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The procedure providing enhanced *Agrobacterium*-mediated transformation of wheat

Nevena MITIĆ[1]*, Branka VINTERHALTER[1], Slavica NINKOVIĆ[1] & Dejan DODIG[2]

[1]*Institute for Biological Research "Siniša Stanković", Department of Plant Physiology, University of Belgrade, Despot Stefan Boulevard 142, 11060 Belgrade, Serbia; e-mail: mitic.nevena@ibiss.bg.ac.rs*
[2]*Maize Research Institute "Zemun Polje", Slobodana Bajića 1, 11185 Belgrade, Serbia*

Abstract: The examinations of conditions for establishing a variety independent *Agrobacterium*-mediated transformation procedure for wheat are preferable since many of cultivars and breeding lines remain recalcitrant for biotechnological manipulation, mainly due to low efficiency of plant regeneration *in vitro*, which is highly genotype specific. This paper describes and discusses an improved protocol for enhanced and low-genotype dependent *Agrobacterium*-mediated transformation using a super-binary vector LBA4404/pTOK233 carrying reporter *gus-intron* gene and hygromycin (*hpt*) and kanamicyn (*npt*II) selectable marker genes. The protocol was optimized on highly responsive common wheat cv. Vesna. Transient expression monitored by the *gus-intron* on explants after 3, 6 and 25 days of co-cultivation, followed by GUS expression and hygromycin resistance in whole plants indicated the protocol including a co-cultivation of freshly isolated immature embryos in the presence of ascorbic acid, and acetosyringone added only in the bacteria-containing infection medium combined with a delayed and stepwise increasing hygromycin B selection procedure significantly enhanced the transformation efficiency in cv. Vesna that exceed 7% of treated explants from previously 0.41%. Explant pre-cultivation did not additionally improve transformation efficiency. The optimized protocol was successful in evoking satisfactory transformation efficiencies from 3.6% to 10.8% in 5 less-responsive wheat genotypes. All 57 T0 hygromycin-resistant and GUS-positive lines were phenotypically normal and fertile. Therefore, the conditions employed in this study may serve as a base to facilitate the transformation in other, particularly recalcitrant wheat cultivars.

Key words: *Agrobacterium*; immature embryos; GUS expression; transformation frequency; regeneration; wheat genotypes.

Introduction

Wheat as one of the most widely grown food crops in the world has been representing for decades the main target for improvement in agronomic and biological properties. Complementary to conventional breeding that reaches a plateau due to limited gene pool available; genetic engineering allows the possibility to exploit genes from different sources beyond the wheat gene pool and to combine them in a desired genotype. Wheat has initially been considered as one of most recalcitrant species for *in vitro* regeneration and genetic transformation and important advances have been achieved by continuous screening of genotypes and tissues for regeneration potential (Bhalla 2006). The first fertile transgenic wheat plants have been obtained by Vasil et al. (1992) using the microprojectile bombardment transformation method. *Agrobacterium*-mediated transformation has been demonstrated as a better alternative to biolistic gun for low copy insertions of T-DNA with minimal rearrangements and degree of gene-silencing phenomenon (Cheng et al. 2004; Jones et al. 2005; Shrawat & Lörz 2006; Tao et al. 2011). Unfortunately, despite the great progress achieved in wheat transformation mediated by *Agrobacterium*, the low transformation efficiencies (in most cases about 1% and rarely exceeding 4%) (Li et al. 2012) and a restricted number of successfully transformed genotypes, screened mainly according to high regeneration potential, remain a major obstacle for the routine application of this transformation method in a wide spectrum of wheat genotypes.

Further optimization of culture conditions to minimize plant cell death after the *Agrobacterium* application and development of the appropriate selection procedure are important to improve the transformation efficiency in less-responsive but agronomically important wheat genotypes. In our previous study (Mitić et al. 2004) we have successfully transformed immature embryos of spring wheat cv. Vesna, which was selected for its high regeneration capacity (Mitić et al. 1999). The average transformation efficiency amounted to 0.41% using the super-binary vector in *A. tumefaciens* strain LBA4404 (Mitić et al. 2004). The obtained data suggest that the transformation of cv. Vesna is feasible but the low transformation frequency

* Corresponding author

represents the major concern and need to be improved.

For this purpose here we report on some modifications of the transformation protocol used before which concern: a) few days of pre-cultivation of freshly isolated immature embryos before inoculation by *Agrobacterium*; b) the addition of ascorbic acid as an antioxidant during co-cultivation; c) the variation of the presence of induction agent acetosyringone in inoculation and co-cultivation media, and d) delayed and stepwise hygromycin B selection procedure. The effect of the transformation protocol on transient GUS expression and stable transformation according to plant hygromycin-resistance was studied. The conditions optimized for highly responsive cv. Vesna were than successfully applied to few less-responsive wheat genotypes.

Material and methods

Plant material

Common wheat genotypes used in this study included commercial spring cv. Vesna, chosen for its high regeneration capacity; advanced winter breeding line ZA-205, winter landrace from Bosnia and Herzegovina BL-100, two CIMMYT cvs. of spring type Inia 66 and Tobari 66 and winter cv. Norin 10 (Japan) selected because of their previously established less regenerative potential compared to cv. Vesna. Cultivars Vesna and ZA-205 have been developed at the Centre for Agricultural and Technological Research (Zaječar, Serbia), while BL-100, Inia 66, Tobari 66 and Norin 10 have been obtained from the core collection of the Institute of Field and Vegetable Crops (Novi Sad, Serbia).

Immature seeds of field grown plants were harvested 15 d after pollination and stored until use at 4 °C for at least 24 h. The seeds were surface sterilized with 70% ethanol for 1 min, followed by commercial NaOCl bleach (8% active chlorine) with a few drops of fungicide Captan® for 30 min, rinsed 4 times with sterile distilled water and blotted dry on a piece of sterile filter paper. Immature embryos, about 1.5–2.0 mm long, were excised aseptically under stereomicroscope and used for co-cultivation in transformation experiments.

Regeneration experiment

Before transformation an experiment was performed to check the regeneration capacity of wheat genotypes. Isolated immature embryos were placed, with scutellar side up, on a 25 mL solid callus induction medium (CIM) in Petri dishes (14–20 embryos per one dish) in 4 replicates per genotype and cultured 2 subcultures in 3 week-intervals. CIM contained MS (Murashige & Skoog 1962) mineral salts and vitamins, 100 mg L^{-1} casein hydrolysate, 30 g L^{-1} sucrose, 0.7% (w/v) agar (Torlak Institute of Immunology and Virology, Belgrade, Serbia), and 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich, St. Louis, MO, USA). Calli were transferred onto the MS plant growth regulator (PGR)-free medium (PRM) for another 2 subcultures for plant regeneration. The cultures were grown at 25 ± 2 °C, in the white fluorescent light, with the irradiance of 47 µmol m^{-2} s^{-1}, and a day/night regime of 16/8 h. The regeneration frequency was calculated as a number of regenerated immature embryos per explant/total number of explants × 100. The mean number of shoots per explant was also counted. The index of shoot-forming capacity (SFC) was used to evaluate a cumulative effect of the two aforementioned variables, and it was calculated as follows: SFC = (mean green spot number per explant) × (% of regenerating explants)/100. The regenerated plants were transferred in culture vessels on PGR-free ½ MS medium for further development and rooting.

Table 1. Treatments for optimization of *Agrobacterium*-mediated transformation of wheat cv. Vesna using LBA4404/pTOK233 vector. Additives A-Acetosyringone and AA-Ascorbic acid in infection (CIM-inf) and co-cultivation (CIM-co) media. [a]FIIE, Freshly isolated immature embryo; PCIE, four-day pre-cultured immature embryos.

Treatment	Explant[a]	Media	
		CIM-inf	CIM-co
FIIEControl	FIIE	A + AA	A + AA
FIIE1	FIIE	A + AA	A + AA
FIIE2	FIIE	A + AA	AA
FIIE3	FIIE	–	–
PCIEControl	PCIE	A + AA	A + AA
PCIE1	PCIE	–	–
PCIE2	PCIE	A + AA	A + AA

Agrobacterium strain and plasmid

An *A. tumefaciens* strain LBA4404 carrying the superbinary plasmid construct pTOK233 (Hiei et al. 1994) with the kanamycin-resistance gene (*npt*II), the hygromycin-resistance gene (*hpt*), and the *β*-glucuronidase (*gus-intron*) gene and extra set of *vir* B, C and G genes was used for plant transformation. The *gus-intron* gene expresses GUS activity in plant cells but not in the cells of *Agrobacterium*. The LBA4404/pTOK233 was maintained on agar solidified AB medium (Chilton et al. 1974), supplemented with 50 mg L^{-1} hygromycin B (Sigma-Aldrich Co.). Prior to inoculation, *Agrobacterium* samples were grown at 27 °C for 48 h. For bacterial suspension, 1 full loop (3 mm diameter) of bacterial culture was scraped from a 3-day-old plate and resuspended in 5 mL of a liquid CIM (CIM-inf) in a 50-mL Falcon tube. The tube was fixed horizontally to a vortex platform and shaken at low speed for 3–5 h prior to infection at room temperature.

Transformation method

The basic transformation method was described in detail in Mitić et al. (2004). Briefly, the explants were immersed in bacterial suspension for 15 min, while the control explants were soaked in bacteria-free liquid CIM. After infection, the explants were blotted dry with sterile filter paper and placed onto CIM for co-cultivation at 27 °C for 3 d in the dark. Then the explants were transferred onto hygromycin B containing CIM-selection 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 onto PRM for shoot regeneration. Regenerated plantlets were cultured on ½ MS PGR-free medium for rooting.

To improve transformation frequency in cv. Vesna in the first transformation experiment the factors that could produce significant differences in T-DNA delivery and plant regeneration varied. The transformation treatments are presented in Table 1. The freshly isolated immature embryos

Table 2. Regeneration potential of immature embryos of six different common wheat genotypes following 3 weeks of cultivation on CIM with 2 mg L^{-1} 2,4-D. SFC, shoot forming capacity. Data indicated the mean ± standard error (SE). Four replicates, each with 14–20 samples were used per genotype. Within a column, means denoted by the same letter are not significantly different according to the LSD test at $P < 0.05$.

Genotype	No. of embryos isolated	Calli (%)	Days to regeneration	Regenerating calli (%)	Mean green spots regenerating callus^{-1}	SFC
Vesna	80	90.5 ± 1.7 ab	8	89.5 ± 1.8 a	5.1 ± 0.01 a	4.3
ZA-205	66	98.9 ± 1.1 a	10	10.6 ± 0.3 c	1.5 ± 0.01 b	0.2
Norin 10	70	84.7 ± 0.3 ab	14	10.2 ± 0.1 c	2.0 ± 0.01 b	0.2
BL-100	58	85.5 ± 0.6 ab	20	28.0 ± 0.2 c	5.8 ± 0.01 a	1.6
Tobari 66	79	77.8 ± 0.3 b	9	58.3 ± 0.1 b	2.0 ± 0.05 b	1.2
Inia 66	80	97.1 ± 1.0 a	14	46.5 ± 1.6 b	6.4 ± 0.07 a	3.0

(FIIE) of spring wheat cv. Vesna as well as the immature embryos pre-cultured for 4 days (PCIE) on CIM containing 2 mg L^{-1} 2,4-D (PCIE) were used as explants (Table 1). The explants were inoculated with *A. tumefaciens* strain LBA4404 and co-cultivated in the presence of 100 mg L^{-1} ascorbic acid as antioxidant. The presence of the induction agent acetosyringone (100 μM) (Sigma-Aldrich Co.) in the inoculation and co-cultivation medium varied (Table 1). *A. tumefaciens* co-cultivated explants were subjected to cefotaxime (500 mg L^{-1}) (Tolycar®, Jugoremedija AD Zrenjanin, Serbia) for 15 days to eliminate *Agrobacterium* cells followed by antibiotic screening on hygromycin B to eliminate untransformed plant tissues. The selection procedure was as follows: 1 subculture (21 days) on medium with 10 mg L^{-1} hygromycin B, 1 subculture on medium with increased (20 mg L^{-1}) hygromycin B concentration and then 3 subcultures on 10 mg L^{-1} hygromycin B-containing media.

Survived shoots were detached from shoot bunches and grown further on PGR-free ½ MS media with 10 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime for a week followed on the same medium without hygromycin B and cefotaxime for additional four weeks. The experiment was performed in 2 successive years.

The most optimal protocol in cv. Vesna was further tested in another 5 wheat genotypes: ZA-205, Norin 10, BL-100, Tobari 66 and Inia 66 to observe whether it could be extrapolated to less-responsive wheat cultivars.

GUS histochemical assay

To evaluate the effect of co-cultivation treatments on transient expression of the *gus-intron* gene, the explants were taken 3, 6 and 25 days after inoculation by *Agrobacterium*. In addition, stable expression of the *gus* gene was detected histochemically in leaves and roots of regenerated T0 wheat plants at the end of the selection procedure. The histochemical staining solution was prepared according to Jefferson et al. (1987). The samples were incubated in 100 μL of 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid cyclohexylammonium salt) (Sigma, USA) solution, pH 7.0 at 37 °C overnight. GUS expression exerted as a blue colour was visualized under a stereomicroscope. Green leaves were soaked in 96% ethanol for removing chlorophyll. The presence of blue sectors was examined under the stereomicroscope.

Molecular analysis of transformed plants by PCR

Genomic DNA was extracted from leaves of the putative transformed and untransformed control plants using a CTAB DNA isolation method (Murray & Thompson 1980). The presence of the *gus* gene was demonstrated by PCR-amplification of a 366-bp fragment using the primer 5'-CCCGGCAATAACATACGGCGTG-3' and the reverse primer 5'-CCTGTAGAAACCCCAACCCGTG-3'. Primers specific for the amplification of the *virG* gene amplified a fragment of 390 bp; the primer forward sequence was 5'-GCCGACAGCACCCAGTTCAC-3', the reverse sequence 5'-CCTGCCGTAAGTTTCACCTCACC-3'. Thermocycling conditions were as follows: denaturation at 94 °C for 5 min; and 35 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. The program was terminated by a final extension step at 72 °C for 10 min. PCR products were separated on 0.9% agarose gels and visualized by ethidium bromide staining (0.5 μg mL^{-1}).

Statistical analysis

Treatments were fully randomised in the experiments. Data were subjected to square root transformation and percentage data to angular transformation prior to the statistical analyses and inversely transformed for presentation. The standard errors of the means were calculated and stated in the figures and tables. Differences between means were evaluated by the *t*-test and Fisher's LSD test at the confidence level of $P < 0.05$. The correlation analysis was performed employing the Pearson's Correlation Test. Statistical analyses were performed using SAS software (SAS Institute, 2002. SAS/STAT, ver. 9.00. SAS Institute Inc., Cary, NC.).

Results

Regeneration

Prior to the transformation, 6 wheat genotypes were checked for their callus and shoot regeneration ability after 3 weeks of cultivation of immature embryos on 2,4-D containing MS medium. According to ANOVA, the genotype significantly influenced callus regeneration and the mean number of green spots per callus, but not the callus formation that in all genotypes exceed 77% (Table 2). The five genotypes displayed variable regeneration ability (SFC 0.2-3.0), all lower comparing to high responsive cv. Vesna (SFC 4.3) (Table 2). Besides, immature embryos of cv. Vesna the earliest started to regenerate buds (Table 2).

Optimization of the transformation protocol

For optimization of the protocol for cv. Vesna transformation, freshly isolated and pre-cultured immature embryos were co-cultivated with super-binary *A. tumefaciens* vector LBA4404/pTOK233. The *β*-glucuronidase (*gus-intron*) gene was used as a marker expressed

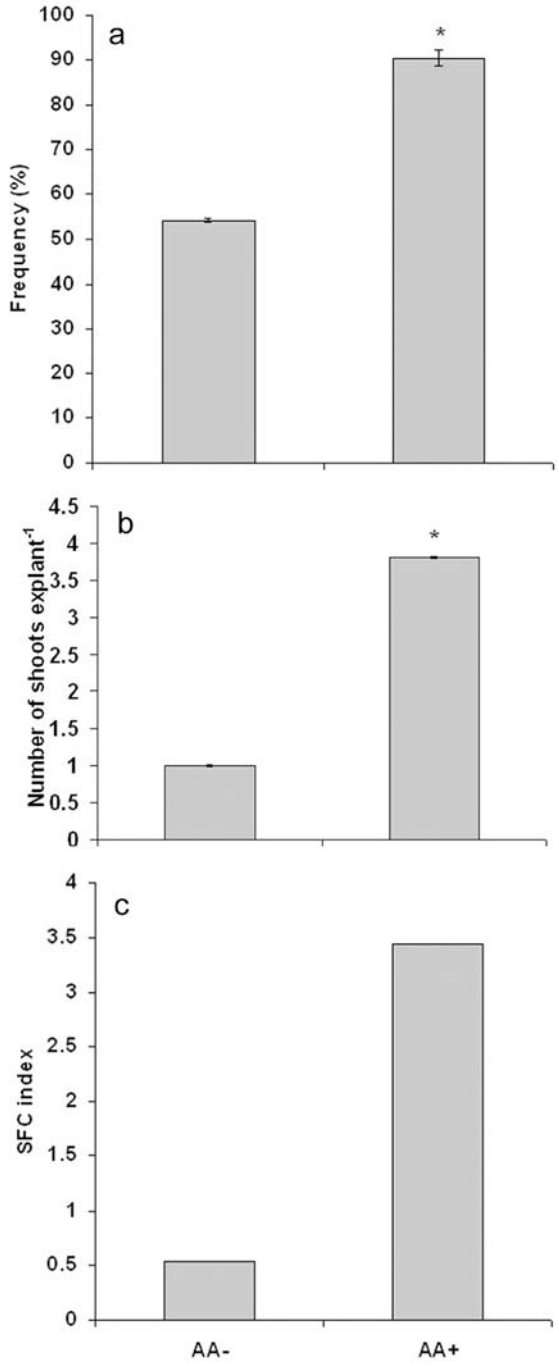

Fig. 1. Regeneration response of cv. Vesna immature embryos after 2 weeks of co-cultivation by *Agrobacterium* using ascorbic acid (AA) at 100 mg L^{-1} as an antioxidant. a – frequency of regenerating calli; b – mean number of shoots per explant, and c – shoot forming capacity (SFC) index. Values represent means ± SE. Means marked by an asterisk (*) are significantly different by *t*-test ($P < 0.05$). AA+, with ascorbic acid; AA-, without ascorbic acid.

only in plant tissue to optimize several parameters with effects on transient (3, 6 and 25 days after co-cultivation) and the GUS expression in whole plants. The most optimal protocol in cv. Vesna was further tested in another 5 wheat genotypes to observe whether it could be extrapolated to less-responsive wheat genotypes (ZA-205, Norin 10, BL-100, Tobari 66 and Inia 66).

Effect of ascorbic acid (AA) on the regeneration of immature embryo explants after Agrobacterium infection

One of the biggest problems in wheat genetic engineering is a low shoot regeneration efficiency and tissue necrosis after the explant treatment by *Agrobacterium.* Thus, in the first experiment with cv. Vesna the influence of an antioxidant ascorbic acid (AA) on regeneration frequency (percent of regenerating calli) and intensity of regeneration (the mean number of shoots per explant) was studied. Results revealed that AA at 100 mg L^{-1} added in both media, CIM-inf and CIM-co, significantly increased regeneration frequency by 1.7-fold (Fig. 1a) and intensity of regeneration by 3.8-fold (Fig. 1b) in *Agrobacterium* treated explants compared to the corresponding control treatment. As was calculated, AA treatment greatly improved (by 6.4-fold) shoot forming capacity (Fig. 1c) and by this reason AA was included in the main experiment in which the influence of other factors on regeneration and transformation was studied.

Effect of pre-culture and acetosyringone on transient GUS expression

The necessity of pre-culture was evaluated in cv. Vesna by examining transformation with or without a pre-incubation procedure. Thus, freshly isolated (not pre-cultured, FIIE) and immature embryos pre-cultured (PCIE) on 2 mg L^{-1} 2,4-D-containing CIM for four days were subjected to the *Agrobacterium* infection method. Acetosyringone, the agent important for induction of transformation, at 100 μM was added or not in either or both CIM-inf and CIM-co. The results obtained are based on the transient GUS expression of inoculated explants recorded after 3, 6 and 25 days of co-cultivation (Fig. 2a–c). The visible GUS expression was observed following 3 days of co-cultivation in all treatments except controls that were not infected by *Agrobacterium* (Table 3). The GUS expression frequency was the highest in FIIE explants (62.5–68.4%), while PCIE explants exhibited significantly lower frequency of GUS expression (41.0 and 43.0%, Table 3). In most cases similar levels of GUS expression was observed after 6 days of co-cultivation in all treatments. However, after 25 days of co-cultivation the highest frequency of GUS expression (over 60%) kept FIIE in treatments in which acetosyringone has been omitted (treatment FIIE3) or added only in CIM-inf medium (treatment FIIE2). When acetosyringone was included in both CIM-inf and CIM-co (treatment FIIE1) the frequency of FIIE expressing GUS considerably decreased (to 11%) comparing with frequencies in same treatments recorded after three and six days. Contrary, PCIE displayed the highest frequency of GUS expression only in the treatment that included acetosiringone (Table 3).

Effects of pre-culture and the influence of acetosyringone on production of transformed cv. Vesna plants

To further examine the effects of pre-culture and the influence of acetosyringone on production of transformed

Table 3. Effect of pre-culture and acetosyringone on transient GUS expression in immature embryo explants of cv. Vesna at different post co-cultivation time by *Agrobacterium*. FIIE – freshly isolated (not pre-cultured) immature embryos; PCIE – immature embryos pre-cultured on 2 mg L^{-1} 2,4-D-containing MS medium for four days. Data represent response of 30 co-cultivated explants per treatment.

Treatment	Acetosyringone at 100 μM Inoculation/co-cultivation medium	GUS expression (%)		
		3 d	6 d	25 d
FIIEControl	+/+	0	0	0
FIIE1	+/+	68.4	66.7	11.1
FIIE2	+/–	66.7	70	66.7
FIIE3	–/–	62.5	80	64.7
PCIEControl	+/+	0	0	0
PCIE1	–/–	41.0	40.7	22.2
PCIE2	+/+	43.0	41.7	62.5

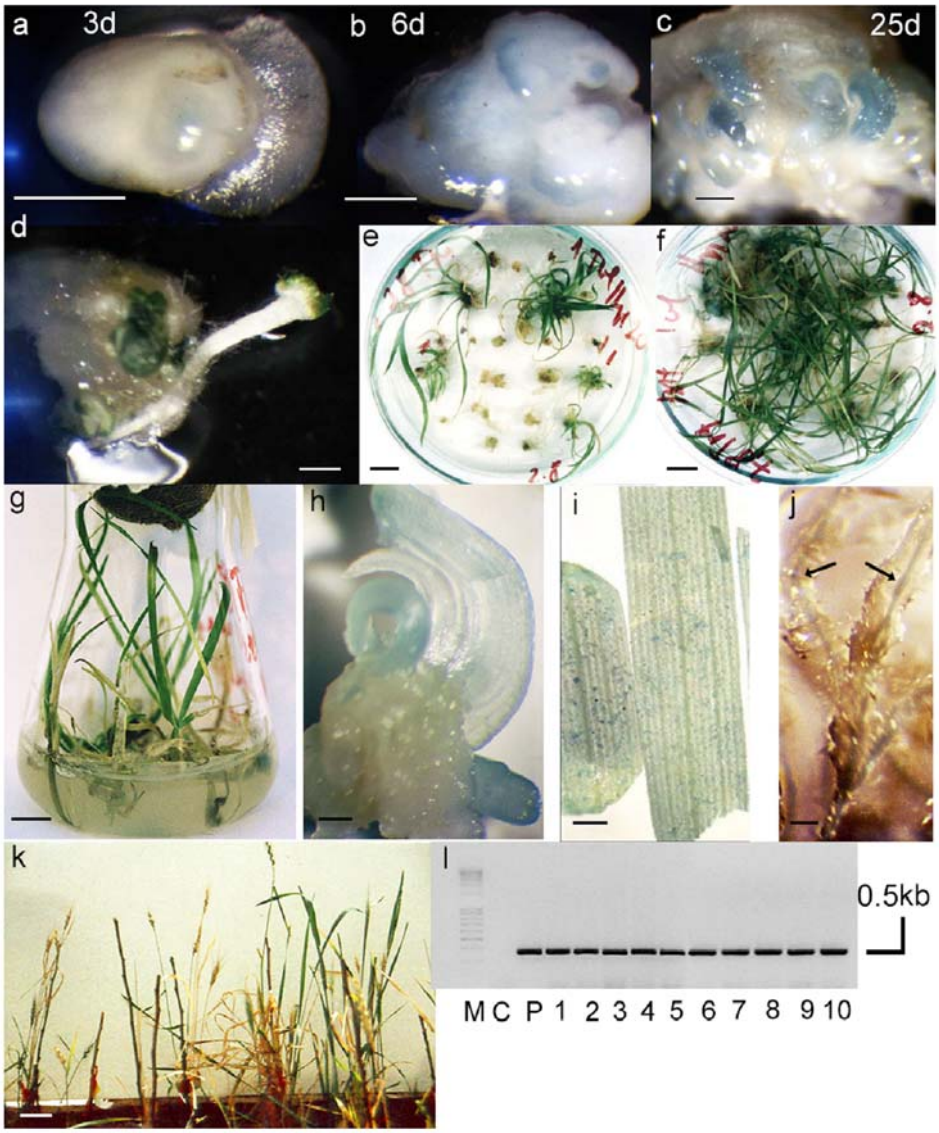

Fig. 2. *Agrobacterium*-mediated transformation of common wheat immature embryos. a-c – transient GUS expression in FIIE explants after 3, 6 and 25 days of co-cultivation, respectively, scale bar = 1 mm; d – regenerative embryogenic callus after 20 d of culture, scale bar = 1 mm; e – shoots regenerated from hygromycin B-resistant callus on medium with 20 mg L^{-1} hygromycin, scale bar = 1 cm; f – elongating shoots on medium with 10 mg L^{-1} hygromycin, scale bar = 1 cm; g – elongated and rooted transformed cv. Vesna plants, scale bar = 1 cm; h–j – GUS histochemical expression in induced shoot bud and in putative T0 cv. Vesna plant in leaf, and root vascular cylinder, respectively, scale bar = 1 mm; k – acclimated transformed Vesna, ZA-205, BL-100, Inia 66, Tobari 66 and Norin 10 plants in greenhouse, scale bar = 10 cm; l – PCR amplification of putative transformed wheat shoots with pTOK233 using *gus* primers showing amplicon of 366 bp. Lane M 1kb – DNA ladder, lane C – negative control, lane P – positive control, lanes 1–8 – two transformed plant lines of each wheat genotype: Vesna, ZA-205, BL-100, Inia 66, respectively, and lanes 9 and 10 – transformed plant line of Tobari 66 and Norin 10, respectively.

Table 4. Optimization of transformation of immature embryos and regeneration of hygromycin-resistant (Hyg^r) and GUS positive (GUS+) plants of wheat cv. Vesna. SFC – shoot forming capacity. *TE-Transformation efficiency = (No. of survived plants/No. of inoculated embryos) × 100, **Survival rate = (No. of survived calli/No. of embryo explants) × 100. Data indicate the mean ± standard error (SE). Five replicates, each with 16–27 samples, were used per treatment. Treatments denoted by the same letter are not significantly different according to the LSD test at $P < 0.05$.

Treatment	No. of embryo explants	Calli (%)	Frequency of regeneration (%)	Mean green spots regenerating callus^{-1}	SFC	No. survived calli (%)	Survival rate** (%)	No. GUS+ Hyg^r lines	TE* %
FIIEControl	100	83	86.3 ± 1.8 a	5.0 ± 0.01 b	4.3	0 c	0.0	0	–
FIIE1	150	81	23.4 ± 0.8 b	2.8 ± 0.01 d	0.7	19(16.8) ab	12.7	6	4.0
FIIE2	125	85	28.9 ± 1.0 b	4.6 ± 0.02 bc	1.3	28(35.9) a	22.4	9	7.2
FIIE3	225	46	21.9 ± 0.3 b	4.3 ± 0.03 bcd	0.9	15(8.4) ab	6.7	12	5.3
PCIEControl	90	100	91.8 ± 0.2 a	6.8 ± 0.01 a	6.2	0 c	0	0	–
PCIE1	100	92	1.0 ± 0.4 c	2.9 ± 0.09 cd	0.03	3(3.8) b	3.0	2	2.0
PCIE2	130	70	4.5 ± 0.8 c	2.3 ± 0.03 d	0.1	3(3.0) b	2.3	5	3.8

cv. Vesna plants, FIIE and PCIE explants subjected to the *Agrobacterium* infection method were subsequently grown and regenerated on selective hygromycin B- containing MS medium. The statistically significant difference between pre-cultured and fresh inoculated explants in their regeneration ability was recorded. As shown in Table 4, low regenerating ability for frequency of regeneration and the mean number of green spots per callus resulting in a low SFC index (only 0.03 and 0.1) characterized pre-cultured explants. On the other hand, FIIE retained significantly higher regeneration ability after the *Agrobacterium* treatment, especially for frequency of regeneration irrespective of the transformation treatment variations, implicating the higher rate of the SFC index (0.7-1.3, Table 4) compared to pre-cultured ones. Moreover, FIIE2 and FIIE3 displayed the statistically insignificant mean number of green spots per callus compared to the bacteria untreated control treatment (Table 4).

After *Agrobacterium*-inoculation, screening of putative transformants based on antibiotic selection is an important step for eliminating untransformed tissues. The application of antibiotic selection immediately after co-cultivation resulted into a high level of suppression of *in vitro* regeneration (data not shown). Therefore, antibiotic selection pressure was applied after 15 days of inoculation by *Agrobacterium* in order to allow and to support the recovery of transformed cells. The threshold sensitivity level of wheat cv. Vesna immature embryos to hygromycin B was 10 mg L^{-1}. Antibiotic screening of transformants was as follows: 1 subculture for 21 days on the medium with 10 mg L^{-1} hygromycin B, 1 subculture on the medium with the increased (20 mg L^{-1}) hygromycin B concentration and then 3 subcultures on 10 mg L^{-1} hygromycin B-containing media. On the onset of the selection cycle, shoot buds developed on regenerating calli that showed embryogenic characteristics (Fig. 2d). The percent of surviving regenerating calli originated from *Agrobacterium* inoculated explants decreased during higromycin selection cycles and after 5 cycles on the hygromycin B-containing medium it ranged from 3.0 to 35.9% (Table 4). Calli regenerated from control untransformed immature embryos survived the first selection round on 10 mg L^{-1} hygromycin but subsequently they stopped growing and finally died. The treatment FIIE2 using fresh immature embryos as explants with the addition of acetosyringone only in CIM-inf had a positive effect on the calli survival rate after the selection regime was completed on hygromycin B-containing media (22.4%, Table 4, Fig. 2e). The regenerating calli and the shoot buds regenerated from them during the selection process were subcultured on PGR-free MS medium where the shoot buds developed into shoots (Fig. 2f). The elongated shoots of 3–4 cm in height were employed for rooting in PGR-free MS medium having 10 mg L^{-1} higromycin B along with 250 mg L^{-1} cefotaxime for 1 week and then they were transferred on hygromycin-, cefotaxime- and PGR-free MS medium (Fig. 2g). In this medium, 100 % rooting was achieved within 15 days of incubation. The histochemical GUS assay was checked at earlier (Fig. 2h) and later stages of the shoot development. In whole hygromycin-resistant plants, the histochemical assay showed blue colour localization in leaf and root tissues (Figs. 2i and 2j) but was absent in untransformed tissue, which indicated stable GUS expression. For resume as shown in Table 4, 34 hygromycin-resistant and GUS-positive plants recovered from 730 initially co-cultivated explants were obtained without escapes. The highest number of hygromycin-resistant plants with stable GUS expression was found in the treatment FIIE1, FIIE 2, and FIIE3, all 3 with fresh immature embryos used as explants. The treatment FIIE2 combining fresh immature embryos with the application of acetosyringone only in infection medium during co-cultivation conferred the highest transformation efficiency of 7.2%. On the other hand, four days of preculture resulted in the lowest transformation efficiency among treatments performed (2.0 and 3.8%, Table 4).

Evaluation of an optimized transformation with five other wheat genotypes

Protocol FIIE2, optimized for enhanced *Agrobacterium*-mediated transformation in highly responsive cv. Vesna, was applied to another 5 wheat genotypes which displayed variable regeneration capacities (Table 2). After the FIIE2 transformation treatment was applied on these genotypes they responded to the transfor-

Table 5. The application of FIIE2 protocol optimized for cv. Vesna transformation to another five wheat genotypes selected for variable regeneration capacities. C, control treatment; T, transformation treatment; TE, transformation efficiency = (No. of survived plants/No. of inoculated embryos) × 100; Survival rate = (No. of survived calli/No. of embryo explants) × 100. Values represent mean ± SE. Mean values within a column denoted by the same letter are not significantly different according to the LSD test at $P < 0.05$.

Genotype/ treatment	No. embryos	Callus formation (%)	Regenerating callus (%)	No. survived calli (%)	Survival rate (%)	No. of Hyg^r and *gus*+ plants	TE %
BL-100/T	64	21.9 ± 0.2 e	30.0 ± 1.8 cd	8 (66.7)	12.5	17	8.6
BL-100/C	40	92.2 ± 0.8 ab	36.3 ± 0.1 bc	0	0	0	0
Norin 10/T	38	27.3 ± 0.1 e	27.5 ± 0.4 cd	4 (33.3)	10.5	6	3.6
Norin 10/C	30	89.2 ± 1.1 ab	13.1 ± 0.1 de	0	0	0	0
ZA-205/T	44	53.2 ± 0.1 cd	17.3 ± 0.1 cde	4 (14.3)	9.1	10	5.9
ZA-205/C	32	76.2 ± 1.9 bc	15.1 ± 0.3 b	0	0	0	0
Tobari 66/T	30	61.3 ± 1.0 cd	70.9 ± 1.2 a	2 (9.1)	6.7	18	10.8
Tobari 66/C	22	65.6 ± 0.2 cd	60.1 ± 1.3 a	0	0	0	0
Inia 66/T	36	50.5 ± 2.2 d	9.7 ± 0.5 e	3 (11.1)	8.3	6	4.5
Inia 66/C	20	96.5 ± 0.8 a	53.0 ± 0.1 b	0	0	0	0

mation conditions by reducing the callus formation (Table 5). Surprisingly, frequency of regeneration in Tobari 66, ZA-205 and Norin 10 was slightly enhanced in *Agrobacterium*-inoculated explants compared to uninoculated (control) ones (Table 5). The callus survival rate on selection media ranged between 6.7% and 12.5% (Table 5) resulting in an average of 9.4%. Among five cultivars BL-100 showed a relatively higher (12.5%) survival rate than the other genotypes (about 10% and below). The number of hygromycin- resistant and GUS positive plants regenerated enabled satisfactory transformation efficiency from 3.6% to 10.8 % (Table 5). Although the Pearson's correlation coefficient showed a positive linear correlation, the relationship between regeneration potential and transformation efficiency was not significant ($r = 0.45$). The highest TEs of 8.6% and 10.8% were displayed BL-100 and Tobari 66, respectively (Table 5), with significantly different regeneration capacity (28.0% and 58.3%, Table 2). When survived shoots were transferred onto PGR-free 1/2MS, finally 57 transformed plant lines (Table 5) were rooted within two weeks and successfully acclimated to non-sterile conditions in the greenhouse. They developed into normal plants that produced spikes and set seeds (Fig. 2k).

Molecular analysis of transformed plants

The PCR analysis detected the presence of the expected 366 bp amplified product corresponding to *gus* gene (Fig. 2l) in transformed shoot. No amplification was detected in the control untransformed shoots. The absence of signals in PCR using *vir*G primers in transformed shoots ruled out artefacts caused by *A. tumefaciens* contamination (data not shown).

Discussion

A successful and efficient transformation protocol depends on several factors including target cells or tissues competent for regeneration and transformation, efficient DNA delivery, stringent system for selection of transformed cells, and ability to recover fertile plants from transformed tissue. For decades wheat has been considered as the most recalcitrant cereal species for regeneration and transformation compared to rice and maize mainly due to low efficiency of plant regeneration under tissue culture conditions. A lot of investigations were carried out on the optimization of conditions for the improved *Agrobacterium*-mediated genetic transformation in this major crop (Weir et al. 2001; Wu et al. 2003; Patnaik et al. 2006; Yao et al. 2007) but the transformation efficiency remained low (mostly around 1%) even the model cultivars were used (Weeks et al. 1993; Nehra et al. 1994; Cheng et al. 1997). Increased frequencies were achieved by introduction of high responsive wheat genotypes such as model cultivar Bobwhite (9.3%) (Zhang et al. 2000) and generally transformation protocols were further optimized using model cultivars with a relatively high embryogenic capacity (Janakiraman et al. 2002). However, established protocols have a restricted application on elite and other wheat cultivars due to a wide range of variability in regeneration and transformation responses between varieties. The fact that a highly responsive wheat genotype such as cv. Vesna displayed transformation efficiency of only 0.41% or 0.1% (Mitić et al. 2004) alludes that a genotype seems important but no crucial factor influencing efficient genetic transformation. We therefore tested several modifications of the previously used protocol to improve transformation of cv. Vesna. For that purpose, freshly isolated and 4-day-precultivated immature embryos, ascorbic acid as an antioxidant compound, modulated application of acetosyringone during a co-cultivation step, and delayed and a stepwise hygromycin B selection regime were used. The super-binary *A. tumefaciens* vector LBA4404/pTOK233, which was designed to transform recalcitrant monocot species (Hiei et al. 1994; Nandakumar et al. 2007) and which previously provided higher transformation efficiency than AGL1/pDM805 in wheat cv. Vesna (Mitić et al. 2004) was applied. Efficiency of the *gus* gene delivery was assessed by scoring GUS expression in co-cultivated immature embryos and in whole plants that survived hygromycin selection. The *gus-intron* (Ohta et al. 1990) was a convenient marker for this purpose since this gene is strongly expressed in

plant tissue but not in *A. tumefaciens* cells that could remain in the plant tissues.

Cell necrosis after the *Agrobacterium* application influenced necrosis and disability of regeneration in even 56% of cv. Vesna immature embryos inoculated (data not shown) and represents one of the basic limiting factors in wheat genetic transformation. Recent research indicates that reactive oxygen species (ROS) such as superoxide radical (O_2^-), the hydrogen peroxide (H_2O_2), the hydroxyl radical (OH'), and the peroxyl radical (RO_2) may be playing an important role in tissue browning and necrosis during transformation (Parrott et al. 2002).

Antioxidant compounds such as ascorbic acid, cysteine, silver nitrate, etc. used during pre-culture, infection, and during and after co-cultivation by *Agrobacterium* were reported to reduce the detrimental effect of a hypersensitive response, which in turn led to an increase in the survival rate of *Agrobacterium*-infected cells and improved the transformation efficiency in rice (Enríquez-Obregón et al. 1999), sugarcane (Enríquez-Obregón et al. 1997), maize (Frame et al. 2002). Some protocols for successful transformation of wheat also mentioned utilization of ascorbic acid before (Hu et al. 2003) and during co-cultivation and regeneration phases of immature embryos (Cheng et al. 1997; Pastori et al. 2001) as well as in microspore culture techniques for the production of doubled haploid wheat plants (Santra et al. 2012). In the present study, we also considered ascorbic acid as a compound in reducing cell necrosis during co-cultivation and found that its application helped to minimize the damage due to oxidative bursts during the immature embryo-*Agrobacterium* interaction enabling most satisfactory regeneration ability in *Agrobacterium* treated explants. According to its *in vitro* function, Dan (2008) classified ascorbic acid into the group of major antioxidants that can both reduce tissue browning and promote organogenesis, somatic embryogenesis, and shoot growth during micropropagation across different plant species. Hence, to facilitate a further development of transformed wheat cells we included ascorbic acid in all further *Agrobacterium*-mediated transformation experiments in wheat. Oppositely, Tao et al. (2011) found that the wheat transformed-calli growth status was not improved by adding anti-oxidants ascorbic acid, cysteine and silver nitrate. Compared to the present study, they used the same concentration of ascorbic acid, but wheat genotypes, transformation vector, concentration of acetosyringone, which increases susceptibility to *Agrobacterium*, and addition of other organic compounds in suspension liquid medium were different. Besides, possible reasons for this discrepancy should be considered in the ambience along with other factors which may be linked to hypersensitive defence reaction in plants to *Agrobacterium* infection. They include physiological status of donor plants as well as tissue manipulation during explant isolation and inoculation period influencing activation of signal molecules, such as salicylic acid, as a response of plant tissue to the given *Agrobacterium* strain (Yuan et al. 2007), and the level of antioxidative activities which may play important role in initiation of defence mechanisms to *Agrobacterium* and on the further regeneration process (Papadakis et al. 2001; Xu et al. 2013).

It has been reported that pre-cultivation of explants on regenerating medium had a positive effect on subsequent transformation efficiencies (Wu et al. 2003; Dutt & Grosser 2009). In this study, the necessity of pre-culture by examining transformation with or without a pre-incubation procedure was evaluated. Pre-cultured explants did not only display a lower percent of transient GUS expression, which dramatically decreased after 25 days of inoculation in the treatment in which acetosyringone was omitted, but also more importantly showed decreased regeneration frequency and survived rate leading to lower transformation frequencies compared to freshly isolated immature embryos. Cheng et al. (1997) found no significant difference in the transformation efficiency among three explant types of wheat cv. Bobwhite, freshly isolated immature embryos, pre-cultured immature embryos and embryogenic calli, although it varied among experiments. Ahsan et al. (2007) also found optimal transformation efficiency in tomato when freshly perforated cotyledonary explants were directly infected with a bacterial solution.

Acetosyringone is known as the most potent phenolic inducer of the *vir* genes of *Agrobacterium* which can increase the transformation efficiencies in many plant species (Costa et al. 2006; Dutt & Grosser 2009), although plant response to its application could be a genotype dependent. Dutt & Grosser (2009) found that divers citrus cultivars differently responded to the addition of acetosyringone to the co-cultivation medium. In wheat, it has also been considered as an important factor for successful *Agrobacterium*-mediated transformation at all (Cheng et al. 1997; Weir et al. 2001; Wu et al. 2003). The addition of acetosyringone was not conclusive for transient GUS expression in FIIE, while it was important for PCIE. On the other hand, its application had a positive effect on surviving of regenerating calli from FIIE, particularly in case of the addition only into the infection medium. Unfortunately, acetosyringone had no significant effect on surviving of regenerating calli from PIIE indicating a predominant influence of a type of the starting material used for transformation.

To procure the selection regime enabling the ability of transformed cells to divide and form regenerating structures seems to be one of the most critical factors making a transformation protocol more efficient. Hygromycin B is a potent antibiotic that has served as a suitable selective agent in transformation studies of a range of the cereal species, including wheat. Unlike DL-phosphinothricin (PPT), also previously used (Mitić et al. 2004), transformed cells are not likely to protect untransformed cells in their vicinity with metabolites that would counteract the effect of hygromycin B (Olhoft et al. 2003). If it was applied immediately after

co-cultivation in the intense selection regime the transformation frequency was rather low (0.41%) despite relatively high frequencies of GUS transient expression (69.9%) in co-cultivated explants (Mitić et al. 2004). One possible explanation for the low transformation efficiency could be that transformed cells surrounded with damaged untransformed ones failed to develop meristematic centres. Therefore, in the present experiment, the putative transformed explants were cultured on the hygromycin B-free medium for 2 weeks, followed by 15 weeks on selection media, including 1 week on the medium with the highest hygromycin B concentration of 20 mg l^{-1}. Co-cultivation of FIIE in the presence of ascorbic acid and acetosyringone, combined with a delayed stepwise increasing selection procedure, resulted in a synergistic increase in the production of transformed cv. Vesna plants to efficiency exceeding 7% of treated explants comparing to previously achieve 0.41% (Mitić et al. 2004).

The effectiveness of this protocol optimized on cv. Vesna was tested with 5 wheat genotypes displaying lower regeneration ability than cv. Vesna. Interestingly, when compared, three spring wheat genotypes (VS, Inia 66 and Tobari 66) exhibited the higher regeneration response than those of winter wheat (BL-100, Norin 10 and ZA-205) representing the prospective material for further examinations of linking QTL markers for regeneration and vernalization. The application of optimized transformation protocol on these genotypes gives rise to transformation efficiencies ranges 3.6-10.8%. This rise was narrowly correlated with genotype regeneration frequency, alluding to a low dependence of the transformation protocol from regeneration potential. This is especially evident in BL-100 and Tobari 66 that displayed the highest TE but at the same time exhibited a significant difference in regeneration capacity. Moreover, after the transformation treatment Tobari 66 demonstrated the highest regeneration frequency comparing to the untreated control treatment that could be assumed as a stress-induced response and would be further examined. In line with this, no correlation between embryogenic capacity and frequency of transformation was also found in Candeza and Canon wheat varieties (Pastori et al. 2001). Iser et al. (1999) demonstrated that there was no parallelism between the frequencies of regeneration and transformation, indicating more fundamental genotype-dependent transformation potential.

In conclusion, optimization of the previously established transformation procedure may significantly increase the transformation efficiency of high responsive wheat cv. Vesna from previous 0.41% to 7.2%. The most favourable procedure to achieve improved transformation efficiency using super-binary *Agrobacterium* vector LBA4404/pTOK233 was the combination as follows: freshly isolated immature embryos as the explants, the addition of ascorbic acid during co-cultivation, the addition of acetosyringone only in the bacteria-containing infection medium, the delayed and stepwise increasing hygromycin B selection procedure. The optimized protocol was less genotype dependent since that was successfully applied to another 5 less-responsive wheat genotypes providing satisfactory transformation efficiencies from 3.6% to 10.8%. Therefore, the conditions appointed in this study may serve as a base to facilitate the transformation in other, particularly recalcitrant wheat genotypes.

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