of *uidA* gene from binary vector AGL1/pDM805 in the PPT-resistant T_0 plant (Fig. 6*A*, *lane 4*), but not in the DNA from the untransformed control (Fig. 6*A*, *lane C*). The T_0 plants were self-pollinated in the greenhouse and the mature T_1 seeds were harvested and germinated on MS plant growth regulator-free media, containing either 25 mg dm^{-3} hygromycin, or 1.0 mg dm^{-3} PPT for seedling selection (Table 3). The presence of GUS activity was confirmed by histochemical analysis of root and leaf segments of the T_1 seedlings. Evidence from PCR analysis also indicated the presence of the *uidA* gene in the genome of T_1 seedlings (Fig. 6*B*,*C*). It is noticeable that the segregation of the *uidA* gene in the T_1 progeny departs from Mendelian ratio 3:1. However, this can be explained by the fact that the proper assessment of the segregation was actually precluded, due to the small number of seeds produced.

Discussion

The results presented here repeatedly provide evidence on the efficiency of *A. tumefaciens* in transforming a cereal species – wheat. The low transformation frequency represents the major concern, although it is still within the range of some other published data (*e.g.* Cheng *et al.* 1997). Several approaches could be envisaged to study this problem. Since the untransformed embryos of the wheat cv. Vesna presented no difficulties in regeneration, it is perhaps necessary to re-evaluate some details of the inoculation, or selection procedure, on the early developmental events. On the other hand, the capacity of the two *A. tumefaciens* vectors to transmit their T-DNA seems to be adequate. This could be inferred from the high percentage of transient GUS expression, which is noted also in some other plant species (*e.g.* cotton, Banerjee *et al.* 2002). The great discrepancy between the high transient GUS expression and the subsequent failure in regenerating a comparable number of plants may have various, as yet unknown, reasons (Khanna and Daggard 2003). McCormac *et al.* (1998) found that wheat scutellum cells, that were transformed, as judged by the presence of visual markers, failed to divide and form cell clusters and regenerating structures. Unless it is shown that the visual markers interfere with cell division, this would mean that the capacity for T-DNA integration and the capacity for regeneration are segregated in different cells. Furthermore, the failure of organized structures to survive may be due to their possible multicellular origin. If the somatic embryos arising on the scutellar surface are composed of transformed and non-transformed cells, the latter may prevail and impair further development on selective media.

In conclusion, these experiments demonstrate that *A. tumefaciens* can be used as vector for genetic transformation of *T. aestivum* cv. Vesna, the study of which was our primary objective. If the genetic engineering of crop plants is meant to improve crops worldwide, it should be applied to a variety of cultivars that are well adapted to local climatic and soil conditions. The results with cv. Vesna seem to provide basis for further work in breeding this and related cultivars.

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