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Efficient Method of *Agrobacterium*-mediated Transformation for Triticale (x *Triticosecale* Wittmack)

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ABSTRACT

Transgenic plants of triticale cv. Wanad were obtained after transformation using three combinations of strain/vectors. Two of them were hypervirulent *Agrobacterium tumefaciens* strains (AGL1 and EHA101) with vectors containing *bar* under maize ubiquitin 1 promoter (pDM805), and both *hpt* under p35S and *npt*II under pnos (pGAH). The third one was a regular LBA4404 strain containing super-binary plasmid pTOK233 with selection genes the same as in pGAH. The efficiency of transformation was from 0 to 16% and it was dependent on the selection factor, auxin pretreatment, and the strain/vector combination. The highest number of transgenic plants was obtained after transformation with LBA4404(pTOK233) and kanamycin selection. Pretreatment of explants with picloram led to the highest number of plants obtained after transformation with both *Agrobacterium*/vector systems LBA4404(pTOK233) and EHA101(pGAH) and selected with kanamycin. Transgenic character of selected plants was examined by PCR using specific primers for *bar*, *gus*, *npt*II, and *hpt* and confirmed by Southern blot hybridization analysis. There was no GUS expression in T_0 transgenic plants transformed with *gus* under p35S. However the GUS expression was detectable in the progeny of some lines. Only 30% of 46 transgenic lines showed Mendelian segregation of GUS expressing to GUS not expressing plants. In the remaining 70% the segregation was non-Mendelian and the rate was much lower than 3:1. Factors that might effect expression of transgenes in allohexaploid monocot species are discussed.

Key words: Triticale; *Agrobacterium tumefaciens*; Cereal transformation; Transgene expression; PCR analysis

INTRODUCTION

Triticale is an allohexaploid species obtained as a result of crossing allotetraploid wheat (AABB, $2n = 4x = 28$) with diploid rye (RR, $2n = 2x = 14$). It was created over 100 years ago, but the first cultivars were only released in the 1970s. Currently used cultivars, the result of intensive breeding programs, are competitive with other cereals and are already widely cultivated. With its modest requirements, the species is a good fit for climatic and soil conditions in Central Europe, and it gives relatively

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high yields. Despite the clear success of classical breeding there are several traits that still need to be modified. Among them is low resistance to preharvest sprouting and improvement of grain quality (for example low level of lysine content or altered gluten composition).

Genetic modification is an important experimental tool that can be used to analyze and understand mechanisms responsible for expression of trans-genes or endogenous genes, as well as to create plants with desired characteristics. Of the two most used genetic transformation techniques, *Agrobacterium*-mediated and biolistic, the latter is still the main method for genetic engineering of allopolyploid cereals. Although, *Agrobacterium*-mediated transformation is the method of choice because of its low level of transgenic DNA rearrangement, low number of introduced copies, stable integration of defined T-DNA fragment, and expected higher level of transgene expression. This was well documented in papers comparing these two techniques in rice (Dai and others 2001) and wheat (Hu and others 2003). However, there are no papers on *Agrobacterium*-mediated transformation of some allopolyploid cereals like triticale or oat, and only recently have three papers been published on wheat (Hu and others 2003; Khanna and Daggard 2003; Wu and others 2003) after an earlier publication by Cheng and others (1997). Up to now, triticale has only been transformed by the biolistic method with 3.3% transformation efficiency (Zimny and others 1995).

We developed a short-term, efficient method of triticale plant regeneration from immature embryos (Przetakiewicz and others 2003). The goal of this research was to establish *Agrobacterium*-mediated transformation of triticale, based on the method of regeneration previously published. The influence of three kinds of auxin used during the pre-culture period on efficiency of transformation with three strain/plasmid combinations was tested. Among 168 selected, PCR-positive plants, expression of GUS was analyzed in offsprings of 46 T_0 transgenic plants.

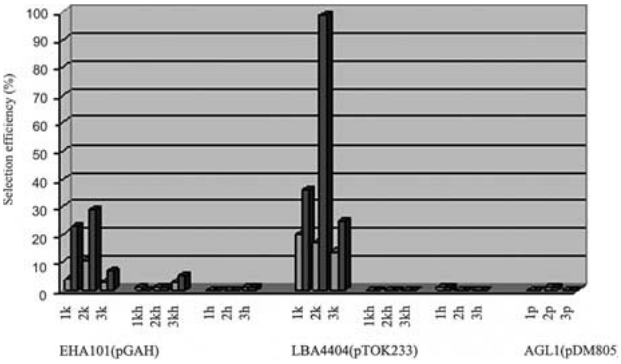

Figure 1. Influence of the three auxins: 2,4-D (1), picloram (2), and dicamba (3) on selection efficiency per explant (gray bar) and rooted plant number (black bar) after transformation with three strain/vector systems and selection on kanamycin (k), hygromycin (h), kanamycin + hygromycin (kh), or phosphinothricin (p). 100 explants were used in each combination.

Materials and Methods

Plant Material and Transformation Procedure

Immature embryos of Polish spring triticale (× *Triticosecale* Wittmack) cultivar Wanad were used for experiments. Donor plants were cultivated in growth chambers at 20/16°C (day/night), 16 h photoperiod, and under 350 μmol $s^{-1}m^{-2}$ light. Immature seeds were collected between 12 and 14 days after anthesis. They were sterilized and cultured according to the procedure described in Przetakiewicz and others (2003).

Isolated immature embryos were precultured on solid MSB medium (Przetakiewicz and others 2003) containing 3 mg l^{-1} of one of the three auxins: 2,4-D (13.6 μM), picloram (12.4 μM), or dicamba (13.6 μM). After three to four days they were inoculated with a drop (about 10 μl) of *A. tumefaciens* suspension that was placed on top of each embryo and cocultured for the next three days on the same medium. Subsequent culture was carried out on the MSB medium containing 2,4-D and picloram 1.5 mg l^{-1} each, 150 mg l^{-1} Timentin and one of the selection agents: kanamycin 50 mg l^{-1}, hygromycin 15 mg l^{-1} or phosphinothricin 2 mg l^{-1}. In one combination both kanamycin and hygromycin were used. After 4 more weeks of culture, the embryogenic calli were transferred on plant regeneration R2-MSB medium (Przetakiewicz and others 2003) containing the same selection agent. Final development and rooting of regenerated plantlets continued under appropriate selection on modified MS medium. Green, well-rooted plants were transferred to soil for further growth and were maintained in the growth chamber under the conditions described above. Mature seeds of self-fertilized plants were collected to produce the first (T_1) generation of T_0 transgenic plants.

Plant transformation efficiency, expressed as a percentage, was the number of independent calli lines, which gave PCR and/or GUS positive, seed setting plants, divided by the number of all transformed explants. They were selected after two to

three months of culture. Selection efficiency per explant (Figure 1) was defined as a percentage of explants regenerating green plants under selection.

Bacterial Strains and Binary Vectors

The following *A. tumefaciens* strains were used: LBA4404 (Hoekema and others 1983) and two hypervirulent strains AGL1 (Lazo and others 1991) and EHA101 (Hood and others 1986). LBA4404 carried superbinary pTOK233 vector provided by Dr. T. Komari, Japan Tobacco Inc. (Hiei and others 1994). The vector contained pnos/*npt*II and p35S/*hpt* for selection and intron *gus* under 35S promoter. Binary vector pDM805 provided by Dr. R. Brettell (Tingay and others 1997) was electroporated into AGL1 strain. T-DNA of the vector contained *gus* under rice actin 1 promoter (first exon and intron of the rice actin 1 gene) and *bar* under maize ubiquitin 1 promoter (first exon and intron of the maize ubiquitin 1 gene). EHA1O1 carried binary pGAH vector provided by Dr. N. Murata (Hayashi and others 1997). The vector contained pnos/*npt*II and p35S/*hpt* selection constructs. The components of T-DNA located between right (RB) and left border (LB) and the direction of transcription (arrows) were according to the scheme presented below:

pTOK233 RB ▶ pnos → *npt*II intron*gus* ← p35S ■ *hpt* ← 35S ◀ LB

pDM805 RB ▶ pnos → *npt*II ■ p35S → *hpt* ◀ LB

pGAH RB ▶ *bar* ← Ubil ■ Actl → *gus* ◀ LB

Agrobacterium AGL1 (pDM805) strain was cultured in MG/L liquid medium supplemented with 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin, strain LBA4404(pTOK233) in AB with 50 mg l^{-1} hygromycin and EHA101(pGAH) in MG/l supplemented with 5 mg l^{-1} kanamycin and 25 mg l^{-1} hygromycin. After 2 days of culture at 28°C, bacteria were resuspended in MSB medium containing 200 μM acetosyringone. The density (OD_{600}) of bacteria in inoculation suspension ranged from 1.0 to 2.0.

PCR Analysis

Genomic DNA was isolated from young leaves of T_0 and T_1 plants using the modified cetyltrimethyl ammonium bromide (CTAB) method (Cheng and others 1997). 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 were as follow: GAGGCTATTCGGCTATGACTG and ATCGGGAGC GGCGATACCGTA for amplification of the 700 bp *npt*II fragment; GGAGTATTGCCAACGAACC and CGCCAGGAGAGTTGTTCATTC for the 606 bp *gus* fragment amplification; TCTGCACCATCGTCAACC ACTACATC and CAGAAACCCACGTCATGCCA GTTC for the 430 bp *bar* fragment amplification; TTTGCCCTCGGACGAGTGCT and GGGAGTTTAG CGAGAGCCTGACCTA for the 778 bp *hpt* fragment; and ACGTCCTGTAGAAACCCCAA and CCCGCTTC GAAACCAATGCC for the 1097 bp *gus* fragment amplification (Jefferson and others 1986). Amplification conditions were: 94°C for 1 min., 62°C (*npt*II), 57°C (606 bp *gus*), 68°C (*bar*), 65°C (*hpt*), or 60°C (1097 bp *gus*) for 1 min and 72°C for 2 min (an exception was 1 min for *hpt*). The number of cycles was 36. The conditions were optimized for each pair of primers separately.

Southern Blot Hybridization

Approximately 35 μg of genomic DNA from T_0 and T_1 plants digested with *Hind*III was electrophoresed together with a molecular weight marker in 0.7% agarose in 1 × TBE buffer. DNA was blotted onto nylon membranes, positively charged (Roche) by vacuum alkaline transfer. The probe was a PCR-amplified (using pTOK233 as a template) 700 bp *npt*II fragment labeled with DIG dNTP (Roche). Prehybridization (1 h) and hybridization (16 h) were performed at 68°C in standard hybridization buffer (Roche). Labeling and detection were done with 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 and Herbicide Resistance

GUS expression was determined in young leaf tissue of T_0 plants and leaf and coleoptile fragments were collected from 5-day old T_1 and T_2 seedlings using histochemical GUS assay (Jefferson and others 1987). Explants were incubated overnight at 37°C in buffer containing 2 mM X-Gluc and 50 mM sodium phosphate buffer pH 7.0.

Herbicide resistance was tested by spraying young seedlings (with 3–5 leaves) with Basta solution containing gluphosinate ammonium 150 mg l^{-1}. Plants were grown in growth chamber under the conditions described above.

Table 1. Effect of Strain/Binary Vector and Selection Agent on Efficiency of Triticale Transformation

Strain (vector)	Applied selection	Number of explants	Regenerating explants (%)		Number of plants.		Transformation efficiency
			4 weeks	10 weeks	Selected	Transg.	
AGL1 (pDM805)	Phosphinothricin	300	21	0.33	1	1	0.33
EHA101 (pGAH)	Hygromycin	300	15	0.33	1	1	0.33
	Kanamycin	300	16	6.0	59	44	6.0
	Hyg. + Kan.	300	16	1.7	6	3	1.7
LBA4404(pTOK233)	Hygromycin	300	53	0.33	1	1	0.33
	Kanamycin	300	41	16.0	161	118	16.0
	Hyg. + Kan	300	38	0.0	0	0	0.0

Results

Three combinations of strain/vector were used to test the capability of *Agrobacterium* system to transform triticale: AGL1(pDM805) (Tingay and others 1997), LBA4404(pTOK233) (Hiei and others 1994), and EHA101(pGAH)(Hayashi and others 1997). The first one contained *bar* under maize ubiquitin 1 promoter and the next two p35S/*hpt* and pnos/*npt*II. According to the specific selection genes present in T-DNA of transformation vectors phosphinothricin, hygromycin, and/or kanamycin selection were tested (Table 1).

The ratio of explants regenerating somatic embryos after 4 weeks of culture was 21% after transformation with AGL1(pDM805), 15% to 16% with EHA101(pGAH) and from 38% to 53% after inoculation with LBA4404(pTOK233) (Table 1). The ratio changed dramatically after longer selection (the next 6 weeks of culture) ranging from 0 to 16%. The highest number of regenerating explants turned brown on media containing hygromycin or both hygromycin and kanamycin. These calli lines regenerated one to several plants resistant to the selecting factor. The highest number of 118 triticale plants were selected on kanamycin containing medium after transformation with LBA4404(pTOK233), equivalent to 16% transformation efficiency. A high rate of transformation was also obtained using EHA101(pGAH) with kanamycin selection. Forty-four plants were regenerated after inoculation with this strain/vector, corresponding to a 6% transformation efficiency (Table 1).

A few plants were selected on hygromycin or hygromycin plus kanamycin, and this result was independent of the combination of strain/vector used for transformation. Only one transgenic plant was obtained after transformation with AGL1(pDM805) and phosphinothricin selection. The rate of transformation efficiencies ranged from 0 to 1.7% for the above combinations (Table 1).

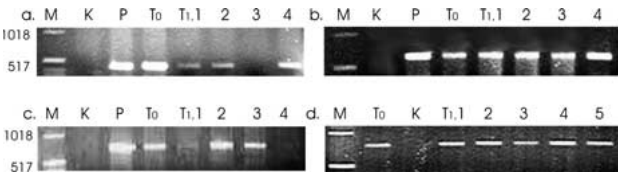

Figure 2. Polymerase chain reaction analysis of transformed T_0 plants and T_1 (1–4) offspring. Amplification of *bar* 430 bp with DNA of 399 line (**a**), *gus* 606 bp with DNA of 48 line (**b**), *npt*II 700 bp with DNA of 297 line (**c**) and *hpt* 778 bp with DNA of 371 line (**d**). M = molecular weight marker; K-control with DNA from untransformed plant; P-plasmid DNA used as a template.

The effects of three auxins, 2,4-D, picloram, and dicamba, used during pre-culture of immature embryos on selection efficiency after transformation with three combinations of strain/vector are presented in Figure 1. The highest number of plants (99 and 29) were obtained after pre-culture on media containing picloram followed by inoculation with either LBA4404(pTOK233) or EHA101(pGAH) and selected on kanamycin. Selection efficiency per explant (number of explants from which plants were obtained), was also high in the same combinations (17 and 11, respectively). Pre-culture on 2,4-D followed by selection on kanamycin gave slightly (in the case of EHA101) or much lower (for LBA4404) numbers of regenerated plants and lower efficiency per explant (for EHA101) compared with the combination of picloram/kanamycin. The lowest results (in terms of both number of plants and selection efficiency) with kanamycin-based selection were obtained after pre-culture of embryos on dicamba-containing medium.

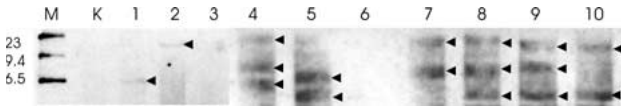

Figure 3. Southern blot analysis of T_0 plants (1–10) showing integration of *npt*II fragment. About 35 μg of DNA from plants transformed with pTOK233 were digested with *Hind*III and probed with 700 bp *npt*II fragment labeled by DIG dNTP. The enzyme cut at T-DNA once, generating a fragment longer than 5.65 kb (= size of *npt*II fragment at the RB). M = DIG-labeled DNA molecular weight marker II; K = DNA from non-transformed plant.

The transgenic character of selected and rooted plants was confirmed by PCR analysis of *bar*, *gus*, *npt*II, and/or *hpt* (Figure 2 a–d). At least two out of four tested pairs of primers were used to test each T_0 plant. Seventy-five percent of plants transformed with EHA101(pGAH) and 73.3% of plants transformed with LBA4404(pTOK233) were PCR-positive. The PCR data were reliable only with selected pairs of starters (Figure 2) and purified (phenol extracted) template DNA. These data were confirmed by Southern blot hybridization (Figure 3).

Function of transgenes in *Agrobacterium* -mediated transgenic triticale plants was assessed by testing GUS expression and Basta resistance (Figure 4a,b; Table 2). Offspring of 46 T_0 plants, out of 118 obtained after LBA4404(pTOK233) transformation, were analyzed for segregation of GUS expression (Table 2). Fourteen lines (30%) showed a segregation ratio of GUS(+) to GUS(−) close to 3:1. The Segregation ratio in progeny of the remaining lines was lower.

There was only one transgenic, adapted to soil, and seed-setting plant obtained after AGL1(pDM805) transformation and phosphinothricin selection. The segregation ratio of Basta-resistant to Basta-susceptible offspring in T_1 was 1:2, and segregation for GUS (+) positive to GUS (−) was 2:1. In the second generation (T_2) of the three Basta-resistant plants, all progeny were GUS (+) but there were almost no Basta-resistant plants. The ratio of resistant to susceptible plants was 1:10.

Seedlings of three T_1 lines (transformed with pTOK233) were tested for GUS activity after germination on kanamycin (Table 3). The ratio of GUS (+) to GUS (−) plants was very low. Germinated seedlings of the same lines without kanamycin treatment showed a higher segregation ratio of GUS expression.

Discussion

Three systems of *Agrobacterium* strain/vector were tested to transform one selected cultivar of triticale. In two of them, hypervirulent strain AGL1 (Lazo and others 1991) carrying binary vector pDM805 (Tingay and others 1997) or EHA101 (Hood and others 1986) carrying pGAH (Onouchi and others 1991) were used. The third system was composed of the commonly used LBA4404 strain and super-binary vector pTOK233 (Hiei and others 1994). The AGL1 (pDM805) and LBA4404 (pTOK233) strains have already been reported to transform other cereals (see review Nadolska-Orczyk and Orczyk 2003). The first combination was used by Tingay and others (1997) to transform barley, and the second was used by Hiei and others (1994) to obtain transgenic rice. The EHA101(pGAH), harboring the *codA* gene instead of *gus*, was used to test expression of this gene in *Arabidopsis thaliana* (Hayashi and others 1997). All three systems were also tested for their ability to transform wheat (Przetakiewicz and others 2004).

Transformation efficiency of barley cv. Golden Promise reported by Tingay and others (1997) was 4.2% and was very high compared with triticale, where one plant from 300 immature embryos was selected (0.33%) (Table 1). The highest rate of transformation, 16% was observed after transformation of triticale with common strain LBA4404 containing the super-binary plasmid pTOK233. This efficiency was lower than that obtained for Japonica rice (Hiei and others 1994) but much higher than in the case of three cultivars of wheat transformed with the same combination of strain/vector (Przetakiewicz and others 2004). Interestingly, the highest transformation rate in the case of wheat was established for EHA101(pGAH). Such high *Agrobacterium*-mediated transformation frequency was only reported in the case of the corn hybrid Hi-II after using a modified pTOK system with pSB11 vector (Zhao and others 2001). In the former paper, however, the figure referred precisely to ''callus transformation frequency,'' defined as the number of immature embryos that generated GUS-positive calli. As it was calculated by the authors plant transformation efficiency was 83.7% of the ''callus transformation frequency''. Despite the efficiencies calculated for the same *Agrobacterium*/ vector combination the system itself was used for very different cereal species. Rice and corn are diploid species representing relatively small (4×10^8 bp) or intermediate (2.4×10^9 bp) genome sizes (Moore 2000). The genome of allohexaploid triticale is probably as large as that of allohexaploid wheat, which is estimated at 16×10^9 bp (Moore 2000). Most of its genomic DNA consists of heterochromatic regions (repetitive sequences) that are expected to influence transformation efficiency as well as gene and transgene expression.

Table 2. Segregation Analyses of GUS Expression and Basta Resistance in T_1 Generation of Selected Transgenic Lines of Triticale cv. Wanad

Strain (vector) construct	Line number	Segregation factor/segregation ratio	χ^2 test for 3:1 segregation ratio	*p*
LBA4404		GUS-positive/GUS-negative		
(pTOK233)	#48	12:4	1.0	1.0
p35S/int*gus*	#49	13:8	0.166	0.41
	#109	15:6	0.705	0.84
	#131	13:3	0.564	0.75
	#132	16:16	0.001	0.03
	#134	13:10	0.04	0.2
	#161	16:5	0.9	0.95
	#162	12:6	0.414	0.64
	#202	14:7	0.378	0.61
	#204	14:7	0.378	0.61
	#241	11:5	0.564	0.75
	#242	16:5	0.9	0.95
AGL1 (pDM805) pUbil/*bar*	#399	Basta (+)/Basta (−) 4:8	0.001	0.03
pActl/*gus*		GUS (+)/GUS(−) 10:5	0.456	0.68

The selection system is one of the most important factors influencing transformation efficiency. In our experiments three selection agents were tested: PPT after transformation with AGL1(pDM805) and kanamycin and/or hygromycin after transformation with EHA101(pGAH) or LBA4404(pTOK233). The *bar* gene was under maize Ubil promoter, *hpt* was under 35S, and *npt*II under nos promoter. As it was clearly shown, kanamycin selection was the most appropriate for triticale compared with hygromycin or phosphinothricin. The only factor effecting *npt*II or *hpt* selection tested in our experiments (using the same *Agrobacterium*/vector combination) was the promoter driving expression of the selection gene. Despite the earlier indications that the 35S promoter gave stronger expression than pnos, we did not notice any positive influence of this promoter on selection efficiency using hygromycin. There were no papers on *Agrobacterium*-mediated transformation of triticale. However, in our experiments on successful *Agrobacterium*-mediated transformation of wheat, another closely related, allohexaploid species, kanamycin selection, was the most appropriate one (Przetakiewicz and others 2004). In another report on wheat, 4% of transformation efficiency was reported. Transgenic plants expressing *npt*II under an enhanced 35S promoter were selected on G418 (Cheng and others 1997). The transformation rate after hygromycin selection was low, and it was similar to the rate obtained for wheat (Przetakiewicz and others 2004). Our data from PPT selection after *Agrobacterium*-mediated transformation were also very low compared with kanamycin and much less efficient than that obtained after microprojectile bombardment of triticale (Zimny and others 1995) with construct, where *bar* was under the control of the 35S promoter (Becker and others 1994). Transformation efficiencies ranged from 0.3% (our report) to 3.3% (comparable to the biolistic method) demonstrated by Wu and others (2003) for wheat transformed with AGL1 harboring the pGreen-based plasmid-containing *bar* under the maize Ubil promoter plus ubiquitin 1 intron. A slightly higher transformation efficiency (4.4%) was demonstrated for wheat transformed by *Agrobacterium* strain C58 (ABI) containing the glyphosate-tolerant *aro*A:CP4 gene under rice actin 1 promoter (Hu and others 2003). This gene was also successfully used as the selectable marker in wheat transformation by microprojectile bombardment (Zhou and others 1995). In other newly published procedures for wheat transformation via *Agrobacterium*, the best result,

Table 3. Segregation of GUS Expression in Seedlings Treated and Not Treated with Kanamycin in T_1 Progeny of Three LBA4404 (pTOK233) Transformed Lines

T_1 line	Kanamycin-treated seedlings GUS(+)/GUS(−)	Water germinated seedlings GUS(+)/GUS(−)
#243	3:13	10:6
#275	2:14	8:8
#278	3:13	12:4

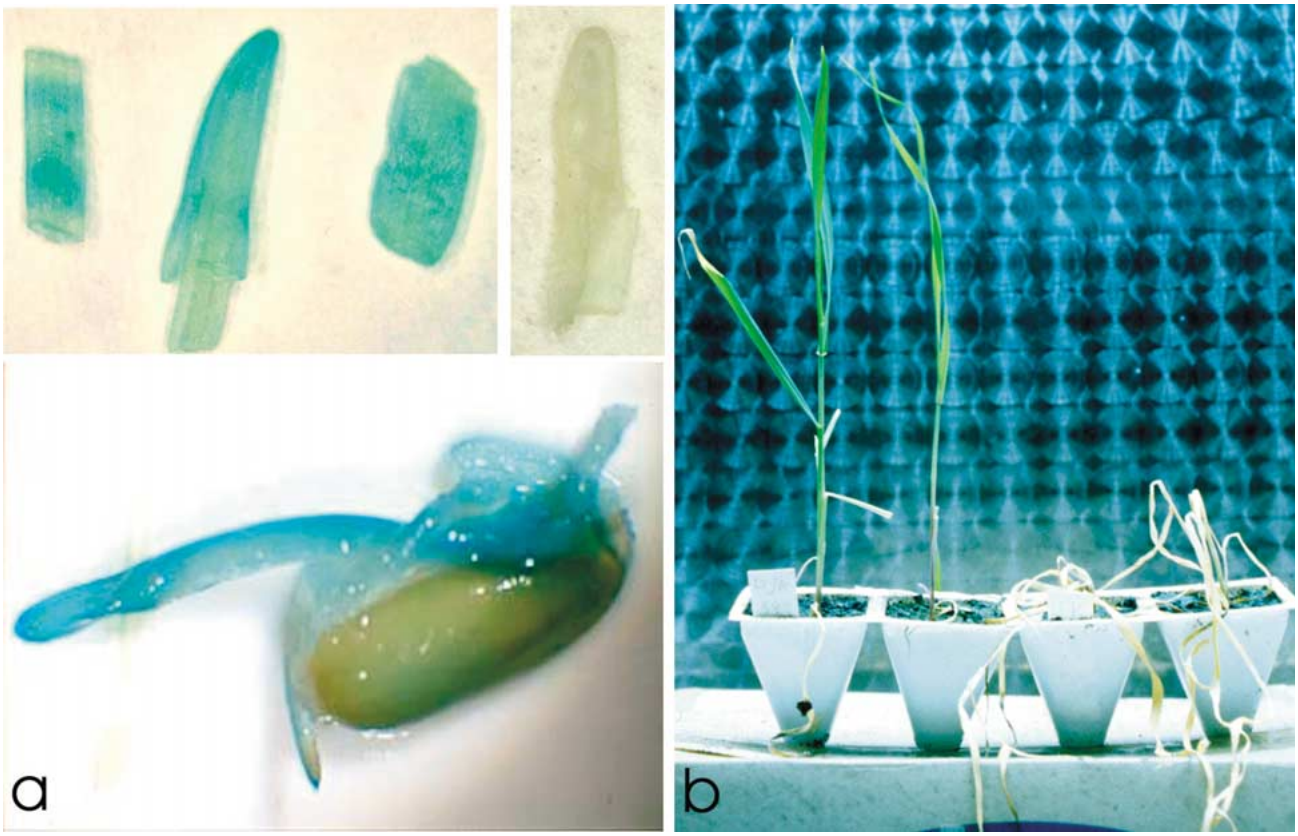

Figure 4. GUS activity in leaf and coleoptile fragments of seedlings (left), control coleoptile (a, right top) and a germinating seedling (**a**, bottom), and Basta- resistant (green) and susceptible control (dried) plants of Wanad after 9 days of herbicide spraying (**b**).

3.9% efficiency, was obtained after transformation with the super-binary pHK21 vector containing *bar* under the intron ubiquitin1 promoter (Khanna and Daggard 2003).

The influences of three auxins, 2,4-D, picloram, and dicamba, on triticale transformation efficiency were obvious in the case of kanamycin selection. The best results were obtained with immature embryos precultured on picloram-containing medium. This observation was correlated with the highest plant-regeneration efficiency obtained on this medium (Przetakiewicz and others 2003). The key role of phytohormones during *Agrobacterium*-mediated transformation has already been demonstrated (Sangwan and others 1992; Villemont and others 1997). As shown in *A. thaliana in vitro* culture, the effect of genotype can be partly overcome by increasing the duration of the phytohormone treatment (Chateau and others 2000). Anyway, this effect, visible only in the case of kanamycin but not in the cases of hygromycin or PPT selection of triticale, suggested that the influence of the selection agent in triticale transformation is more important.

Transgenic character of obtained plants and their progeny was confirmed by polymerase chain reaction (PCR) and Southern hybridization. Some pairs of primers selected for efficient amplification on a control plasmid DNA template gave very low amplification with some of the tested plants (not shown). In such cases, very weak amplification signals had to be visualized by Southern hybridization, even though the presence of the transgene was confirmed in the progeny of the plants. There are some possible causes for the problem. The ratio of single transgene to genome is very low. This may be particularly important in *Agrobacterium*-mediated transformation, where the number of integrated copies is very low too. For plants with very large genomes, such as wheat, one copy transgene might be about a 8 to 16 $\times$ 10^{-6} fraction of the whole genome. Secondary structures of regions where the transgene was integrated can make the target less accessible and/or amplification less efficient. Cases of amplification of an unspecific product of similar size to the expected one indicated that the specificity of PCR products obtained in polyploid cereal

species with large genomes should be confirmed with hybridization. Another possibility is to choose better working pairs of primers and highly purified plant DNA for PCR amplification.

In most of the papers describing transformation of polyploid, cereal species, segregation of transgenes was tested by expression analysis (Cheng and others 1997; Hu and others 2003; Zimny and others 1995). However, in one of the most recent papers on *Agrobacterium*-mediated transformation of allohexaploid wheat (Khanna and Daggard 2003) segregation of the *bar* gene in the T_1 generation was tested by PCR, giving reliable Mendelian segregation scores.

The transgenic character of selected plants and their offspring was confirmed by expression analysis. According to our results, only 30% of 47 lines tested showed Mendelian segregation of GUS expressing to GUS non-expressing plants, indicating the presence of a single integration locus. In the remaining 70% the segregation was non-Mendelian and the rate was much lower than 3:1. Segregation of transgene expression after *Agrobacterium*-mediated transformation is expected to be closer to Mendelian-type ratios than after biolistic transformation. This notion relies on a model of T-DNA integration in the *Agrobacterium*-mediated system (Komari and others 1996; DeNeve and others 1997) compared with an integration of foreign DNA delivered by the biolistic method (Dai and others 2001; Kohli and others 1998). High numbers of transgenic lines showing non-Mendelian segregation were observed in allohexaploid oat after biolistic transformation (Pawlowski and others 1998; Svitashew and others 2000). Part of this segregation was caused by the integration of the transgenic loci on the border of the translocation sites (Svitashew and others 2000). T-DNA integration in rice was observed in regions rich in genes (Barakat and others 2000). We suppose that the non-Mendelian segregation pattern of expression in triticale transformed by *Agrobacterium* is not directly related to the structure and location of the transgenic loci, but rather to the T-DNA composition (promoters, enhancers) as well as mechanisms of transgene silencing in this allohexaploid species.

Gene dosage conferred by the ploidy level in polyploid species leads in general to higher levels of gene expression (Osborn and others 2003). Stable and strong GUS expression was observed in transgenic wheat containing more than 10 copies of a transgene (Stoger and others 1999). Positive correlation between transgene dosage and level of expression was visible in our experiments. GUS expression, frequently invisible in T_0, hemizygotic triticale plants, was visible in part of homozygotic progeny. This observation is in contrast to the commonly accepted notion for diploid species, that a single or low copy number is needed to maximize expression and minimize silencing (Kooter and others 1999; Matzke and Matzke 1995). We would suggest that one of the causes for higher expression levels of transgenes in polyploid cereals, more frequently reported after biolistic than *Agrobacterium* transformation, was the result of higher copy number integration. An integration of a single-copy transgene, also in agreement with our results, is very typical for *Agrobacterium*-mediated transformation. Frequently about 30% (up to 60%) of T_0 plants contained single inserts (see review by Nadolska-Orczyk and others 2000).

Low GUS expression or lack of GUS expression in transgenic triticale could also be the result of the type of promoter used. *Gus* was usually driven by 35S. This commonly used, constitutive promoter considered as strong in dicotyledonous plants, was reported to be weak in monocots, especially in wheat (Chen and others 1998, 1999). In opposition to low or lack of expression of 35S/*gus*, the expression of this marker was detectable in T_0 and stable in T_1 and T_2 of one triticale plant, when the Act1 promoter was used. All these factors might have contributed to a low expression level, which could be the main cause of difficulties in developing a reliable method of *Agrobacterium*-mediated transformation of polyploid cereals.

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