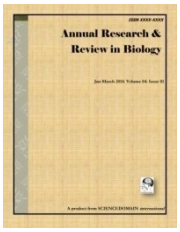

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Immature Embryo-Derived of Two Bread Wheat (*Triticum aestivum* L.) Varieties Transformation Using Particle Bombardment Method

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Authors' contributions

This work was carried out in collaboration between all authors. Author DCTE worked in the practical part and wrote the first draft of the manuscript. Authors DI, RA and SMU managed the analyses of the study. Authors MNB and MME managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Wheat cultivation is still difficult to control because it faces several biotic and abiotic stresses. The transfer of resistance in wheat plants to these stresses through traditional approaches remains limited. However, genetic transformation allows the acquisition of this tolerance while overcoming the difficulties of classical improvement.
Methodology: In this context, immature embryos of two Moroccan bread wheat varieties ('Massira' and 'Arréhane') and the pBY520 plasmid containing the HVA1 gene for drought tolerance as well as the gene bar selection marker for resistance to the herbicide phosphinothricin were used in this study for bombardment protocol.
Results: The percentage of calli bombarded with regenerated plantlets was 23.92 % for the variety 'Massira' and 5.26% for the variety 'Arréhane'. The selection was carried out

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on ½ MS rooting medium lacking hormones and supplemented with phosphinothricin (3 mg l^{-1}); 4.36% and 6% of plantlets of varieties 'Massira' and 'Arréhane' respectively survived. The resistant plantlets were transferred to the greenhouse and the evaluation of the expression of the bar gene in leaves was positive about 60%. The confirmation by molecular analysis revealed only a transformation efficiency of 0.52 % for the variety 'Arréhane'.

Keywords: Bar gene; genetic transformation; bread wheat; plasmid pBY520.

ABBREVIATIONS

ASP: Asparagin; CTAB: Cetyl trimethylammonium bromide; LB: Luria Bertani; MS: Murashige and Skoog; PPT: Phosphinothricin; rpm: rotation per minute.

1. INTRODUCTION

Hexaploid wheat, *Triticum aestivum* L., a foundation of human nutrition, is among the most widely grown annual crops cultivated in many areas of Morocco and many other countries in the world for making noodles, bread, cake, etc. Its production in a wide range of climates and different regions comes with frequent and various stresses that severely limit crop growth and yield [1,2]. Therefore, it is very important to improve wheat resistance.

Genetic engineering makes it feasible. In fact, genetic engineering has undoubtedly opened a new avenue to overcome crop losses due to various biotic and abiotic stresses prevalent in the agricultural ecosystems [1,2,3,4,5,6]. The successful employment of genetic engineering for crop improvement and for basic studies related to gene expression requires a simple and reproducible genetic transformation procedure [7]. The ability to deliver and express foreign genes in plant cells are important steps in genetic engineering of plants. The genetic transformation of wheat, while it is one of the most important cereals, is still far from being a routine procedure. The particle bombardment method (biolistic method) has been accepted as a breakthrough since genetic transformation with this method became almost a routine process in many important crop species, including cereals and legumes, which are known to be recalcitrant for transformation with other techniques [8].

Even though it was the first successful method for genetic transformation of wheat, particle bombardment still appears to be the most commonly used procedure for wheat transformation [9]. Several factors have been described to influence the applicability and efficiency of biolistic gene transfer. Genotypes had been shown to be crucial [10,11,12]. In addition, the regeneration of whole plants from selected transgenic tissues is required for the successful application of genetic engineering breeding in wheat improvement. Tissue culture responses which include callus induction and regeneration capacity of wheat are influenced by many factors such as explants source [13]. In fact, wheat has remained to be difficult in the transgenic study mainly due to the lack of explants with high regeneration efficiency [14,15]. Immature wheat embryos have generally been considered to be optimum explants for plant regeneration of wheat; they have been widely used as explants for transformation in various protocols [16,17,18,19]. Therefore, the main objective of this study was to transfer the HVA1 gene for drought tolerance into two Moroccan bread wheat varieties. We adapted a protocol for genetic transformation and regeneration of viable shoots from immature embryos by particle bombardment method.

2. MATERIALS AND METHODS

2.1 Plant Materials

A Moroccan bread wheat variety 'Massira' and 'Arréhane', procured from Experimental Research Station of INRA at Marchouch, Morocco, was grown in greenhouse. Based on it in vitro differentiation response this variety was chosen for genetic transformation experiments. The immature seeds collected 12-16 days post-anthesis were surface rinsed with 70% (v/v) ethanol for 3 minutes, followed by a bath of sodium hypochlorite (2.4%) containing a few drop of tween 20 for 15 minutes. Thereafter, they were rinsed 3 times with sterile distilled water.

Immature embryos were aseptically dissected away from the caryopses and the remaining endosperm and radical removed to prevent early germination. The embryos were placed in induction medium (MS Asp) [20] in a petri dish (12 replications for each variety, each replication with 20 embryos). The medium was solidified with 2.5 g l^{-1} phytagel and pH was adjusted to 5.7 before autoclaving at 120ºC for 20 min. The Petri dishes were incubated at 25ºC in the dark for 3-5 days. The embryos whose cells started rapid division were selected for subsequent transformation and subsequent subculturing. Three extra plates of 20 immature embryos were prepared for unbombarded controls of each variety.

2.2 Bacteria Materials and Genetic Construction

The plasmid used for bombardment pBY520 contained the linked selectable marker/herbicide resistance bar (phosphinothricin acetyl transferase) gene (driven by cauliflower mosaic virus 35S promoter and the nopaline synthase nos terminator) and the barley HVA1 gene (driven by rice Act1 promoter and terminated by the potato protease inhibitor pin II).

The *Escherichia coli* strain carrying plasmid pBY520 harboring HVA1 gene construct were grown over night in LB medium at 37ºC and 150 rpm shaking. To extract plasmid DNA from bacterial cell suspensions, the alkaline lysis procedure was used [21,22]. The plasmid DNA pellet was washed with 70% ethanol and dried and solubilized in 100 µl sterile distilled water. The gold particles for the bombardment were prepared according to Iraqi et al. [20].

2.3 Production and Selection of Transgenic Lines

Three to five days after placing the immature embryos from 'Massira' and 'Arréhane' varieties on callus induction medium, proliferation of callus tissue was observed. At this stage, the embryos were placed in the center of a petri dish for 4 hours in the osmotic MS medium supplemented with 15% mannitol, and bombarded twice (first at around 9 cm and second at around 13 cm) with 1µm gold particles [20] coated with the plasmid DNA (plasmid pBY520) containing HVA1 gene at 1100 psi a pressure of generated by Helium.

After the bombardment embryos were left in the same medium in the dark for 16 hours at 25ºC and subsequently transferred to the MS Asp medium without mannitol for a period of 40 days. The calli were then transferred to the regeneration medium [20]. The selection of the regenerated shoots was carried out on rooting induction MS half-strength medium lacking hormones and supplemented with 3 mg l^{-1} of PPT (Phosphinothricin).

2.4 Histochemical Analysis of Transgenic Plants

Herbicide resistance of the putative transgenic wheat plants was determined by painting leaves of plants at the fifth or sixth leaf stage 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 [23].

2.5 DNA Analysis of Transgenic Plants

Total genomic DNA was extracted from the putative transgenic plants using a modified CTAB procedure [24]. The detection of the bar gene and 35S promoter sequences in all the putative transgenic plants by PCR amplification was performed in 20 µl containing 50 ng DNA template, 1x *Taq* DNA polymerase buffer, 200 µM of each dNTP; 0.5 pmol of the respective primers, and 0.6 unit of *Taq* DNA polymerase (Promega). Primers pairs used to detect the bar gene (402 pb) were 5' GTCTGCACCATCGTCAACC 3' (forward) and 5' GAAGTCCAGCTGCCAGAAAC 3' (reverse). Primers pairs used to detect the 35S (195 pb) gene were 5' GCACAATCCCACTATCGTTCGC 3' (forward) and 5' TCCGTCCACTCCTGCGGTTC 3' (reverse). DNA amplifications were performed in a thermo-cycler (Master Cycler, Eppendorff, Germany) using initial for both primers denaturation at 94ºC for 4 min, followed by 30 cycles of 1 min at 94ºC, 1 min at 58ºC, 2 min at 72ºC, and a final 10 min extension at 72ºC. The reaction mixture was loaded directly onto a 1.2% (w/v) agarose gel, stained with ethidium bromide, and visualized with UV light (306 nm). The amplified transgene product size was compared with the positive control.

3. RESULTS AND DISCUSSION

3.1 Bombardment, Callus Induction, Regeneration and Selection of Putative Transformed Plantlets

A total of 400 embryos were bombarded from these two varieties (Table 1). After bombardment, the embryos were transferred to callus recovery and induction medium without selection for 40 days. After this the percentage of callus induction (around 81%; Table1) were recorded. Callus was then transferred onto the regeneration medium (Fig. 1a). Three weeks after incubation of callus on regeneration medium, we observed a differentiation of callus tissue with green spot (Fig. 1b). Three to four weeks later, we obtained regenerated plantlets (Fig. 1c, 1d) The percentage of regenerable callus lines (number of calli with plant regenerated / total number of calli transferred in regeneration medium x 100) [25] was not significantly different for the variety 'Massira' (23.92%) and his unbombarded control (25%). However, for the variety 'Arréhane' the percentage of regenerable callus lines of bombarded explants (5.26%) was very low compared to his unbombarded control (29.64%). This can be explained by the fact that the variety 'Arréhane' was more sensitive to the contact of the gold particles on the target tissue whose shock caused damage that reduced the ability of shoot regeneration. These results confirm the works of Rasco-Gaunt et al [26] who observed that, for some varieties, unbombarded tissues showed better shoot regeneration than those bombarded. Therefore, it is important to minimize the amount of gold particles used for bombardment in order to reduce the damage sustained by the target tissues [26].

Table 1. Transformation experiments of two bread wheat varieties. Callus induction, regeneration and selection of transgenic plants and transformation efficiency

Treatment	Number of bombarded (B)* / unbombarded (UB)** explants	Callus induction (%)	Regenerable callus lines (%)	Regenerated plantlets	Independent PPT-resistant plantlets lines (%)	Bar gene expression (%)	Plants containing transgenes (%)	Transformation efficiency (%)
Massira (B)*	210	85.71	23.92	321	4.36	60	0	0
Massira (UB)**	60**	88.32	25	65	0	0	-	-
Arréhane (B)*	190	81.57	5.26	50	6	66.66	33.33	0.52
Arréhane (UB)**	54**	83.33	29.63	83	0	0	-	-

Plantlets were then transferred to the rooting medium (half-strength MS) containing 3 mg l-1 of PPT for selection. Untransformed plantlets and plantlets from control treatment were not able to grow under the selection conditions and died whereas transformed plantlets survived and stayed Green and vigorously (Fig. 1e; Table 1). The regenerated putative transformed plantlets were transferred to pots in the green-house for acclimatation (Fig. 1f and 1g). The resulting plants were normal, fertile and without anomalies.

Recognizing the fact that the selection system could permit false positive plants, a second selection was done by painting the leaves with 0.3% of PPT to demonstrate the expression of the PPT herbicide-resistance gene bar [27]. After 7 days, for around 60% (Table 1) of putative transformants of both varieties, the painted leaves stayed green whereas for the control, they became yellow and dead (Fig. 1h).

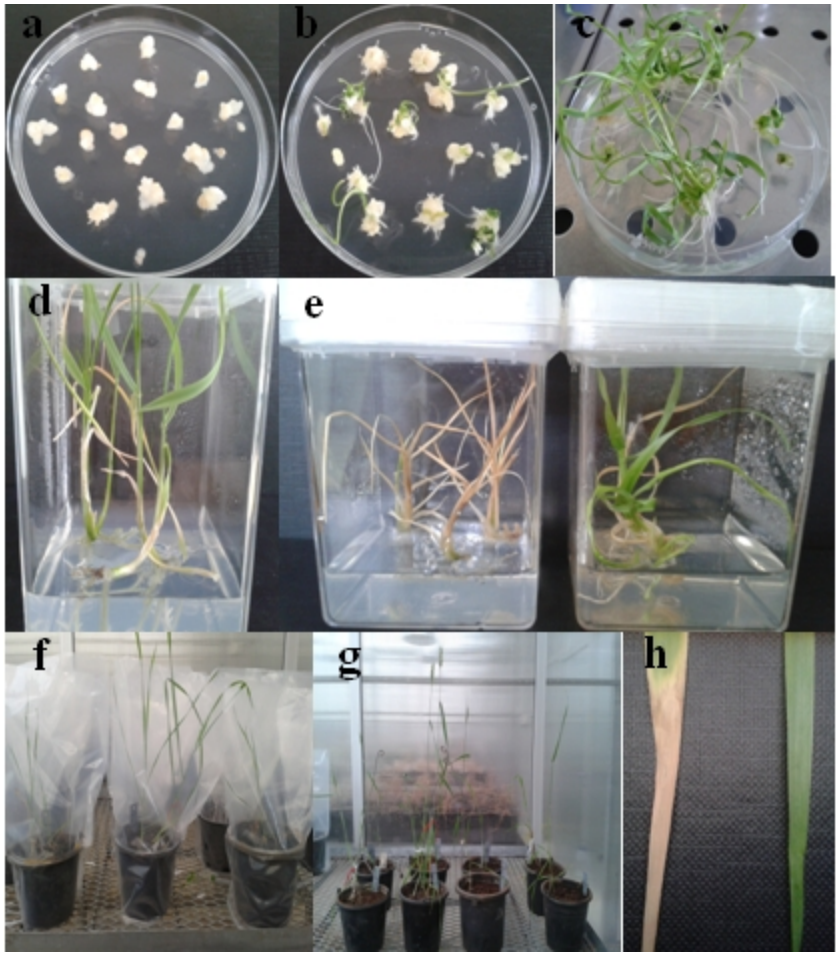

Fig. 1. a: Calli after 40 days of bombardment. b: Differentiation of bombarded calli after 3 weeks on regeneration medium. c and d: Regeneration of putative transgenic plantlets. e: Plantlets after selection with 3 mg l^{-1} of PPT on rooting medium; untransformed plantlets (left), transformed plantlets (right). f and g: Acclimatation of putative transformes plants. h: PPT leaf paint assay; control (left; untransformed) and transformant (right)

3.2 Confirmation of T_0 Transgenic Plants

To confirm the presence of plasmid pBY520 in the transgenic plants T_0, all the independent putative transformants generated were analyzed by PCR amplification of genomic DNA using primers specific to bar gene (Fig. 2b) and 35S (Fig. 2a). PCR analysis amplified the expected sizes for both bar gene (402 pb) and 35S (195 pb) promoter in 33.33% of transgenic plants of variety 'Arréhane' (Table 1) and positive controls (pBY520), whereas, the negative controls (non transformed plants, distilled water) and putative transformed T0 plants of variety 'Massira' did not amplified the desired bands.

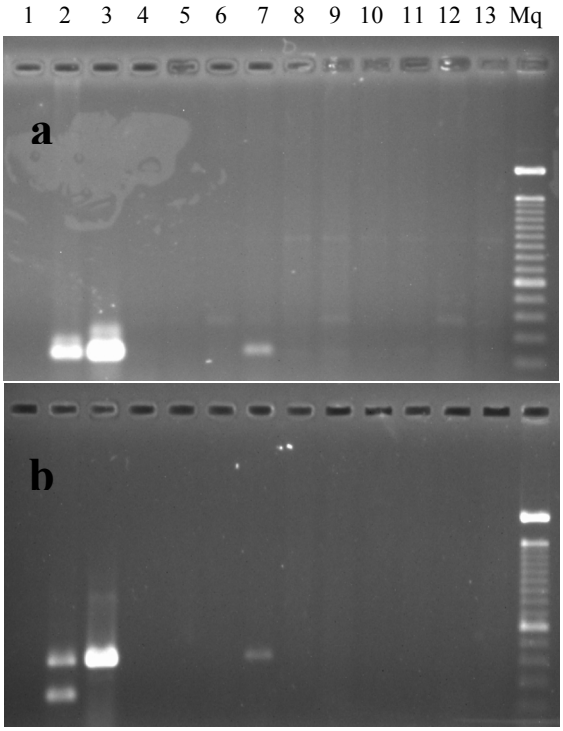

Fig. 2. PCR analysis of T_0 transgenic plants with 35S primers 195 bp (a) and bar primers 402 bp (b). 1: water negative control; 2: positive control; 3: plasmid pBY520; 4: 'Arréhane' negative control; 5-6: 'Arréhane' non transformed plants; 7: 'Arréhane' transgenic plant; 8: 'Massira' negative control; 9-13: 'Massira' non transformed plants; Mq: Marker 100 bp

Transformation efficiency was calculated as the number of transgenic plants obtained (plants surviving PPT-selection and PCR-positive for the bar gene and the 35S) per number of bombarded embryos x 100 [17,23]. 'Arréhane' variety showed 0.52% as transformation efficiency. This value is superior to the range (0.1-0.4%) obtained by some workers for wheat immature embryos [10,27-33]. However, other workers observed higher efficiency of 0.7-0.9%, compared to our study [10,7,34-37].

3.3 Genotype-Dependent Regeneration Response and Genotype-Dependent Transformation Response

In our study, the significant difference observed on transformation efficiency (Table 1) reflects the effect of genotype. Similar results have been reported that the variability of transformation was more due to the genotypic and physiological status of the donor plants than due to the efficiency of the biolistic procedure [10,11,12,19,38]. Moreover, in this study, we observed a highly significant difference in the percentage of embryogenic callus (regenerable callus lines) of around 23.92% and 5.26% respectively for the varieties 'Massira' and 'Arréhane' while transformation rates were 0% and 0.52% respectively. Our results clearly showed that there was no parallelism between regeneration capacity and transformation frequencies. This finding confirms the works of Galovic et al. [19] in bombardment of wheat embryos, where, the varieties which had one of the highest induction capacities exhibited the lowest transformation efficiency. They noted that transformation efficiency was not significantly linked to the induction capacity of embryogenic calli; it was observed to depend significantly on the genotype.

4. CONCLUSION

In the present study, we have been able to successfully introduce plasmid pBY520 via particle bombardment into bread wheat. Since the transgenic plants developed in this study contained barley *HVA1* gene, further analysis for tolerance to water stress tolerance and salt tolerance in T1 and subsequent generations will be performed. On the other hand, we were able to highlight some key points involved in the successful transformation of wheat by particle bombardment. Firstly, we found that the damage caused by the penetration of gold particles into the targeted cells depended on the variety. This damage gave rise in some cases to a significant reduction in the regenerative capacity of bombarded tissues. Secondly, transformation efficiency was highly genotype dependent. The third observation was that there was no significant link between regeneration capacity and the rate of transformation. Existing scientific and technical obstacles must be overcome in order to bring the technology to its full potential to achieve high transformation efficiency.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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