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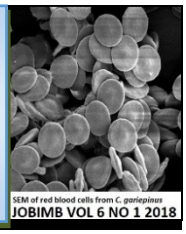

Regeneration and Genetic Transformation Systems of Durum Wheat, *Triticum durum*

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ABSTRACT

Wheat is a major cereal crop for humans but quite recalcitrant in transformation. Establishment of regeneration system in wheat using immature embryos is not easy and time/cost-consuming. Herein, we developed a regeneration and transformation system using mature seeds in four pasta wheat cultivars. The MS medium with 2.0 mg/l 2,4-D and 2 mg/l BA was the optimum medium for developing shoots from calli. Wheat cultivars showed different regeneration frequencies response due to their genetic makeup. The cultivar Sohag-3 produced the highest regeneration frequency (93.2%) among the tested cultivars. Developed cultivars Sohag-3 and ACSAD1105 mature embryos were co-cultivated with *Agrobacterium tumefaciens* strain GV3101 with the binary vector pISV2678 harboring the *bar* gene and *β-glucuronidase* (*gus*) gene. The transformation efficiencies were 12.3 and 9.1% for cultivars Sohag-3 and ACSAD1105, respectively. The polymerase chain reaction (with specific primers for the transgenes) and the dot blot hybridization were used to confirm the integration of the transgene in transformed plants. The transformation percentages were reduced according to their expression and reached 5.6 and 4.6% for cultivars Sohag-3 and ACSAD1105, respectively. RT-PCR and northern blot analysis confirmed the expression of the *gus* gene only in the transgenic plants. The procedures developed in this study demonstrate the ability to produce transgenic wheat plants expressing the *gus* gene; hence, this protocol could be used to regenerate transgenic wheat plants expressing desirable and selective genes

INTRODUCTION

Wheat is a globally important food crop with the highest protein content amongst cereals. It offers 20% of the daily protein and provides the food calories for 4.5 billion people [1]. Despite of wheat economic importance, various factors, such as biotic and abiotic stresses, affects its productivity [1]. Conventional breeding may help in wheat improvement, but it is rather slow, due to difficulties in crossing with wild relatives and limited hereditary base [1,2]. Transformation of wheat was the bottommost among monocots, due to its complicated genome (2n = 28 for *Triticum durum* and 2n = 42 for *Triticum aestivum*) as it is rich in repetitive sequences causing difficulties in regeneration and transformation [3–5].

Totipotent cells are crucial for regeneration, but not all cells in cereal have totipotent capacity, as it depends on the cell type and the cultivar [6]. Success of transformation system basically depends on the adapting of regeneration system, therefore, it is important before starting plant transformation to select the appropriate genotype and/ or explant [7]. Generally, there are several factors that influence plant regeneration such as type of explant, media composition and genetic background. Different types of explants including mature embryo [8,9], immature embryo [10,11], shoot meristem [12] and leaf bases [13] have been successfully implemented for *in vitro* wheat regeneration. Immature-embryos have the highest regeneration capability for *in vitro* regeneration of wheat [14,15], however, these explants require controlled conditions in greenhouse, and personal skills to isolate them at a suitable stage, in addition they are not available all the year round [16]. On the other hand,

use of mature embryo as explant although it is time-consuming, but available all the year round in high quantities and cost-effective procedures [17,18].

Agrobacterium co-cultivation is a highly recommended tool for plant transformation due to its high efficiency and low cost for gene delivery. The most widely method for gene transfer is *Agrobacterium*-mediated gene transfer and has been extensively applied to many crops including wheat. *Agrobacterium* have many advantages; including the capability of transferring large segments of DNA, stable transgenes integration with low copies without the vector backbone [19]. *Agrobacterium*-mediated gene transfer in wheat was first reported in early 1990s [20,21]. For durum wheat, He *et al.* [22] obtained 121 independent transgenic lines from using *Agrobacterium*-mediated transformation with the strain AGL1 containing the super binary vector pGreen/pSoup. They reported that high acetosyringone implementation increased the efficiency up to 6.3%. Wang *et al.* [23] successfully transformed Chinese hexaploid wheat cultivars with efficiency of up to 37.7% using *Agrobacterium*-mediated method. The current study aimed to develop an efficient procedure for regeneration and *Agrobacterium* transformation of four wheat cultivars (*T. durum*) using mature seeds as explants.

MATERIALS AND METHODS

Plant material

Four tetraploid *T. durum* cultivars namely, Bani-Sewef 1, Bani-Sewef 3, Sohag-3 (obtained from Horticulture Institute, Agriculture Research Center, Giza, Egypt) and ACSAD1105 (obtained from The Arab Center for the Studies of Arid Zones and Dry Lands, Syria) were used in this work. The mature seeds of the four cultivars were sterilized using 70% ethanol for 1 min, with 20% sodium hypochlorite (NaOCl) for 25 min, and finally rinsed thrice with sterile H_2O. Seeds were embedded overnight in distilled H_2O at 25ºC.

Callus induction

Mature embryos were carefully removed with a scalpel from the embedded seeds for callus induction. In each experiment, 10 embryos were cultured per sterile 90 mm Petri dishes. Isolated mature embryos were transfer on MS medium [24], including different concentrations (1-6 mg/l) of 2,4-dichlorophenoxy acetic acid (2,4-D). An amount of 30 g/l sucrose was added for the media, adjusted to pH 5.8 and solidified by adding 7 g/l agar. Prepared media were sterilized by autoclaving at 121ºC for 20 min, and 1.1 kg/cm^2 pressure.

Shoot induction

Calli that showed signs of proper formation were transferred to the shoot induction medium which consist of MS medium supplied with different concentration of BA (1-4 mg/l). The cultures were incubated at 25°C under a 16/8 h day/night photoperiod (1000-Lux). Plant materials were subcultured every 3-4 weeks until shoots reach suitable height for rooting.

Rooting and acclimatization

Plantlets of 3.0 cm height were hand-transferred to rooting medium, which contain 1/2 MS in 200 ml flasks. After 3-4 weeks, shoots with well-developed roots were transferred and cultivated in pots containing a mixture of sand and peat moss (1:1 v/v). Pots were covered with transparent polyethylene bags to increase humidity and placed in temperature-controlled greenhouse conditions. One week later, the covers were removed gradually.

Bacterial strain and vector

Agrobacterium tumefaciens strain GV3101 harboring the binary vector pISV2678 was used for establishing wheat transformation. The plant vector pISV2678 carries the reporter system *gus*-intron gene under the control of 35S promoter and *nos* terminator and a plant selectable marker gene *bar* fused to the AMV leader, *nos*-promoter and pAg7 terminator was employed. The vector was kindly provided by Dr. Pascal Ratet (ISV - CNRS, France) (**Fig. 1**).

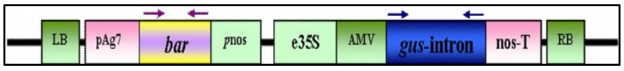

Fig. 1. The T-DNA region of the constructed pISV2678 vector. RB: right border, LB: left border.

Agrobacterium co-cultivation

The *Agrobacterium* culture (OD_{600}) were mixed thoroughly with the explants (calli) through deep immersion for 10 min. The explants were blotted on sterile Whatman filter papers to remove excess culture. Explants were incubated for 72 h in dark on MS medium supplied with suitable concentration of BA. After co-cultivation for three days, the explants were transferred to selection media containing the regenerated medium with suitable concentration of BA, 500 mg/l cefotaxime and 3 mg/l bialaphos. Weekly, the explants were sub-cultured on freshly prepared media.

GUS assay

For screening the expression of β-glucurodinase (GUS) activity in putative transgenic wheat plants, GUS histochemical assay was performed as per the method described by Jefferson *et al.* [25]. Analysis were carried out on developed calli on selection media and the developed blue spots were recorded after final incubation for 24 h in the reaction buffer at 37°C.

PCR confirmation

DNA was extracted from transformed and non-transformed (control) plantlets according to the methods reported by Rogers and Bendich [26]. The PCR reaction (20 µl) was included 10 ng DNA, 1 µM primer, 0.5 units of Red Hot *Taq* polymerase, 10-X *Taq* polymerase buffer (AB-gene Housse, UK) and 200 µM dNTPs. Three pairs of primers were designated to amplify fragments of the *bar*, e35S promoter and *gus* genes (Table 1). The reaction profile was 94 ºC for 3 min; 35 cycles of denaturation at 94 ºC; annealing as describe in **Table 1**; and extension at 72 ºC for 2 min each, followed by 7 min extension at 72 ºC. Amplicons were recorded after running on agarose gel 1.5% and staining with ethidium bromide.

Table 1. The primers sequences of transgenes used for plant transformation.

Genes	Sequences	Ann-ealing temp.	Amp-lified size
bar	5`AAAAGCTTCCACCATGAGCCCAGAACG ACG 3` 5`AAGGATCCTCAGATCTCGGTGACGG 3`	55	540 bp
e35S promo-ter	5`AAAGGAAGGTGGCTCCTACAAAT 3` 5' CCTAGTAAAGTAAACCTCTCC 3`	56	250 bP
uidA	5`AGTGTACGTATCACCGTTTGTGTGAAC 3' 5`TCGCCGCTTTGGACATACCAT CCGTA 3`	60	750 bp

Dot blot confirmation

Isolated DNA were denaturated by heating over 90 ºC for ten min, spotted onto nylon membrane followed by 1 min crosslink for fixation. Labeling of the probes (amplified *gus* gene),

hybridization and detection were carried out using the Biotin Chromogenic Detection kit #K0661, #K0662 (Ferments Life Sciences, USA) according to manufacturer instructions.

Reverse Transcription PCR (RT-PCR)

PCR positive plants were used to isolate total RNA with SV Total RNA Isolation System (PROMEGA, cat. #Z3100 USA). RT-PCR analysis was performed using RevertAid™ First Strand cDNA Synthesis Kit (Ferments Life Sciences, USA). The analyses were carried out on both putative transformed (PCR positive) and non-transformed plants using the *gus* specific primers and the PCR products were visualized on 2% agarose gels.

Northern blot analysis

Isolated RNA from both putative transformed and negative control plants were electrophoretically separated and transferred onto Hybond NC nylon membrane (Amersham). Prehybridization and hybridization conditions were carried out as provided by the manufacturer's recommendations. PCR produced from *gus* gene was used as a probe (PCR produced from *gus* gene). Hybridization and detection was carried out with the Biotin Chromogenic Detection kit (#K0661, Ferments Life Sciences, USA) according to the supplier's instructions (Ferments Life Sciences, USA).

RESULTS

Callus induction

For callus induction, six different concentrations of 2,4-D acid were used from the mature embryos of the four wheat (*T. durum*) cultivars. On week old incubated explants start showing initial swelling than the callus start formation. Results showed that the number of induced calli increased gradually and reached the maximum when the 2,4-D reaching 2 mg/l. However, raising the concentration more than 2 mg/l decreased the percentage of callus induction. The different cultivars also affect the percentage of callus induction (**Fig. 2**). The data indicated that, the highest callus induction percentage was obtained with the cultivar Sohag-3 (57 %), followed by ACSAD1105 (50.4 %) then Bani-Sewef 3 (33.4 %) and the lowest percentage was recorded for the cultivar Beni-Sewef 1 (27 %) when using 2 mg/l 2,4-D MS medium.

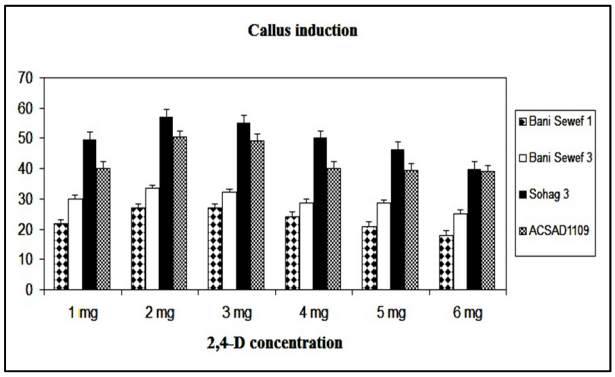

Fig. 2. Calli induction frequency of four wheat cultivars under different 2,4-D concentrations MS medium.

Plant regeneration

Fifteen days old embryogenic calli developed from mature embryo were subcultures on regeneration medium for shoot induction. The regeneration medium consisted of MS basal medium containing different concentration of benzyladenine (1, 2, 3 and 4 mg/l BA) (**Fig. 3**). Data showed that the four wheat cultivars differ for their response to the BA and the shoot induction increased with the increasing of BA concentration to 2 mg/l for all tested cultivars, but decreased with higher concentrations. The data indicated that the highest shoot induction percentage were obtained with the cultivar Sohag-3 (63.5 %) followed by ACSAD1105 (57 %), then Bani-Sewef 3 (50.4 %) and the lowest percentage was recorded for the cultivar Beni-Sewef 1 (47 %).

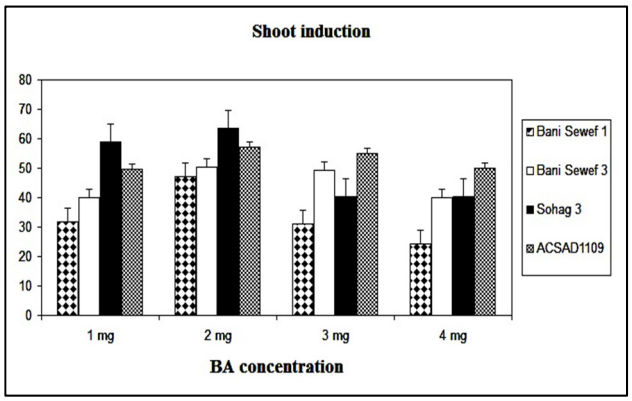

Fig. 3. Shoot induction frequency of four wheat cultivars on MS supplemented with different concentration of BA.

During regeneration, the nodular structures of the embryogenic calli developed into adventitious shoots within two weeks when sub-cultured on the medium containing 2 mg/l BA. The somatic embryos straight arise from the calli explants. The different stages of the plantlets developmental are showed in **Fig. 4**. Regeneration frequencies of the different *T. durum* cultivars were listed in **Table 2**. Data showed that the number of regenerated plants were different among the different cultivars. The cultivar Sohag-3 displayed the highest regeneration percentage (93.2 %) followed by ACSAD1105 (82.7 %) then cultivar Beni-Sewef 3 (78.8) while the cultivar Beni-Sewef 1 induced the lowest regeneration frequency (65.9%). The developed shoots were transferred into half strength MS medium for allowing root induction. Well-developed shoots were observed within 2-3 weeks. Acclimatization was carried out as previously described in the greenhouse. The present data indicates studying the effects of cultivar type on the regeneration capacity. The obtained data from the regeneration method is repeatable and can be used to develop a transgenic *T. durum* wheat plants.

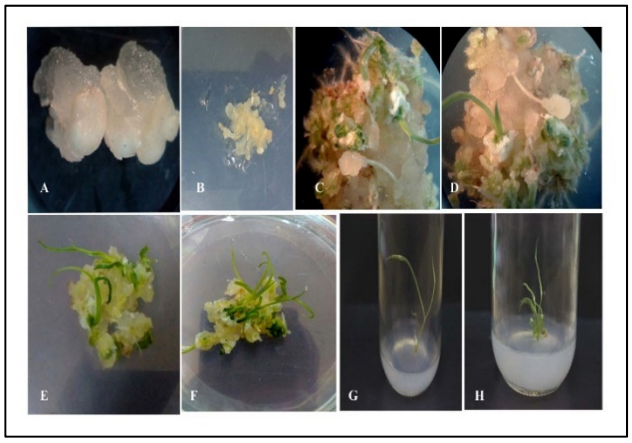

Fig. 4. Somatic embryogenesis in regenerated wheat. A: calli induction, B: embryogenic calli, C and D shoot regeneration.

Table 2. The regeneration frequencies of the mature embryo derived from the four wheat cultivars.

Cultivars	No. of explant	Explants forming					
		callus inducing explants	% Callus induction	No. of shoot initiation	% Shoot induction	No. of Reg. plants	Reg. %
Beni-Sewef 1	100	45	45	44	44.0	29	65.9
Beni-Sewef 3	100	48	48	52	52.0	41	78.8
Sohag-3	100	85	85	89	89.0	83	93.2
ACSAD1105	100	61	61	87	87.0	72	82.7

Agrobacterium transformation

The cultivars Sohag-3 and ACSAD1105, that showed highest regeneration percentage (93.2 and 82.7 %, respectively), were used for *Agrobacterium* transformation. The calli of the two wheat cultivars, Sohag-3 and ACSAD1105, were co-cultivated with *Agrobacterium tumefaciens* GV3101 harboring the binary vector pISV2678. After 3 days co-cultivation, the inoculated calli were transferred to MS medium with low selection agent. The developed shoots (putative transgenic) were regenerated after one month on the selection MS medium including 2 mg/l BA medium and 3 mg/l bialaphos, to reduce the number of non-transgenic tissues. During selection process, survived calli continued to grow well to produce shoot initiations, whereas the non-transformed ones failed to form shoots became of necrotic development within 21 days. The numbers of putative transgenic plants for the wheat cultivars developed on the selection medium were 89 and 87 for cultivars Sohag 3 and ACSAD1105, respectively (**Table 3**).

Table 3. The percentages putative transformed wheat cultivars survived on selection medium after co-cultivated with *Agrobacterium.*

Cultivars	No. Exp-lants	Callus inducing explants	Shoot ind-uction	Analysis of *Agrobacterium* transformed wheat			
				+ve PCR (*gus*, bar & e35S promoter)	% +ve PCR (*gus*, bar & e35S promoter)	+ve RT-PCR & Northern	% Transgenic plants
Sohag-3	100	85	89	11	12.3	5	5.6
ACSA-D1105	100	61	87	8	9.1	4	4.6

The histochemical *gus* assay confirmed the presence and the stable expression of the integrated *gus* gene into the transgenic plant genome. Blue color cells could be detected only in the transgenic calli while the non-transgenic showed colorless cells (**Fig. 5**).

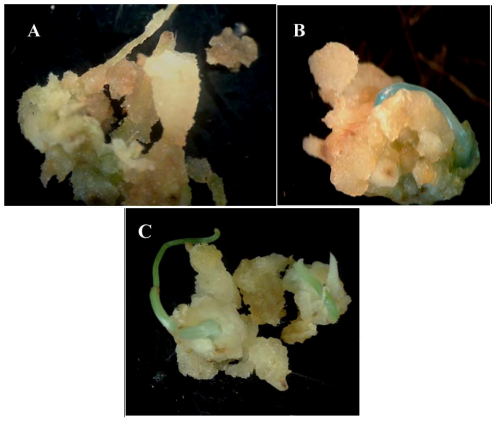

Fig. 5. Histochemical GUS assay showing gene expression in transgenic plant callus (B and C) while no expression can be detected in non-transgenic plant (A).

PCR analyses were performed on total DNA extracted from T_0 plantlets, in order to confirm the presence of the transgenes in the regenerated plants. Three sets of primers specific for the *gus* gene, *bar* gene as well as for the 35S-promoter were used for evaluation. The PCR analysis for regenerated plants developed on the selection medium showed that 11 out of 89 for cultivar Sohag-3 and 8 out of 87 for cultivar ACSAD1105 (Table 3). Positive PCR lines produced clear bands corresponding to the expected size from using the three primer sets with a molecular weight of 750 bp, 540 bp and 250 bp for the *gus* gene*, bar* gene and the 35-S-promoter, respectively (**Fig. 6**).

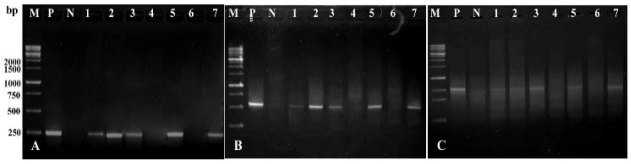

Fig. 6. PCR analysis confirming the transformation of T-DNA into wheat genome. (A) The detection of the 35-S-promoter in the transformed plants. (B) The detection of the *bar* gene into transformed wheat the genome. (C) The detection of the *gus* gene in the transformed plants M: 1 Kb DNA ladder.

For further examination for the presence of the *gus* gene in the putative transgenic wheat lines, genomic DNA of PCR positive lines and non-transgenic plants for both cultivars were blotted and hybridized with the *gus* probe. Results obtained from the dot blot analysis indicated that *gus* gene is presented in the genome of the PCR positive lines (**Fig. 7**).

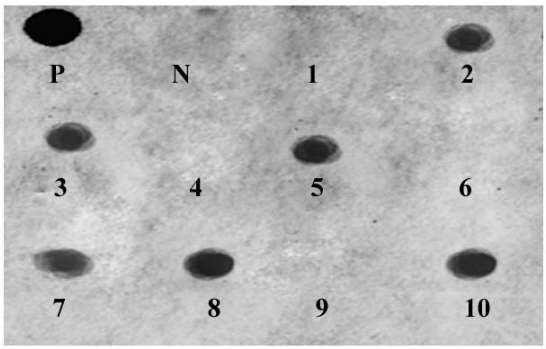

Fig. 7. Dot blot hybridization using *gus* gene specific probe

The stable expression of the *gus*-intron gene in the transgenic wheat was confirmed by using both RT-PCR analysis and northern blotting analysis. Total mRNA was isolated from PCR-positive putative transgenic lines and also from the non-transgenic plants (N). The RT-PCR analysis for the wheat plants showed the occurrence of the mRNA for the *gus*-intron gene in 5 out of 11 PCR positive plants for cultivar Sohag-3 and 4 out of 8 with PCR positive for cultivar ACSAD1105 (**Fig. 8**). For northern hybridization, total RNA was hybridized with the *gus* probe.

The data obtained confirmed the presence of mRNA in the same RT-PCR positive transgenic plants (**Fig. 9**). These results confirm the correct integration and expression of the transgenes into the genome of 5 lines of cultivar Sohage-3 and 4 lines of cultivar ACSAD1105. According to data obtained from Northern and RT-PCR analysis, the percentages of transgenic were 5.6 and 4.6 for cultivars Sohage-3 and ACSAR1105.

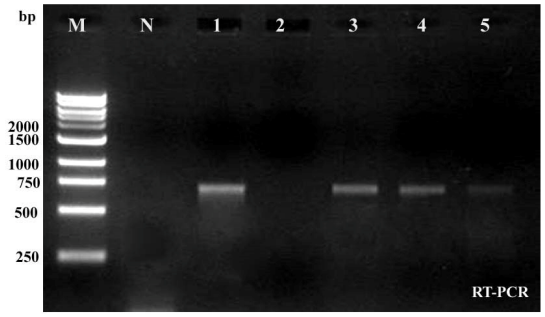

Fig. 8. Confirmation of the transcript of wheat plant expression *gus* gene. The RT-PCR confirming *gus* gene expression in four transgenic lines of wheat. N: non-transgenic and line 1-5 transgenic lines.

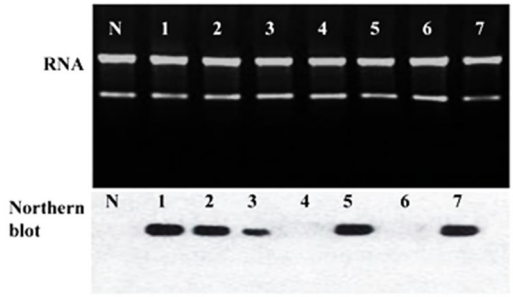

Fig. 9. Confirmation of the transcript of wheat plant expression *gus* gene. Northern blot analysis confirming *gus* gene expression in five transgenic lines of wheat. N: non-transgenic and line 1-7 transgenic lines.

DISCUSSION

Biotechnology has provided additional means for improving plant production under different environmental conditions. This could be performed through integration and expression of specific genes into plant cells that can be regenerated into whole plant. To obtain a successful transformation system, we should first establish a suitable method for regeneration. The present investigation aimed to improve an effective regeneration and *Agrobacterium* gene delivery protocol for durum wheat. The mature embryo was chosen as explant because it is available all the year round and it is easy to extract. Immature embryos are difficult to obtain immature embryos all year round, and their isolation demands high skill persons.

Many factor influence the induction of callus and the regeneration capacity of wheat including the genotype, type of explant and physiological status of the mother plants, the culture medium [27]. One of the common factors that affect embryogenic callus formation is the explant maturity stage and therefore scientists have tried to develop a system of callus induction using mature embryos [28]. The present investigation describes an established method for mature embryos regeneration for four durum wheat cultivars. The formation of callus is affected by the type and level of the auxin presented in culture medium. Most of the previous studies on cereal regeneration using mature embryos, the 2,4-D is commonly used as exogenous growth regulator [29,30]. The callus induction percentage decreased with increasing 2,4-D concentration in the culture medium. Several authors [31-33] had agreed in the superiority of using 2,4-D for embryogenic callus induction for wheat with frequency peak with 2.0 mg/l 2,4-D in the induction medium.
In this investigation, also MS containing 2 mg/l BA was the best as regeneration medium for the four tested cultivars. However, our present data showed that the four wheat cultivars differ genetically on their response and the cultivar Sohag-3 gave the highest regeneration percentage (93.2%).

The previous investigations obtained a lower regeneration capacity than the obtained from this work. Delporte *et al.* [34] obtained an average of 11% regeneration, while, Özgen *et al.* [27] obtained an average of 70.4%. Delporte *et al.* [34] reported that regeneration frequency differs according to the genotype, ranged between 30-60%. The present investigation indicates that the cultivars differ genetically for their regeneration capacity. The reported regeneration protocol is repeatable and can be used to regenerate transgenic wheat plants.

Genetic engineering in plants depends on the development of an efficient and reliable plant regeneration system. Wheat is among the top list of the most important crop species; therefore, the regeneration and transformation systems of wheat have been extensively investigated by Nasircilar *et al.* [35]. The biolistic and the *Agrobacterium* transformation are mainly the two common methods that used for gene transfer in wheat [22]. In early time, cereal transformation using *Agrobacterium,* was considered unmanageable and, the first successful report of fertile transgenic wheat plants were conducted by Cheng *et al.* [36]. Successful wheat transformation *via Agrobacterium* depends on various factors and several workers explored in a quest to achieve higher transformation efficiency [20,37,38].

Agrobacterium-mediated approach has many advantages over other methods of transformation, including the ability to transfer large segments of DNA, transfer fewer copies, develops simple segregation pattern, illuminate vector backbone sequences and higher efficiency of transformation with minimal cost. Thereby, *Agrobacterium*-mediated transformation approach is considered as the optimum technique for plant transformation [39]. For establishing transformation system, embryonic calli derived from mature embryos of the wheat cultivars Sohag-3 and ACSAD1105 were co-cultivated with *Agrobacterium tumefaciens* GV3101 harboring the binary vector pISV2678. The vector contains *gus*-intron gene to insure the expression in plant cells and illuminate any background from bacterial expression. Putative transformed plants were evaluated for detecting the transgenes by PCR and dot blot hybridization and the results were same for both tests.

For cultivar Sohag-3, 11 lines were positive for both tests but for cultivar ACSAD1105, only 8 lines were positive for both tests. According to these tests, the putative transformation percentages were 12.3% and 9.1% for the cultivars Sohag-3 and ACSAD1105, respectively (**Table 3**). For further confirmation, gene expression was carried out using RT-PCR and northern blot hybridization. Obtained results showed reduced in the number of transformed plants to reach 5 and 4 for cultivars Sohag-3 and ACSAD1105, respectively and the percent of transformation were reduced to 5.6 and 4.6 for both cultivars. The decrease in the number from evaluating analysis may due to the presence of the DNA in the tested lines but not expressed. We considered the positive lines for RT-PCR and northern analysis as true transgenic plants.

The differences between transformation percentages for Sohag-3 and ACSAD1105 may reply to the different cultivars. Wu *et al.* [37] reported that many factors influence the

transformation efficiency in wheat, such as genotype, type of explant, inoculation conditions and co-cultivation with *Agrobacterium*, callusing and plant regeneration, vector and *Agrobacterium* strain, etc.

CONCLUSION

The regenerated wheat plants were different among different tested cultivars and gave a reasonable number that could be used for developing transgenic plants. However, the transformation percentages were 5.6 and 4.6% for cultivars Sohag-3 and ACSAD1105, respectively according to their expression. Therefore, the developed regeneration and transformation protocols are recommended for wheat improvement, as they are reproducible.

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