Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants

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Abstract: The efficiency of wheat biolistic transformation systems strongly depends on the bombardment parameters, the condition of the donor plant, and the plant genotype chosen for the transformation process. This paper analyzes the transformation efficiency of the 129 wheat sister lines generically called 'Bobwhite', originally obtained from the cross 'Aurora'//'Kalyan'/'Bluebird 3'/'Woodpecker'. A number of factors influencing the transformation were examined, such as the ability to produce embryogenic callus, regeneration in selection medium, and overall transformation performance. Of the 129 genotypes evaluated, eight demonstrated transformation efficiencies above 60% (60 independent transgenic events per 100 immature embryos bombarded). Among the eight genotypes identified, we studied agronomic characteristics such as earliness to identify the most adaptable line(s) for different lab conditions. 'Bobwhite' SH 98 26 was identified as a super-transformable wheat line.

Key words: wheat transformation, 'Bobwhite', genotype effect.

Résumé : L'efficacité de la transformation biolistique chez le blé est largement déterminée par les paramètres du bombardement, l'état de la plante-mère et le génotype employé. Cet article rapporte les résultats d'une analyse de l'efficacité de la transformation réalisée sur 129 lignées soeurs collectivement appelées 'Bobwhite' et dérivées d'un croisement 'Aurora'//'Kalyan'/'Bluebird 3'/'Woodpecker'. Plusieurs facteurs influençant la transformation ont été examinés tels que l'aptitude à produire des cals embryogènes, la régénération en milieu sélectif, et l'efficacité globale de transformation. Des 129 génotypes examinés, huit ont montré un taux de transformation dépassant 60 % (60 transformants indépendants pour 100 embryons immatures bombardés). Pour ces huit génotypes, des caractères agronomiques, tels que la maturité, ont été examinés afin d'identifier les lignées les mieux adaptées aux conditions de laboratoire. Bobwhite SH 98 26 a été identifiée comme étant une lignée de blé ayant une aptitude exceptionnelle à la transformation.

Mots clés : transformation du blé, 'Bobwhite', effet génotypique.

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Introduction

The goal of various plant tissue culture techniques and transformation processes is to generate novel genetic material by the direct introduction of useful and novel genes into important crops, thus accelerating or complementing their respective breeding programs. Generally, genetic engineering programs focus on establishing reliable and straightforward plant regeneration and genetic transformation systems for the species under investigation; cloning of potentially useful genes and promoters; and generation and characterization of transgenic plants with improved agronomic characteristics. Currently the transformation system widely used in wheat is based on the particle gene-gun transfer system, in which naked DNA is physically introduced into cells by microprojectile bombardment. This process involves high-velocity acceleration of gold microprojectiles carrying foreign DNA, penetration through the cell wall and membrane by microprojectiles, and DNA delivery into plant cells (He and Lazzeri 1998).

For wheat, the prerequisite for genetic manipulation is the establishment of a routine and simple plant regeneration system through tissue culture. Various transformation and regeneration systems have been developed and tried (Altpeter et al. 1996; Barro et al. 1998; Becker et al. 1994; Fennel et al. 1996; Weeks et al. 1993), using a multitude of different wheat genotypes with a complex array of results, with the

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only common factors being the difficulty and genotype dependency of the technique (Jordan 2000; Nehra et al. 1994). One of the most responsive materials reported to be transformable by several laboratories are the sisters of the 'Bobwhite' family (Bleach 1998; Bliffeld et al. 1999). However, the name 'Bobwhite' represents a group of 129 accessions in the CIMMYT (Centro International de Milioramento de Mais y Trigo) ex situ wheat collection with different characteristics and agronomic behaviors, creating problems in the reproducibility of the experiments and confusion between wheat transformation groups. The sister lines were generated by the CIMMYT wheat program in the mid-1970s from the cross CM 33203 with the pedigree 'Aurora'// 'Kalyan'/'Bluebird 3'/'Woodpecker'; the segregating generations were selected and released in the late 1970s. These Bobwhite lines have become important germplasm in many different programs, and between 1984 and 1991, 16 of the sister lines were released as commercial cultivars in 16 locations in Africa, South America, and the Middle East (Souza et al. 1998).

The objectives of this study were (*i*) to use the available transformation protocols and genotype data to screen all 129 'Bobwhite' accessions for their transformation ability and (*ii*) to identify the most transformable and responsive accessions based on their ability to regenerate, to adapt to tissue culture, and on their agronomic characteristics.

Materials and Methods

Plant material

The 129 'Bobwhite' accessions were grown in a greenhouse under controlled conditions with day temperatures of 24–28°C and night temperatures of 15–18°C. To ensure continuous production of immature embryos, seeds of each variety were planted every 2–3 days. Fifteen days after heading, the immature seeds were harvested, sterilized, and a minimum of 200 immature embryos/accession were isolated for bombardment. Particular care was given to maintaining the greenhouse conditions at the same level for all the experiments, and to avoid any stress to the donor plants. Every 15–20 days, routine pesticide and fertilizing treatments were applied to the donor plants. The transformation experiments were done during the dry season in central Mexico, with a maximum light intensity ranging between 700 and 1100 MJ/m^{2}s.

DNA plasmid vector and preparation of the microcarrier

The plasmid used in this experiment was developed from pGEM3Zf(+), containing the selectable *bar* gene that confers resistance to the herbicide Basta (Hoechst, Frankfurt, Germany) under control of the maize ubiquitin promoter (Christensen et al. 1992) cloned into the *Hin*dIII site (pUbibar). Five micrograms of plasmid DNA were precipitated onto gold particles following the protocol described by Pellegrineschi et al. (2000). For each bombardment, 10 µL of microparticle DNA were placed onto the macrocarrier. Bombardments were conducted at a distance of 5 cm from the stopping plate using a PDS 1000/He microprojectile gun (Bio-Rad, Hercules, Calif.) with 900 psi (1 psi = 6.895 kPa), according to the procedure described by Pellegrineschi et al. (2000).

Culture conditions and recovery of transformed plants

A minimum of 200 freshly isolated embryos (between 0.7 and 1.2 mm long) from each accession were isolated and the zygotic meristem then removed. Fifty isolated scutella per petri dish were placed on osmotic basal salt medium (Murashige and Skoog 1962) without modifications (except for the addition of 15% w/v maltose) for 4 h before bombardment. As a control for each experiment, 50 additional scutella from the same accession were transferred to the osmotic medium without bombardment. The day after bombardment, the embryos were placed on MS medium (Murashige and Skoog 1962) containing 2.5 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/L, 30 g sucrose/L, and 8 g Bacto-Agar for somatic embryo induction. Between 12 and 14 days after transfer to the induction medium the induced embryos were scored for the somatic embryo formation. The induced explants were then transferred in MS medium containing 5 mg PPT/L (DL-phosphinothricin; Sigma, St. Louis, Mo.) to a growth chamber with a photoperiod of 16 h light : 8 h dark at 25°C for selection. The light was provided by white cool florescent tubes (Solar) and the light intensity was 200 MJ/m^{2}s. After 30 days, healthy, fully differentiated plantlets were scored and transferred to the same medium for further selection during development. Surviving green-rooted plantlets were transferred to a soil mixture and placed in the growth room. Herbicide resistance of the putative transgenic wheat plants was determined by spraying the leaves of plants at the fifth or sixth leaf stage twice with Basta (0.3% w/v) with 7 days between applications to minimize escapes. Plants were scored as susceptible or resistant according to the degree of leaf desiccation after 7 days.

Molecular analyses

A polymerase chain reaction (PCR) was performed on plant DNA extracted from young leaves using the Nucleospin DNA Purification Kit (Clontech, Palo Alto, Calif.), following the manufacturer's protocol. All of the Basta-resistant plants that survived to tissue culture selection were analyzed by PCR for the presence of the *bar* gene. The pair of specific primers used were 5′-GTCTGCACCATCGTCAACC-3′ (forward) and 5′-GAAGTCCAGCTGCCAGAAAC-3′ (reverse). The PCRs were carried out in a total volume of 25 µL, consisting of 10 ng wheat genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 3 mM $MgCl_2$, 0.1% Triton X-100, 0.24 mM of each dNTP, 0.04 U *Taq* DNA polymerase, and 0.16 µmol of each primer. For PCR analysis of the *bar* gene, DNA was denatured at 94°C for 1 min (hot start), followed by 30 amplification cycles of 94°C for 30 s., 64°C for 2 min, and 72°C for 2 min. For Southern blot analysis, the genomic DNA was isolated from 1g fresh-weight leaf material using the Nucleon Phytopure Plant DNA Extraction Kit, according to the manufacturer's protocol (Amersham Life Sciences, Arlington Heights, Ill.). A 50-µg aliquot of DNA was digested overnight at 37°C with the appropriate restriction enzyme. The digested DNA was fractionated in a 1.0% agarose gel, transferred to a positively

charged nylon membrane (Boehringer Mannheim, Indianapolis, Ind.) and hybridized to digoxigenin-dUTP labeled probes according to the manufacturer's instructions (Boehringer Mannheim). The DNA probe, a fragment of the *bar* gene, was labeled with digoxigenin-11-dUTP (Roche). Detection was achieved using the DIG Luminescent Detection Kit (Boehringer Mannheim) and the hybridization signals were visualized by exposure to Fuji X-ray film at 37°C for 40 min.

Analyses of the progeny

Selfed seeds (T_1 and T_2 generation) of the transformants were sterilized and the embryos excised. The sterile embryos were then transferred to MS medium with 5 mg PPT/L. One week later, the embryos were scored for their survival ability. Ratios were calculated to determine if they fit expected Mendelian segregation ratios. The resistant plantlets were then transferred to a soil mixture and tested for the presence of the transgene using PCR.

Statistical analyses

The first selection of material was conducted with a minimum of 200 embryos (four bombardments with 50 scutella each; each bombardment was considered one repetition). For each experiment, the minimum, maximum, average, and standard deviation of the embryogenesis, regeneration, and transformation efficiencies were calculated. After the first selection, the best performance lines were tested again with three repetitions of 2000 embryos each (40 bombardments of 50 scutella each). Transformation efficiency was calculated as the total number of transgenic plants divided by the total number of embryos bombarded. A plant was defined as transgenic when it demonstrated resistance to the herbicide treatments and showed a positive signal for the *bar* gene when analyzed by PCR. The percentage of somatic embryogenesis development was calculated by dividing the total number of the embryos that differentiated (somatic embryos) by the total number of embryos bombarded. Regeneration efficiency was calculated by dividing the number of embryos that produced plantlets in selective medium by the total number of embryos bombarded.

Where the effects of treatment (bombarded scutella and control) and lines (BW) were considered to be fixed effects and replicates were considered to be random effects, the SAS macro for a generalized mixed linear model (GLINMIX) (Littell et al. 1996) was used. A binomial distribution with a logit function was considered.

Results

Somatic embryo induction

Cultures were checked for somatic embryo formation before transfer to selective medium. The effect of genotype on scutellum embryogenesis is summarized in Table 1. The majority of the 129 genotypes tested produced somatic embryos, with only 18 not reacting to the induction (Table 1). Accessions with the highest yield (nearly 100%) of embryos producing embryogenic callus were SH 98 15, SH 98 88, SH 98 103, SH 98 108, SH 98 116, SH 98 117, SH 98 118, SH 98 119, SH 98 120, SH 98 121, and SH 98 126. All other accessions gave different efficiencies of somatic embryo formation, ranging from 0 to 96%. During this stage of the transformation process, the non-bombarded embryos (controls) generally gave a more efficient formation of somatic embryos (Table 1). There were no distinct differences in stage development, with the exception of the number of scutella-differentiating somatic embryos. Generally, first globular stage somatic embryos were observed 4–5 days after the transfer, and the globular stage was usually formed directly from the scutellum. This was followed by a high frequency of repetitive somatic embryogenesis. Early globular stages were followed by the full differentiation of the somatic embryo.

Transformation efficiency and selection efficiency

The first test of the transformation efficiency was an evaluation of the regeneration performance on the selective medium. Healthy, fully differentiated embryogenic calli were scored (1 per embryo; Table 1) as the number of regenerating calli divided by the total number of immature embryos bombarded (one regenerating callus was scored as 1). The somatic embryo germination frequency in selective medium ranged from 0 to 89%. The different accessions responded in four different ways to the selection and bombardment, as follows: (*i*) no regeneration; (*ii*) tolerant to herbicide and susceptibile to the bombardment process (e.g., lines SH 98 18, SH 98 88, SH 98 113); (*iii*) sensitive to herbicide selection, tolerant to the bombardment process, and highly regenerable (e.g., lines SH 98 26, SH 98 56); and (*iv*) sensitive to herbicide selection and tolerant to the bombardment process, but low regeneration (e.g., SH 98 6, SH 98 42, SH 98 46) (Table 1, Fig. 1). Transformation efficiency was calculated as the effective number of transgenic plants obtained divided by the total number of immature embryos bombarded. The most efficient lines were SH 98 26 and SH 98 56 (Table 1). Other accessions (SH 98 15, SH 98 88, and SH 98 121) gave a higher frequency of regeneration but a lesser overall efficiency because of several escapes (defined as plants that survived the selection process but that were not transgenic). The accessions SH 98 26, SH 98 29, SH 98 56, SH 98 96, SH 98 97, SH 98 110, SH 98 128, and SH-98 129 were chosen as the best lines for transformation and were tested further as described in Material and methods; the results are shown in the Table 2.

Molecular screening of transgenic plants

Shoot tissue harvested from PPT-resistant plants was screened with PCR to verify the presence of the *bar* gene in the plant genome (Fig. 2). Results indicated that among the plants analyzed from all experiments, all contained the *bar* gene. The amplified DNA fragments (approximately 400 nucleotides) from the transgenic plants were identical in size to the controls and all hybridized with the plasmid probe. Twenty independently transformed plants from each accession that successfully produced plants both resistant to the herbicide treatment and positive in the PCR analyses were analyzed for *bar*-gene copy number by Southern blot analysis, in which a gene copy reconstruction lane was also included. In cases where the Southern analyses indicated that there were multiple copies of the transgene (Figs. 3A and

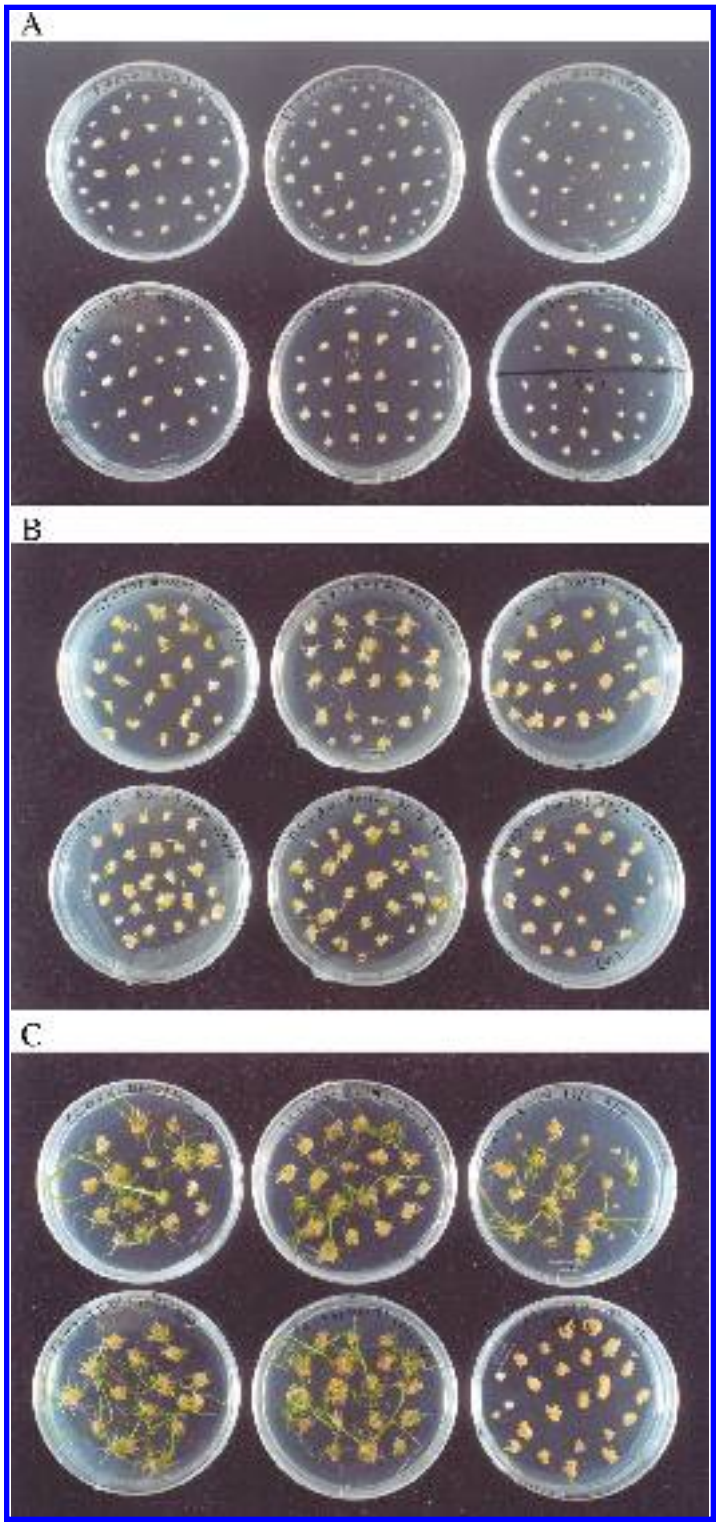
Table 1. Responses of the 129 'Bobwhite' sister lines to somatic embryogenesis induction medium, regeneration, and the effective transformation efficiency of each 'Bobwhite' line.

'Bobwhite' line	Somatic embryogenesis formation (%)		Regeneration efficiency (%)		Transformation efficiency (%)	Bobwhite line	Somatic embryogenesis formation (%)		Regeneration efficiency (%)		Transformation efficiency (%)
	Bombarded	Control	Bombarded	Control			Bombarded	Control	Bombarded	Control	
SH 98 01	50.35	51.86	49.15	1.20	47.94	SH 98 66	41.90	42.74	28.26	13.64	14.62
SH 98 02	66.26	69.57	62.55	3.70	58.85	SH 98 67	60.59	59.98	36.88	23.71	13.17
SH 98 03	33.91	34.59	22.31	11.59	10.72	SH 98 68	29.60	29.89	26.09	3.51	22.58
SH 98 04	36.99	36.62	29.30	7.69	21.61	SH 98 69	37.13	41.74	37.13	0.00	37.13
SH 98 05	33.83	34.17	28.99	4.84	24.16	SH 98 70	41.35	42.67	41.35	0.00	41.35
SH 98 06	0.80	0.90	0.80	0.00	0.80	SH 98 71	66.92	68.92	40.67	26.25	14.42
SH 98 07	40.98	42.30	40.98	0.00	40.98	SH 98 72	88.09	92.49	32.20	55.88	0.00
SH 98 08	7.41	7.63	7.41	0.00	7.41	SH 98 73	84.11	85.79	51.85	32.26	19.59
SH 98 09	61.79	64.88	55.77	6.02	49.75	SH 98 74	61.64	61.02	53.57	8.06	45.51
SH 98 10	38.12	38.88	38.12	0.00	38.12	SH 98 75	64.08	64.72	58.90	5.17	53.73
SH 98 11	39.20	38.80	28.22	10.98	17.25	SH 98 76	67.77	76.17	59.26	8.51	50.75
SH 98 12	8.34	8.42	6.45	1.89	4.56	SH 98 77	49.56	51.15	43.68	5.88	37.80
SH 98 13	23.25	26.13	20.78	2.47	18.31	SH 98 78	47.01	48.42	35.90	11.11	24.79
SH 98 14	97.68	100.00	76.53	21.15	55.38	SH 98 79	57.95	60.85	55.45	2.50	52.95
SH 98 15	100.00	100.00	71.65	37.50	34.15	SH 98 80	32.57	33.22	32.57	0.00	32.57
SH 98 16	89.59	94.07	71.61	17.98	53.63	SH 98 81	80.77	79.96	50.00	30.77	19.23
SH 98 17	24.34	24.83	5.19	19.15	2.05	SH 98 82	0.00	0.00	0.00	0.00	0.00
SH 98 18	37.50	37.13	0.00	37.50	0.00	SH 98 83	79.14	88.95	54.55	24.59	29.96
SH 98 19	48.52	49.00	39.90	8.62	31.28	SH 98 84	62.34	64.34	52.97	9.38	43.59
SH 98 20	3.85	4.32	3.85	0.00	3.85	SH 98 85	43.72	45.03	35.38	8.33	27.05
SH 98 21	43.64	45.03	43.64	0.00	43.64	SH 98 86	35.48	37.26	35.48	0.00	35.48
SH 98 22	54.85	56.49	46.85	8.00	38.85	SH 98 87	46.04	46.96	43.82	2.22	41.60
SH 98 23	47.89	50.28	47.89	0.00	47.89	SH 98 88	100.00	100.00	54.41	80.00	0.00
SH 98 24	56.72	57.85	56.72	0.00	56.72	SH 98 89	95.45	100.00	50.00	45.45	4.55
SH 98 25	20.00	19.80	20.00	0.00	20.00	SH 98 90	0.00	0.00	0.00	0.00	0.00
SH 98 26	73.81	74.55	73.81	0.00	73.81	SH 98 91	50.00	49.50	50.00	0.00	50.00
SH 98 27	16.39	18.42	8.70	7.69	1.00	SH 98 92	94.75	95.70	68.09	26.67	41.42
SH 98 28	0.00	0.00	0.00	0.00	0.00	SH 98 93	54.05	60.75	51.72	2.33	49.40
SH 98 29	62.16	64.03	62.16	0.00	62.16	SH 98 94	42.29	43.14	35.54	6.76	28.78
SH 98 30	48.12	50.53	48.12	0.00	48.12	SH 98 95	74.13	73.39	45.10	29.03	16.07
SH 98 31	22.41	22.86	22.41	0.00	22.41	**SH 98 96**	72.20	72.92	70.54	1.67	68.87
SH 98 32	61.79	61.18	45.13	16.67	28.46	**SH 98 97**	90.92	100.00	77.19	13.73	63.47
SH 98 33	48.61	49.10	34.88	13.73	21.16	SH 98 98	68.93	71.14	44.44	24.49	19.95
SH 98 34	30.62	34.42	24.56	6.06	18.50	SH 98 99	61.95	63.81	50.36	11.59	38.76
SH 98 35	14.03	14.48	5.80	8.24	0.0	SH 98 100	37.95	39.84	28.57	9.38	19.20
SH 98 36	22.92	23.60	22.92	0.00	22.92	SH 98 101	55.20	56.31	36.15	19.05	17.11
SH 98 37	22.48	23.60	22.48	0.00	22.48	SH 98 102	81.00	80.19	57.60	23.40	34.20
SH 98 38	57.03	58.17	42.32	14.71	27.62	SH 98 103	100.00	100.00	80.85	35.21	45.64
SH 98 39	23.03	22.80	23.03	0.00	23.03	SH 98 104	79.99	83.99	62.14	17.86	44.28
SH 98 40	38.88	39.27	11.96	26.92	0.00	SH 98 105	59.08	60.26	45.56	13.51	32.05

SH 98 41	0.02	0.03	0.02	0.00	0.02	SH 98 106	67.12	66.45	55.36	11.76	43.59
SH 98 42	0.03	0.04	0.03	0.00	0.03	SH 98 107	96.26	97.22	58.76	37.50	21.26
SH 98 43	0.00	0.00	0.00	0.00	0.00	SH 98 108	98.81	100.00	66.46	32.35	34.10
SH 98 44	0.05	0.05	0.05	0.00	0.05	SH 98 109	22.10	21.88	13.94	8.16	5.78
SH 98 45	0.00	0.00	0.00	0.00	0.00	**SH 98 110**	82.10	82.92	71.43	10.67	60.76
SH 98 46	0.26	0.26	0.26	0.00	0.26	SH 98 111	73.74	82.89	40.41	33.33	7.08
SH 98 47	0.00	0.00	0.00	0.00	0.00	SH 98 112	81.87	84.49	51.26	30.61	20.65
SH 98 48	0.00	0.00	0.00	0.00	0.00	SH 98 113	92.71	95.49	30.80	61.90	0.00
SH 98 49	25.00	25.80	25.00	0.00	25.00	SH 98 114	94.35	99.07	71.28	23.08	48.20
SH 98 50	0.16	0.17	0.16	0.00	0.16	SH 98 115	0.00	0.00	0.00	0.00	0.00
SH 98 51	0.00	0.00	0.00	0.00	0.00	SH 98 116	100.00	100.00	83.81	50.00	33.81
SH 98 52	0.09	0.10	0.09	0.00	0.09	SH 98 117	100.00	100.00	89.47	47.06	42.41
SH 98 53	0.00	0.00	0.00	0.00	0.00	SH 98 118	100.00	100.00	81.71	37.04	44.67
SH 98 54	0.00	0.00	0.00	0.00	0.00	SH 98 119	100.00	100.00	80.87	40.91	39.96
SH 98 55	24.91	28.00	24.02	0.89	23.13	SH 98 120	100.00	100.00	85.07	37.50	47.57
SH 98 56	73.47	75.82	73.47	0.00	73.47	SH 98 121	100.00	100.00	87.50	41.67	45.83
SH 98 57	34.28	35.31	28.66	5.62	23.04	SH 98 122	0.00	0.00	0.00	0.00	0.00
SH 98 58	0.00	0.00	0.00	0.00	0.00	SH 98 123	25.64	25.38	25.64	0.00	25.64
SH 98 59	39.32	40.10	39.32	0.00	39.32	SH 98 124	0.00	0.00	0.00	0.00	0.00
SH 98 60	48.20	47.71	34.20	14.00	20.20	SH 98 125	0.00	0.00	0.00	0.00	0.00
SH 98 61	0.00	0.00	0.00	0.00	0.00	SH 98 126	100.00	100.00	86.62	23.26	63.36
SH 98 62	71.34	80.19	49.68	21.67	28.01	SH 98 127	0.00	0.00	0.00	0.00	0.00
SH 98 63	0.00	0.00	0.00	0.00	0.00	**SH 98 128**	93.38	96.18	76.71	16.67	60.05
SH 98 64	58.28	60.03	47.37	10.91	36.46	**SH 98 129**	82.92	87.06	72.92	10.00	62.92
SH 98 65	62.28	65.39	49.55	12.73	36.82						

Note: The accessions chosen for their high transformation efficiency (SH 98 26, SH 98 29, SH 98 56, SH 98 96, SH 98 97, SH 98 110, SH 98 128, and SH 98 129) appear in bold. The transformation efficiency has been calculated by dividing the total number of transgenic plants obtained by the total number of embryos bombarded.

Fig. 1. Response of 'Bobwhite' line SH 98 26 to the tissue culture process. (A) Somatic embryogenesis induction. (B) Plant regeneration on selective medium (5 mg PPT/L) after 15 days selection. (C) Plant regeneration on selective medium (5 mg PPT/L) after 30 days selection. Controls C were placed in the lower right corner in all photos.



3B), all of the copies appeared to cosegregate, thus yielding progenies with either all or none of the copies. This suggests that all of the copies of the transgene were inserted at the same genetic locus. The *bar* transgene was inherited and expressed in the T_1 and T_2 generation lines tested (Table 3). In the case that more than one plant was selected from the same callus, Southern analysis was performed immediately. In the case that the band segregation patterns in the Southern blot were similar (Fig. 4), the plants were counted as one.

Fertility of transgenic wheat plants

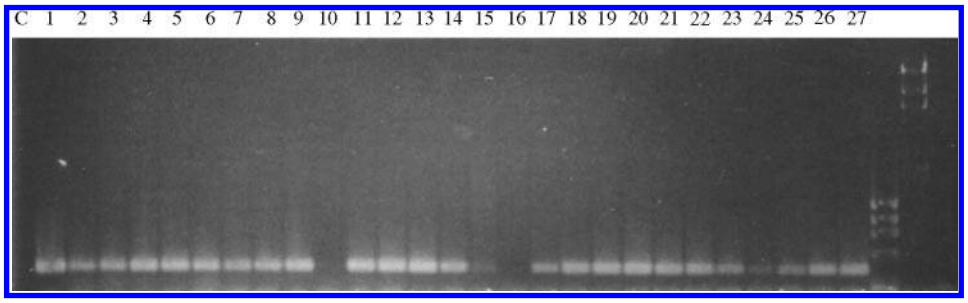
The initial transgenic wheat plants presented a broad

Table 2. Results of the statistical analyses of the best eight 'Bobwhite' lines for embryogenesis, regeneration in selective medium, and effective transformation efficiency.

	Somatic embryogenesis formation (%)		Regeneration efficiency (%)		Transformation
'Bobwhite' line	Bombarded	Control	Bombarded	Control	efficiency (%)
SH 98 26	72.09±13.34	75.35±13.39	71.17±14.68	0	70.86±14.48
SH 98 29	59.23±15.56	63.49±18.22	61.90±11.29	0.00	60.92±11.58
SH 98 56	70.34±9.82	70.14±5.98	70.34±9.83	0.00	69.02±6.94
SH 98 96	69.17±9.92	69.97±4.73	69.17±9.93	2.43±1.55	66.96±6.14
SH 98 97	90.96±5.95	93.33±4.92	76.63±9.02	13.98±4.54	66.96±4.53
SH 98 110	81.07±7.15	81.25±5.23	70.95±6.32	10.77±4.87	60.80±6.28
SH 98 128	91.15±4.85	93.54±4.22	78.13±8.21	20.13±5.61	58.27±10.40
SH 98 129	80.55±7.01	85.59±6.11	71.82±9.61	10.08±3.58	60.04±9.61

Note: The data were pooled from three repetitions with more than 2000 embryos/transformation experiment.

Fig. 2. PCR analyses of some of the transformants of the line SH 98 26. DNA was extracted from leaves as described in Materials and methods. Each lane represents an independent event. Plants 10 and 16 did not survive the Basta treatment.



range of fertility, although most were at least partially fertile. Only a few of the plants were completely sterile. Fertility was usually restored in the subsequent generations, indicating that partial sterility observed at the T_0 generation was not an inherited trait in most cases.

Inheritance of the marker gene

The selected progeny were evaluated again for resistance to PPT. Resistant and sensitive seedlings were clearly distinguishable after spraying with 0.3% Basta. A segregation ratio of 3:1 was observed for 500 of 600 independent transgenic events tested. Twenty lines (randomly taken) were studied for inheritance of the transgenes as shown in the Table 3. The Basta-resistant progeny of the plants that gave segregation ratios of 3:1 or 1:0 were analyzed by PCR and Southern hybridization. All of the Basta-resistant progeny contained bands that hybridized with the probe for the *bar* gene, whereas none of the sensitive progeny hybridized to the probe and thus may have been segregants or escapes (data not shown).

Statistical analyses results

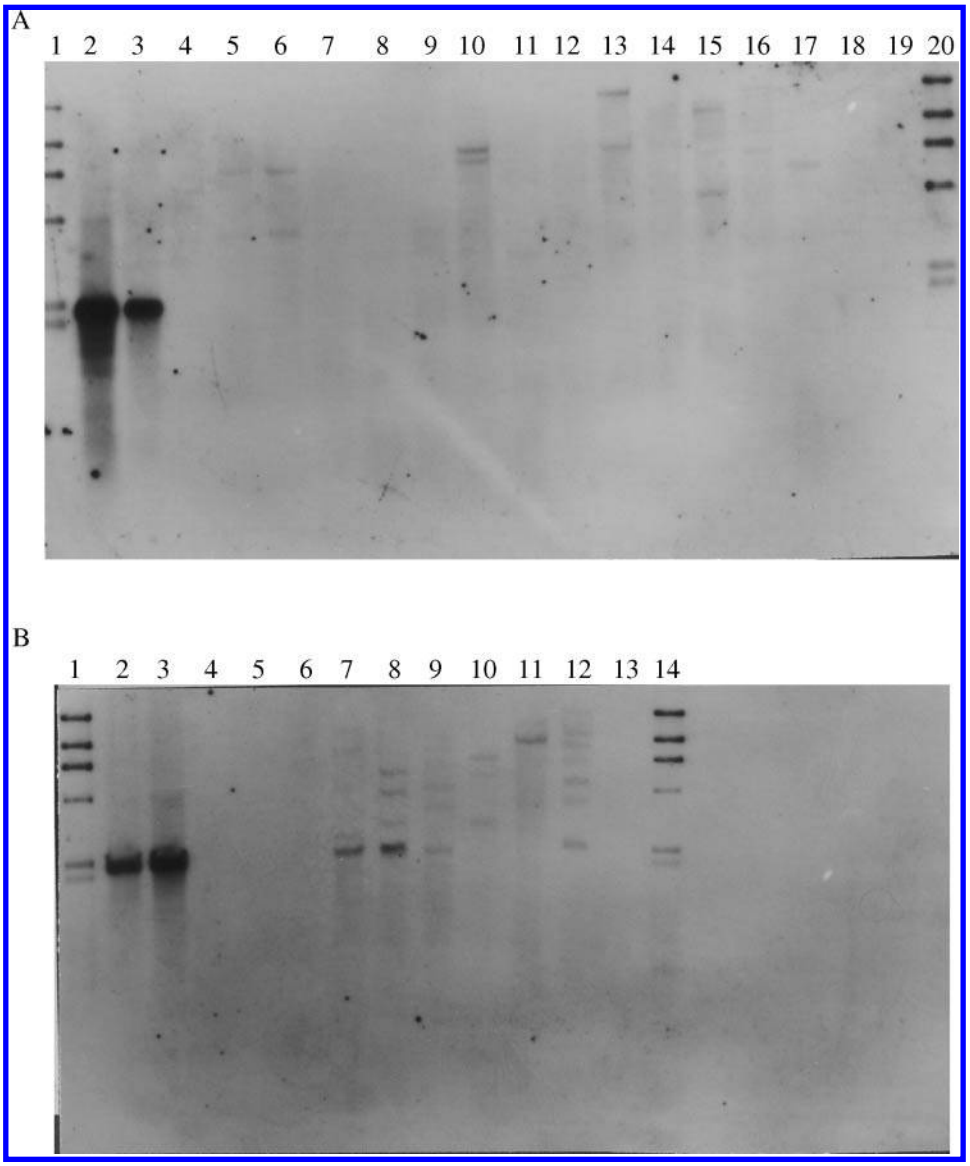
The results of the minimum, maximum, average, and standard deviation of the embryogenesis, regeneration, and transformation efficiencies are shown Tables 1 and 3. Results of the analysis of the eight best 'Bobwhite' accessions showed highly significant differences between bombarded scutella and controls ($P < 0.05$). This analysis was done excluding the 'Bobwhite' line × treatment interaction because of an imbalance in some levels of the main effects of the 'Bobwhite' line that was not estimable. The main effects of treatment on bombarded scutella versus control were highly significant with an estimated proportion of regeneration of 0.72 vs. 0.07. There were significant differences among the top eight 'Bobwhite' lines. Line SH 98 128 showed the highest proportion of regenerated plants (0.48) and line SH 98 29 showed the lowest proportion (0.19).

Discussion

The goal of many genetic engineering efforts is to contribute to sustained production of affordable and high-quality food by introducing novel characteristics into plant varieties. For CIMMYT, the aim is to work on problems that affect a large number of resource-poor farmers in developing countries and to apply biotechnology in such a way that it augments conventional breeding efforts. This goal can be reached only if the novel characteristics introduced into crops such as wheat are used by the breeding programs. In turn, these novel characteristics will advance in breeding only if they are stable and effective. This currently requires the production of a collection of transgenic plants for each novel trait to permit the selection of the most appropriate individuals for the breeding program. Initially, this requires an efficient gene transfer protocol.

The efficiency of a wheat biolistic transformation system strongly depends on the bombardment parameters, the condition of the donor plant, and the plant genotype chosen for the transformation process (Witrzens et al. 1998). A highly responsive wheat genotype is advantageous because it can enhance the efficiency of biolistic transformation and may be useful in other protocols such as *Agrobacterium*-mediated transformation. To identify highly responsive genotypes, it is necessary to optimize and standardize tissue-culture conditions and transformation efficiency (Pellegrineschi et al.

Fig. 3. Southern blot analysis of regenerated T_0 plants from the line SH 98 26 after *Sma*I restriction digest of the plasmid (unique site on Ubibar plasmid). The DNA was digested with *Sma*I and then probed with the digoxygenin-labeled *bar* gene. Lanes 1 and 20 in A and lanes 1 and 14 in B contain a λ ladder. Lanes 2 and 3 in both A and B are the copy number references (copy numbers 10 and 5, respectively). Lanes 5–19 in A and lanes 7–12 in B are from Basta-resistant plants. Controls are in lanes 4-6 in 3B.



2000), as well as identify the specific physiological conditions of the starting material for transformation.

For screening the material, the standardization of the physiological status of the donor plants was one of the most critical factors for comparing the transformation abilities among the 'Bobwhite' accessions. After testing under various conditions (data not shown), a uniform non-stressed growth environment was selected for the optimal growth of the donor plants.

The choice of the zygotic embryo development stage was another important factor. Various development stages were screened for their response to the transformation process. The dimension of the embryo (1 mm on the longest side) was taken as standard in all accessions regardless of the number of days after pollination because at this developmental stage the scutella are more responsive to the tissue culture (e.g., rapid bevelopment of embryogenic callus). In the transformation experiments, the accessions SH 98 26 and SH 98 56 were slightly more efficient than the other accessions, with an overall efficiency of more than 70%, although their ability to differentiate somatic embryos was less than other accessions (Table 1). The performance of these accessions could be explained by their high sensitivity to herbicide selection (non-transformed controls were not able to produce plants under selection conditions), as compared with accessions such as SH 98 121, in which nearly 100% of the immature embryos produced somatic embryos. However, the controls for SH 98 121 also produced plantlets during the selection process, thus increasing the number of escapes.

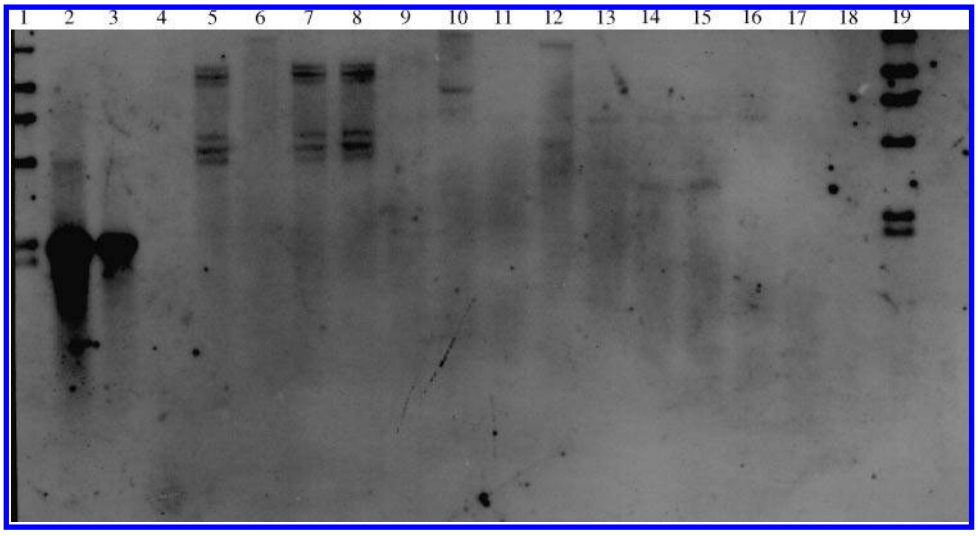
Between the two high performing varieties, we determined the variety SH 98 26 to be super transformable because of its early maturity (under the right conditions, this

Table 3. Segregation of the Basta-resistant phenotype in T_1 and T_2 generation on randomly selected transgenic plants.

	T_1 plant tested for herbicide spray				T_2 plant tested for herbicide spay			
Event No.	Resistant	Sensitive	R:S	χ^2	Resistant	Sensitive	R:S	χ^2
5508	20	11	3:1	0.2555	25	5	3:1	0.1563
5650	28	7	3:1	0.0656	24	6	3:1	0.0563
5679	12	23	SD	4.3513	34	8	3:1	0.1116
5971	32	5	3:1	0.3661	22	6	3:1	0.0268
5993	50	9	3:1	0.4203	26	5	3:1	0.183
5994	25	8	3:1	0.0014	30	10	1:0	0
6003	27	8	3:1	0.0121	23	6	3:1	0.0404
6035	19	5	3:1	0.0313	21	3	3:1	0.2813
6067	34	0	1:0	0	20	0	1:0	0
6070	30	8	3:1	0.0444	25	6	3:1	0.0741
6074	7	6	3:1	0.4363	23	5	3:1	0.1071
6160	26	19	SD	1.001	45	12	3:1	0.0666
6163	25	1	SD	0.8726	10	2	3:1	0.0625
6205	22	0	1:0	0	20	0	1:0	0
6206	21	8	3:1	0.0145	20	4	3:1	0.125
6246	45	0	1:0	0	32	0	1:0	0
6255	12	10	SD	0.6903	32	8	3:1	0.075
6256	10	32	SD	8.2545	16	4	3:1	0.0375
6940	5	13	SD	3.0104	20	8	3:1	0.0268
6942	4	15	SD	4.1472	22	7	3:1	0.0016

Note: Mendelian segregation of this trait was observed in 12 lines on the T_1 generation and in all 20 in the T_2 generation. SD, substantial deviation from the expected segregation ratio

Fig. 4. Southern blot analysis of T_0 plants regenerated from the same callus. The DNA was digested with *Sma*I and then probed with the digoxygenin-labeled *bar* gene. Lane 1, λ molecular weight ladder; lanes 2 and 3, 10 and 5 copies of the plasmid, respectively; lane 4, non-transgenic plant control; lanes 5–10, plantlets derived from the same callus (plants 5, 7, and 8 were considered a single event, plants 6 and 9 were considered to be escapes, and plant 10 was considered an independent event); lanes 12–16, plants derived from one callus; lanes 17 and 18, non transgenic controls.



variety could produce 4 generations in a year), because it does not have the T1BL.1RS translocation, and because it could be suitable as a parent for breeding programs. Genetic analyses of the T_1 and T_2 progeny provided conclusive evidence of the incorporation of the *bar* transgene into wheat chromosomes. The *bar* gene, in the majority of cases, was inherited with a Mendelian ratio of 3:1. However, in some progeny Basta resistance was expressed in the T_1 generation with an unusual pattern of segregation, but the T_2 generated from Basta resistant T_1 plants segregated in the expected Mendelian ratio (3:1 or 1:0 for homozygous plants).

The ability of the particle gun to consistently transform wheat has been previously reported (Lonsdale et al. 1998; Vasil et al. 1992; Witrzens et al. 1998). However, it has been noted that cereal transformation is still difficult because of the number of parameters involved in the technique (Lonsdale et al. 1998; Vasil et al. 1992; Lörz et al. 1996; Uze et al. 1999; Vasil et al. 1993). In deference to this constraint, we

attempted to reduce the number of parameters, particularly the time in tissue culture, and modify the selection pressure.

In conclusion, we have identified a 'Bobwhite' accession (SH 98 26) that is highly efficient for the recovery of transgenic plants. With this variety, we were able to achieve a transformation efficiency that consistently exceeded 70%, based on the average of several transformation experiments. We believe that the use of this variety will enable the constant production of transgenic wheat plants and allow the latest discoveries in biotechnology (genes, promoters, new possibilities of modifying metabolic pathways) to be routinely applied. Further research is in progress to reduce the complexity of the insertions thus producing transformed plants suitable for wheat breeding programs.

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