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Agrobacterium-MEDIATED GENETIC TRANSFORMATION OF BREAD WHEAT (*Triticum aestivum* L.) USING IMMATURE EMBRYOS

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ABSTRACT

Wheat is considered the most important food crop globally. Genetic transformation provides a method for genetic manipulation of wheat to enhance its agronomic performance. Wheat is a difficult crop for genetic transformation. *Agrobacterium*-mediated transformation facilitates precise integration of genes into the plant genome compared to biolistic approach. In the present research, we studied the effect of acetosyringone concentrations, genotypes and embryo explant types (intact or longitudinally halved-embryos or fragmented latitudinal pieces) on transformation frequency for immature embryos derived from four different genotypes of bread wheat (Achtar, Amal, Mehdia, and Rajae). Embryo explant types were inoculated with *A. tumefaciens* strain EHA101 harboring the plasmid vector pTF101.1 carrying the barley *HVA1* drought tolerance gene and *bar*-selection marker for resistance to the herbicide phosphinothricin. The effects of the genotype, embryo explant type, and acetosyringone concentrations on the frequency of phosphinothricin resistant bread wheat plantlets regeneration after transformation with *Agrobacterium* were significant. Transgenic plants were generated from longitudinally-halved immature embryo and intact-immature embryos, whereas, no transgenic plantlets were obtained from fragmented immature embryos. Based on the PCR analysis, the transformation efficiency of T_0 plants for the two genotypes varied from 0.66% ('Rajae' from the longitudinally halved-immature embryo cultured with no acetosyringone) to 0.33% ('Achtar' genotype from intact-immature cultured with 200 µM of acetosyringone).

Keywords: wheat immature embryo, acetosyringone, plasmid pTF101.1, genetic transformation, *bar* gene.
Abbreviations: CTAB: Cetyl trimethyl ammonium bromide; MS: Murashige and Skoog; PPT: Phosphinothricin.

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is one of the most important global food crops. Genetic transformation provides a method for genetic manipulation of wheat to enhance its agronomic performance and resistance to biotic and abiotic stresses (Sahrawat et al., 2003). Wheat is a difficult crop genetically to transform, with the preferred target tissues being immature embryos that in certain cultivars are highly regenerable and amenable to transformation.

Late embryogenesis abundant (LEA) proteins were first characterized in cotton as a set of proteins that are highly accumulated in the embryos at the late stage of seed development (Dure et al., 1989). Subsequently, many LEA proteins or their genes have been characterized from different plant species collated by (Dure, 1992) including in barley *HVA1* gene. LEA protein may play a protective role in plant cells under various stress conditions; more- over, this protective role may be essential for the survival of the plant under extreme stress conditions (Dure et al., 1989; Chandler and Robertson, 1994). Transformation of barley *HVA1* gene in rice resulted in increased tolerance to water deficit and salt stress (Xu et al., 1996; Chen et al., 2015).

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Agrobacterium-mediated transformation methods are preferred than the direct DNA transfer methods such as particle bombardment because they offer advantages such as low numbers of transgene copies and less rearrangement of the introduced DNA (Travella et al., 2005). However, even with these initial developments, progress in wheat genetics has been slow (Przetakiewicz et al., 2004), even though significant improvements in the efficiency of gene transfer and the range of transformable genotypes were made for other cereals (Hiei et al., 2006). *Agrobacterium*-mediated transformation, however, facilitates precise integration of genes into the plant genome (Karami et al., 2009), but the system is affected by a number of factors including tissue culture conditions, genotype, acetosyringone concentration, and explant source (Briza et al., 2008). Here, we exploited different types of immature embryo explants, genotypes, and acetosyringone concentrations to investigate factors affecting *Agrobacterium*-mediated transformation of bread wheat.

MATERIAL AND METHODS

Plant materials

Four Moroccan bread wheat genotypes (‘Achtar’, ‘Amal’, ‘Mehdia’ and ‘Rajae’) were procured from the Experimental Research Station of National Institute of Agronomic Research (INRA) at Marchouch, Morocco and grown in pots containing soil and peat in the green-house. These genotypes were previously tested for regeneration efficiencies *in vitro* (Aadel et al., 2016) and were also chosen for transformation experiments.

Gene construct

The *HVA1* gene construct *pBY520* (*HVA1* cloned in pBluestript vector) was kindly provided by Prof R. Wu, Cornell University, Ithaca, USA. The construct *pBY250* contains cassette consisting of the rice Act1 promoter, the *HVA1* sequence, and Pin3 terminator. In order to insert a novel construct containing *HVA1* sequence, double Cauliflower mosaic virus (*CaMV*) *2x35S* promoter and Nos terminator was subcloned in a gateway binary vector pTF101.gw3 by using the cloning technique “multisite gateway”. The binary vector generated is pTFGat101.11 containing *HVA1* gene was subsequently introduced into *EHA101* strain of *Agrobacterium tumefaciens*. This strain contains a 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 the rice *Act1* promoter and terminated by the potato protease inhibitor *pin*-II).

Immature embryo explants and treatments for inoculation with *Agrobacterium*

The immature wheat seeds 12-16 days post anthesis were collected from green house and surface-sterilized by washing in running tap water two times, sterilized with 70% (v/v) ethanol for 3 min, then immersed in sodium hypochlorite (2.4%) with a few drops of Tween-20 for 15 min and rinsed 3 times with sterile distilled water. The immature embryo tissues were given the following three treatments under aseptic conditions for co-inoculation with *Agrobacterium* cells (Aadel et al., 2018):

- Intact-immature embryos were isolated from the spikes 14-16 days after pollination with a sharp knife. The intact-immature embryos were placed on induction medium (Iraqi et al., 2005) with modified M_2 (Aadel et al., 2016) to start initiation of callus formation for 4 days in the dark at 25°C before co-inoculation with *Agrobacterium* supplemented with different concentrations of acetosyringone.
- Immature embryos were cut into two halves longitudinally along the embryonic axis, and used directly for co-inoculation with *Agrobacterium* supplemented with different concentrations of acetosyringone.
- Immature embryos were gently fragmented into four latitudinal pieces with a sharp blade and cultured on induction medium used for the intact-immature embryos for 7 to 10 days in the dark at 25°C before co-inoculation with *Agrobacterium*

supplemented with different concentrations of acetosyringone.

Preparation of bacterial culture for co-inoculation

Transformation was carried out with *Agrobacterium tumefaciens* strain EHA101 containing binary vector pTF101.1 as described in detail by Abdelwahd et al. (2013). This strain contains a 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 the rice *Act1* promoter and terminated by the potato protease inhibitor *pin*-II). Transformation was performed according to Wang et al. (2009), with modifications for the treatments analyzed according to Aadel et al. (2018). Briefly, *Agrobacterium* suspensions were prepared using a liquid medium with different concentrations of acetosyringone (0, 100, 200 and 400 μM). The medium used for inoculation and co-cultivation was the same as in Wang et al. (2009). The immature embryo halves were immediately immersed in *Agrobacterium* suspensions with different concentrations of acetosyringone for 15 min at room temperature. The callus tissues derived from fragmented embryo and callus prepared from intact-immature embryos were immersed for 15 min in bacterial suspensions supplemented with acetosyringone added immediately before inoculation, while control explants were soaked in free MS liquid medium without bacteria and acetosyringone. After infection, the explants from the three embryo types were blotted dry with sterile filter paper and transferred to fresh co-cultivation medium for 3 days in the dark at 28°C.

Selection and regeneration

After co-cultivation with bacteria, explants from the three treatments were cultured for callus induction on selection medium (MS supplemented with 2 mg/L picloram, 100 mg/L casein hydrolysate, 3 mg/L phosphinothricin (PPT), and 250 mg/L of the antibiotic cefotaxime) and cultured at 25°C in the dark for 40 days. Resistant calli were transferred to selective regeneration medium containing 2 mg/L kinitine and 0.1 mg/L acide geberilique and moved to light conditions for shoot differentiation. Then, the surviving regenerated plantlets were cultured on 1/2 MS-free medium for rooting supplemented with 3 mg/L PPT. Plants showing resistance to PPT were transferred to soil and peat in pots in the glasshouse.

Leaf painting assay

Herbicide resistance of the putative transgenic wheat plants was determined by painting leaves of plant at the fifth leaf stage with basta (0.3%), and observations were recorded after 7 days. Plants were scored as susceptible or resistant according to the degree of leaf desiccation after 7 days (Pellegrineschi et al., 2002).

Statistical analysis

A randomized complete block design (RCBD) was used with four genotypes, four concentrations of acetosyringone, and three embryo explant types in triplicate with 100 embryos for each genotype, concentration of acetosyringone, and embryo explant type. To analyze survived calli and plantlets to the selection agent (PPT), analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure in SAS (SAS Institute, 1985).

DNA analysis of transgenic plants

Genomic DNA was isolated from the young leaves of putative transgenic plants T_0 and their first generation T_1 using a modified CTAB (Udupa et al., 1998). The *bar* gene, 35S promoter sequences, and *HVA1* gene was detected by PCR amplification in 20 μl solution 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 units of *Taq* DNA polymerase (Promega).

Primers pairs for the *bar* gene (402 bp) were: F, 5'GTCTGCACCATCGTCAACC3' and R, 5'GAAGTCCAGCTGCCAGAAAC3'

(reverse primer).

Primers pairs for 35S (195 bp) gene were: F, 5'GCACAATCCCACTATCGTTCGC3' (forward primer) and R, 5'TCCGTCCACTCCTGCGGTTC3' (reverse primer).

Primers pairs for the *HVA1* gene (290 bp) were: F, 5'AGCTAGATCGTGAGACGAAGATG3' (forward primer) and R, 5'CCTGCGCCGTCTCGTACGTCTTGG3' (reverse primer).

DNA amplifications were performed in a thermo-cycler (Master Cycler, Eppendorff, Germany) using initial denaturation at 94°C for 4 min followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C, and a final 10 min extension at 72°C for the *bar* gene and 35S. The PCR program for *HVA1* was: 94°C for 3 min for one cycle followed by 35 cycles of 30 s at 94°C, 30 s at 55.5°C, and 45 s at 72°C; one cycle at 72°C for 10 min; and the final cycle at 4°C. The reaction mixture was loaded directly onto 1% (w/v) agarose gels and 8% (w/v) poly acrylamide gel, stained with ethidium bromide, and visualized with UV light (306 nm). The amplified transgene product size was compared with positive controls and plasmid pTF101.1.

RESULTS AND DISCUSSION

Effects of different treatments on regeneration of the immature embryos after *Agrobacterium* infection and selection on phosphinothricin

This study focused on determining the role of three factors that may affect the transformation efficiency using three immature embryo explant types (intact or longitudinally halved-embryos or fragmented into four latitudinal pieces), four Moroccan genotypes and four concentrations of acetosyringone. The frequencies of resistant calli were highest using intact-immature embryo ranged from 16 to 44%, followed by longitudinally halved-embryos, which varied from 16.33% to 35.66% (Table 1). Our observations indicate that the 'Rajae' genotype was more sensitive to *Agrobacterium* infection using longitudinally halved-embryos range from 17.66 to 35.66%, (Table 1). Furthermore, intact-immature embryo could be used for the 'Achtar' genotype and proved to be a good acceptors for *Agrobacterium* infection, range from 21.33 to 44% (Table 1). The addition of 200 µM acetosyringone to *Agrobacterium* culture significantly improved callus resistant frequencies (44%) for 'Achtar' with intact-immature embryo whereas no significant effect of acetosyringone on callus resistant frequencies (35.66) for 'Rajae' genotype variety longitudinally halved-embryos on the medium without acetosyringone (Table 1).

Regeneration and molecular characterization of T_0 transgenic plants

The resistant plantlets regenerated after 3 to 4 days post incubation in the selective regeneration medium (Figure 1c). The frequencies of resistant plantlets for the four genotypes using intact-immature embryo varied from 0.33 to 2.66% (Table 1). For the longitudinally halved-immature embryos, the frequencies of the resistant plantlets were varied from 0.98 to 3.04% (Table 1). No plantlet regeneration was observed in recipient tissues prepared from fragmented-immature embryos (4 pieces) with the four tested genotypes (data not shown). The medium without acetosyringone, resulted in significantly highest level of resistant plantlets with 'Rajae' genotype from longitudinally halved-immature embryo (3.04%) (Table 1). On the other hand, highest levels of resistant plantlets were obtained using 200 µM of acetosyringone concentration in the wheat genotype of 'Achtar' with intact-immature embryos (2.66%) (Table 1).

A total of 25 PPT-resistant plantlets were finally regenerated from the resistant calli, 15 plantlets were derived from intact-immature embryos. 10 plantlets derived from the longitudinally halved-immature embryos were transplanted into the soil in the green-house for acclimatization (Figure 1g). The upper surfaces of leaflets were painted with Basta (PPT herbicide 0.3%). After 7 days, we demonstrated the expression of the PPT herbicide-resistance *bar* gene. Among the

25 plants, 9 painted plants remained green, while the untransformed controls showed necrosis and yellowed and died (Figure 1i). The PCR analysis amplified (Figures 2 and 3) the expected sizes for the *bar* gene (402 bp), 35S gene promoter (195 bp) and the *HVA1* gene (290 bp). Two T_0 plants regenerated from the longitudinally halved-immature embryo of genotype 'Rajae' cultured with no acetosyringone, and one T_0 plants regenerated from the intact-immature embryo of genotype 'Achtar' with 200 µM of acetosyringone were positive for PCR analysis and carried the desired the *HVA1* gene. The transformation efficiency was calculated as the number of transgenic plants obtained (plants surviving PPT-selection and PCR-positive for the *bar* gene, 35S, and *HVA1* divided by the total number of immature seeds used to prepare the recipient tissues × 100 (Pellegrineschi et al., 2002). Based on the PCR analysis, the transformation efficiency of T_0 plants for the two genotypes varied from 0.66% ('Rajae' from the longitudinally halved-immature embryo cultured with no acetosyringone) to 0.33% ('Achtar' genotype from intact-immature cultured with 200 µM of acetosyringone).

Table 1. Transformation efficiency of bread wheat genotypes using intact-immature embryos and longitudinally halved-immature embryos as explant source with different concentrations of acetosyringone after *Agrobacterium* infection with strain EHA101 (pTF101.1) and selection on phosphinothricin (PPT) medium

Genotypes	No. ET	Treatment cultured	Calli survival rate (%)				PPT-Number of plants selected (%)				PPT-Number of plants selected by panting (%)				Positive T_0 plants tested by PCR				TE (%)
			Concentrations of acetosyringone (µM)																
			0	100	200	400	0	100	200	400	0	100	200	400	0	100	200	400	
Achtar	300	Intact-immature embryos	26.00c*	37.00b	44.00a	21.33c	1.69b	1.700b	2.66a	1.03b	-	-	3b	-	-	-	1	-	0.33b
Amal	300		20.33bc	24.66b	34.33a	16.00c	0.81a	0.81a	0.87a	0.77a	-	-	-	-	-	-	-	-	-
Mehdia	300		20.66bc	23.66ab	27.66a	18.00c	0.66a	0.66a	0.66a	0.33a	-	-	-	-	-	-	-	-	-
Rajae	300		20.66b	21.33b	26.33a	19.00b	0.33a	0.66a	0.66a	0.33a	-	-	-	-	-	-	-	-	-
Achtar	300	Immature embryo halves	19.33b	24.33b	24.667a	19.33b	2.53ab	2.19a	2.25a	1.25b	-	-	-	-	-	-	-	-	-
Amal	300		25.00ab	25.66ab	28.66a	22.00b	1.00a	1.08a	1.10a	0.98a	-	-	-	-	-	-	-	-	-
Mehdia	300		19.00b	20.33a	25.667a	16.33b	1.667a	2.33a	2.16a	1.33a	-	-	-	-	-	-	-	-	-
Rajae	300		35.66a	19.00b	19.867b	17.66b	3.04a	1.63b	1.97b	1.52b	6a	-	-	-	2	-	-	-	0,66a

* The Means value followed by the same letter is not significantly different according to the test t (LSD) at α = 0.05; No. ET: Number of explants tested; TE: Transformation efficiency.

Analysis of progeny T_1 plants

The seeds from transformed T_0 wheat plants that showed positive results with PCR analysis were germinated in petri dishes containing absorbent paper pre-wetted with 700 to 1000 µl sterile water at room temperature, and then planted in pots in a green-house to produce T_1 plants. PCR reactions were used to assess the inheritance of *HVA1* gene in the T_1 generation of bread wheat derived from genotypes 'Achtar' and 'Rajae' respectively. The results of PCR analysis for *HVA1* using genomic DNA of T_1 plants confirmed the inheritance of *HVA1* gene (Figure 4). The T_1 plants displayed better growth compared to T_0 transformants plants. The number of seeds set by the transgenic T_1 plants was much higher than for their parents T_0. The T_1 progeny appeared to be normal phenotypically and similar in morphology to seed-derived control plants.

Figure 1. Agrobacterium transformation of immatures embryos with phosphinothricin selection.
a - Co-cultivation of calli derived from intact-immatures embryos with *Agrobacterium* on co-cultivation medium, b - Resistant embryogenic calli produced on selection medium, c, d - Resistant plantlets produced on selection medium, e - In *vitro* rooting of phosphinothricin-resistant plantlets, f - No transformed plantlets on selection medium, g - Acclimation of putative transformed plants, h - Fertile regenerated plant in green-house, i - PPT paint assay of leaves from T_0 transgenic plants (outside - control untransformed, in the middle - transgenic plantlets), j - Morphologically normal transformed T_0 seeds, k - No transformed seeds from the control bread wheat

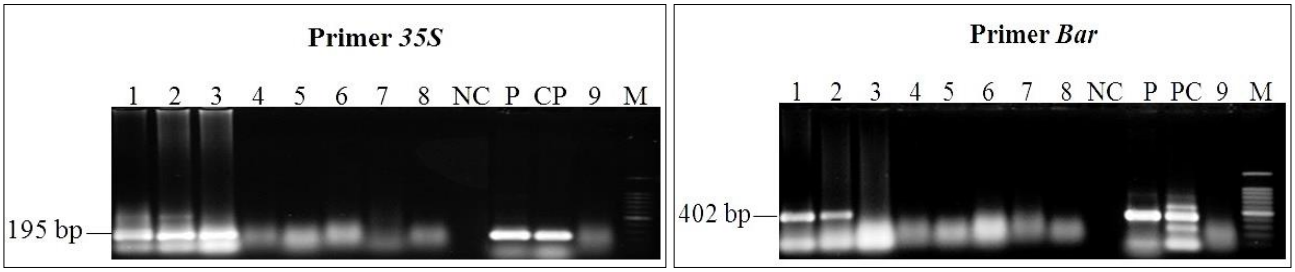

Figure 2. PCR analysis of genomic DNA of T_0 transformants of immature embryos using primers specific for 35S (195 bp) and bar (402 bp). The T_0 transformants were obtained by co-cultivation of immature embryos with *Agrobacterium* strain EHA101. M - 100 bp marker; 1 - Genomic DNA from leaf tissues of T_0 transgenic plants of 'Achtar' genotype with intact-immature embryos; 2, 3 - Transgenic plants from 'Rajae' genotype with halved - immature embryos; 4-8 - non-transformed plants from 'Amal' and 'Mehdia' genotype; NC - water (negative control); P - plasmid (pTF101.1); PC - positive control; 9 - non-transformed plant from 'Achtar' genotype

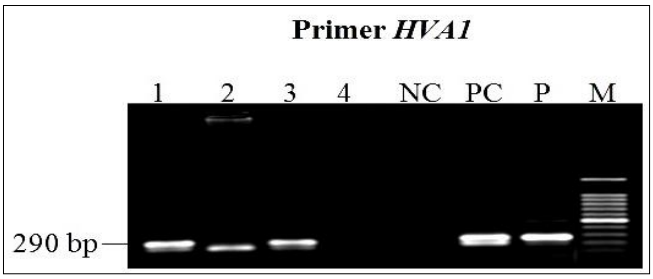

Figure 3. Molecular confirmation of genetic transformation of T_0 transgenic for 'Achtar' and 'Rajae' genotypes using target primer HVA1. 1 - genomic DNA from leaf tissues of T_0 transgenic plants of 'Achtar' genotype with intact-immature embryos; 2, 3 - transgenic plant from 'Rajae' genotype with halved-immature embryo; 4 - 'Rajae' genotype negative control; NC - Water (negative control); P - Positive control; 7 - Plasmid (pTF101.1); M - 100 bp marker

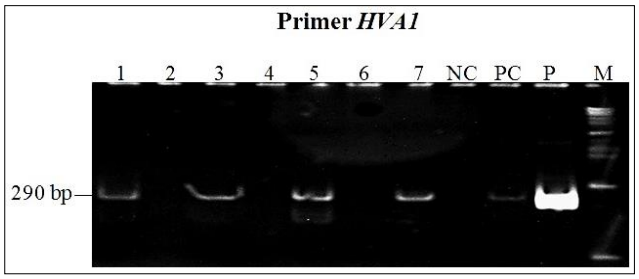

Figure 4. PCR analysis of T_1 transgenic plants using HVA1 gene specific primer. M - Marker 50 pb; P - plasmid pTF101.1; PC - positive control; NC - water (negative control); 1, 3 - Genomic DNA from leaf tissues of two T1 transgenic plants of 'Achtar' genotype; 5, 7 - Genomic DNA from leaf tissues of two T1transgenic plants of genotypes 'Rajae'; 2, 4, 6 - Genomic DNA from non-transgenic plant as a negative control from 'Achtar' and 'Rajae' genotypes

Although cereals are not natural hosts for *Agrobacterium*, many studies have been performed to optimize *Agrobacterium*-mediated wheat transformation using local genotypes (Jones et al., 2005). The genotype proved to be one of the key factors influencing tissue culture and plant regeneration during wheat transformation. Immature embryos from different genotypes with different treatments showed various levels of sensitivity and regeneration potential after infection with *Agrobacterium*. There were significant differences observed between the different genotypes, with two transformation treatments used to prepare the explants in terms of resistant calli frequencies and plantlets recovered. Of the tested genotypes, 'Rajae' derived from immature halved- embryos and 'Achtar' from intact-embryo produced superior calli and maximum regenerative ability after selection. Our results are in line with findings from earlier studies on wheat transformation of 129 genotypes with respect to their ability to produce embryogenic tissues, regenerate in selection medium, and overall transformation efficiency (Pellegrineschi et al., 2002). The significant role of the genotype in transformation efficiency has also been shown in the sunflower (Gürel and Kazan, 1999).

According to our results, only 'Achtar' and 'Rajae' genotypes showed high sensitivity to *Agrobacterium* and good regeneration ability after selection on selection media. We also addressed one of the biggest problems in wheat genetic engineering, which is the low efficiency of plant regeneration in wheat tissue culture after transformation with *Agrobacterium*. There were significant differences in the transformation ability of the explants with the two types of embryo explant.

Acetosyringone is one of the most potent phenolic inducers of the *vir* genes of *Agrobacterium* and can increase transformation efficiencies in many plant species (Costa et al., 2006); although plant response to its application could be genotype dependent. Dutt and Grosser (2009) found that various citrus cultivars differentially responded to the addition of acetosyringone to the co-cultivation medium. It has also been considered as an important factor for successful *Agrobacterium*-mediated transformation in wheat (Cheng et al., 1997). Our results showed that the addition of acetosyringone was not conclusive for the survival of plantlets derived from 'Rajae' with halved-immature embryo as explants but it was important for calli derived from 'Achtar' with intact-immature embryos with 200 μM of acetosyringone. Our results are similar to these of Amoah et al. (2001), who added acetosyringone at a final concentration of 200 μM and obtained increased transformation efficiency. Conversely, its application had a positive effect on the survival of regenerating calli prepared from

intact-immature embryos. Unfortunately, acetosyringone had no significant effect on the survival of regenerating calli from halved- immature embryo, indicating that the type of starting material used in transformation has the most influence on outcomes.

The PCR data showed that the transformation efficiency is somewhat higher than that reported for *Agrobacterium*-mediated transformation of wheat using immature embryos as recipient tissues (Miti´c et al., 2004), and is also greater than that obtained by some scientists for immature wheat embryos (Pellegrinesch et al., 2002).

CONCLUSIONS

From this study we can conclude that, we successfully introduced plasmid pTF101.1 via *Agrobacterium* into immature embryos from recalcitrant bread wheat genotypes for the first time in Morocco. Since the transgenic plants contained the barley *HVA1* gene, further water stress tolerance and salt tolerance analyses in T_2 and subsequent generations will be performed. The obtained results showed that the genotype, recipient tissue, and concentration of acetosyringone had effects on resistant callus and plantlet recovery. Further optimization of the experiments described in this work may eventually lead to the development of an efficient *agrobacterium*-based transformation protocol that uses immature embryos as recipient tissues and is suitable for transforming diverse wheat genotype.

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