Plant Cell Rep (2008) 27:1721–1729
DOI 10.1007/s00299-008-0593-y

GENETIC TRANSFORMATION AND HYBRIDIZATION

Agrobacterium-mediated transformation of oat (*Avena sativa* L.) cultivars via immature embryo and leaf explants

Sebastian Gasparis · Cezary Bregier · Waclaw Orczyk · Anna Nadolska-Orczyk

Received: 17 April 2008 / Revised: 16 June 2008 / Accepted: 25 July 2008 / Published online: 9 August 2008

Abstract This paper reports on the successful *Agrobacterium*-mediated transformation of oat, and on some factors influencing this process. In the first step of the experiments, three cultivars, two types of explant, and three combinations of strain/vectors, which were successfully used for transformation of other cereals were tested. Transgenic plants were obtained from the immature embryos of cvs. Bajka, Slawko and Akt and from leaf base explants of cv. Bajka after transformation with *A. thumefaciens* strain LBA4404(pTOK233). The highest transformation rate (12.3%) was obtained for immature embryos of cv. Bajka. About 79% of the selected plants proved to be transgenic; however, only 14.3% of the T_0 plants and 27.5% of the T_1 showed GUS expression. Cell competence of both types of explant differed in terms of their transformation ability and transgene expression. The next step of the study was to test the suitability for oat transformation of the pGreen binary vector combined with different selection cassettes: *npt*II or *bar* under the nos or 35S promoter. Transgenic plants were selected in combinations transformed with nos::*npt*II, 35S::*npt*II and nos::*bar*. The highest transformation efficiency (5.3%) was obtained for cv. Akt transformed with nos::*npt*II. A detailed analysis of the T_0 plants selected from a given callus line and their progeny revealed that they were the mixture of transgenic, chimeric-transgenic and non-transgenic individuals. Southern blot analysis of T_0 and T_1 showed simple integration pattern with the low copy number of the introduced transgenes.

Keywords Oat · Polyploid cereals · *Agrobacterium* · Binary vectors · Transgene expression

Communicated by W. Harwood.

S. Gasparis · C. Bregier · W. Orczyk · A. Nadolska-Orczyk (✉)
Plant Transformation and Cell Engineering Department, Plant Breeding and Acclimatization Institute, Radzikow, 05-870 Blonie, Poland
e-mail: a.orczyk@ihar.edu.pl

Introduction

Genetic modification is an important experimental tool that can be used to analyze and understand the mechanisms responsible for the expression of transgenes or endogenous genes, and to create plants with the desired characteristics. Of the two basic methods of genetic transformation—biolistic and *Agrobacterium*-mediated—the latter is more reasonable for most of the applications, like cotransformation or stable gene silencing by RNAi. The use of RNA interference technology is especially important in the case of polyploid species, mainly because it enables the repression of multiple orthologues (Lawrence and Pikaard 2003).

Although *Agrobacterium*-mediated transformation is the most widely and successfully applied technique for genetic transformation of plants, its adaptation for use with allohexaploid cereals is far from universal. It has been successfully developed only for the agronomically important wheat (Cheng et al. 1997; Hu et al. 2003; Khanna and Daggard 2003; Wu et al. 2003; Przetakiewicz et al. 2004) and more recently for triticale (Nadolska-Orczyk et al. 2005). Oat (*Avena sativa* L.) is another allohexaploid species thus far transformed only via the biolistic method. Its genetic transformation was accomplished using embryogenic calli or cell suspensions derived from immature embryos (Kuai et al. 2001; Pawlowski and Somers 1998; Perret et al. 2003; Somers et al. 1992;

Torbert et al. 1995, 1998a), mature embryos (Kaeppler et al. 2000; Torbert et al. 1998b), leaf base segments (Gless et al. 1998b), cultures from seeds (Cho et al. 1999), and shoot apical meristems (Cho et al. 2003; Maqbool et al. 2002; Zhang et al. 1999). Obtaining transgenic plants from leaf base segments has thus far only been possible for oat; it is not yet applicable to other allohexaploid cereals (Sticklen and Oraby 2005). The most probable reason is the ability and efficiency of the oat plant to regeneration from this type of explant. The system was efficient for oat leaf bases (Gless et al. 1998a; Nuutila et al. 2002 and this paper) and inefficient for wheat (Wang and Wei 2004) and triticale (Nadolska-Orczyk, not published).

We have developed procedures of oat regeneration from two different types of explants: immature embryos and leaf base segments. Immature embryos are composed of highly totipotent, meristematic cells, whereas leaf base segments consist of differentiated cells, which have to undergo dedifferentiation before somatic embryo development and plant regeneration. This difference encouraged us to test cell-competence to *Agrobacterium*-mediated transformation and transgene expression. Our study also focused on preliminary testing suitability of pGreen (Hellens et al. 2000) vector system containing different selection cassettes for the effective transformation of this species.

Materials and methods

Plant material and in vitro culture

Immature embryos and leaf base segments of three Polish spring oat cultivars—cv. Bajka, cv. Slawko and naked cv. Akt—were used. The donor plants for the immature embryos were grown in a growth chamber at 20/16°C (day/night), with a 16-h photoperiod (150 µmol s^{-1} m^{-2} light). Immature seeds were collected 10–14 days after anthesis and sterilized in 0.1% $HgCl_2$ for 4 min and washed with sterile distilled water for 3, 6 and 20 min. Immature embryos were excised and placed scutellum side-up on callus induction medium. To obtain leaf-base explants, mature seeds were sterilized (0.1% $HgCl_2$) and placed on ½ MS (Murashige and Skoog 1962) medium without vitamins, and with 20 g l^{-1} sucrose and 2 g l^{-1} gelrite (Duchefa Biochemie). After 4 days of culture, the base part of the leaf, about 4–5 mm in length, was cut from each seedling and placed on the medium. Both types of explant were cultured on solid MSB medium containing Murashige and Skoog's (1962) macro- and microelements, Gamborg's et al. (1968) vitamins, 30 g l^{-1} sucrose and 2 g l^{-1} gelrite and 3 mg l^{-1} of 2,4-D (callus induction medium). After 4 weeks, a subsequent culture was performed on MSB medium containing 0.2 mg l^{-1} IAA and 1.0 mg l^{-1} BAP, and plantlets were developed on MS medium (Przetakiewicz et al. 2003). Well-rooted plants were planted in soil and grown to maturity.

Agrobacterium strains and vectors

We used four combinations of *Agrobacterium* strain and vector [LBA4404(pTOK233), EHA101(pGAH), AGL1(pDM805), AGL1(pGreen)] containing different cassettes of promoter/selection gene/reporter gene. pTOK233 (Hiei et al. 1994) was provided by Dr. T. Komari from Japan Tobacco Inc., pDM805 (Tingay et al. 1997) by Dr. R. Brettell from CSIRO (Australia), pGAH (Hayashi et al. 1997) by Dr. N. Murata (Japan), and the pGreenII system (Hellens et al. 2000) by the John Innes Centre (www.pgreen.ac.uk). Both pTOK233 and pGAH contained *npt*II under the nos promoter and *hpt* under the 35S promoter. Additionally, pTOK233 carried *gus* with an intron under 35S (Fig. 1a). pDM805 contained *bar* under the ubiquitin 1 promoter and *gus* under the actin 1 promoter. The LBA4404(pTOK233) strain was cultured in AB medium with 50 mg l^{-1} hygromycin (Hiei et al. 1994), and EHA101(pGAH) in MG/L (Garfinkel and Nester 1980) supplemented with 5 mg l^{-1} kanamycin and 25 mg l^{-1} hygromycin (Hayashi et al. 1997).

Four types of selection cassette were cloned into the T-DNA of pGreen. The first vector contained 35S::*npt*II, the second nos::*npt*II, the third 35S::*bar*, and the fourth nos::*bar*. The reporter cassette, 35S::GUS containing an intron, was cloned close to the RB (*Stu*I site) of these vectors (Fig. 1b). The pGreen vectors were co-electroporated with pSoup plasmid (containing RepA) to *Agrobacterium tumefaciens*, strain AGL1. The bacteria were cultured in MG/L liquid medium (Garfinkel and Nester 1980) supplemented with 50 mg l^{-1} rifampicin (*Agrobacterium* strain) and 50 mg l^{-1} kanamycin (pGreen) or 5 mgl^{-1} tetracycline (pDM805). A single colony derived suspension of *A. tumefaciens* was divided into 100 µl aliquots, frozen and used as an *inoculum* of bacterial culture. After 2 days of culture at 28°C, the bacteria were resuspended in MS medium containing 200 µM acetosyringone at an OD_{600} from 0.6 to 1.0.

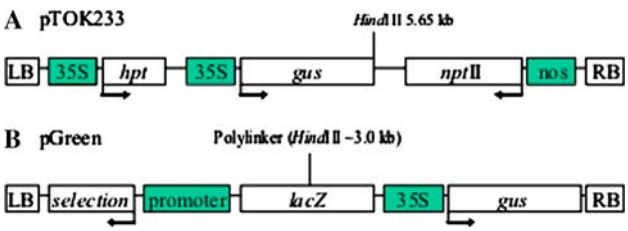

Fig. 1 The schematic structure of the T-DNA of the pTOK233 (**a**) and pGreen (**b**) vectors

Transformation was performed according to the already published procedure for triticale (Nadolska-Orczyk et al. 2005). After 3 days of preculture on callus induction medium, the explants were inoculated with a drop (about 10 µl) of *A. tumefaciens* suspension, and co-cultured for the next 3 days on the same medium. Next, they were transferred to that medium supplemented with 150 mg l^{-1} Timentin, and one of two selection agents: kanamycin (50 mg l^{-1}) or phosphinothricin (2 mg l^{-1}). The subsequent culture was performed on the same media with the same selection agent added.

PCR analysis and Southern hybridization

Genomic DNA was isolated from the young leaves of putative transgenic plants (T_0) and their first generation (T_1) using a modified version of Murray and Thompson's (1980) CTAB method. The PCR amplification was carried out in a 25 µl reaction mixture containing 240 ng of template genomic DNA, 100 µM of each dNTP, 3 µM of each primer, 1 U Taq DNA polymerase (Promega), 2 mM $MgCl_2$ and 1× DNA polymerase buffer. The sequences of the primers for the amplification of the 700-bp *npt*II gene fragment were: 5′-GAGGCTATTCGGCTATGACTG-3′ and 5′-ATCGGGAGCGGCGATACCGTA-3′. For the 1,097-bp *gus* gene fragment amplification, they were: 5′-ACGTCCTGTAGAAACCCCAA-3′ and 5′-CCCGCT TCGAAACCAATGCC-3′. For the 778-bp *hpt* fragment amplification, they were: 5′-TTTGCCCTCGGACGAGTG CT-3′ and 5′-GGGAGTTTAGCGAGAGCCTGACCTA-3′. For the 430-bp *bar* fragment amplification, they were: 5′-TCTGCACCATCGTCAACCACTACATC-3′ and 5′-CA GAAACCCACGTCATGCCAGTTC-3′. The control primers for the sequences specific to *Agrobacterium* (T_0 plants) were: 5′-CTTCTTGAACTCGCCACCGC-3′ and 5′-AAG ATCTGATCGATAATGAG-3′ for the 284-bp *virA* fragment amplification. The amplification conditions were: 94°C for 1 min, 62°C (*npt*II), 60°C (*gus*), 68°C (*bar*), 65°C (*hpt*) or 55°C (*virA*) for 1 min, and 72°C for 2 min (except in the case of *hpt*, 1 min). The number of cycles was 36. The conditions were optimized for each pair of primers separately.

From the T_0 plants, 40 µg of genomic DNA digested with *Hin*dIII was used for Southern blot analysis. Electrophoresis was in 0.8% agarose and 1× TBE buffer. The DNA was blotted onto nylon membranes, positively charged (Roche) using vacuum alkaline transfer. The probe was a PCR-amplified 700-bp *npt*II fragment labeled by DIG dNTP (Roche) using pTOK233 as a template in the case of T_0 hybridization and pGreen as a template in the case of T_1 hybridization. Prehybridization (0.5 h) and hybridization (16 h) were performed at 68°C in a standard hybridization buffer (Roche). Labeling and detection were done using a non-radioactive method according to the manufacturer's protocol. CSPD was used as a chemiluminescent substrate, and the light signals were detected on X-ray film.

Histochemical GUS assay

GUS expression was determined in the young leaves of T_0 plants and in leaf and coleoptile fragments collected from 5-day old T_1 seedlings using a modified histochemical GUS assay (Jefferson et al. 1987). The seedlings were germinated in 8 cm diameter Petri dishes covered with filter paper dripped with water. Explants were incubated overnight at 37°C in a buffer containing 2 mM X-Gluc, 50 mM sodium phosphate, 50 mM potassium ferricyanide and 50 mM potassium ferrocyanide at pH 7.0.

Results

The two types of explant for plant regeneration and *Agrobacterium*-mediated transformation

Regeneration procedures via somatic embryogenesis were established for two types of explant from three cvs. Bajka, Slawko and Akt. The explants from leaf base fragments were cut from seedlings and from immature embryos. Both had a high level of regeneration ability, yielding several plants per explant, depending on the cultivar (data not shown). On average, 90% of the immature embryo explants and 48% of the leaf base fragments regenerated plants.

In the preliminary experiments of *Agrobacterium*-mediated transformation of oat, we tested three combinations of strain and vector that had been successfully used for other cereals. There were: LBA4404(pTOK233), EHA101 (pGAH) and AGL1(pDM805). Embryogenic callus was developed after 3–4 weeks of culture on selection media (Fig. 2a) and plantlets were visible after a further 1–3 weeks (Fig. 2b, c). Transgenic plants were obtained from both types of explant—immature embryos (IE) and leaf base fragments (LB) of Bajka cultivar after LBA4404(pTOK233) transformation and kanamycin selection (Table 1; Fig. 2d, e). The transformation rate was estimated as a percentage of the selected, independent callus lines (each from one explant) which gave rise to at least one fertile transgenic plant. It was highest for the two types of explant from cultivar Bajka. The transformation efficiencies for immature embryos were 12.3% and for leaf-base fragments, 8.2%. Much lower transformation rates were obtained for the immature embryos of cvs. Slawko and Akt transformed with LBA4404(pTOK233): 1.1 and 3.4%. There were no transformants selected from leaf base explants of Slawko and Akt transformed with the

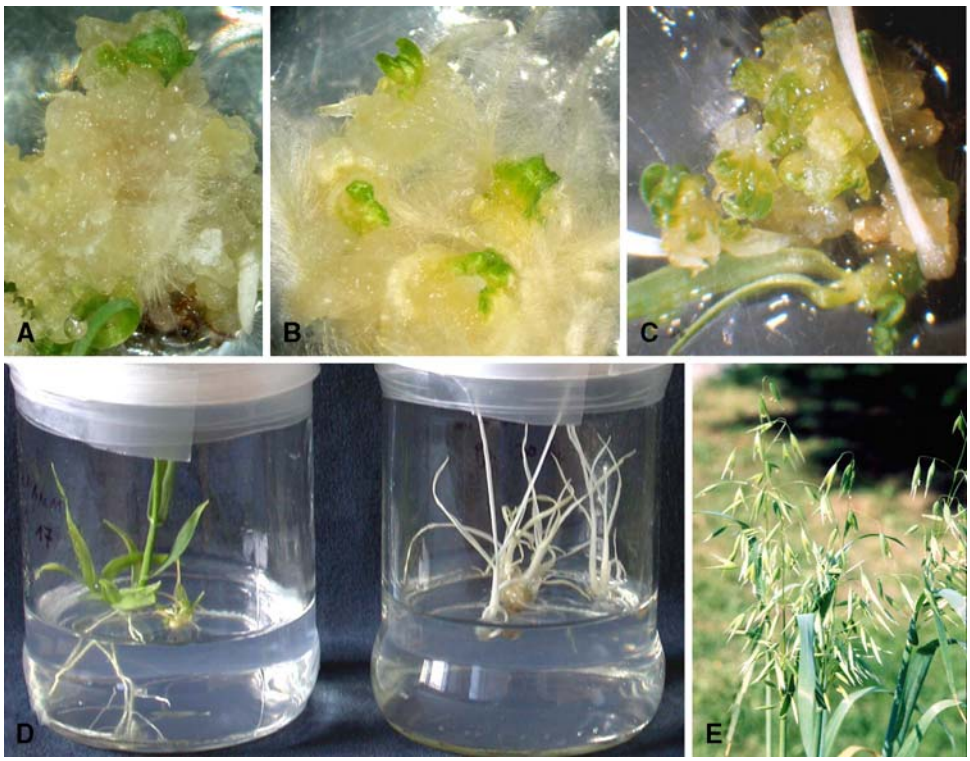

Fig. 2 Somatic embryogenesis and transgenic plant selection. Kanamycin selection of embryogenic callus (**a**) and green plantlets (**b**) from immature embryos and leaf base explants (**c**). Green, rooted (*left*) and white, not rooted (*right*) plants under kanamycin selection (**d**). Mature, seed setting, transgenic oat plant (**e**)

Table 1 The selection of green, rooted plants (kanamycin selection) and transformation efficiencies from two types of explant transformed with LBA4404(pTOK233)

Cultivar/ explant	Number of explants[a]	Explants forming embr. calli (%)	Number of selected plants[b]	Number of callus lines producing transgenic plants	Transformation efficiency (%)
Bajka					
IE	138	14.5	65	17	12.3
LB	134	28.0	53	11	8.2
Slawko					
IE	174	15.0	7	2	1.1
LB	144	5.1	0	0	0.0
Akt					
IE	174	15.5	17	6	3.4
LB	166	0.0	0	0	0.0
Sum	930		142	36	

IE immature embryos, *LB* leaf base fragments

[a] The sum from two experiments

[b] Only seed-setting plants

same combination of strain and vector (Table 1). Transformation of 408 immature embryos of Bajka, Slawko and Akt with EHA101(pGAH) and 582 immature embryos of the same cultivars with AGL1(pDM805) did not result in the selection of any transformed plants.

Immature embryos of Bajka transformed with pTOK233 regenerated an average of 3.8 plants (65/17) and the leaf base fragments 4.8 (53/11; Table 1). However, the rate of PCR-positive T_0 plants selected from the two types of explant differed considerably and was 79 and 36.5%, respectively (Table 2). Additionally, 9 (14.5%) out of 61 plants selected from leaf base explants died in the soil before maturation. Only 11.7% of the IE plants and 9.6% of the LB plants were GUS positive (Fig. 3a–e). The GUS activity in the T_1 plants was higher; the mean percentage of GUS-positive progeny of immature embryo-derived plants

Table 2 The rate of PCR-positive (PCR+) and GUS-expressing (GUS+) plants of Bajka, Slawko and Akt transformed with LBA4404(p-TOK233), and the segregation of GUS expression in T_1

Cultivar/explant type	Number of tested T_0 plants	PCR+ number (%)	GUS+ of PCR+ number (%)	Number of tested T_1 lines	T_1 GUS + number (%)
Bajka/IE	62	49 (79.0)	7 (14.3)	7	119 (27.5)
Slawko/IE	2	1 (50.0)	0 (0.0)	0	0
Akt/IE	13	10 (76.9)	2 (20.0)	2	23 (51.1)
Sum/mean	77	60 (77.9)	9 (15.0)	9	142 (29.8)
Bajka/LB	52 (9 failed)	19 (36.5)	5 (26.3)	13	77 (24.6)

IE immature embryos, *LB* leaf base fragments

PCR+ at least two positive PCR amplifications with two different pairs of primers

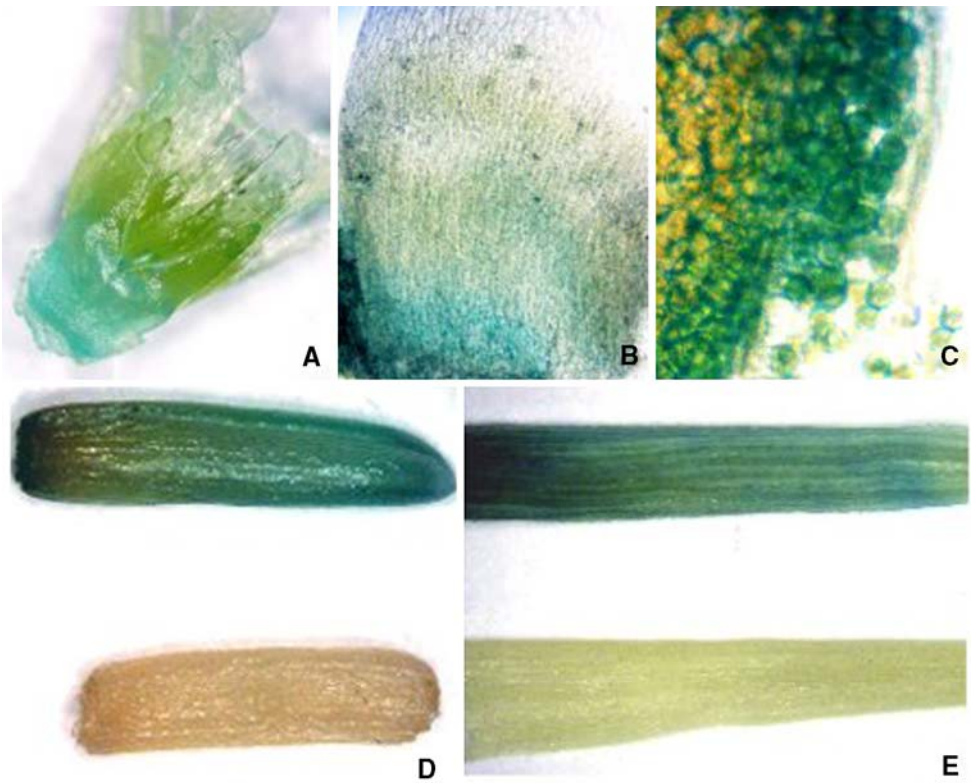

Fig. 3 The expression of the *gus* gene in different tissues of oat. GUS staining in the ovule (**a**), tissue of a hull (**b**), pollen grains in a sac (**c**), coleoptile (**d**) and leaf (**e**) of a transgenic (*top*) and control (*bottom*) plant

from Bajka and Akt was 29.8. However, none of the tested 9 lines showed Mendelian segregation. In total, 24.6% of the progeny of 13 Bajka lines selected from the leaf base explants were GUS positive, and the segregation for one line was Mendelian.

Southern hybridization proved that the selected, PCR-positive oat plants transformed with pTOK233 were transgenic (Fig. 4). The positive control, which was an 8.8 kb fragment of pTOK233 plasmid containing 0.8 pg of a fragment hybridizing with the probe, contained the equivalent of one copy of the *npt*II. The hybridization pattern for the DNA from the transgenic plants of immature embryo origin, representing independent callus lines (Fig. 4, lines 79–55) were simple, with 1–3 copies of the transgene per line. The restriction pattern of the DNA from plants of leaf base origin (Fig. 4, lines 2.5–12.7) differed from those of immature embryo origin with a higher number of bands observed.

pGreen binary vector containing the appropriate selection cassette is suitable for *Agrobacterium*-mediated transformation of oat

To test the suitability of the pGreen vector system for oat transformation, four selection cassettes were cloned into the T-DNA of the vector: *npt*II under the nos or 35S promoter and *bar* under nos or 35S. All the pGreen vectors had *gus* under 35S and were transformed into the AGL1 strain. Putative transgenic plants were selected from the immature embryos of both cultivars after transformation with vectors

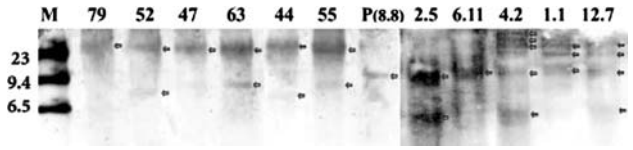

Fig. 4 Southern blot analysis of transgenic oat plants regenerated from immature embryos (*lines 79–55*) and leaf base fragments (*lines 2.5–12.7*). The DNA was digested with *Hin*dIII and hybridized with a PCR-amplified 700 bp *nptII*, DIG-labeled probe. The expected bands' lengths were over 5.65 kb. *M* DIG molecular weight marker; *P* 8.8-kb fragment of pTOK233 containing a 0.8-pg fragment hybridizing with the probe, equivalent to one copy of *npt*II

containing: 35S::*npt*II, nos::*bar* (Bajka) or nos::*npt*II (Akt) selection cassettes (Table 3). The transformation efficiency ranged from 0 to 5.3%.

The inoculation of 1,317 immature embryos of cvs. Bajka and Akt with AGL1 containing the pGreen vector led to the selection of 17 callus lines (Table 4) and regeneration of 91 plants (Table 3). Six lines regenerated single plants, and 11 lines regenerated from 2 to 9 plants. Only 11% of them expressed GUS, and 49.3% were PCR-positive. Transgenic plants were found in 14 out of the 17 tested lines. The number of PCR-positive plants per line was always lower than the number of all the selected plants. Most (73 plants out of 91) set from 1 to 292 seeds (with a mean of 31.7 seeds per plant). Eighteen plants, including the eight regenerated from two callus lines of Akt transformed with nos::*npt*II, did not set seeds. For 15 plants, the number of seeds was lower than 10.

GUS expression in the T_1 generation of the PCR-positive plants was observed in 24.2% of the progeny, and the segregation was non-Mendelian. Only for one plant were there more GUS-positive progeny than GUS-negative, but it was impossible to verify the type of segregation, because the plant set only six seeds. The percentage of PCR-positive plants in the first generation was 47.5% and, as in the case of GUS expression, segregation was non-Mendelian. Southern blot analysis of the T_1 plants (Fig. 5) confirmed the results of the PCR analyses. The tested transgenic lines showed one or two transgenic loci and the same pattern of inheritance in the progeny of one line. The number of seeds set by the transgenic T_1 plants ranged from 17 to 250 with a mean of 133, which was much higher than for their parents (T_0), and similar to the control R_1 plants.

Discussion

Competence of immature embryos and leaf-base fragments for transformation

In the case of oat, in vitro plant regeneration and genetic transformation is not only possible from the highly morphogenic, undifferentiated cells of immature embryos, as with other plants, but also from differentiated cells. The latter have to undergo dedifferentiation to be able to form somatic embryos and develop plants. The genetic and physiological background of these cells is different. To determine plant cell competence for transformation and transgene expression, two kinds of explant were used, immature embryos and leaf base fragments. The cells of the IE were more effective at producing transgenic plants

Table 3 Plant selection and transformation efficiency from immature embryos of cvs. Bajka and Akt transformed with pGreen containing different selection cassettes (AGL1 strain)

Cultivar/selection/ reporter cassettes	Number of immature embryos[a]	Explants forming embr. calli (%)	Number of selected plants[b]	Number of callus lines producing PCR+ plants	Transformation efficiency (%)
Bajka					
nos::nptII/35S::gus	133	21.8	0	0	0
35S::nptII/ 35S::gus	142	73.9	3	2	1.4
nos::bar/35S::gus	238	68.1	11	2	0.8
35S::bar/35S::gus	92	6.5	0	0	0
Akt					
nos::nptII/35S::gus	244	48.4	76	13	5.3
35S::nptII/ 35S::gus	196	8.7	1	0	0
nos::bar/35S::gus	168	9.5	0	0	0
35S::bar/35S::gus	104	13.5	0	0	0
Sum	1,317		91	17	

[a] The sum from two experiments

[b] Only seed-setting plants

PCR+ at least two positive PCR amplifications with two different pairs of primers

Table 4 The rate of GUS-expressing (GUS+) and PCR-positive (PCR+) plants of cvs. Bajka and Akt among T_0 and T_1 progeny transformed with pGreen

Selection cassettes	Number of regenerating lines under selection	Number of tested plants	T_0 GUS+		T_0 PCR+		Number of tested T_1 lines		T_1 GUS+:GUS−	T_1 PCR+:PCR−
			Lines	Plants	Lines	Plants	GUS	PCR		
nos::*npt*II	13	60	5	8	11	30	8	3	36:111	19:21
35S::*npt*II	2	4	0	0	1	1	0	0	–[a]	–
nos::*bar*	2	9	0	0	2	5	1	0	2:8	–
Sum	17	73	5	8	14	36	9	5	38:119	19:21
Mean per line		4.0				2.0				
Positive (%)			29.4	11.0	82.4	49.3			24.2	47.5

pG pGreen

[a] Not tested

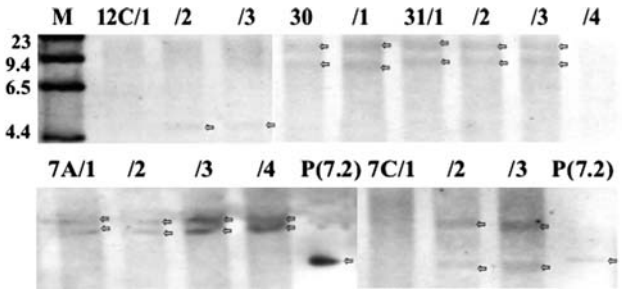

Fig. 5 Southern blot analysis of the T_1 progeny of the *12C*, *30*, *31*, *7A* and *7C* plants (*7A* and *7C* were from the same callus line *7*). The DNA was digested with *Hin*dIII and hybridized with a 700-bp *nptII* PCR-amplified and DIG-labeled probe. The enzyme cuts pGreen in a polylinker site once. The expected bands' lengths were over 1.8 kb. *M* DIG molecular weight marker; *P(7.2)* linear, 7.2-kb of pGreen containing 4 and 1 pg of the DNA hybridizing with the probe, equivalent to one copy of *npt*II

in all three of the tested cultivars. The number of selected plants per callus line was higher in the case of the leaf base explants of cv. Bajka; however, the percentage of transgenic plants obtained was less than half as many as with IE cells. The percentage of GUS-expressing plants in both the T_0 and T_1 generations was higher in the group of IE plants. Undifferentiated oat cells from immature embryos proved to be more competent for *Agrobacterium*-mediated transformation and transgene expression than differentiated, leaf base segment cells.

Biolistic versus *Agrobacterium*-mediated transformation of oat

In cereals, *Agrobacterium*-mediated transformation is now routinely used for rice, some genotypes of maize and barley, and wheat. The success of using this method for other than wheat allohexaploid cereals is still limited, so biolistic gene delivery is commonly used. The biolistic technique is well optimized and permits good transformation efficiency to be obtained (Svitashev et al. 2000). However, it leads to the integration of complex transgenic loci frequently interspersed with genomic DNA (Svitashev and Somers 2001; reviewed by Somers et al. 2003), cosegregation (Kohli et al. 1998; Leggett et al. 2000), low fertility (Cho et al. 1999), and frequent chromosomal aberrations (Choi et al. 2000). We showed simple integration patterns of transgenic loci produced by *Agrobacterium*-mediated transformation with low transgene copy number. Additionally, almost all the plants regenerated from immature embryos and 85% of the plants obtained from leaf bases were fertile in the first experiment. Good fertility was also proved in the second experiment with the pGreen vectors. Furthermore, the best combinations of the tested factors allowed transgenic plants to be obtained in a short time with a high degree of efficiency.

Other factors influencing *Agrobacterium*-mediated transformation of oat

The combination of the *Agrobacterium* strain and vector was important. Of the three used in the first experiment, only LBA4404(pTOK233) was effective in the transformation of oat cultivars. The hypervirulent AGL1 strain carrying the pGreen binary vector system was also able to transform oat cultivars. As in the case of allohexaploid wheat (Przetakiewicz et al. 2004) all the cultivars used were transformed by *Agrobacterium*. The strain/vector combination, which was the best for oat, was also the best for allohexaploid triticale (Nadolska-Orczyk et al. 2005), although it was less efficient for wheat (Przetakiewicz et al. 2004).

To test selection cassettes we used two typical selection genes under the control of two promoters. The nos promoter was effective at driving the *npt*II in kanamycin-selected plants of Bajka, Sławko and Akt (pTOK transformation; repeated for Akt after pGreen) and *bar* gene in phosphinothricin-selected Bajka plants. The *npt*II regulated

by the 35S promoter enabled selection of transgenic Bajka plants. Other selection cassettes were reported as effective after particle bombardment transformation of oat. The most frequently used were the hygromycin- or herbicide-resistant under-monocot promoters Ubi1 and Act1 (Perret et al. 2003; Cho et al. 1999; Cho et al. 2003). In other successful biolistic protocols, *bar* was driven by 35S (Maqbool et al. 2002) and *npt*II by Ubi1 (Cho et al. 2003). We already proved for other allohexaploid cereals (Nadolska-Orczyk et al. 2005; Przetakiewicz et al. 2004) that the nos and 35S promoters could be successfully used in *Agrobacterium*-mediated transformation. Selection genes driven by these promoters were effective at the embryonic callus level and for plantlet selection.

Transgene expression

Only one line out of the 21 tested in the first experiment showed the Mendelian type of segregation for *gus* expression. A similar result was observed in the second experiment. Additionally, as shown in the second experiment, the number of GUS positive plants in T_0 and T_1 was always lower than the number of PCR-positive plants. We suppose that at least part of the problems with the lack of *gus* expression in T_0 or its non-Mendelian segregation in T_1 was caused by the weak promoter. In this case, the relationship between the hemi- and homozygous transgene expression level, already documented for rice (James et al. 2002) could explain the distorted segregation.

The irregular pattern of transgene expression and non-Mendelian inheritance of the trait in oat transformed by the biolistic method was also reported for transgenes driven by the Adh1, 35S (Pawlowski et al. 1998) and Act1 (Zhang et al. 1999) promoters. However, in these reports, some of the instabilities were caused by the method itself. The instability of transgene expression is often attributable to the transgene copy number, the position of transgene integration and/or the degree of homology to endogenous genes (Matzke and Matzke 1995) and genomic instability (genetic and/or epigenetic) induced during the in vitro culture and transformation process (Zhang et al. 1996), and finally to the structure of the transgenic locus (Pawlowski and Somers 1998). Mittelsten-Scheid et al. (1996) showed that a change in ploidy could also modify epigenetic silencing. As discussed by Pawlowski et al. (1998), allohexaploid oat may possess a mechanism preventing unwanted gene expression, and as few as two copies of the transgene could trigger silencing. Epigenetic silencing correlated with ploidy level could be another explanation for the non-Mendelian pattern of transgene expression observed by us in the allohexaploid cereals oat, triticale (Nadolska-Orczyk et al. 2005) and wheat (Przetakiewicz et al. 2004) transformed by *Agrobacterium tumefaciens*.

The multiple plants selected from a given callus line are a mixture of transgenic, chimeric-transgenic and non-transgenic individuals

Detailed analysis of the T_0 plants selected from particular callus lines revealed that only some of them were transgenic. The non-Mendelian segregation of the transgenic progeny in T_1 suggested that their parents were transgenic chimeras. Obtaining chimeric plants was possible because the regeneration procedure was very short. Somatic embryos were formed during the first 3–4 weeks and plantlets developed from them during the next 1–3 weeks of culture, and were probably of multicellular origin. The different ratio of PCR- and GUS-positive to negative progeny in the plant lines originating from the same callus line suggested that the T_0 parents were not clonally multiplied, but that they instead developed from different somatic embryos. Both the non-Mendelian segregation ratio and the different origin of plants from the same callus line were shown by Southern analysis. The positive hybridization control represented the equivalent of a single copy of the *nptII* gene in the plant DNA. Therefore, the results suggested that the transgenes integrated within the plant DNA at one or two insertion sites, predominantly as single copies.

Acknowledgments This research was supported by the Polish Ministry of Science and Higher Education, grant PBZ-KBN_089/P06/2003, Scientific Network "Genomika i transgeneza roslin uzytkowych" and the statutory IHAR project 1-1-01-4-04.

References

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol 115:971–980

Choi H-W, Lemaux PG, Cho M-J (2000) High frequency of cytogenetic aberration in transgenic oat (*Avena sativa* L.) plants. Plant Sci 156:85–94

Cho M-J, Jiang W, Lemaux PG (1999) High-frequency transformation of oat via microprojectile bombardment of seed-derived highly regenerative cultures. Plant Sci 148:9–17

Cho M-J, Choi HW, Okamoto D, Zhang S, Lemaux PG (2003) Expression of green fluorescent protein and its inheritance in transgenic oat plants generated from shoot meristematic cultures. Plant Cell Rep 21:467–474

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Garfinkel M, Nester EW (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumourigenesis and octopine catabolism. J Bacteriol 144:732–743

Gless C, Lorz H, Jahne-Gartner A (1998a) Establishment of a highly efficient regeneration system from leaf base segments of aot (*Avena sativa* L.). Plant Cell Rep 17:441–445

Gless C, Lorz H, Jahne-Gartner A (1998b) Transgenic oat plants obtained at high efficiency by microprojectile bombardment of leaf base segments. J Plant Physiol 152:151–157

Hayashi H, Alia , Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J 12:133–142

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol Biol 42:819–832

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hu T, Metz S, Chay C, Zhou HP, Biest N, Chen G, Cheng M, Feng X, Radionenko M, Lu F, Fry J (2003) *Agrobacterium*-mediated large-scale transformation of wheat (*Triticum aestivum* L.) using glyphosate selection. Plant Cell Rep 21:1010–1019

James VA, Avart C, Worland B, Snape JW, Vain P (2002) The relationship between homozygous and hemizygous transgene expression levels over generations in populations of transgenic rice plants. Theor Appl Genet 104:553–561

Jefferson RA, Kavanagh A, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kaeppler HW, Menon GK, Skadsen RW, Nuutila AM, Carlson AR (2000) Transgenic oat plants via visual selection of cells expressing green fluorescent protein. Plant Cell Rep 19:661–666

Khanna HK, Daggard GE (2003) *Agrobacterium tumefaciens*-mediated transformation of wheat using a superbinary vector and a polyamine-supplemented regeneration medium. Plant Cell Rep 21:429–436

Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc Natl Acad Sci USA 95:7203–7208

Kuai B, Perret S, Wan SM, Dalton SJ, Bettany AJE, Morris P (2001) Transformation of oat and inheritance of *bar* gene expression. Plant Cell Tissue Organ Cult 66:79–88

Lawrence RJ, Pikaard CS (2003) Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. Plant J 36:114–121

Leggett JM, Perret SJ, Harper J, Morris P (2000) Chromosomal localization of cotransformed transgenes in the hexaploid cultivated oat *Avena sativa* L. using fluorescence in situ hybridization. Heredity 84:46–53

Maqbool SB, Zhong H, El-Maghraby Y, Ahmad A, Chai B, Wang W, Sabzikar R, Sticklen MB (2002) Competence of oat (*Avena sativa* L.) shoot apical meristems for integrative transformation, inherited expression, and osmotic tolerance of transgenic lines containing *hva1*. Theor Appl Genet 105:201–208

Matzke MA, Matzke AJM (1995) How and why do plants inactivate homologous (trans)genes? Plant Physiol 107:679–685

Mittelsten Scheid O, Jakovleva L, Afsar K, Maluszynska J, Paszkowski J (1996) A change of ploidy can modify epigenetic silencing. Proc Natl Acad Sci USA 93:7114–7119

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Murray AA, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res 8:4321–4325

Nadolska-Orczyk A, Przetakiewicz A, Kopera K, Binka A, Orczyk W (2005) Efficient method of *Agrobacterium*-mediated transformation for triticale (x *Triticosecale* Wittmack). J Plant Growth Regul 24:2–10

Nuutila AM, Villiger C, Oksman-Caldentey KM (2002) Embryogenesis and regeneration of green plantlets from oat (*Avena sativa* L.) leaf-base segments: influence of nitrogen balance, sugar and auxin. Plant Cell Rep 20:1156–1161

Pawlowski WP, Somers DA (1998) Transgenic DNA integrated into the oat genome is frequently interspersed by host DNA. Proc Natl Acad Sci USA 95:12106–12110

Pawlowski WP, Torbert KA, Rines HW, Somers DA (1998) Irregular patterns of transgene silencing in allohexaploid oat. Plant Mol Biol 38:597–607

Perret S, Valentine J, Leggett JM, Morris P (2003) Integration, expression and inheritance of transgenes in hexaploid oat (*Avena sativa* L.). J Plant Physiol 160:931–943

Przetakiewicz A, Orczyk W, Nadolska-Orczyk A (2003) The effect of auxin on plant regeneration of wheat, barley and triticale. Plant Cell Tissue Organ Cult 73:245–256

Przetakiewicz A, Karas A, Orczyk W, Nadolska-Orczyk A (2004) *Agrobacterium*-mediated transformation of polyploid cereals. The efficiency of selection and transgene expression in wheat. Cell Mol Biol Lett 9:903–917

Somers DA, Rines HW, Gu W, Kaeppler HF, Bushnell WR (1992) Fertile, transgenic oat plants. Biotechnology 10:1589–1594

Somers DA, Torbert KA, Svitashev SK (2003) Genetic transformation of oat (*Avena sativa* L.). In: Jaiwal PK, Singh RP (eds) Plant genetic engineering, vol 2, Improvement of food crops. Sci Tech Publishing, LLC, pp 141–155

Sticklen MB, Oraby H (2005) Shoot apical meristem: a sustainable explant for genetic transformation af cereal crops. In Vitro Cell Dev Biol Plant 41:187–200

Svitashev SK, Somers DA (2001) Genomic interspersions determine the size and complexity of transgene loci in transgenic plants produced by microprojectile bombardment. Genome 44:691–697

Svitashev S, Ananiev E, Pawlowski WP, Somers DA (2000) Association of transgene integration sites with chromosome rearrangements in hexaploid oat. Theor Appl Genet 100:872–880

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. Plant J 11:1369–1376

Torbert KA, Rines HW, Somers DA (1995) Use of paromomycin as a selective agent for oat transformation. Plant Cell Rep 14:635–640

Torbert KA, Gopalraj M, Medberry SL, Olszewski NE, Somers DA (1998a) Expression of the *Commeliana* yellow mottle virus promoter in transgenic oat. Plant Cell Rep 17:284–287

Torbert KA, Rines HW, Somers DA (1998b) Transformation of oat using mature embryo-derived tissue cultures. Crop Sci 38:226–231

Wang C-T, Wei Z-M (2004) Embryogenesis and regeneration of green plantlets from wheat (*Triticum aestivum*) leaf base. Plant Cell Tissue Organ Cult 77:149–156

Wu H, Sparks C, Amoah B, Jones HD (2003) Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. Plant Cell Rep 21:659–668

Zhang S, Warkentin D, Sun B, Zhong H, Sticklen M (1996) Variation in the inheritance of expression among subclones for unselected (*uidA*) and selected (*bar*) transgenes in maize (*Zea mays* L.). Theor Appl Genet 92:752–761

Zhang S, Cho M-J, Koprek T, Yun R, Bregitzer P, Lemaux PG (1999) Genetic transformation of commercial cultivars of oat (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) using in vitro shoot meristematic cultures derived from germinated seedlings. Plant Cell Rep 18:959–966