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The Role of virulence gene inducing factors in the improvement of *in planta* transformation of wheat (*Triticum aestivum*)

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Abstract

Agrobacterium tumefaciens-based plant transformation is a natural and ideal way of introducing genes into plant genomes. The system is based primarily on tissue culture techniques, including cell differentiation and plant regeneration, which might produce somatic cell mutations. In the present research, wheat plants were transformed by a non-tissue culture approach. Accordingly, mature embryos were inoculated with *A. tumefaciens* at an early stage of germination. A variety of different treatments, such as *Agrobacterium* strains (EHA105 and LBA4404 harboring pCAMBIAl105.1R), levels of glucose, concentrations of acetosyringone (0, 50, 100 and 200 µM), and types of wheat cultivars (Azar2, Alvand, and Sardari) were studied. The transformation efficiency was determined by using the number of resistant leaves to hygromycin, histochemical GUS analysis of leaf tissues, as well as PCR analysis of three transgenes located within the T-DNA region. The results showed that the maximum efficiency was obtained when 200 µM acetosyringone was used in combination with 15% glucose in the induction medium. In addition, the relative expression analysis of virulence genes by qRT-PCR revealed that *Vir*B2 and *Vir*D2 were significantly up-regulated when 200 µM acetosyringone and 15% glucose were used as inducers and carbon source, respectively. Therefore, *Vir* genes inducing factors as well as a three-step culture of *Agrobacterium* should be considered as major elements for any wheat transformation protocol. The putative transgenic T_1 seeds were soaked and germinated in hygromycin solution, and the seedlings were further analyzed with the aforementioned methods. Based on the results, a modified *Agrobacterium*-mediated transformation protocol is presented. The current results suggest an inexpensive, quick and efficient approach for wheat genome transformation.

Key words: Acetosyringone, glucose, Histochemical GUS assay, Hygromycin resistance, Real-Time PCR.

INTRODUCTION

It is estimated that wheat production around the world in 2019/2020 was 766 million tons, which could represent a 4.8-million-ton increase over the last year production, or 4.50% (FAO, 2021). Maintaining and improving crop productivity levels is an important challenge in today's agriculture, and mitigation strategies must streamline in order to boost yield under limited resources. Utilizing genetic variation in the gene pool and identifying desirable traits that lead to stress tolerance are two key principles of crop improvement (Allard, 1960; Abhinandan *et al*., 2018). Genetic engineering has facilitated the transfer of genes from distantly related species to target crops, mainly through *Agrobacterium*-mediated and microparticle bombardment methods (Gao, 2021). The majority of wheat transformation protocols published so far are based on microparticle bombardment (Hwang *et al*., 2017). Comparing the two methods, *A. tumefaciens* has several advantages as it allows for a more stable integration of defined and large segments of DNA

(150-200 kb) into the plant genome, transferring a lower transgene copy number and fewer chromosomal rearrangement. *A. tumefaciens* is a natural vector capable of permanently transferring a transgene to plants where it can be expressed continuously (Gheysen *et al*., 1998). In addition, many scientists regard *Agrobacterium*-mediated transformation as a desirable method due to its cost-effectiveness (Gould, 1996; Hamilton *et al*., 1996; Hiei *et al.,* 1997; Gelvin, 2006; Thompson *et al.,* 2020).

Wheat *Agrobacterium*-mediated transformation was performed as a tissue culture-dependent process using immature seed cultures, with an efficiency of approximately 4 to 6% (Cheng *et al*., 1997). Several disadvantages of this method have been noted, including low callus regeneration, frequent chimeric plants, somaclonal variations, as well as high costs and expensive equipment (Finnegan *et al*., 2000; Mohan, 2001). Callus induction is required for tissue culture-based methods (Lin *et al*., 2009), while the ability of transformed callus to generate a complete plant is a major limitation for monocots (Singh *et al*., 2015). During regeneration induction, transformed plants may lose their reproductive potential and/or be exposed to somaclonal variations (Larkin and Scoweroft, 1982), which can cause a failure in transgene expression.

Agrobacterium-mediated *in planta* transformation is another alternative transformation method (Risacher *et al*., 2009; Singh *et al*., 2015). By using this tissue culture-independent approach, fertile transformed plants can be obtained with minimum labor, cost and no possibility of somaclonal variation in short periods of time. A number of economically and agriculturally important crops have been transformed using the *in planta* method such as rice (Lin *et al*., 2009; Naseri *et al*., 2012) corn (Chumakov *et al*., 2006) buckwheat (Bratic *et al*., 2007) sugar cane (Mayavan *et al*., 2013) and hybrid Populus (Takata and Eriksson, 2012).

The present study examined the use of an *Agrobacterium*-mediated transformation of wheat using a non-tissue culture approach. On the other hand, the indirect effects of virulence (*vir*) gene inducing factors such as glucose and acetosyringone; as well as type of wheat genotype on the number of transgenic events were assessed statistically. A direct effect of *vir* genes inducing factors on the upregulation of *vir*B2 and *vir*D2 genes was also quantified by qRT-PCR. The transformation efficiency was calculated based on the analysis of T_1 generation.

MATERIALS AND METHODS

Agrobacterium strains and plant materials

The experiment was conducted in the PC2 growth chamber of the Biotechnology laboratory, the College of Agricultural Sciences at the University of Guilan, Rasht, Iran. In this study two *Agrobacterium* strains (EHA105 and LBA4404) harboring pCAMBIAl105.1R vector were used to transform wheat germinating seeds. The vector contains hygromycin resistant (*hph*) and β-glucuronidase (*gus*-Plus)™ genes with chalcone synthases intron near 5′ terminal plus a constitutive promoter of CaMV 35S in T-DNA region (www.cambia.org; Figure 1). The seeds of cultivars Azer2 (Az), Alvand (Al) and Sardari (S) were obtained from the Sanandaj seed research institution.

Inoculation of wheat Seeds with *Agrobacterium* strains

To sterilize, the wheat seeds were immersed in 90% ethanol for 1 min., followed by submerging in 1.5% sodium hypochlorite for 5 min. and finally, rinsed three times with sterilized distilled water. They were kept in Petri dishes in dark for 1 day at 20 °C till the appearance of the radicle tip.

A. tumefaciens strains were cultured in YEP liquid medium (tryptone 20 g/l, yeast extract 5 g/l, NaCl 0.5 g/l, pH=7) with rifampicin (100 µg/ml) and spectinomycin (100 µg/ml) antibiotics in a shaking incubator for 16 h at 28 °C and 250 rpm. About 500 µl of bacterial suspension was added to a 50 ml AB medium (50 ml sterile 20×AB buffer [60 g/L of K_2HPO_4 and 20 g/L of NaH_2PO_4, pH=7] and 50 ml sterile 20×AB

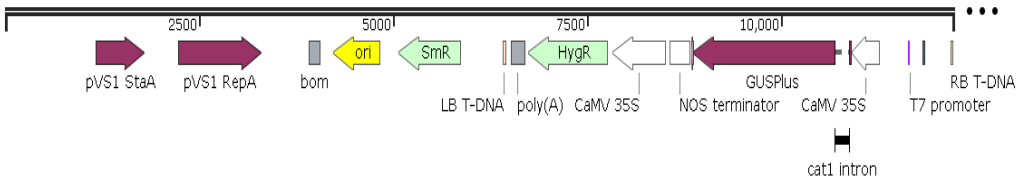

Figure 1. The schematic diagram of pCAMBIA1105.1R plasmid (AF354045). LB, left border for T-DNA repeat; RB, right border for T-DNA repeat; HygR: hygromycin resistance gene; GUSPlus: β-glucuronidase gene (includes catalase intron; cat1); CaMV 35S: cauliflower mosaic virus 35S promoter (The map of the vector has drawn by SnapGene®).

salts [20 g/L NH_4Cl, 6 g/L $MgSO_4 \cdot 7H_2O$, 3 g/L KCl, 0.2 g $CaCl_2$ and 50 mg $FeSO_4 \cdot 7H_2O$] with 900 ml sterile sucrose-water (0.5%) containing the above antibiotics and were cultured overnight (OD_{600}=0.8). The bacteria were pelleted (centrifugation for 5 min at 9000 *g*) and resuspended in 2 vol of the induction medium [1×AB salts, 2 mM phosphate buffer (pH=5.6), 50 mM 2-(4-morpholin) ethansulfonic acid (MES) and 0.5% glucose] plus different concentration of acetosyringone (AS). The bacteria were shaken very gently (approx. 50 rpm) for 14–24 h at room temperature, were pelleted, and resuspended in ½ MS and were used as an inoculation medium (Gelvin, 2006).

To conduct AS tests, either different concentrations of AS (0, 50 and 100 µM) or 100 µM AS in combination with vacuum or without vacuum were used in the inoculation medium. The vacuum was applied using Eppendorf Concentrator Plus (Eppendorf) in ambient temperature.

To inoculate seeds with *Agrobacterium*, the embryonic region of germinated seeds was punctured about 1 mm by a needle (0.5 mm, ⌀) and covered with bacteria. The inoculated seeds were kept in a growth chamber at 23 °C in darkness for 2 days to germinate. In order to remove the bacterium, the inoculated seeds were treated by cefotaxime antibiotic (1000 mg/l) for 1 h (Bechtold *et al.*, 1993). Germinated seedlings were placed in pots containing peat moss and were transferred to a growth chamber kept at 23 °C and 16 h photoperiod.

Virulence genes expression analysis using Real-Time PCR

To study the virulence **(***vir*) genes induction, the *A. tumefaciens* C58 was employed. The combinations of different concentrations of AS (0, 100 and 200 µM) and glucose (0%, 0.5% and 1.5%) were used according to the above-mentioned methods. The bacterial suspension (2 ml) was harvested 48 h later and was centrifuged at 3000 rpm. The culture medium was removed and RNA was extracted using RNA-extract kit specific to prokaryotes (Roche) based on the manufacturer manual. RNA quantity and integrity were estimated using Nanodrop and agarose gel. cDNA was prepared using total RNA (1 µg), dNTPs, random hexamer primers and MMLV Reverse transcriptase (Thermo Fisher Scientific) following manufacturer's protocol. Real-Time PCR was carried out using Sybr Green florescent (Bio-Rad) based on manufacturer protocol. The expression of *virD2* and *virB2* genes was quantified using 16S RNA expression for normalization. The primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0). The relative expression of RT-qPCR products was determined using the $2^{-\Delta\Delta}$ CT method (Livak and Schmittgen, 2001). Detailed information on primers used in this experiment is listed in Table 1.

Hygromycin resistance leaf assay

Hygromycin resistant gene (*hph*) is a common selectable marker in plant transformation. To prove genetic transformation of seedlings, resistance of detached leaves to hygromycin antibiotic was tested simultaneously in both liquid and solid culture media (Ming-Bo and Waterhouse, 1997). First, some pieces with 3-4 cm in length were prepared from leaf tip. They were then placed in non-sucrose MS medium supplemented with 6-benzylaminopurine (6-BAP; 1 mg/L) and different concentrations of hygromycin (0, 100 and 150 mg/l). The specimens were kept at 25 °C and 16 h photoperiod for 7 days.

Hygromycin resistance seed assay in T_1 genotypes

Following transformation and primary analyses, putative transgenic plants were transferred to the greenhouse. T_0 seeds were collected, disinfected and soaked between filter papers in a petri dish. In this test non-transgenic seeds and putative transgenic T_0 seeds were soaked in a hygromycin solution (30 ppm). Non-transgenic seeds were dampened with demineralized water as a control. Digital images were taken from radicles, and their length was measured by Image J software (http://rsbweb.nih.gov/ij/).

***GUS* Histochemical Assay**

Leaf segments of one cm long were placed in tubes containing 1 ml GUS solution (8.9 ml sterilized water, 1 ml phosphate buffer, 0.5 mM NaPO4, 10 µl Tween 20 and 200 µl x-gluc 2 mM). The tubes were placed in a shaking incubator at 150 rpm and 37 °C overnight. Then, the specimens were decolored using ethanol (100%) till no longer dark green pigments left, and they were kept in 70% ethanol and examined for the presence of cells with blue color (Bratels, 2011).

PCR Reactions

Genomic DNA was extracted from the seedlings using a CTAB based method (Murray and Thompson, 1980). The *gus*-Plus™ and *hph* gene-specific primers were designed using Primer3 program (http://frodo.wi.mit.edu; Table 1). The PCR reactions were set up as follows: 1 µl template DNA (1 µg), 1 µl of each forward and reverse primers (10 pmoles), 1 µl dNTPs (10 mM, 200 µM final concentration of each dNTP), 1.5 µl $MgCl_2$ (50 mM), 10×standard *Taq* reaction buffer and 0.3 µl of 1 unit *Taq* DNA polymerase (Thermo-Scientific) in a final volume of 25 µl. The PCR cycles for *GUS*-Plus

Table 1. The characteristics of primers used in the analysis of integration of T-DNA into rice genomes and the study of *vir* genes induction in *Agrobacterium tumefaciens*.

Primers	Sequences (5´ → 3´)	Tm (°C)	Amplicon length (bps)
F-Gus-Plus	CCG TCC CAA GCA GTT ACA A	55.1	455
R-Gus-Plus	GGT CAC AAC CGA GAT GTC C	55.5	
F-Hyg	GAT GTT GGC GAC CTC GTA T	54.7	450
R-Hyg	GTG CTT GAC ATT GGG GAG T	55.3	
F-virD2	GGA CAG GGC TGG CTG AAG	58.6	173
R-virD2	GCG CAG GGC GTC GTA AT	58.8	
F-virB2	GGC CAG TCA GTC GCT GTTCT	59.8	156
R-virB 2	TGA AGC GCG ACC GAA CAT	57.6	
F-16SRNA	ACC CAT CTC TGC GGA ATA GC	57	154
R-16SRNA	GCT CAT CCA TCC CCG ATA AA	54.5	

and *hph* genes were carried out as follows: 94 °C for 5 min; followed by 30 cycles at 94 °C for 30s, 55 °C for 30s, 72 °C for 40s; plus, a final extension at 72 ºC for 10 min.

Statistical analysis of transformation efficiency

The experiment was performed using two *Agrobacterium* strains, three AS concentrations, three glucose levels and three wheat cultivars. The experiment was performed in 3 technical and 2 biological replications; each treatment contained 50 seeds. The statistical analysis was carried out in a factorial design. Due to the discrete data, non-parametric SPSS method was applied where each factor was defined as a group. The percentage of transformation efficiency (TE) was calculated as follows: The numbers of transgenic seedlings in each treatment/total numbers of inoculated seeds×100.

RESULTS AND DISCUSSION

Analysis of transformation efficiency in needle inoculation method

Seed transformation analysis in non-vacuum condition exhibited a significant difference among AS levels in non-parametric method. The maximum TE (0.55%) was attained in EHA105×200 µM AS combination with Az and S cultivars according to Duncan test at $P<0.05$. High TEs (0.45% and 0.50%) were also obtained using LBA4404×200 µM AS combination with S and Al cultivars (Figure 2). The minimum TEs were observed at 0 µM AS with all combinations of *Agrobacterium* strains and cultivars, where practically no transgenic plants were identified as mentioned by Wise *et al*. (2006). There are limited reports regarding the interaction of *Agrobacterium* strains×cultivars×AS concentrations. According to our results, the compatibility between wheat cultivars and *Agrobacterium* strains depends on wheat cultivar, since *Agrobacterium* strain EHA105 had the highest TE with

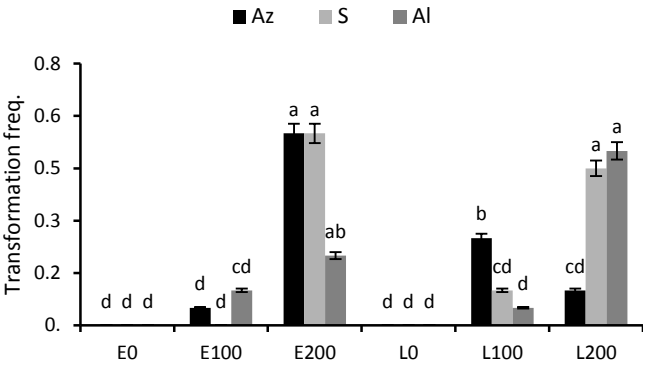

Figure 2. A comparative study of transformation efficiency of wheat cultivars using needle method. The combination of two strains of *Agrobacterium*, three concentrations of AS (0, 100 and 200 µM) and three wheat cultivars was studied. LBA4404 (L) and EHA105 (E) strains of *A. tumefaciens* were used for transformation of Azar (Az) Alvand (AL) and Sardari (S) cultivars.

Az and LBA4404 strain had the highest TE with Al. In both bacterial strains, the results of TE were the same for S cultivar. These results suggest that the TE by the *Agrobacterium* strain can be different depending on the wheat cultivar as reported by Park *et al*. (2021) in their investigation on pepper.

According to the results, TE of all three cultivars was improved as concentration of AS boosted from 100 to 200 µM in both bacterial strains (Figure 2). However, the TE was reduced when AS concentrations increased from 100 to 200 µM in LBA4404×Az combination (Figure 2). Similarly, Gharari *et al*. (2020) reported that TE was improved when AS concentration increased, but, it declined above 100 µM. The relatively low TE in Az cultivar at 200 µM AS (0.2%) might be due to the suppression of virulence genes (Godwin *et al*., 1991) as, no toxic effects on vegetative growth of the bacteria was observed at this specific AS concentration (Gharari *et al.*, 2020). Therefore, it can be deduced that combinations of *Agrobacterium* strains, different concentrations of AS and different cultivars should be studied individually for each case.

However, regarding the cultivars×strain interactions, the average TE in S cultivar was significantly high in both L and H strains (45% and 55%). The highest TE of Al cultivar was recorded when L strain was used (0.5%). The TE in Az cultivar was significantly higher in E×200 AS combination than L×200 AS (0.55% and 0.01%).

Analysis of transformation efficiencies in needle and vacuum inoculation methods

In this part of the experiment, 100 µM AS was used for both needle (N) and vacuum (V) inoculation. Statistical analysis using Duncan test (P<0.05) showed the best combinations of strains and inoculation techniques in turn for S, Az and Al cultivars is EHA×V, LBA×N and LBA×V in which the TEs were 0.25%, 0.4% and 0.25%, respectively. For the remaining treatments, the differences between vacuum and needle inoculation at 100 µM AS were not statistically significant.

As Figure 3 displays, the two best results were obtained when young radicles were inoculated using the vacuum method. In light of the practical advantages of easy handling, low labor costs, and low cost, vacuum might be the best method to try first. In theory, inoculation using vacuum device imposes less physical harm to the growing embryos, tissues and further permeation of bacterium into tissues. Physical treatments, such as vacuum infiltration and wounding are the most important elements for successful infection by *Agrobacterium* (Nanasato and Tabei, 2020). The application of vacuum infiltration to wounded explants or tissues allows *Agrobacterium* to gain access to deeper layers of cells.

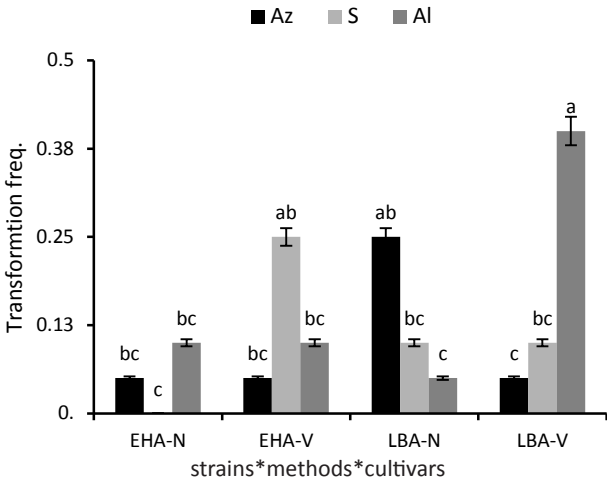

Figure 3. A comparative study of transformation efficiency using needle (N) and vacuum (V) methods. LBA4404 (LBA) and EHA105 (EHA) strains of *A. tumefaciens* used for transformation of Azar (Az) Alvand (Al) and Sardari (S) wheat cultivars.

The results of comparative studies showed that LBA4404 strain performed significantly better than EHA105. There were differences in efficiency of transformation among some combinations of cultivars, inoculation methods, and bacterial strains, but they were not statistically significant (Figure 3).

In another study, the highest TE was obtained when 200 µM AS and vacuum were applied for wheat transformation (Ashrafi-Dehkordi *et al.*, 2021). Additionally, according to Sedaghati *et al*. (2021) and Ishida *et al.* (2015) the highest TEs were obtained at 100 µM AS. Sparks *et al.* (2014) suggest the use of 200 µM to 800 µM AS, and concluded that even though the AS concentration is important, it does not seem to be the key factor to be adjusted. However, some reports indicate no pre-induction or co-cultivation with AS is required (Wroblewski *et al.*, 2005).

Assay of wheat leaves with hygromycin

Leaf specimens from T_0 and T_1 plants were cultured on solid (Figure 4) and liquid (Figure 5) selective MS medium containing 0, 100 and 150 mg/l of hygromycin. The leaf tissues from putative transgenic plants remained green after a week whereas samples from non-transgenic or wild-type plants lost their chlorophyll and turned brown and white in the same period.

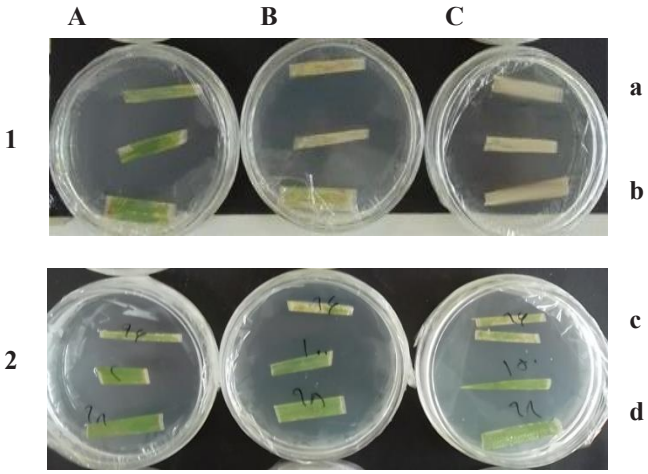

Figure 4. Leaf assay for hygromycin resistance on the solid MS selective medium. **1:** Leaves from non-transgenic and **2:** putative transgenic seedlings were cultured on **A:** 0, **B:** 100 and **C:** 150 mg/L hygromycin. Non-transgenic controls had a noticeable symptom of bleaching 7 days later. The genotypes were considered as **a:** non-transgenic, **b:** transgenic, **c:** with relatively low and **d:** high in expression of *hph* gene.

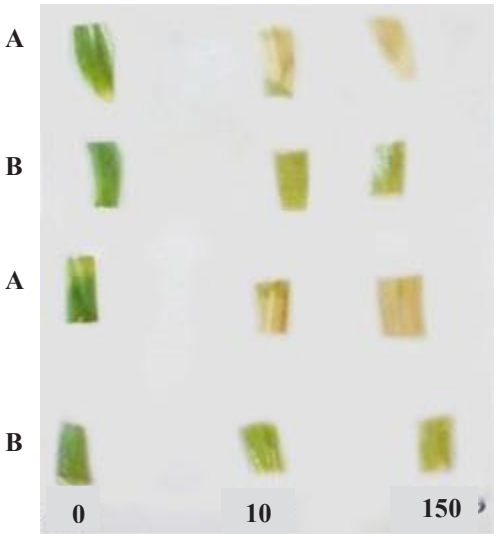

Figure 5. Hygromycin leaf assay on the liquid selective MS medium. Wheat leaves after 7 days on 0, 100 and 150 mg/l hygromycin. **A:** Non-transgenic controls lost the chlorophyll and turned white after 7 days, **B:** while transgenic samples stayed green.

We observed that screening transgenic plants using a solid medium, in addition to easy handling, was relatively straightforward and took a week to complete. However, screening was found more reliable and/or repeatable in the liquid selective medium. Based on these results, hygromycin resistance gene (*hph*) may be a suitable selectable marker for wheat with no detrimental effects on growth and fertility

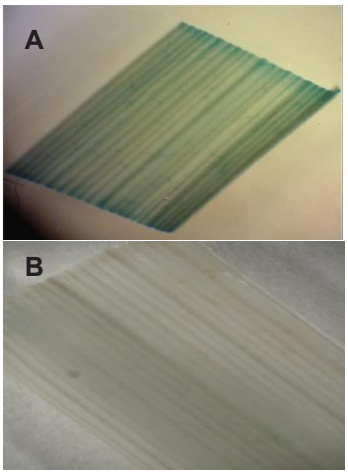

Figure 6. Histochemical GUS expression in wheat leaves. Deposition of blue indigo dye in putative **A:** transgenic, leaves compared with **B:** non-transgenics.

(Pazuki *et al*., 2015). Hygromycin is one of the most popular selection agents applied to wheat genetic transformation system, and the *hph* gene was found to be a more effective selection marker than the *bar* gene (Ortiz *et al.*, 1996).

Histochemical GUS assay

The histochemical GUS test was conducted only on plants with hygromycin positive results. As a result of the test, blue color was developed inside the putative transformed leaf cells (Figure 6). Neither *gus* nor *hph* marker genes had any apparent effect on development and morphology of the transgenic wheat plants as all plants in the greenhouse demonstrated normal morphology and growth behavior (Miki and McHugh, 2004).

β-glucuronidase enzyme is the product of *GUS*-Plus gene that finally hydrolyse X-gal into an insoluble blue compound similar to indigo dye. The test is relatively quick (24 h test) and precise (<1% false negative and <5% false positive). GUS expression indicated the transgene integration in the wheat genome (Jefferson *et al*., 1987, Vitha, 2007).

PCR analysis of the genes

Putative positive transgenic T_0 and T_1 plants for hygromycin and/or GUS were further analyzed using PCR technique. The *hph* gene-specific primer pair used for this study can amplify a 450 gDNA region in transgenic plants (Figure 7).

Using *GUS*-Plus gene-specific primer pair, a 455 bp fragment was amplified in putative transgenic plants (Figure 8).

Two PCR products from each *hph* and *GUS*-Plus genes were randomly selected for sequencing (Bionner, South Korea). The sequencing results confirmed that these two gene-specific primers are suitable for screening of a wheat population using PCR technique.

Hygromycin screening of potential transgenic seeds

The length of radicles in putative T_1 transgenic and non-transgenic seedlings were measured in the presence of hygromycin, 7 days after imbibition. Data variance analysis showed that the simple factor effect was significantly different (P<0.05) between transgenic and non-transgenic seedlings (Figure 9). The effect of hygromycin simple factor and water was significantly different (P<0.01) as well.

The mean length of radicles in putative transgenic seedlings was significantly longer than their length in non-transgenics (6.5 versus 1.7 mm). Therefore, seed imbibition and germination in hygromycin solution provides a simple and fast screening method at an early stage of seedling growth. The test can drastically reduce the size of the screening population in the following generation, which can save time and labor.

The effect of carbohydrates and phenolic compounds on *Vir* gene expression induction

To measure the molecular effects of inducing agents on *vir*B2 and *vir*D2 induction, quantitative Real Time PCR (q-PCR) was employed. The results indicated that *vir*B2 and *vir*D2 relative expression levels at 200 µM AS were 2.8 and 5.68 folds higher than their expression at 0 µM AS treatment (Figure 10). The results are comparable to the TE in the *in planta* experiment, where 200 mM AS resulted in nearly 3-4 times more transgenic plants than a mock treatment.

Regarding the type and concentration of carbohydrates used in this experiment, 1.5% glucose induced *vir*B2 and *vir*D2 up to 3.4 and 3.9 fold compared to their expression at 0 µM glucose in control samples (Figure 11). Induction of *Agrobacterium* strains with 10 mM D-glucose and 100 µM acetosyringone also showed higher rates of efficiency in an *in planta* experiment compared to other treatments in *Dunaliella* (Srinivasan *et al*., 2016).

Following molecular elucidation of *A. tumefaciens* pathogenesis, the bacteria have become an essential tool in agricultural biotechnology. A boundless amount of development has been made to improve the efficiency by which *A. tumefaciens* introduces DNA into the host and expand the host range. Numerous external factors drastically influence *Agrobacterium*-mediated transformation including, tissue type, antioxidant addition (Dutt *et al.*, 2011), duration of agroinfiltration (Xu *et al.*, 2014), method of agroinfiltration

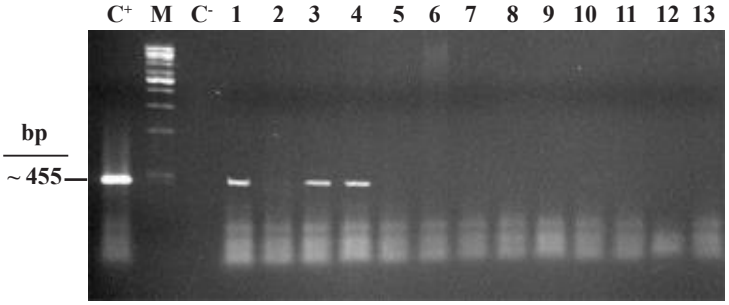

Figure 7. Polymerase chain reaction analysis showing integration of *GUS* coding region (455 bp) in the genome of randomly selected putative transgenic wheat plants. M: DNA size marker; Lanes 1–13: analyzed plants; C^+: pCAMBIAl105.1R as a positive control; C^-: non-transformed plant as a negative control.

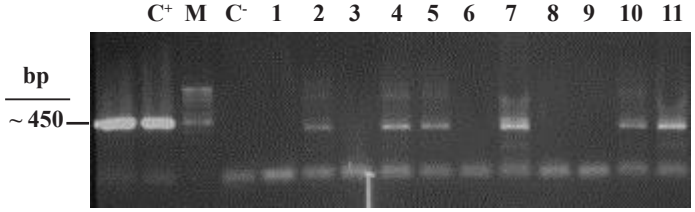

Figure 8. PCR analysis showing integration of *HPH* coding region (450 bp) in the genome of randomly selected putative transgenic wheat plants. M: DNA size marker; Lanes 1–11: analyzed plants; lanes C^+: pCAMBIAl105.1R as a positive control; C^-: non-transformed plant as a negative control.

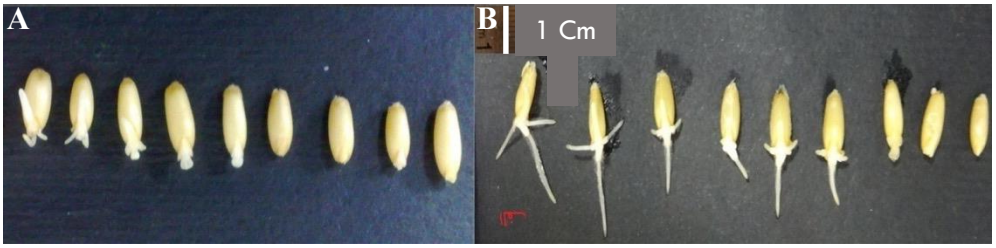

Figure 9. Length of radicles following germination of T_1 putative transgenic seeds on selective medium. in **A:** non-transgenic control and **B:** putative transgenic 7 days after imbibition in 300 ppm hygromycin solution. Transgenic seedlings showed significantly longer radicles than non-transgenic seedlings.

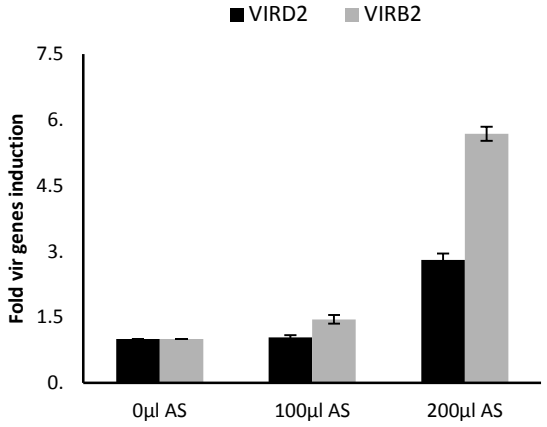

Figure 10. Expression of *Agrobacterium VirB2* and *VirD2* genes in the putative transformed plants treated with different concentrations of acetosyringone (AS). Acetosyringone at 200 µl gave the highest expression compared to the control.

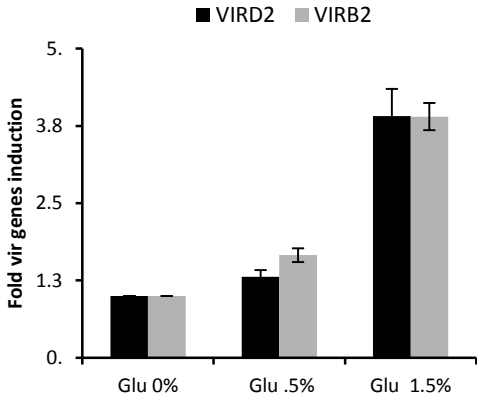

Figure 11. Expression of *Agrobacterium virB2* and *virD2* genes in different concentrations of glucose. The highest induction was obtained at 1.5% glucose compared to control. The induction was achieved at 1.5% glucose compared to control.

(Subramanyam *et al.*, 2011), day-night cycling (Kim *et al.*, 2009), the age of the plant (Chakrabarty *et al.*, 2014) and plant tissue pretreatment (Gurel *et al.*, 2009). However, modifying the pathogenicity of the bacterium has been confirmed extremely valuable in the expansion of the utility of *A. tumefaciens* as a means for both transient expression and stable genetic integration (Thompson *et al.*, 2020). Accordingly, the induction of *vir*B2 and *vir*D2 were considered in the present experiment in order to improve wheat TE, directly measured by means of qRT-PCR and indirectly quantified through analyzing the number of transgenic seedlings.

The *vir* genes are regulated by the key regulator two-component structure VirA (the sensor kinase) and VirG (the response regulator), which are able to incorporate various environmental signals. The VirA sensor is believed to be directly activated by plant-derived phenolic compounds, such as acetosyringone (Raineri *et al.*, 1993) but can indirectly sense plant-derived monosaccharides via the help of the chromosomally encoded periplasmic sugar-binding protein ChvE (Hu *et al.*, 2013) and pH via the chromosomally encoded ChvG/I TCS (Li *et al.*, 2002). These environmental elements provide the induction of the *vir* genes and also to an increase in the copy number of pTi itself.

All of the VirB proteins are essential for the formation of the transport complex and the T pilus (Fullner *et al.*, 1996; Lai *et al.*, 2000). This extracellular assembly is mainly composed of processed VirB2 proteins (Lai *et al.*, 1998). VirD2 is one of the key *A. tumefaciens* proteins involved in T-DNA processing and transfer (Mysore *et al.*, 1989). The synthesis of the T-DNA from the pTi plasmid involves the cleavage of the DNA by VirD2 at the two T-DNA borders. Following the cleavage, VirD2 remains covalently bound to the 5′ end of the single-stranded T-DNA via a phosphotyrosine bond and protects the DNA from exonucleolytic degradation. The NLS domain of VirD2 is important for import of the complex into plant nuclei, and also facilitates the import of T-DNA into plant nuclei *in vitro* (Tinland *et al.*, 1995; Ziemienowicz *et al.*, 1999;

Ziemienowicz *et al.*, 2001). The *vir*B2 and *vir*D2 genes are both inducible, and therefore, their expression might provide clues regarding the improvement of the TE in plant-microbe interaction (Gelvin, 2006).

Some scientists have suggested high glucose concentration (20%) for the induction of *Vir* genes (Wise *et al*., 2006), which was approved in the current experiments. At the same time, inducing *Vir* genes occurs at pH ~5.5 that is the acidity environment around rhizosphere where *Agrobacterium* is active (Fierer and Jackson, 2006).

Regarding target cells or tissues for transgene integration, meristematic cells located in the apical dome of nodal buds have been considered ideal recipients for transgenes because, both growth and development occur in this area. Furthermore, some cell layers of the apex will eventually contribute to the germline and are thus transmitted to the sexual offspring (Hansen and Wright, 1999; Desfeux *et al*., 2000). Though, much still needs to be learned about the mechanism of T-DNA integration in plants (Gelvin, 2017).

Transgenic approaches are required for gene functional studies, crop improvement, and the delivery of components for new breeding technologies such as gene overexpression studies, gene complementation, RNA interference (RNAi), and genome editing based on clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) in wheat (Borrill *et al*., 2019, Smedley *et al*., 2021). As such, a high TE is extremely desirable from a plant scientist's point of view (Hu *et al*., 2017).

Several transformation protocols for wheat have significantly higher TE compared to the current procedure; however, tissue culture is a key component of their techniques (Risacher *et al*., 2009; Hayta *et al*., 2021). The advantages of the current protocol compensate for the higher TE of tissue culture-based methods.

CONCLUSION

The purpose of the current study was to investigate the role of different *vir* genes inducing factors in *Agrobacterium*-mediated *in planta* transformation approach, which is a year round, easy handling, effective selection technique (Mayavan *et al*., 2013; Subramanyam *et al*., 2013). In this regard, the *Agrobacterium* was cultured in two stages with different growth conditions including vegetative and induction phases. As a result, a correlation was found between relative *vir*B2 / *vir*D2 expression and *in planta* TE for different treatments. The treatments have the potential to prolong and promote active cell division (Villemont *et al*., 1997), and might ultimately improve T-DNA integration into plant genomes.

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CONFLICTS OF INTEREST

The authors hereby declare that the study was carried out without any financial and/or commercial relationship that could result in a potential conflict of interest.

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