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GENETIC TRANSFORMATION AND HYBRIDIZATION

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The effect of pretreatment with mild heat and drought stresses on the explant and biolistic transformation frequency of three durum wheat cultivars

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Abstract The use of transgenic plants in durum wheat breeding makes possible the utilization of genes not previously available in the wheat genetic pool. However, to effectively use this technology, it is necessary to be able to continuously produce large numbers of transgenic plants. This paper describes the development of a routine biolistic transformation technology for durum wheat and its adaptation to the mass production of transgenic plants. The method involves subjecting the donor plant to mild drought stress 1 day before the transformation experiment, subsequent bombardment of the immature embryos harvested from these plants with plasmid DNA, and reduced levels of tissue culture manipulation. The in vitro culture response of bombarded immature embryos and the production of transgenic plants were investigated in three durum wheat cultivars: Mexicali, D5c31YN S74, and D5c31YN S48. In all three genotypes, immature embryos (1.0 mm long) were transformed with a plasmid containing a phosphinothricin-resistant gene (*bar*) under the control of the maize ubiquitin promoter. The transformation frequency was highest in Mexicali, with a range of 0–6.46% transformation efficiency. The data suggest that variations in transformation were frequently influenced by differences in the genotypes and physiological status of the donor plant, rather than by the efficiency of the particle bombardment procedure.

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Introduction

Triticum turgidum L. var. durum, commonly called "durum wheat," is a staple food crop of the Mediterranean basin and is cultivated on approximately 17 million hectares worldwide. Its production is concentrated in West Asia and North Africa, Mediterranean Europe, Ethiopia, and the Asian subcontinent. In the North and South American continents, durum production is located in Argentina, Chile, the Andean region, Mexico, the United States, and Canada.

While the average yield on a world-wide scale is around 1.7 t/ha, in developing countries it is substantially lower (1.2 t/ha). This is despite the fact that considerable progress has been made on increasing yields using classical breeding, and modern short-statured, high-yielding durum materials now have a genetic yield potential higher than that of bread wheat. The main problems for durum wheat production in the marginal areas are biotic stresses, such as the fungal diseases, and general abiotic stresses such as drought.

The prospects are good for developing wheat transgenics that increase resistance to fungal diseases (Clausen et al. 2000; Lorito et al. 1998) and improve or modify drought tolerance (Satoshi et al. 2000; Yoshiba et al. 1999). These production constraints are particularly important for people dependent on wheat as a staple food and for obtaining export quality products.

Substantial data have been generated recently on wheat transformation protocols (Liu et al. 2000; Lorz et al. 1996; Sorikin et al. 2000; Vasil et al. 1992; Witrzens et al. 1998; Zhang et al. 2000), and gene expression (Muller et al. 1996; Rooke et al. 2000; Takumi et al. 1997), thereby providing an essential background for the development of a system for the mass production of bread wheat transgenic plants. For durum wheat, however, the rate of success has been substantially lower, with

only a few investigations to be found in the published literature (Bommineni et al. 1997; He et al. 1999; Martin et al. 1999; Tosi et al. 2000). These studies focused on proving that it is possible to transform durum wheat, and although they are useful scientific contributions, due to the low transformation rates and small number of plants produced, the information provided falls far short of meeting the practical needs of plant breeders. Consequently, there is clearly a need to develop a dependable method of transformation for this species, thereby encouraging the use of genetic transformation as an everyday tool for durum wheat breeders.

The objectives of the study reported here were to develop a robust transformation system for durum wheat based on available wheat transformation protocols and to determine the potential for the mass production of transgenic plants.

Materials and methods

Plant material

Three durum wheat cultivars (Mexicali, D5c31YN S74, and D5c31YN S48) were used for the transformation experiments based on their regeneration ability (A. Pellegrineschi, unpublished data). The plants were grown in a screen-house at daytime temperatures of 24°–28°C and nighttime temperatures between 15°C and 18°C. To ensure a continuous production of immature embryos, we planted seeds of each variety every 2–3 days. The donor plants were transferred to a growth chamber 13, 14, and 15 days after heading and stressed according to the conditions described below.

Growth chamber conditions of plant material

Drought and heat conditions were created in a growth chamber (Onviron, Controlled Environments, Winnipeg, Man., Canada) to replicate these stress conditions in a controlled environment. Fifty plants at the same heading stage were subjected to drought conditions by withholding water for 1, 2, and 3 days to test for suitable levels of stress induction. For all treatments, 16-day-old immature seeds were then harvested and subsequently sterilized, and the embryos isolated. The photoperiod was set at 16/8 h (light/dark), the temperature at 23°C, and the humidity at 75%. To evaluate the effect of heat stress, we maintained the donor plants in water-saturated soil and stressed them at 40°C during the daytime, again for 1, 2, and 3 days. The experiments were repeated three times for each treatment. The moisture level was measured with a soil moisture meter (Lincoln Irrigation) using a scale from 0 to 10 (0–2=0–20% total moisture; 2–4=20–40% total moisture; 4–6=40–60% total moisture; 6–8=60–80% total moisture; 10=100% total moisture). A standard soil mix was used in all experiments (peat/sand/soil: 1/1/1).

Transformation procedure

The plasmid used in this experiment was developed from pGEM3Zf(+) (Promega, Madison, Wis.) and contained the selectable *bar* gene that confers resistance to the herbicide Basta under control of the maize ubiquitin promoter (Christensen et al. 1992) cloned at the *Hin*dIII site (pUbi-*bar*). Five micrograms of plasmid DNA was precipitated in a mixture consisting of 2 mg of gold particles (0.6 µm in diameter), 50 µl 2.5 *M* $CaCl_2$, and 20 µl 100 m*M* spermidine-free base. After microprojectile-DNA precipitation, the supernatant was discarded. Particles were washed in 250 µl absolute ethanol and resuspended in 250 µl absolute ethanol. For each bombardment, 10 µl of microparticles-DNA were placed on 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.) set at 900 psi. Immature embryos (1 mm long) or the isolated scutella were excised and directly transferred into a MS (Murashige and Skoog l962) basal medium (Sigma, St. Louis, Mo.) supplemented with 15% maltose, left to dehydrate for 4 h, and then bombarded. Eight hours after bombardment, the embryos were placed on MS medium containing 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 30 g l^{-1} sucrose, and 8 g Bacto-Agar for somatic embryo induction. Twenty days later, the treated embryos were evaluated for somatic embryo production and then transferred into MS medium containing 5 mg l^{-1} PPT (phosphinothricin, the active compound of the herbicide Basta). After a further 30 days, green regenerating shoots surviving the treatment were transferred again to the same medium. Twenty days later, surviving green-rooted shoots were transferred to soil.

Molecular identification of transgenic plants

DNA was extracted from young leaves using the Nucleospin DNA purification kit from Clonetech (Palo Alto, Calif.) following the manufacturer's protocol. All of the Basta-resistant plants were analyzed by the polymerase chain reaction (PCR) for the presence of the *bar* gene. The sequence of the forward primer was 5′-GTCTGCACCA TCGTCAACC-3′ and of the reverse primer 5′-GAAGTCCAGCTGCCAGAAAC-3′. The PCR reactions were carried out in a total volume of 25 µl comprising 10 ng of wheat genomic DNA, 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.8), 3 m*M* $MgCl_2$, 0.1% Triton X-100, 0.24 m*M* each dNTP, 0.04 U *Taq* DNA polymerase and 0.16 µmol of each primer. The DNA was denatured at 94°C for 1 min. (hot start), followed by 30 amplification cycles (94°C for 30 s, 64°C for 2 min, 72°C for 2 min).

Southern blots were carried out with total genomic DNA isolated from young leaves using the Nucleon Phytopure Plant DNA Extraction Kit according to the manufacturer's protocol (Amersham Life Sciences, Arlington Heights, Ill.). DNA was digested with *Sma*I, and the resulting fragments were separated by electrophoresis on a 1.0% agarose gel. The DNA was then transferred to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, Mo.) and hybridized to digoxigenin (DIG)-dUTP labeled probes according to the manufacturer's instructions (Boehringer Mannheim). The entire plasmid was DIG-labeled by nick translation and used as a probe. 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 2 h.

Phenotypical analyses

Herbicide resistance of the putative transgenic wheat plants was determined by spraying applications of Basta (0.3% w/v) onto the leaves of plants at the fifth-leaf stage. Plants were scored as susceptible, partially resistant, or resistant, according to the degree of leaf desiccation after 7 days.

Analyses of the progeny

Some seeds of the selfed generations (T_1 and T_2) of the transgenic plants were sterilized and the embryo excised. The sterile embryos were then transferred to MS medium with 5 mg/l PPT. One week later, the embryos were scored for the survival ratio (segregation of the *bar* phenotype). The resistant plantlets were then transferred to soil and tested for transgene presence using PCR.

Statistical analyses

For the drought and heat treatment analyses, 2,000 embryos were utilized (40 bombardments with 50 embryos or scutella per bom-

Table 1 Effects of drought (A) or heat stress (B) treatments on the ability of cultured embryos and scutella to produce somatic embryos. Values are the percentage of cultured embryos or scutella that gave rise to embryogenic callus (± standard deviation), and the data were pooled from more than 2,000 embryos per experiment

	One-day drought (moisture level 3–4)[a] Percentage		Two-day drought (moisture level 1–3)[a] Percentage		Three-day drought (moisture level 1–2)[a] Percentage		Control (moisture level 6–8)[a] Percentage	
	Embryo	Scutellum	Embryo	Scutellum	Embryo	Scutellum	Embryo	Scutellum
A								
Mexicali	47.63±4.01	36.02±3.24	43.56±5.76	31.34±5.02	40.12±3.10	29.84±4.01	32.12±2.46	27.00±1.75
D5c31YN S48	29.32±3.23	23.52±2.85	21.02±5.60	22.07±4.45	20.93±3.45	19.10±3.98	18.05±1.38	13.04±4.10
D5c31YN S74	28.02±3.75	21.02±2.67	24.02±4.68	21.08±4.12	19.33±2.27	15.24±3.31	18.34±2.02	12.98±3.56
B								
Mexicali	45.20±3.01	38.02±2.03	41.00±5.76	26.78±6.32	37.82±4.30	26.45±4.84	31.56±1.34	26.48±2.00
D5c31YN S48	30.47±3.23	20.56±4.13	25.04±6.78	18.28±6.05	22.02±3.25	22.10±3.98	17.85±1.02	14.34±2.40
D5c31YN S74	26.45±4.82	19.78±3.02	24.80±6.02	23.02±4.50	18.04±2.05	12.44±5.02	19.84±3.04	13.41±2.16

[a] 0–2, 0–20% total moisture; 2–4, 20–40% total moisture; 4–6, 40–60% total moisture; 6–8, 60–80% total moisture; 10, 100% total moisture

bardment), and each bombardment was considered to be a repetition. For each experiment, the average, standard deviation, maximum and minimum of the somatic embryogenic formation (number of embryos producing embryogenic callus per total number of embryos treated), surviving ratio in selective medium (number of embryos producing PPT-resistant plantlets per total number of embryos bombarded), and the transformation efficiency (number of transgenic plants obtained per total number of embryos bombarded) were calculated.

Results

Tissue culture

Twelve to 14 days following their transfer to the induction medium, the induced embryo/scutella from the stressed plants and from the control plants were scored for somatic embryo formation (Table 1). Average somatic embryo formation in 1-day drought-stressed plants of all varieties tested was higher than in non-stressed plants – 47.63% for Mexicali and 29.32% and 28.02% for varieties D5c31YN S48 and D5c31YN S74, respectively. The induced scutella gave 36.02%, 31.34%, and 29.84% somatic embryo formation in induced Mexicali scutella depending on the length of the drought stress induction (1, 2, and 3 days, respectively) in contrast to the 27% observed for the non-stressed control plants (Table 1). Varieties D5c31YN S48 and D5c31YN S74 had a similar level of response to the drought stress treatments, with var. D5c31YN S48 showing a maximum somatic embryo formation of 29.32% with 2 days of stress (Table 1).

The heat shock also induced differences on somatic embryo formation (Table 1), with a higher formation of somatic embryos on the scutella derived from plants stressed for 1 and 2 days (Table 1). Following 1 day and 2 days of heat shock, the embryos of all varieties tested developed more somatic embryos than the controls (Table 1).

There were no distinct differences with respect to stage development of the somatic embryos, with the exception of the number of embryos or scutella differentiating somatic embryos. The globular stage of the somatic embryos was observed 7–8 days after the transfer onto induction medium, and the somatic embryos were usually formed directly from the scutellum. Based on tissue culture results, the entire embryo was chosen as the standard transformation target.

Transformation frequency and selection efficiency

The explants that induced somatic embryos were transferred to MS medium containing 5 mg l^{-1} PPT for selection to allow resistant somatic embryos to germinate without hormonal pressure. Culture on this selective medium resulted in a high mortality among the somatic embryos – only about 10–15% of the total number of somatic embryos developed into PPT-resistant plantlets (Fig.1). After 30 days, healthy, vigorously growing plantlets were again scored (Table 2) and these then transferred to the same medium to eliminate any eventual formation of shoots derived from the zygotic meristem. The frequencies of transformation related to the stress treatments (calculated as the number of independently regenerated PPT-resistant plants divided by the total number of immature embryos bombarded) were calculated from 84 transformation experiments (Table 2). This frequency varied from 0.00% to 6.46% and was obtained with immature embryos isolated from plants stressed by withholding water for 1 day (Table 2). With this stress condition, an average of 2.86% for Mexicali (range: 0.20–6.46%), 1.34% for D5c31YN S48 (range: 0.00–4.23%), and 0.92% for D5c31YN S75 (range: 0.00–4.53%) was obtained, as shown in Table 2. Most of the transgenic plants produced were recovered from different embryos, while some were physically separated from the same explant. The selection system for theses

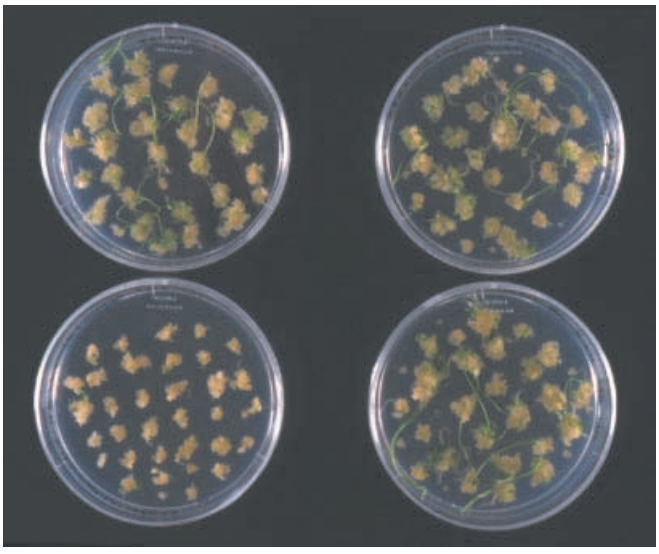

Fig. 1 Regeneration on selective medium of embryogenic calli derived from bombarded embryos of cv. Mexicali. Non-bombarded control embryos are at the *lower left*

wheat transformation experiments employs the *bar* gene and the PPT herbicide. It was found that selection based upon the presence of 5 mg l^{-1} PPT in the medium, especially during the stage of regeneration and plantlet growth, was critical to reducing the growth of non-transformants.

Molecular screening of transgenic plants

Shoot tissues harvested from PPT-resistant plants were screened using PCR to verify insertion of the *bar* gene into the plant genome. The results indicated that among the 259 plants analyzed from 84 experiments, all contained the *bar* gene. The amplified DNA fragments obtained from these transgenic plants, approximately 400 bp in length, were identical in size to the amplified plasmid controls and hybridized with the plasmid probe. Fifteen independently transformed plants were analyzed for copy number (*bar* gene) by Southern blot analyses in which three different gene copy reconstruction lanes were included (Fig. 2). DNA gel blots were hybridized with a *bar*-specific probe to determine the structure of the transgene inserted. Figure 2 shows the results obtained by hybridizing the *bar* probe with the genomic DNA digested with *Sma*I. These hybridizations showed a variety of insertions of the transgene that varied from a simple insertion (two copies) to multicopy insertion (Fig. 2). In the cases in which the Southern analyses indicated that there were multiple copies of the transgene, all of the copies were cosegregating.

Table 2 Average transformation efficiency of the three durum wheat lines studied stressed with mild drought (A) and a 1-day heat shock (B)

	Average[a] (%)	Maximum[a] (%)	Minimum[a] (%)
A			
Mexicali	2.86±1.32	6.46	0.20
D5c31YN S48	1.34±1.78	4.23	0.00
D5c31YN S75	0.92±1.20	4.53	0.00
B			
Mexicali	1.62±0.52	4.03	0.00
D5c31YN S48	0.82±0.58	2.20	0.00
D5c31YN S75	0.76±0.20	1.94	0.00

[a] For each line, the average (± standard deviation), maximum and minimum of the transformation efficiency (number of transgenic plants obtained per total number of embryos treated) were calculated

Inheritance of the marker gene

With a few exceptions, the transgenic T_0 plants tested were at least partially fertile, displaying a broad range of fertility that ranged from the production of a single spike

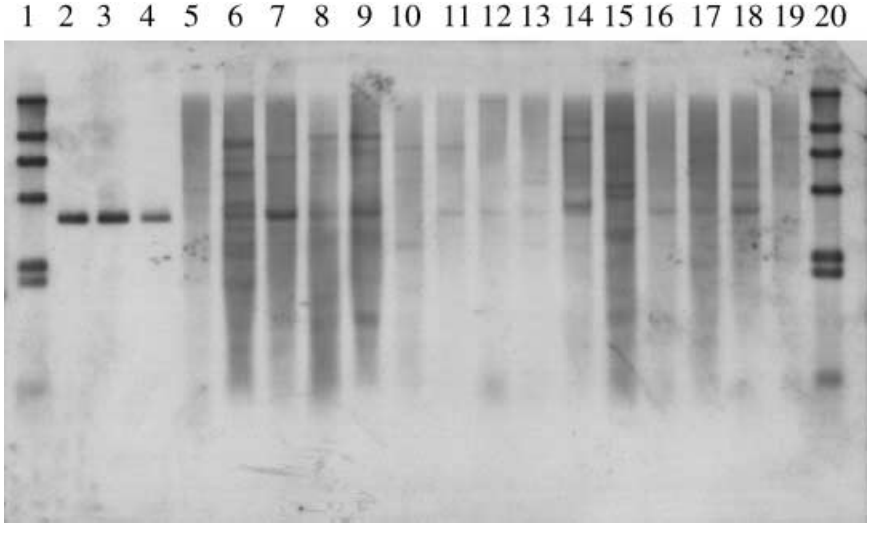

Fig. 2 Southern analyses of transformants (T_0 generation). DNA samples (15 µg) from *bar* transformed plants digested with *Sma*I (*lanes 5–19*) and the resulting fragments were separated by electrophoresis and transferred to a membrane. The membrane was hybridized with a DIG-labeled DNA probe corresponding to the *bar* gene. The expected band size was around 3,200 bp. *Lanes 1, 20* 1-kb ladder, *lanes 2, 3, 4* UbiBar plasmid (copy numbers of 10, 5 and 1, respectively)

Table 3 Segregation analysis of *bar* gene and PPT-resistant phenotype on 20 transgenic plants (randomly taken) from the transformation experiment

Test number	Plant number	T_0 PCR – results of Southern blots	Segregation ratio pattern of the *bar* gene (T_1)	Segregation ratio pattern of the *bar* gene phenotype (T_1)
1	7229	*bar* gene present	T_0 sterile	T_0 Sterile
2	6803	*bar* gene present	3:1	3:1
3	7309	*bar* gene present	Not present	0:1
4	7327	*bar* gene present	3:1	3:1
5	7315	*bar* gene present	3:1	1:5
6	6833	*bar* gene present	3:1	0:1
7	7288	*bar* gene present	Not present	0:1
8	7290	*bar* gene present	3:1	3:1
9	7295	*bar* gene present	3:1	1:20.
10	7299	*bar* gene present	Not present	0:1
11	5219	*bar* gene present	3:1	1:5.
12	7417	*bar* gene present	3:1	1:9.
13	7412	*bar* gene present	3:1	1:5.
14	5220	*bar* gene present	3:1	3:1
15	5221	*bar* gene present	3:1	1:13
16	5315	*bar* gene present	3:1	1:1
17	7404	*bar* gene present	T_0 sterile	T_0 sterile
18	7413	*bar* gene present	3:1	0:1
19	7425	*bar* gene present	3:1	3:1
20	7406	*bar* gene present	3:1	1:5

to a normal plant phenotype. Fertility was restored in subsequent generations, indicating that any partial sterility observed at the T_0 generation was a product of the tissue culture process and not caused by the inserted gene itself.

The selected progeny were also evaluated for resistance to PPT. The segregation patterns of the offspring of 20 plants from ten independent transformation experiments (randomly taken) are shown in Table 3. Resistant and sensitive seedlings were clearly distinguishable following spraying with 0.3% Basta, and a segregation ratio of 3:1 was observed for 8 of 20 independent T-plants (Table 3). Progenies of some of the other plants showed segregation of *bar*-positive and *bar*-negative plants; however, the segregation ratio did not follow a 3:1 Mendelian ratio (Table 3). The remainder of the plants were either sterile or did not transmit the transgene to the progeny.

Discussion

This paper describes a method of using the biolistic procedure for durum wheat transformation as a means of obtaining a constant and consistent production of transgenic plants. The study strongly suggests that donor plants should be treated with a mild drought stress to optimize transformation efficiency. Subjecting the donor plants to controlled abiotic stress markedly improves results when compared to currently available methods (Bommineni et al. 1997). Bommineni et al. (1997) obtained six transgenic plants from 245 bombarded embryos, for a transformation efficiency of 2%. Although this efficiency was correctly derived, the calculation was based on a single experiment and may not be replicable given the low number of transgenic plants produced. He et al. (1999) also used particle bombardment to develop transgenic durum wheat; however, the transformation efficiency reported was 0.6%, and again the number of transgenic lines produced was low. With the method described in this study, transgenic plants have been routinely produced from CIMMYT elite genotypes D5c31YN S48, D5c31YN S75, and Mexicali, and an average transformation efficiency of 1.7% was observed, with a maximum frequency of transformation of 6.46% being obtained with Mexicali.

Plants showing resistance to the herbicide Basta also showed a banding pattern in Southern blot analyses that varied among the transformants, which is consistent with random chromosomal integration (Fig. 2). In this study, our research team focused on the characterization of the *bar* gene as a standard for its transformation work, but in the future it intends to use better marker genes as they become available.

Using immature entire embryos, the total time required for the process (from the immature embryo bombardment to the transgenic plants) is about 100 days, which is similar to other protocols proposed for wheat transformation (Becker et al. 1994; Nehera et al. 1994; Vasil et al. 1993).

The “non-Mendelian” segregation of herbicide resistance that was observed in several transgenic plants was observed only for the T_1 generation and has already been reported by Bommineni et al. (1997). The T_1 herbicide-resistant plants produced a normal segregating ratio in the following generations.

An interesting conclusion drawn from this work is that when the donor plants are subjected to a mild heat or drought stress, better responses to the transformation procedure are obtained, with the production of abundant formations of embryogenic callus (Table 1). There are several possible explanations for this observation. It could simply be that the embryos are already dehydrated and less stressed during the transfer to the maltose medi-

um before the bombardment and, consequently, react faster to embryogenic induction in vitro. A second hypothesis suggests that water reduction could stop or reduce the photosynthetic action of the plants and thus produce the right induction signal to the embryo for the development of somatic embryos. This could be confirmed by observing the response of embryos from mother plants treated with excessive light intensity or reduced CO_2, thereby duplicating a reduction in photosynthetic action. Finally, it could be hypothesized that the response is related to an increase in abscisic acid in the stressed plants, which would increase the osmolarity of the cells and again induce a better adaptation to the tissue culture. Experiments are in progress to confirm or reject these hypotheses.

In summary, we have developed a method for the constant production of transgenic durum wheat. The transformation efficiency of 1.70% is based on the average of the transformation experiments and the different cultivars. The transformed plants showed a normal phenotype but displayed a range of Basta resistance and fertility. The ultimate goal of our efforts is to contribute to a future sustained production of affordable and high-quality food for poor farmers in developing countries by augmenting conventional plant breeding with applied biotechnology. We can reach this goal only if the novel characteristics we introduce into wheat are used in the breeding programs. In turn, the novel characteristics will only be useful in breeding if they are stable and effective. This requires that breeders be provided with a collection of many transgenic plants for each novel characteristic in order to permit selection of the most appropriate individuals for the breeding program. It follows that this requires a more efficient gene transfer protocol than those currently available.

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References

Becker D, Brettschneider R, Lorz H (1994) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J 5:299–307

Bommineni VR, Jauhar PP, Peterson TS (1997) Transgenic durum wheat by microprojectile bombardment of isolated scutella. J Hered 88:474–481

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: genes, structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplast by electroporation. Plant Mol Biol 18:675–689

Clausen M, Kräuter R, Schachermayr G, Potrykus I, Sautter C (2000) Antifungal activity of a virally encoded gene in transgenic wheat. Nat Biotechnol 18:446–449

He GY, Rooke L, Lazzeri PA (1999) Transformation of pasta wheat (*Triticum turgidum* L. var. durum) with high-molecular-weight glutenin subunit genes and modification of dough functionality. Mol Breed 5:377

Liu Y-G, Nagaki K, Fujita M, Kawaura K, Uozumi M, Ogihara Y (2000) Development of an efficient maintenance and screening system for large-insert genomic DNA libraries of hexaploid wheat in a transformation-competent artificial chromosome (TAC) vector. Plant J 23:687–695

Lorito M, Woo SL, Fernandez IG, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc Natl Acad Sci USA 95:7860–7865

Lorz H, Becker D, Lutticke S (1996) Molecular wheat breeding by direct gene transfer. In: Braun HJ, Altay F, Kronstad WE, Beniwal SPS, McNab A (eds) Wheat: prospects for global improvement. Proc 5th Int Wheat Conf. Kluwer, Dordrecht, pp 285–289

Martín A, Alvarez JB, Martín LM, Barro F, Ballesteros J (1999) The development of *Tritordeum*: a novel cereal for food processing. J Cereal Sci 30:85–95

Muller E, Lorz H, Lutticke S (1996) Variability of transgene expression in clonal cell lines of wheat. Plant Sci 114:71–82

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

Nehra N, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M, Kartha KK (1994) Self fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J 5:285–297

Rooke L, Byrne D, Salgueiro S (2000) Marker gene expression driven by the maize ubiquitin promoter in transgenic wheat. Ann Appl Biol 136:167–172

Satoshi I, Masatomo K, Shinozaki K (2000) A stress-inducible gene for 9-cis-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. Plant Physiol 123:553

Sorokin AP, Ke X-Y, Chen D-F, Elliott MC (2000) Production of fertile transgenic wheat plants via tissue electroporation Plant Sci 156 227–233

Takumi S, Otani M, Shimada T (1997) The rice Act1 promoter gave high activity of transient gusA expression in callus, immature embryos and pollen embryoids of common wheat and its relatives following particle bombardment. Wheat Inf Serv 84:25–32

Tosi P, Napier JA, D'Ovidio R, Jones HD, Shewry PR (2000) Modification of the LMW glutenin subunit composition of durum wheat by microprojectile-mediated transformation. R Soc Chem 261:93–96

Vasil V, Castillo AM, Fromm ME, Vasil IK (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Biotechnology 10:667–674

Vasil V, Srivastava V, Castillo AM, Fromm ME, Vasil IK (1993) Rapid production of transgenic wheat plants by direct bombardment of fertile transgenic plants. Biotechnology 10:667–674

Witrzens B, Brettell RIS, Murray FR, McElroy D, ZhongYi Li, Dennis ES (1998) Comparison of three selectable marker genes for transformation of wheat by microprojectile bombardment. Aust J Plant Physiol 25:9–44

Yoshiba Y, Nanjo T, Miura S, Yamaguchi Shinozaki K, Shinozaki K, (1999) Stress-responsive and developmental regulation of Delta(1)-pyrroline-5-carboxylate synthetase 1 (P5CS1) gene expression in *Arabidopsis thaliana*. Biochem Biophys Res Commun 261:766–72

Zhang L, Rybczynski JJ, French R (2000) An efficient wheat transformation procedure: transformed calli with long-term morphogenic potential for plant regeneration. Plant Cell Rep 19:241–250