Original Papers

Plant Biotechnology, 14(3), 151-156(1997)

Genetic Transformation of Durum Wheat (*Triticum durum* Desf.) through Particle Bombardment of Scutellar Tissues

Shigeo TAKUMI* and Takiko SHIMADA

Research Institute of Agricultural Resources, Ishikawa Agricultural College, Nonoichi, Ishikawa 921, Japan

Received 8 May 1997; accepted 1 September 1997

Abstract

This is the first report of the production of transgenic durum wheat. A transformation system for common wheat through particle bombardment of scutellar tissues of immature embryos was successfully applied to emmer wheat. Three emmer wheats, *Triticum durum* var. *reichenbachii*, *T. durum* var. *agricunum* and *T. aethiopicum* Jakubz., were used in the transformation experiments. The scutellar tissues isolated from immature seeds were bombarded with a plasmid, pDM302 containing the bialaphos-resistant gene (*bar*) under control of the rice actin 1 gene (*Act1*) promoter. In *T. durum* var. *agricunum*, 25 bialaphos-resistant plants were independently regenerated from each green-spotted calli. On the other hand, no bialaphos-resistant plants were recovered from the other two emmer wheat, *T. durum* var. *reichenbachii* and *T. aethiopicum*. Most regenerated plants grew into mature and fertile plants. The integration and inheritance of the *bar* gene were confirmed by the PCR amplification and Southern analysis in T_0 and T_1 plants. All 25 plants showed the positive band of the *bar* gene. The frequency of transformation was 1.17 % (25 independent transformants/2144 immature embryos).

1. Introduction

Both *Triticum durum* Desf. and *T. aethiopicum* Jakubz., relative species to common wheat (*T. aestivum* L.), are classified into emmer wheat ($2n=4x=28$, genome constitution AABB) and belong to the tetraploid group; *T. durum* is one of the most important monocotyledonous crops as a raw material of pasta products. Of all wheats, *T. durum* is the second in importance after the common wheat ($2n=6x=42$, genome constitution AABBDD) [1]. However, genetic transformation in emmer wheats has not yet been reported.

The microprojectile bombardment system is a powerful technique for the genetic transformation of common wheat. The scutellar tissues of immature embryos have been successfully used as a target to deliver a foreign DNA through particle bombardment in common wheat [2-6]. In durum wheat, Bennici *et al.* [7] studied the callus formation of immature, 9- to 21-day-old, embryos and plant regeneration of the calli. The genotypic effects on immature embryo culture were reported in durum wheat [8]. He *et al.* [8] noted that six cultivars of *T. durum* formed embryogenic callus from scutellar tissues of immature embryos at the induction frequency of 44 % to 89 %, but no embryogenic calli were induced from epiblast tissues. Culture conditions also affected the callus induction and plant regeneration. Among four cultivars of durum wheat, the culture condition of immature embryos has been recently optimized using Murashige-Skoog [9] medium [10]. Moreover, immature embryos of durum wheat were cultured for callus induction on Linsmaier-Skoog (LS) medium [11] containing 2 mg/*l* 2,4-dichlorophenoxyacetic acid (2,4-D) and 30 g/*l* sucrose, and for plant regeneration on a phytohormone-free LS medium [12]. These conditions for immature embryo culture are similar to our conditions for common wheat [6]. Immature embryos seemed to be the most appropriate explants for plant regeneration from callus cultures in durum wheat [12] and to produce transgenic plants of durum wheat by the microprojectile bombardment system. Here, we report the successful production of transgenic plants in durum wheat by the same method as in common wheat [6].

2. Materials and Methods

2. 1 *Plant materials and culture of immature embryos*

Immature embryos of six durum wheat accessions were used to estimate the ability of green-spot formation. These durum wheats were grown in a greenhouse in Ishikawa, Japan. Immature seeds sterilized

* Pesent address: *Department of Biological and Environmental Science, Faculty of Agriculture, Kobe University, Nada-ku, Kobe 657, Japan*

in 70 % ethanol for 5 min. and immature embryos isolated were placed with scutellar tissues exposed on the LS medium containing 2 mg/*l* 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 % (w/v) Gelrite (Merck). Immature embryos of three emmer wheats (*T. durum* var. *reichenbachii*, *T. durum* var. *agricunum* and *T. aethiopicum* Jakubz.) were used to examine the integration of a marker gene.

2.2 Plasmid DNAs

The plasmids p*Act1*-F and pDM302 were used. p*Act1*-F [13] includes the *gus* coding region controlled by the 1.3 kb 5′ region of the rice actin 1 gene (*Act1*). pDM302 contains the *bar* (phosphinothricin acetyltransferase) gene placed after the rice *Act1* promoter [14] as a selectable marker gene. The rice *Act1* promoter showed high activity of transient *gus* expression in wheat cultured cells [15] and scutellar tissues [6]. Recombinant plasmids were amplified in liquid cultures of *Escherichia coli*, isolated by alkaline lysis, and purified twice by CsCl-density centrifugation [16].

2.3 Particle bombardment, selection of transformants and enzyme assay

Plasmid DNAs were adsorbed to gold particles (1.6 μm diam.) according to the protocol for the Biolistic^{R} PDS-1000/He Particle Delivery System (Bio-Rad) and delivered to the target embryo tissues [15]. Immature embryos were transferred to selection medium comprised of LS medium containing 2 mg/*l* 2, 4-D and 5 mg/*l* bialaphos two days after bombardment. After about 1 month, the bialaphos-resistant tissues were transferred to 2,4-D free LS medium containing 4 mg/*l* bialaphos for regeneration. Following bombardment, immature embryos were incubated at 26°C for two days prior to the assay for GUS activity. GUS activity was histochemically assessed as described previously [15]. The numbers of blue spots showing transient *gus* expression per embryo were counted in two separate experiments. In each experiment, more than 20 immature embryos were used.

2.4 Molecular analysis

Plant genomic DNA was extracted from leaf tissues (0.5 to 1 g) according to the minipreparation procedures of Mettler [17]), as modified by Liu *et al.* [18]. One primer set (BAR7 and BAR8) was synthesized based on the *bar* sequence to use for the primary PCR amplification. BAR7 and BAR8 are 5′-GATCCATGAGCCCAGAACGAC-3′ and 5′-TTGCGGGTATGCCAGTTGAGT-3′, respectively. Thirty cycles of PCR were performed in a programmed temperature control system (PC700, Astec). A single cycle of the primary PCR amplification consisted of the following steps; denaturation at 94°C for 1 min., annealing at 57°C for 1 min., and DNA synthesis at 72°C for 1 min. Other conditions were described previously[6]. For the secondary amplification using nested primers, another primer set (BAR5 and BAR6), which were designed and synthesized based on the sequence of the *bar* gene, were expected to produce a 402 bp product after PCR amplification[6]. In the nested PCR, one microliter of the primary PCR products was used as a template. Thirty cycles of PCR were performed, and a single cycle of the nested PCR consisted of the following steps; denaturation at 94°C for 1 min., annealing at 60°C for 1 min., and DNA synthesis at 72°C for 1 min. Amplified DNAs were analyzed by EtBr staining after 1.8 % agarose gel electrophoresis at 50 V. PCR products after electrophoresis were transferred to nylon membrane (Hybond-N^{+}, Amersham). The major fragments were identified by Southern hybridization against a probe of the *bar* gene using the ECL system (Amersham). Moreover, total DNAs (20μg) of transformants were digested by *Hin*dIII. The methods of electrophoresis, Southern blotting, hybridization with ^{32}P-labelled probes, and autoradiography were the same as those reported by Liu *et al.* [18].

2.5 Seed fertility

Selfed seed fertility (%) was estimated by the seed setting rate of the first and second florets of ten spikelets in two to three ears per plant. Non-transformed plants were used as controls.

3. Results and Discussion

3.1 Immature embryo culture

In common wheat, He *et al.* [8] proposed a classification based on the five morphological stages of embryo development and concluded that the suitable stages for induction of scutellum callus were stages I, II, and III (about 10-16 days after anthesis). In most of the genotypes used in this study, the developmental stage III of embryo was observed 13 to 15 days after anthesis. Calli were induced from all the genotypes and the frequencies in most genotypes were as high as 100 %. Most calli derived from scutellar tissues at the suitable stages exhibit localized chlorophyll synthesis (green spots) with occasional formation of many green shoots [19]. However, the frequency of green-spot formation varied from 18.5 % to 92.1 % (**Table 1**) and markedly differed with the genotype. Similar results have been reported in a study using randomly chosen 35 cultivars of *T. aestivum* and six cultivars of *T. durum* [8] and in other studies using eight genotypes of *T. aestivum*

Table 1.
Variation in callus induction and plant regeneration from immature embryos of durum wheats.

Accession	Direct Germination (%)	Callus Induction (%)	Green Spot Formation (%)
T. durum var. *reichenbachii*	1.9	98.7	44.9
T. durum var. *agricunum*	0	100.0	92.1
T. durum var. *murciens*	0	100.0	27.8
T. durum var. *valenciae*	0	100.0	56.4
T. durum var. *affine*	0	100.0	88.9
T. durum var. *leucurum*	0	100.0	18.5

Around 50 immature embryos were cultured on the LS medium containing 2 mg/*l* 2,4-D.

Table 2.
Culture response in four developmental stages of immature embryos from three emmer wheats.

Accession	Green spot formation (%)			
	Stage I	Stage II	Stage III	Stage IV
T. durum var. *reichenbachii*	3.0	16.7	35.7	7.0
T. durum var. *agricunum*	11.8	78.4	91.9	84.2
T. aethiopicum	17.5	35.9	38.9	37.8

The four developmental stages (I -IV) were according to He *et al.* [8]. Around 50 immature embryos were cultured on the LS medium containing 2 mg/*l* 2,4-D.

[20] and ten genotypes of *T. aestivum* [21]. Obviously, there is no correlation between callus induction and plant regeneration as suggested by Sâgi *et al.* [12]. *T. durum* var. *agricunum* showed the highest frequency of green-spot formation among the six genotypes. In common wheat, the importance of the developmental stage for embryogenesis has been reported with several explants including immature embryos [22]. In all three accessions of emmer wheat, the immature embryos in stage II and III formed green spots more efficiently than those in other stages (**Table 2**), which indicates that the suitable stage for induction of regenerable calli was stage II and III in emmer wheat.

3. 2 Transient expression of gus *gene*

The scutellar tissues of wheat immature embryos (stage III) that had been cultured for one, five and nine days were bombarded with the particles coated with p*Act1*-F to estimate the transient expression. The rice *Act1* promoter, which showed a high level of transient expression in cultured cells of durum wheat [15], efficiently yielded the transient *gus* expression in scutellar tissues of emmer wheat (**Fig. 1-a**). The activity of the gus expression in the embryos of *T. durum* var. *reichenbachii* incubated for five days before bombardment was higher than that of the embryos incubated for one or nine days. The GUS activity of the embryos of the two other emmer wheat accessions incubated for nine days was slightly higher than that of the embryos incubated for five days. No clear correlation was recognized between the culture duration before bombardment and transient *gus* expression in any of the three emmer wheats (**Fig. 2**). *T. durum* var. *agricunum* gave the highest activity of transient *gus* expression.

3. 3 Selection and analysis of transformants

The scutellar tissues of the immature embryos cultured for five days were bombarded with pDM302 to obtain the transgenic plants of three emmer wheats. Then, the cultured immature embryos were transferred to selection medium. After culture for about thirty days, some calli with green spots were observed. These green-spotted calli were transferred to regeneration medium containing 4 mg/*l* bialaphos. In *T. durum* var. *agricunum*, 25 bialaphos-resistant plants were independently regenerated from each of the green-spotted calli (**Fig. 1-b**). On the other hand, no bialaphos-resistant plants were recovered from the other two emmer wheats, *T. durum* var. *reichenbachii* and *T. aethiopicum*.

The integration of the *bar* gene in T_0 bialaphos-resistant plants was assessed by the combination of PCR amplification and Southern hybridization analysis. The nested PCR products from the plantlets integrating the *bar* gene showed the 402 bp fragment, and the identity of the observed 402 bp fragment was determined by Southern hybridization to the labelled *bar* gene. All 25 plants showed the positive band of the *bar* gene (**Fig. 3**). Most regenerated plants grew into mature plants (**Fig. 1-c**). The selfed seed fertility of 18 fertile transgenic plants varied from 60. 0 % to 90. 7 % (**Fig. 4**) and the average was 80 .1 %. The fertility was lower in transgenic plants than in non-transformed plants, which may be due to the stress

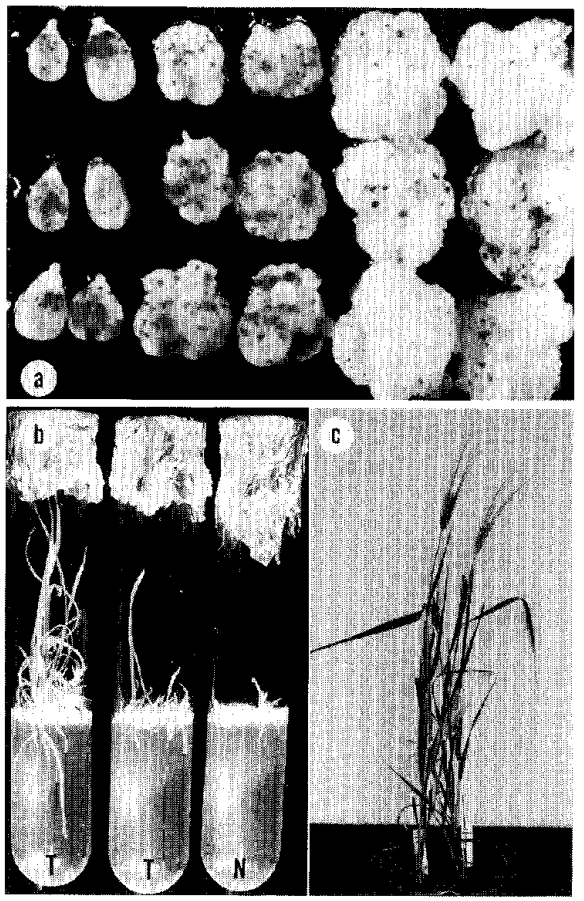

Fig. 1 (a) Transient *gus* expression in immature embryos of three emmer wheat accessions, *T. durum* var. *reichenbachii* (top), *T. durum* var. *agricunum* (middle) and *T aethiopicum* (bottom).
These immature embryos were bombarded with p*Act1*-F after one-day (left two), five-days (middle two) and nine-days (right two) incubation on LS medium containing 2 mg/*l* 2,4-D and then transformed cells (blue spots) were detected in *in situ* enzyme assay. (b) Regeneration of transformants on medium containing 5 mg/*l* bialaphos in test tubes. N, non-transformant as a negative control; T, transformed wheat plant. (c) Mature fertile transgenic durum wheats (T_0).

during the immature embryo culture.

The inheritance of the *bar* gene into T_1 progeny was tested by PCR amplification using two lines of transgenic plants. The 402 bp fragment in the *bar* gene was detected in seven of nine T_1 plants from one transformant and in four of six from another. The *bar* gene was integrated and segregated in T_1 progenies, which suggested the inheritance of the transgene into the next generation. The total DNAs from five *bar*-positive T_1 plants from one transformant were used for genomic Southern analysis. The Southern blot pattern is shown in **Fig. 5**. The frequency of transformation was 1.17 % (25 independent transfromants/2144 immature embryos). In common wheat, the transformation frequency obtained by

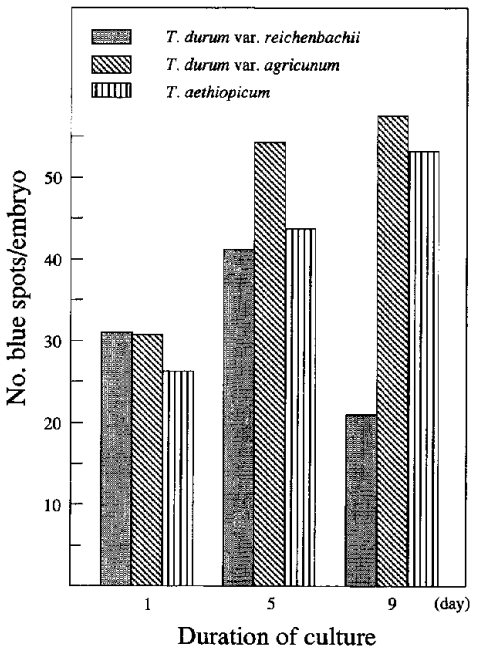

Fig. 2 Influence of the culture duration prior to bombardment on the transient *gus* expression in immature embryos of three emmer wheat accessions.

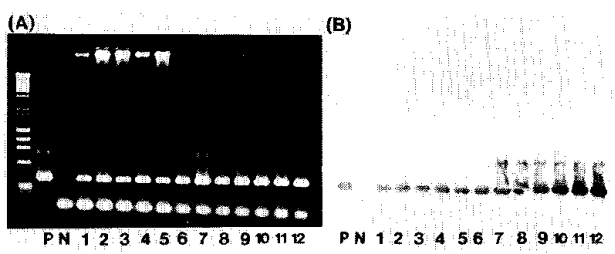

Fig. 3 (A) PCR amplification of the *bar* gene from total DNA isolated from the bialaphos-resistant plants (T_0) of *T. durum* var. *agricunum*. EtBr staining pattern after agarose gel electrophoresis. Two primers recognized the *bar* gene and should amplify a 402 bp fragment. (B) Results of Southern hybridization after transfer of the DNA shown on the agarose gel of (A).
P, pDM302 as a positive control; N, non-transformant as a negative control; lane 1-12, regenerated plants (T_0).

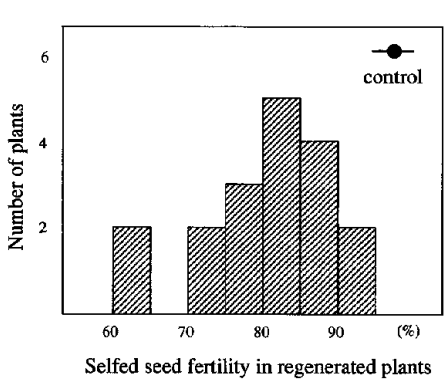

Fig. 4 Selfed seed fertility (%) of transgenic plants (T_0 generation).

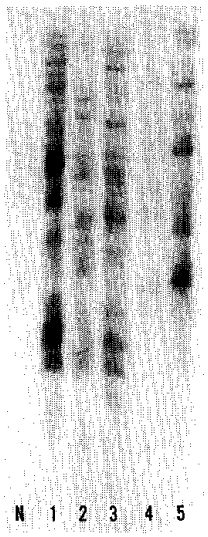

Fig. 5. Autoradiograms of Southern blots of *bar*-positive T_1 plants from one transformant with the ^{32}P-labelled *bar* gene.
Each lane contains 20μg of total DNA. The used restriction emzyme was *Hin*dIII.
N, non-transformant as a negative control; lane 1-5, *bar*-positive T_1 plants.

Weeks *et al.* [2] and Nehra *et al.* [5] was about 1%, which was similar to that we obtained in durum wheat.

Transgenic plants could be produced in durum wheat by the same method as in common wheat, and this procedure can be used as a routine system for gene transfer in durum wheat. However, several problems remain to be solved. First, transgenic plants were generated from *T. durum* var. *agricunum*, but not from other emmer wheats, which indicated that the genotypes from which transformants could be obtained were limited. No transformed plants were recovered in *T. durum* var. *reichenbachii* and *T. aethiopicum*, because their regeneration frequencies from cultured immature embryos were low. The efficient production of transgenic wheat plants requires the induction of green-spotted calli from more than 90% of immature embryos [23]. For the production of transgenic plants in these varieties, the *in vitro* culture system should be improved to increase the regeneration frequency markedly. Recently, Bommineni and Jauhar [10] generated the mature, fertile durum wheat plants from immature embryos of four durum wheat cultivars and they established an *in vitro* culture procedure for rapid regeneration. Second, the frequency of transformation was low. However, Nehra *et al.* [5] noted that the use of geneticin as a selection agent gave a higher transformation frequency in common wheat. Moreover, osmotic treatment of cultured immature embryos and improvement in the procedure used for culture and selection allowed increase of transformation frequency [24]. Therefore, transformed plants of durum wheat are expected to be more efficiently produced in future.

Acknowledgements

We are grateful to Dr. McElroy and Dr. R. Wu for the plasmids p*Act1*-F and pDM302. We thank Meiji Seika Kaisha for the gift of bialaphos. We also thank Dr. K. Yamaguchi for his kind help.

References

[1] Bennici, A., 1986. In "Biotechnology in Agriculture and Forestry 2. Crops I." (ed. by Bajaj, Y. P. S.), p. 89-104, Springer-Verlag, Berlin, Heidelberg.
[2] Weeks, J. T., Anderson, O. D., Blechl, A. E., 1993. Plant Physiol., **102**: 1077-1084.
[3] Vasil, V., Srivastava, V., Castillo, A. M., Fromm, M. E., Vasil, I. K., 1993. Bio/Technol., **11**: 1553-1558.
[4] Becker, D., Brettschneider, R., Lörz, H., 1994. Plant J., **2**: 299-307.
[5] Nehra, N. S., Chibbar, R. N., Leung, N., Caswell, K., Mallard, C., Steinhauer, L., Baga, M., Kartha, K. K., 1994. Plant J., **5**: 285-297.
[6] Takumi, S., Shimada, T., 1996. J. Plant Physiol., **149**: 418-423.
[7] Bennici, A., Caffaro, L., Dameri, R. M., Gastaldo, P., Profumo, P., 1988. Euphytica, **39**: 255-263.
[8] He, D. G., Yang, Y. M., Dahler, G., Scott, K. J., 1988. Plant Sci., **57**: 225-233.
[9] Murashige, T., Skoog, F., 1962. Physiol. Plant., **15**: 473-497.
[10] Bommineni, V. R., Jauhar, P. P., 1996. Plant Sci., **116**: 197-203.
[11] Linsmaier, E. M., Skoog, F., 1965. Physiol. Plant., **18**: 100-126.
[12] Sági, F., Beke, B., Sági, L., 1990. In "Biotechnology in Agriculture and Forestry 13. Wheat." (ed. by Bajaj Y. P. S.) p. 494-510, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.
[13] McElroy, D., Zang, W., Cao, J., Wu, R., 1990. Plant Cell, **2**: 163-171.
[14] Cao, J., Duan, X., McElroy, D., Wu, R., 1992. Plant Cell Rep., **11**: 586-591.
[15] Takumi, S., Otani, M., Shimada, T., 1994. Plant Sci., **103**: 161-166.
[16] Maniatis, T., Fitsch, E. F., Sambrook, J., 1982. In "Molecular Cloning: A laboratory Manual." Cold Spring Harbor Laboratory, New York.
[17] Mettler, I. J., 1987. Plant Mol. Biol. Rep., **5**: 346-349.
[18] Liu, Y. G., Mori, N., Tsunewaki, K., 1990. Jpn. J. Genet., **65**: 367-380.
[19] Shimada, T., 1978. Jpn. J. Genet., **53**: 371-374.
[20] Mathias, R. J., Simpson, E. S., 1986. Plant Cell

Tiss. & Org. Cult., **7**: 31-37.

[21] Carman, J. G., Jefferson, N. E., Campbell, W. F., 1987. Plant Cell Tiss. & Org. Cult., **10**: 101-113.

[22] Scott, K. J., He, D. G., Yang, Y. M., 1990. In "Biotechnology in Agriculture and Forestry 13. Wheat." (ed. by Bajaj, Y. P. S.), p. 46-67, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

[23] Takumi, S., Shimada, T., 1997. Genes Genet. Syst., **72**: 63-69.

[24] Altpeter, F., Vasil, V., Srivastava, V., Stöger, E., Vasil, I. K., 1996. Plant Cell Rep., **16**: 12-17.