Pak. J. Bot., 48(6): 2467-2475, 2016.

COMPARISON OF *AGROBACTERIUM* MEDIATED WHEAT AND BARLEY TRANSFORMATION WITH NUCLEOSIDE DIPHOSPHATE KINASE 2 (*NDPK2*) GENE

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Abstract

An efficient and reliable transformation system is imperative for improvement of important crop species like barley and wheat. Wheat transformation is complex due to larger genome size and polyploidy while barley has a limitation of genotypic dependency. The objective of current study was to compare the relative transformation efficiency of wheat and barley using specific expression vector pBRACT 214-*NDPK2* constructed through gateway cloning carrying Nucleoside Diphosphate Kinase 2 (*NDPK2*) gene. The vector was used to compare the transformation response in both crops using immature embryos through *Agrobacterium* mediated transformation. Both wheat and barley showed different responses towards callus induction and regeneration. Immature embryos of 1.5 to 2 mm in diameter was found optimum for wheat callus induction while 1 to 1.5 mm for barley. Both embryogenic and non-embryogenic calli were found in wheat with significantly greater tendency for embryogenecity in barley. The overall regeneration response was found different for all transformed wheat and barley cultivars. Wheat cultivars showed good response initially that drastically slowed down in later stages with the exception of Fielder that reached to the green shoots with good roots. The barley transformed lines showed good regeneration response as compared to wheat. PCR analysis of putative transformants using genomic DNA showed a maximum of 27% transformation efficiency in barely. No true transformation response was obtained in all cultivars of wheat used in this study. The protocol developed for wheat and barley transformation will greatly be helpful in crop improvement programme through genetic engineering especially in diploid relatives of cereals.

Key words: Callogenesis, Embryogenic, *Agrobacterium*, *NDPK2*, Wheat, Barley.

Introduction

The family *Triticeae* includes some of the most economically important crop plants including wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare*). Bread wheat ranked third in world food crop production and makes an essential source for our everyday diet throughout the globe. Barley holds the fourth position among important cereal crops in terms of production. As well as being an important crop in its own right, it is used as a diploid model for the more complex polyploid cereals such as hexaploid wheat. Domestication history of *Triticeae* is thousands years old, therefore, its members contains a complex genetic record (Purugganan & Fuller, 2009). Divergence of wheat and barley is almost 11.6 million years back and from that time continued into species and sub species (Chalupska *et al*., 2008). Both wheat and barley have complex genomes of large size, high repeat contents and complex transposable element structure (Eilam *et al*., 2007; Wicker *et al*., 2011). Wheat, a hexaploid, has a larger genome of ~17 Gb, whereas barley is a diploid and contains genome of ~5 Gb (Pickering & Johnston, 2005).

Both wheat and barley are being investigated for successful and efficient transformation potential. Between the two, barley transformation efficiency is quite promising as compared to the wheat that is more difficult to transform due to low efficiency, genotype dependency and its complex genome (Li *et al*., 2012). Therefore, barley could be used as a model for wheat because of its better transformation efficiency due to less complex and smaller genome as compared to wheat (Harwood, 2011). In this study, the transformation efficiency of both wheat and barley was tested with *NDPK2* a regulatory protein that retains the ability to induce tolerance against multiple abiotic stresses, using *Agrobacterium*-mediated transformation.

An efficient transformation requires the cloning of gene of interest into a suitable plasmid vector (Clough *et al*., 1998; John *et al*., 2014; Abbasi *et al*., 2016). Traditional methods of cloning including DNA restriction and ligation is quite lengthy and less efficient (Goderis *et al*., 2002; Tzfira *et al*., 2005). In recent times, Gateway® cloning technology (Invitrogen Co.) has proven its role for fast and promising cloning that is based on the bacteriophage λ site-specific recombination system (Hartley *et al*., 2000). When compared to conventional cloning methods, this method is more convenient and efficient because it can clone the gene of interest into larger binary vectors (5 to 12 kb) (Curtis & Grossniklaus, 2003) and does not involve either DNA digestion or ligation (Karimi *et al*., 2007; Xu & Li, 2008) that can impede the cloning process. Many Gateway® compatible binary vectors have been made available (Karimi *et al*., 2007; Curtis & Grossniklaus, 2003). In this study plant expression vector carrying *NDPK2* gene under the control of maize ubiquitin promoter with hygromycin resistance gene was constructed using gateway cloning method for barley and wheat transformation.

Materials and Methods

A. Construction of plant expression vector

In order to construct the plant expression vector, full length cDNA (accession no. AF017640) of Nucleoside Diphostase Kinase 2 (*NDPK2*) gene from *Arabidopsis* was used and cloned into the pBRACT 214 expression vector under the control of ubiquitin promoter in which the selectable marker encodes hygromycin resistance using gateway cloning technology (Invitrogen). Plant expression vector was transformed into the AGL1 *Agrobacterium* strain through electroporation. Transformation cassette formed in this study was used for *Agrobacterium* based transformation in barley and wheat.

B. Plant transformation

Plant material and growth conditions: Wheat cultivars Fielder, Siran, Atta Habib, Marvi 2000, MH 97 and barley cultivar Golden Promise were used in the study. Donor plants of both wheat and barley were grown in a controlled environment in growth room at John Innes Center (JIC), Norwich, UK, for the collection of immature embryos. Growth conditions were maintained at 24°C day and 18 °C night temperatures (for wheat) and 24°C day and 18 °C night (for barley), 80% relative humidity and light levels of 500 µmol/m^2 /s^1 at the mature plant canopy level. Light was provided by metal halide lamps (HQI) supplemented with tungsten bulbs. The plants were watered regularly and were not sprayed with any fungicide. Immature embryos were collected when they reached to 1.5 to 2 mm in diameter in wheat while 1 to 1.5 mm for barley (Fig. 2a). The immature embryos were transformed by two protocols of both barley (Adopted from Harwood *et al*., 2009) and wheat under strict aseptic conditions.

Media used in transformation: For media preparation, phytagel was used as gelling agent, prepared at 2x the required concentration and was autoclaved prior to adding into the media. All the tissue culture media was sterilized through steritop 0.22 µm filter except the stocks and were added in double concentration to that of required concentration. After warming at 60^0 C the 2x media and 2x phytagel was mixed well and poured on to the petri plates. During transfer to regeneration and rooting medium, the plants were transferred to either deep plates or glass tubes. For barley and wheat transformation, different but standard plant tissue culture media were used for each stage including inoculation, callus induction, selection and regeneration (Tables 1 and 2).

Growth of *Agrobacterium* tumefaciens strain AGL1: *Agrobacterium* culture was prepared from glycerol stock without any antibiotics. After overnight incubation at 28°C with shaking (200 rpm) the bacteria were collected by centrifugation at 2200 rpm for 10 min at 4^oC. For barley full strength *Agrobacterium* culture was used while for wheat, cells were re-suspended into the inoculation medium and left for 3 hours at room temperature with slight shaking until the desired $OD_{0.4}$ was achieved.

Table 1. Media composition for wheat transformation.

Media	Composition
Liquid inoculation	4.3 g/L MS Basal Salt + 1 ml/L MS vitamins + 10 g Glucose + 0.5 g/L MES + 10 ul Acetosyringone, pH 5.8
Co-cultivation	4.3 g/L MS Basal Salt + 1 mL/L MS vitamins + 10g Glucose + 0.5 g/L MES + 500 ul Acetosyringone + 2000 ul $AgN0_3$ + 2000 ul $CuSO_4$ 5 H_20 + Agarose 8g, pH 5.8
Resting	4.3 g/L MS Basal Salt + 1 ml/L MS vitamins + 0.5 g/L Glutamine + 0.1 g/L Casein Hydrolysate + 0.75 g/L MgCL2-6H20 + 1.95 g/L MES + Maltose 40 g/L + 0.5 mg/L 2,4-D + 2.2 mg/L Picloram + 160 mg/mL Timentin + 0.84 mg/L AgNO3 + 0.100 mg/L Ascorbic Acid + 5 g Agarose, pH 5.8
Selection	4.3 g/L MS Basal Salt + 1 ml/l 100X MS vitamins + 0.5 g/L Glutamine + 0.1 g/L Casein Hydrolysate + 0.75 g MgCl2-6H20 + 1.95 g/L MES + 0.5 mg/L 2,4-D + 0.5 mg/L Picloram +160 mg/mL Timentin + 0.42 mg/L $AgNO_3$ + 0.05 g/L Ascorbic Acid + 40 g/L Maltose + 15 mg/L Hygromycin for Ist selection and 30 mg/L for 2nd selection + 5 g Agarose, pH 5.8
Regeneration	4.3 g/L MS Basal Sal + 10 ml 100X Modified LS vitamins + 20 g/L Sucrose + 0.5 g/L MES+ 0.0025 g/L $CuSO_4.5H_2O$ + 400 ul Zeatin + 160 mg/mL + Timentin + 30 mg/L + 5g Agarose, pH 5.8
Rooting	4.3 g/L MS Basal Salt Mixture (M0221) + 10 ml Modified LS vitamins + 15 g Sucrose + 0.5 g/L MES+ 0.2 mg/L IBA+ 160 mg/mL Timentin + 30 mg/l + 5 g Agarose, pH 5.8

Table 2. Media composition for barley transformation.

Media	Composition
Callus induction	4.3 g/L MS plant salt base + 30 g/L maltose, 1.0 g/L casein hydrolysate + 350 mg/L myoinositol + 690 mg/L proline + 1.0 m g/L thiamine HCl + 2.5 m g/L dicamba + 1.25 m g/L + $CuSO_4$.$5H_2O$ + 3.5 g/L Phytagel. pH 5.8
Transition	2.7 g/L Murashige and Skoog modified plant salt base (without NH_4NO_3) + 20 g/L maltose + 165 mg/L NH_4NO_3 + 750 mg/L glutamine + 100 mg/L myoinositol + 0.4 mg/L thiamine HCl, + 2.5 mg/L 2,4-D, 0.1 mg/L 6-benzylaminopurine (BAP) + 1.25 mg/L $CuSO_4.5H_2O$ + 3.5 g/L Phytagel. pH 5.8
Regeneration	2.7 g/L Murashige and Skoog modified plant salt base (without NH_4NO_3) + 20 g/L maltose + 165 mg/L NH_4NO 3 + 750 mg/L glutamine + 100 mg/L myoinositol, 0.4 mg/L thiamine HCl, 50 mg/L Hygromycin + 60 mg/L Timentin + 3.5 g/L Phytagel. pH 5.8
Selection	2.7 g/L Murashige and Skoog modified plant salt base (without NH_4NO_3) + 20 g/L maltose + 165 mg/L NH_4NO_3 + 750 mg/L glutamine + 100 mg/L myoinositol + 0.4 mg/L thiamine HCl + 3.5 g/L Phytagel , pH 5.8

Sterilization of wheat immature embryos: For sterilization, immature seeds of wheat were treated with 70% ethanol for two minutes followed by three washes in sterile distilled water and 20% sodium hypochlorite stock solution for 7 minutes, followed by a rinse of four changes of sterile distilled water. For sterilization of barley immature embryos a protocol of Harwood *et al*. (2014) was adopted. All the sterilization processes were performed in the laminar flow hood under aseptic conditions.

Transformation procedure for barley and wheat

i. Inoculation and co-cultivation with *Agrobacterium*: Sterilized immature embryos of wheat cultivars were introduced into the inoculum medium containing 200 µM acetosyringone and centrifuged at 14409 rpm at 4°C. The inoculum medium was replaced by the *Agrobacterium* solution and incubated in the dark for 10 min. The explants were taken out and placed on the sterilized filter paper to remove the excessive *Agrobacterium* solution and dry out.

Next, the explants were placed on the co-cultivation medium with the scutellum side down in order to maximize the chances of scutellum contact with the *Agrobacterium*. Further, the medium was also supplemented with 200 uM acetosyringone. This is very important to induce the bacterial vir gene as the cereals are known to be inferior in inducing phenolic compounds. Twenty to twenty five embryos were placed on each 9 cm plate. Following two days co-cultivation, the embryonic axis was removed from the embryo using the fine forceps under microscope (Fig. 2b). Embryos were placed scutellum side down onto resting medium for five days, in dark at 25°C.

In barley for inoculation, 200 µL of full strength *Agrobacterium* culture was dropped onto the surface of about 25 embryos per plate (Fig. 3a). After drying the embryos, the plate was tilted to run excessive Agro solution; the explants were transferred to the callus induction medium with their scutellum side down.

ii. Regeneration and selection: After five days resting period, wheat explants were transferred to first selection medium supplemented with 15 mg/L hygromycin. In addition, the antibiotic timentin was also added at 160 mg/L during all selection stages in deep petri plates and placed in dark for two weeks at 25°C. Viable calli were selected and transferred to a second selection with the same medium but adding the double concentration of 30 mg/L hygromycin for further three weeks in the same conditions as was for the first selection. The calli that survived from the second selection were transferred to the regeneration medium supplemented with 0.04 mg/L of zeatin but with the same level of antibiotics. Onto the regeneration medium the transformed calli were identified by producing green shoots. After two weeks, the plantlets were transferred to the rooting medium supplemented with the 0.2 mg/L indole butyric acid (IBA).

In barley, selection proceeded in three selection steps each with 50 mg/L of hygromycin and 160 mg/L of timentin at two weeks interval. During transfer the entire embryo was shifted and handled as a single experimental unit. After giving the selection pressure of six weeks the explants were transferred to the transition medium for further two weeks. The selected calli were shifted to the regeneration medium. Regeneration medium was supplemented with the same concentration of hygromycin but was devoid of any growth regulators. On the formation of roots and when the shoots attained a height of about 2-3 cm, the plantlets were shifted to the glass tubes in callus induction medium containing the timentin and hygromycin at the same level but devoid of dicamba and growth regulators.

iii. Plantlets transfer to soil: Plantlets of both wheat and barley with strong root systems in hygromycin were considered as transformed. Selected plants were removed from the glass tubes gently with the help of long forceps. The entire medium from the plants was washed away under a running tap and the plants were transferred to the soil in the respective compost of both wheat and barley. In order to make the plants acclimatize, they remained covered with propagators for one week after transfer to soil.

iv. Analysis of transgenic plants by PCR: Genomic DNA extraction was done from the collected samples of both wheat and barley using Extract-N-Amp kit (Sigma-Aldrich, Germany). None transformed wheat and barley DNA was used as a negative control. Putative transformants were detected by PCR screening. PCR amplification was performed as: Initial denaturation at 94°C for 3 min, followed by 33 cycles of denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute, and extension at 72° C for 1 min followed by the final extension for 10 minutes. PCR products were checked on 1% agarose gel by ethidium bromide staining.

Results and Discussion

Wheat and barley transformation: Immature embryos of barley and wheat were transformed with *Agrobacterium* harboring pBRACT 214-*NDPK2* vector (Fig. 1). Both wheat and barley showed different response towards different stages of transformation including callus induction, regeneration and selection. Transformation efficiency in both crops found dependent on many factors including starting material, media composition and the protocols adopted.

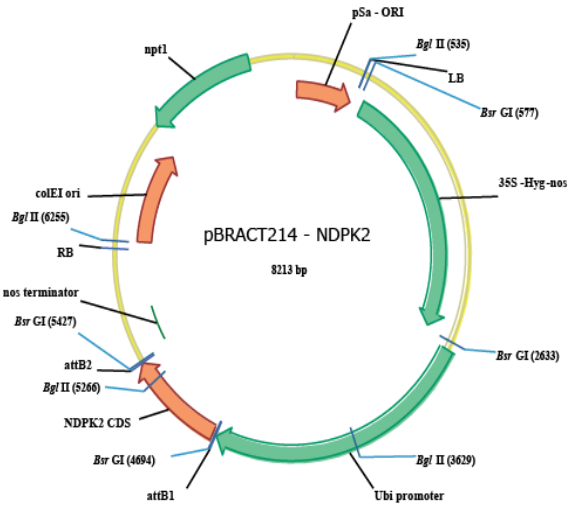

Fig. 1. Schematic representation of pBRACT 214 *NDPK2* vector.

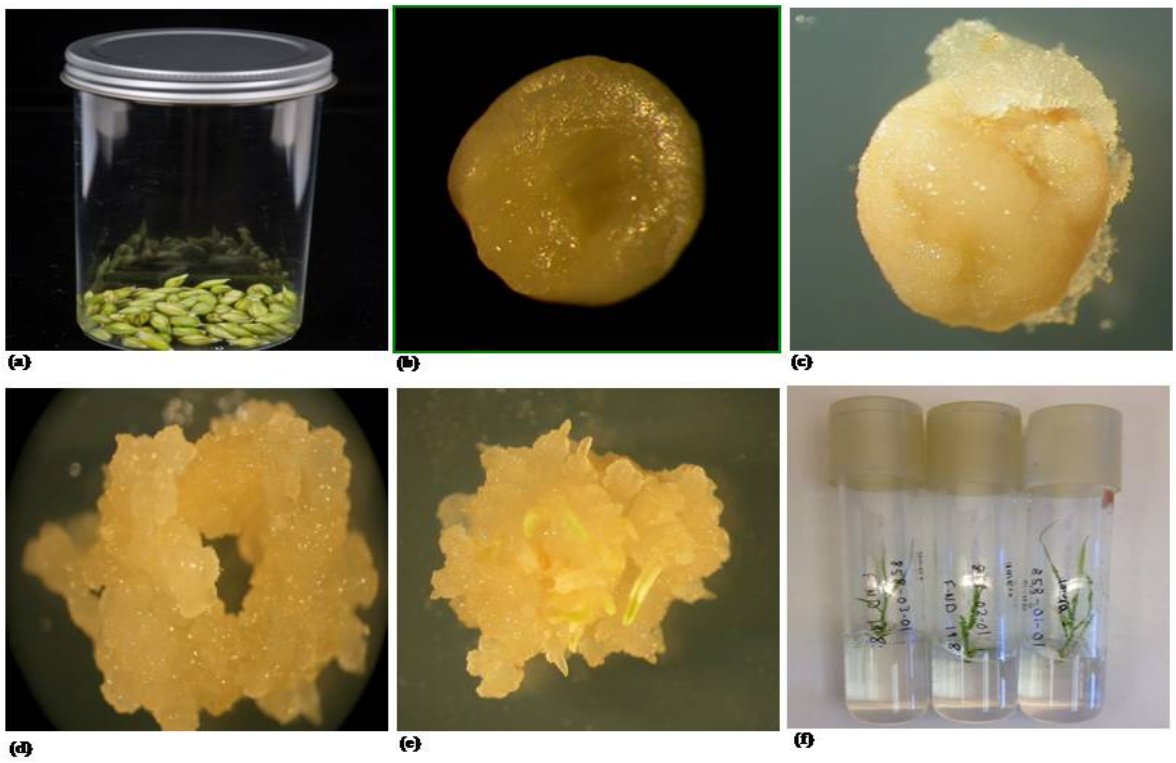

Fig. 2. Different stages of the wheat transformation process. (a) Sterilized wheat seeds before immature embryo isolation (b) an immature embryo after embryonic axis removed (c) Initiation of callus formation on callus induction medium (d) embryogenic callus formation (e) Initiation of green shoots on regeneration medium (f) Plantlet rooting in glass culture before shifting to soil.

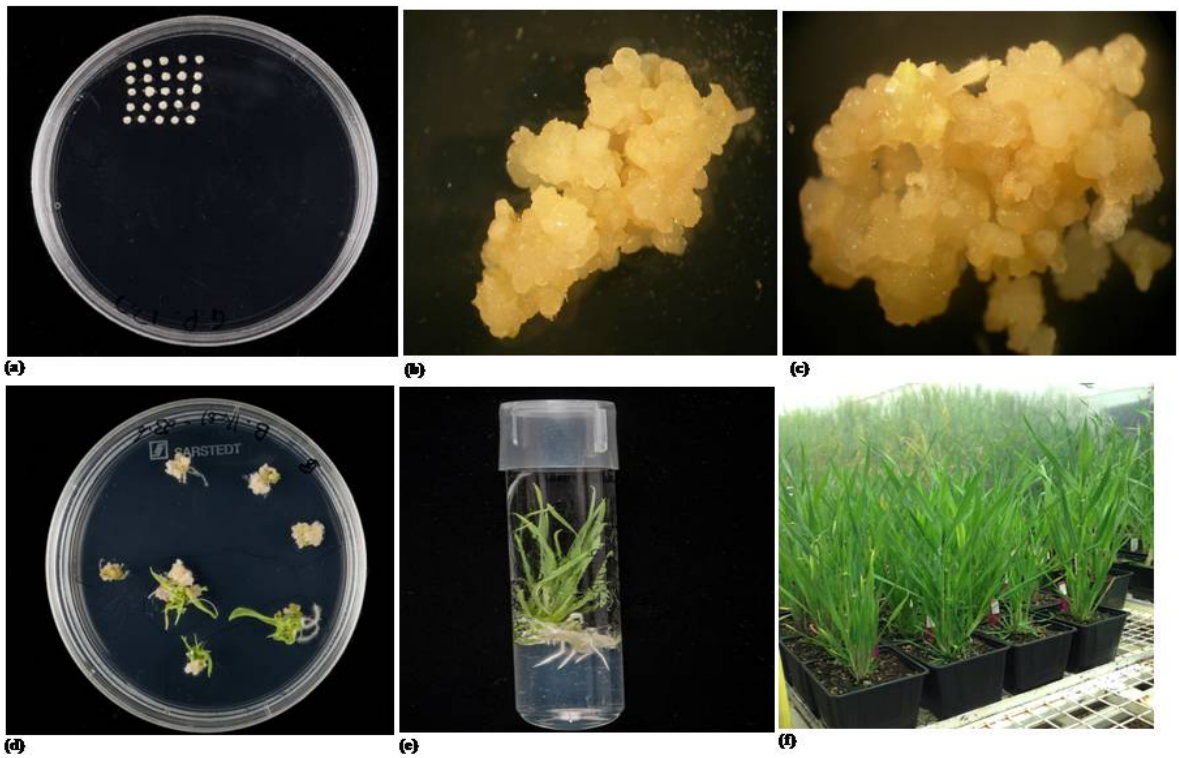

Fig. 3. Different stages of the barley transformation process. (a) Arrangement of Immature for inoculation (b) formation of embryogenic callus (c) the initiation of green spots on callus (d) regeneration of transgenic plantlets on regeneration medium (e) plantlet rooting in glass culture tubes (g) transgenic barley growing to maturity in the glasshouse.

Role of starting material: Immature embryos were used as the explant source. For each individual experiment a minimum of 100 embryos were collected as the starting material for both barley and wheat. Care was taken in selecting the starting material because appropriate size of the explant is of critical importance in inducing the good callus. Embryos smaller than the appropriate size could not induce high quality callus while larger embryos were difficult to handle and also produced poor quality callus. The spikes were isolated at the stage when the immature embryos were 1.5 to 2 mm in diameter for wheat while 1 to 1.5 mm for barley.

Previous studies also emphasized that successful transformation events are based on the availability of the explants with appropriate sizes and age. In wheat most of the transformation experiments the explant choice was mainly the freshly isolated immature embryos or the callus derived from such embryos. First successful wheat transformation also witnessed the use of immature embryos (Cheng *et al*., 1997). From the time onwards other researchers also tested immature embryos for wheat transformation but could not produce the desirable results (Uze *et al*., 2000; Sarker & Biswas, 2002).

There are different types of explants that are in constant use in barley transformation including microspore (Yao *et al*., 1997), shoot meristems tissue (Zhang *et al*., 1999) and mature embryos (Park *et al*., 2006; Um *et al*., 2007; Shah *et al*., 2009) but immature embryos remained as good choice of explant for transformation. The early studies on the both *Agrobacterium*-mediated and biolistic techniques of barley transformation, also reports the successful use of immature embryos as starting material (Wan & Lemaux, 1994; Tingay *et al*., 1997). An improved transformation efficiency in wheat using immature embryos as explants, is based on the fact that such embryos retains higher rate of cell division that ultimately enhance the delivery of T DNA (Cheng *et al*., 1997; Hu *et al*., 2003; Wu *et al.,* 2003) and also positively effects the recovery of the stable transgenic wheat plants.

In addition to the type of explant used another important and determining factor is its age and size. It was investigated that embryos size determines the transfer of T-DNA, transgene expression and regeneration rate as well. The large sized embryos (larger then 2 mm) though shows a marked transient expression but low regeneration rate during wheat transformation (Wu *et al*., 2003). Most of the studies recommend the use of immature embryos isolated in early stage of development i.e. 14 to 16 days of anthesis with a size of 0.8 to 1.5 mm (Jones, 2005). Weir *et al*. (2001) produced desirable transformation results by using the nine days old immature embryos. These reports are in line with the current studies where the immature embryos were collected carefully at specific developmental stage and size for both barley and wheat.

In case of barley the optimum size of the immature embryos was found to be as 1 to 1.5 mm. This development period was found to be the optimum for both calli induction and as well as for the better transformation efficiencies in barley previously (Sharma *et al*., 1995; Caswell *et al*., 2000).

Embryogenic callus induction: Callus induction response was different in both barley and wheat in terms of callus quality and its growth period. Among wheat cultivars, Fielder initiated callus induction during the first week (Fig. 2c) while Siran and Atta Habib took two weeks to initiate the calli induction. Under the microscope, callus growth clearly indicated the embryogenic and non-embryogenic structures in wheat. Embryogenic calli were found to be pale in color smooth and compact structures with globular notches (Fig. 2d and 3b**)**. On the other hand, non-embryogenic calli were found to be of cream in color with watery appearance. Compactness and presence of globular notches or ridged structure are some of the true signs of embryogenic callus formation (Satyavathi *et al*., 2004, Abid *et al*., 2014). At the end of second selection, maximum callus induction was recorded as 52 % in model cultivar Fielder while Atta Habib produced a maximum of 40% similarly 46, 37 and 24 % was recorded for Siran, Marvi 2000 and MH 97 respectively (Table 3).

Barley cultivar Golden Promise showed healthy, compact, nodular and more conspicuous embryogenic notches on callus surface (Fig. 3c). Barley showed a very promising callus induction response with a maximum of 87 % (Table 4). The callus size of barley was also larger as compared to wheat. While among wheat, cv. Fielder showed the great embryogenecity followed by the Siran and Atta Habib. The embryogenic notches turned into green leafy structures in later period in both barley and wheat cultivar (Fig. 2e, 3c). Among wheat cultivars only Fielder showed a maximum appearance of green notches, other Pakistani cultivars though did not produce the green spotting. On the other hand a great trend of embryogenecity was seen in the cv. golden promise of barley (Fig. 3c). Such differences in the observations of callus induction clearly indicate that tissue culture response is greatly dependent upon the genotypes. Similar findings were also seen in the previous studies conducted by Mahmood *et al*. (2012).

Regeneration capability: The more resistant, embryogenic and healthy calli with green notches of both barley and wheat were selected for regeneration. The model cultivar Fielder was among the first cultivar to develop small green shoots on regeneration medium in the first week of regeneration period followed by the cultivar Siran in the late first week (Fig. 2e). While cv. Atta Habib showed good embryogenic callus but did not develop green shoots till the second regeneration week. Regeneration response was found greatly dependent on genotype. Green shoots producing calli was quite low in number as compared to the callus induction frequency. Fielder showed the maximum number of calli producing green shoots in the regeneration medium in comparison with other wheat cultivars (Table 3). Previous reports also observed the genotype dependency of regeneration in wheat (Aydin *et al*., 2011; Khalid *et al*., 2013).

Table 3. *Agrobacterium* mediated transformation of wheat.

Experiment No.	Genotype	No. of embryos inoculated	Callus induction frequency (%)	%Calli showing regeneration potential	Putative transformants	*NDPK2* positive plants
1	Fielder	100	40	11	1	0
2		200	52	16	0	0
3		200	45	22	2	0
1	Atta Habib	100	20	6	0	0
2		200	40	5	0	0
3		100	32	3	0	0
1	Siran	200	32	6	0	0
2		200	46	12	0	0
3		100	39	11	0	0
1	Marvi 2000	150	37	0	0	0
2		150	30	1	0	0
3		150	29	0	0	0
1	MH 97	200	20	5	0	0
2		100	21	0	0	0
3		100	24	2	0	0

Table 4. *Agrobacterium* mediated transfromation of barley.

Exp. No.	Genotype	No. of inoculated embryos	Callus induction freq. (%)	% Calli showing regeneration potential	*NDPK2* positive plants	Total transformation efficiency
01	Golden Promise	150	87%	40	40	27%
02	Golden Promise	100	85%	30	27	27%

Wheat cultivars showed rooting after almost six days of regeneration. Although most of the explant developed roots on regeneration medium, but Pakistani cultivars developed roots rapidly as compared to the Fielder. Out of 100 embryos as the starting stuff set in one individual experiment, only 25 to 30 showed green spots at the early stages of the regeneration, where a only 5 or 6 embryos reached to the shoot and roots stage (Fig. 2f). After two weeks, the green shoots were shifted to the rooting medium, with 0.2 mg/L indole butyric acid with hygromycin concentration of 15 mg/L keeping the timentin concentration the same as that of selection medium. Development of roots prior to shoot formation is not a satisfactory indication for the explants to develop into the plantlets. Siran, Atta Habib and Marvi 2000 though showed a good response till the regeneration medium, but these cultivars in the later stages shed off shoots and developed roots. Among all wheat cultivars, only the model cv. Fielder reached to a stage with green shoots and roots and was selected for shifting to the soil.

On the other hand, transformed lines of barley grew very rapidly on the regeneration medium. Transformed plants were recognized by the presence of strong roots, as good root development in the presence of antibiotic is used to test transgenic plants (Fig. 3e). When the barley shoots attained a height of about 2–3 cm in length and roots were formed, small plantlets were transferred to the culture tubes containing 10 to 12 mL of callus induction medium containing antibiotic at same level as on selection medium but without dicamba and growth regulators. Plants growing well on rooting medium were transferred to barley compost respectively (Fig. 3f). Once plants were established in soil, leaf samples were collected for molecular analysis.

PCR based confirmation of putative transgenic plant: PCR analysis showed that out of 150 embryos of barley from one individual experiment, forty independent putative transformed plants were achieved; this gives a transformation efficiency of 27% (Table 4). Out of forty plants, twenty two were selected for PCR analysis for both the transformed genes *NDPK2* and hygromycin. Seventeen plant samples were found PCR positive for *NDPK2* gene (Fig. 4a and b) while twenty plants samples were found positive for the hygromycin (Fig. 5a and b). In contrast wheat showed no true transformants (Tables 3 and 4).

Barley has been explored for biolistic transformation methods for transfer of useful genes. Previous studies indicated that overall transformation efficiency was higher from the *Agrobacterium* mediated transformation as compared to the other methods of transformation. In addition, transgene silencing was quite frequent in the progeny raised by particle bombardment as compared to the *Agrobacterium* mediated transformation outcomes (Travella *et al*., 2005).

However, Um *et al.* (2007) tested barley transformation with plant expression vector provided with *NDPK2* gene under the control of stress inducible *SWAP2* promoter of sweet potato and *bar* gene as plant selection marker. From this study, a total of 20 plantlets could be regenerated from a 400 immature embryos that were cultured on the selection medium. 0.15% transformation efficiency was achieved from the two barley cultivars that were tested for transformation response. In the current study, regeneration rate was quite higher, 67 independent lines were obtained from a total of 250 and molecular analyses revealed that 27 of 40 plants analyzed, were positive transgenic plants.

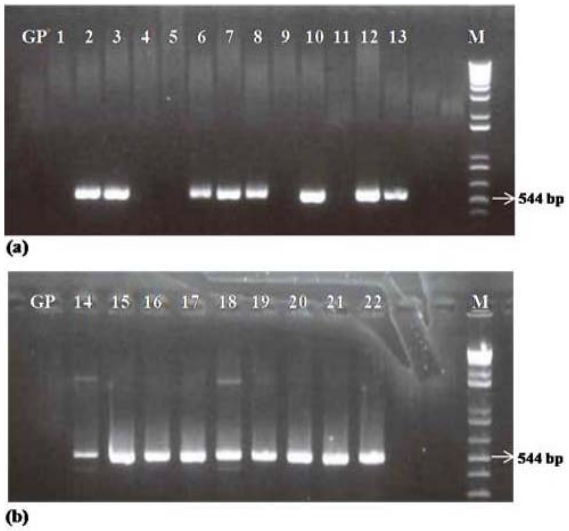

Fig. 4. PCR amplified bands of *NDPK2* gene in barley plants regenerated following *Agrobacterium* inoculation. A negative control (non-transgenic wild type plant DNA of cv Golden Promise (GP), molecular weight markers (M) (Invitrogen Lambda DNA markers, 1 kb plus ladder), and the expected *NDPK2* PCR product (544 kb) are indicated. DNA samples used for the PCR reaction were taken from plants regenerated from immature embryos (a) (Lanes 1-13) and (b) (Lanes 14-22). *NDPK2* positive plants exhibiting bands of expected size.

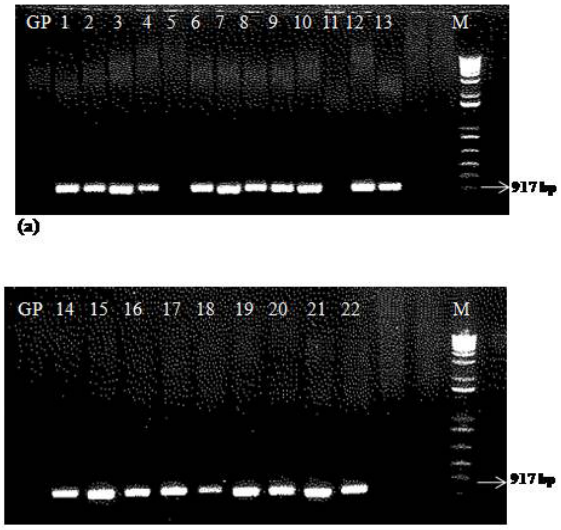

Fig. 5. PCR amplified bands of hygromycin gene in barley plants regenerated following *Agrobacterium* inoculation. A negative (-) control (non-transgenic plant DNA of cv Golden Promise), molecular weight markers (M) (Invitrogen PCR markers 1-kb ladder), and the expected PCR *hph* product (353 bp) are indicated. DNA samples used for PCR reaction were taken from plants regenerated from immature embryos (a) (Lanes 1-13) and (b) (Lanes 14-22). Hygromycin positive plants exhibiting bands of expected size.

The improved transformation efficiency is due to many factors, including transformation method, the protocol and the promoter in the construct. Hygromycin was used in the present study as plant selection marker. The hygromycin selection process was found to be very effective during many cereals transformation, as this selection system allows rare chances of escapes during plant selection (Bartlett *et al.,* 2008). In addition, the promoter used in the current study was of monocot origin that generally gives better transformation efficiency as compared to the driving promoter of dicot origin. Above all, the *Agrobacterium*-mediated transformation was successfully used in the current study to transform gene that was well known for its economic importance in comparison to biolistic transformation in addition to the efficient transformation efficiency.

In case of wheat, five putative transformants were checked through PCR but no true transformants could be recovered. Negative transformation response in wheat can be attributed to many factors. One of the most important factors is that over expression of the targeted gene *NDPK2* may have interfered with the growth of wheat plants in some way that they could not be recovered. Therefore the only plants recovered on regeneration medium were escapes. This is because some of the stress responsive transcription factors pose negative or unwanted side effects in the host plants, like yield loss and retardation in growth (Kasuga *et al*., 1999; Hsieh *et al*., 2002; Zhang *et al*., 2004; Waheed *et al*., 2015). Another possible factor can be the use of selection system. Hygromycin selection system has been explored for barley transformation previously (Harwood *et al*., 2009) and also proved good for selection of barley in the current study as well. On contrary, for wheat, the hygromycin selection though been in constant use (Hauptmann *et al*., 1987) but it is less efficient and plants can still occasionally grow on hygromycin leading to the regeneration of 'escape' plants.

Conclusion

Gateway cloning method proved to be reliable and efficient strategy for vector construction. Transformation rate in barley was promising. The negative wheat transformation may be due to the transcription factor *NDPK2*. The constitutive expression of *NDPK2* might have caused lethal effect on the regeneration. The protocol developed and optimized for wheat and barley transformation will greatly help crop improvement programmes through genetic engineering especially in diploid relatives of cereals.

Acknowledgment

The authors greatly appreciate and acknowledge the contributions of Higher Education Commission (HEC) of Pakistan for generously funding the project under International Research Support Initiative Programme (IRSIP) to accomplish this research at John Innes Center, UK.

References

Abbasi, A.Z., M. Bilal, J. Hussain, M.M. Shah, A. Hassan, S.Y. Kwon, Shafiq-ur-Rehman and R. Ahmad. 2016. Robust regeneration protocol for the *Agrobacterium tumefaciens* mediated transformation of *Solanum tuberosum*. *Pak. J. Bot.*, 48(2): 707-712.

Abid, N., A. Maqbool and K.A. Malik. 2014. Screening commercial wheat (*Triticum aestivum* L.) varieties for *Agrobacterium* mediated transformation ability. *Pak. J. Agri. Sci.,* 51(1): 83-89.

Aydin, M., M. Tosun and K. Haliloglu. 2011. Plant regeneration in wheat mature embryo culture. *Afr. J. of Biotechnol*., 10(70): 15749-15755.

Bartlett, J.G., S.C. Alves, M. Smedley, J.W. Snape and W.A. Harwood. 2008. High-throughput *Agrobacterium*-mediated barley transformation. *Plant Methods*, 4: 22.

Caswell, K.L., N.L. Leung and R.N. Chibbar. 2000. An efficient method for *In vitro* regeneration from immature inflorescence explants of Canadian wheat cultivars. *Plant Cell Tiss. Org. Cult*., 60(1): 69-73.

Chalupska, D., H.Y. Lee, J.D. Faris, A. Evrard, B. Chalhoub and R. Haselkorn. 2008. Hexaploid wheat (*Triticum aestivum* L.) and validation of genome coverage using locus specific primers. *Genome*, 46: 870-878.

Cheng, M., J.E. Fry, S.Z. Pang, H.P. Zhou, C.M. Hironaka, D.R. Duncan, T.W. Conner and Y.C. Wan. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol*., 115: 971-980.

Clough, S.J. and Bent A.F. 1998. Floral dip: A simplified method for *Agobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.,* 16(6): 735-43.

Curtis, M.D. and U.A. Grossniklaus. 2003. Gateway cloning vector set for high throughput functional analysis of genes in planta. *Plant Physiol.,* 133: 462-469.

Eilam, T., Y. Anikster, E. Millet, J. Manisterski, O. Sagi Assif and M. Feldman. 2007. Genome size and genome evolution in diploid *Triticeae* species. *Genome*, 50: 1029-1037.

Goderis, I.J., M.F. De Bolle, I.E. François, P.F. Wouter, W.F. Broekaert and B.P. Cammue. 2002. A set of modular plant transformation vectors allowing flexible insertion of up to six expression units. *Plant Mol. Biol.*, 50(1): 17-27.

Hartley, J.L., G.F. Temple and M.A. Brasch. 2000. DNA cloning using *in vitro* site-specific recombination. *Genome*, 2000, 10: 1788-1795.

Harwood, W.A., J. Bartlett, S. Alves, M. Perry, M. Smedley, N. Leyland and J.W. Snape. 2009. Barley transformation using *Agobacterium*-mediated techniques. In: *Methods Mol. Biol.* (Eds.): H.D. Jones and S.R. Peter. Transgenic Wheat, Barley and Oats, 478(9): 137-147.

Harwood W.A. 2011. Advances and remaining challenges in the transformation of barley and wheat. *J. Exp Bot*., 63: 1791-1798.

Harwood, W.A. 2014. A protocol for high-throughput *Agrobacterium*-mediated barley transformation. *Methods Mol. Biol*., 1099: 251-260.

Hauptmann, R.M., P. Ozias-Akins, V. Vasil, Z. Tabaeizadeh, S.G. Rogers, R. Horsch, I.K. Vasil and R.T. Fraley. 1987. Transient expression of electroporated DNA in monocotyledonous and dicotyledonous species. *Plant Cell Rep*., 6: 265-270.

Hsieh, T.H., J.T. Lee, Y.Y. Charng and M.T. Chan. 2002. Tomato plants ectopically expressing *Arabidopsis CBF1* show enhanced resistance to water deficit stress. *Plant Physiol.,* 130: 618-62.

Hu, P.J. M.B. Isakson, S.G. Lawton, C.B. Metz, D. Rempel, A.P. Ryerson, A.L. Sansone, R.J. Shook, J.M. Starke, S.A. Tichota and Valenti. 2003. Field efficacy assessment of transgenic Roundup Ready wheat. *Crop Sci.,* 43: 1072-1075.

Jones, H.D. 2005. Wheat transformation: current technology and applications to grain development and composition. *J Cereal Sci*., 41: 137-147.

John, E., A. Maqbool and K.A. Malik. 2014. Optimization of *Agrobacterium tumefaciens* mediated transformation in *Populus deltoids*. *Pak. J. Bot*., 46(3): 1079-1086.

Karimi, M., A. Bleys, R. Vanderhaeghen and P. Hilson. 2007. Building blocks for plant gene assembly. *Plant Physiol*., 145: 1183-1191.

Kasuga, M., Q. Liu, S. Miura and K. Yamaguchi-Shinozaki. 1999. Improving plant drought, salt and freezing tolerance by gene transfer of a single stress inducible transcription factor. *Nat. Biotechnol.*, 17: 287-292.

Khalid, M., M. Aarshad, G.M. Ali and A. Razzaq. 2013. Tissue culture responses of some wheat (*Triticum aestivum* L.) cultivars grown in Pakistan. *Pak. J. Bot*., 45(SI): 545-549.

Li, J., X. Ye, B. An, L. Du and H. Xu. 2012. Genetic transformation of wheat: current status and future prospects. *Plant Biotechnol. Rep*., 6: 183-193.

Mahmood, I., A. Razzaq and Z.U. Khan. 2012. Evaluation of tissue culture responses of promising wheat (*Triticum aestivum* L.) cultivars and development of efficient regeneration system. *Pak. J. Bot*., 44(1): 277-284.

Park, T.I., M.O. Um, Y.J. Kim, J.J. Hwang, K.G. Choi and S.J. Yun. 2006. Somatic embryogenesis and plant regeneration in barley (*Hordeum vulgare* L). *J Plant Biotechnol.* 33: 237-242.

Pickering, R.A. and P.W. Morgan. 1985. The influence of temperature on chromosome elimination during embryo development in crosses involving *Hordeum* spp., Wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.). *Theor. Appl. Genet.,* 70: 199-206.

Purugganan, M.D. and D.Q. Fuller. 2009. The nature of selection during plant domestication. *Nature*, 457:843-848.

Sarker, R.H. and A. Biswas. 2002. *In vitro* plantlet regeneration and *Agrobacterium*-mediated genetic transformation of wheat (*Triticum aestivum* L.). *Plant Tissue Cult*., 12: 155-165.

Satyavathi, V.V., P.P. Jauhar, E.M. Elias and B. Rao. 2004. Effects of growth regulators on *in vitro* plant regeneration in durum wheat. *Crop Sci.,* 44: 1839-1846.

Shah, M.M., Q. Khalid, U.W. Khan, S.A.H. Shah, S.H. Shah, A. Hassan and A. Pervez. 2009. Variation in genotypic responses and biochemical analysis of callus induction in cultivated wheat. *Genet. Mol. Res*., 8(3): 783-793.

Sharma, V.K., R. Hansch, R.R. Mendel and J. Schulze. 1995. Comparison of development stages of inflorescence for high frequency plant regeneration in *Triticum aestivum* L. and *Triticum durum* L. Desf. *Plant Cell Rep.,* 15: 227-231.

Tingay, S., D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton and R. Brettell. 1997. *Agrobacterium* tumefaciens-mediated barley transformation. *Plant Journal*, 11: 1369-1376.

Travella S., S.M. Ross J. Harden, C. Everett, J.W. Snape and W.A. Harwood. 2005. A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. *Plant Cell Rep.,* 23:780-789.

Tzfira, T., G.W. Tian, B. Lacroix, S. Vyas, J. Li, Y. Leitner-Dagan, A. Krichevsky, T. Taylor, A. Vainstein and V. Citovsky. 2005. pSAT vectors: a modular series of plasmids for auto fluorescent protein tagging and expression of multiple genes in plants. *Plant Mol. Biol.,* 57: 503-516.

Um, M.O., T.I. Park, Y.J. Kim, H.Y. Seo, J.G. Kim, S.Y. Kwon, S.S. Kwak and D.J. Yun. 2007. Particle bombardment-mediated transformation of Barley with an *Arabidopsis* N*DPK2* cDNA. *Plant Biotechnol Rep*., 1: 71-77.

Uze, M., I. Potrykus and C. Sautte. 2000. Factors influencing T-DNA transfer from *Agrobacterium* to precultured immature wheat embryos (*Triticum aestivum* L.). *Cereal Res. Commun*., 28: 17-23.

Waheed, U.K., R. Ahmed, I. Shahzadi and M.M. Shah. 2015. Some important factors influencing tissue culture response in wheat. *Sarhad J. Agric*., 31: 199-209.

Wan, Y. and P.G. Lemaux. 1994. Generation of large numbers of independently transformed fertile barley plants. *Plant Physiol*., 104: 37-48.

Weir, B., X. Gu, M. Wang, N. Upadhyaya, A.R. Elliott and R.I.S. Brettell. 2001. *Agrobacterium* tumefaciens-mediated transformation of wheat using suspensión cells as a model system and green fluorescent protein as a visual marker. *Funct Plant Biol*., 28: 807-818.

Wicker, T., K.F. Mayer, H. Gundlach, M. Martis, B. Steuernagel, U. Scholz, H. Simkova, M. Kubalakova, F. Choulet and S. Taudien. 2011. Frequent gene movement and pseudo gene evolution is common to the large and complex genomes of wheat, barley and their relatives. *Plant Cell*., 23: 1706-1718.

Wu, H., C. Sparks, B. Amoah and H. Jones. 2003. Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. *Plant Cell Reports*, 21: 659-668.

Xu, R. and Li, Q.Q. 2008. Protocol: Streamline cloning of genes into binary vectors in *Agrobacterium* via the Gateway® TOPO. *Plant Methods*, 4: 4.

Yao, Q.A., E. Simion, M. William, J. Krochko and K.J. Kasha. 1997. Biolistic transformation of haploid isolated microspores of barley (*Hordeum vulgare* L.). *Genome,* 40: 570-581.

Zhang, J.Z., R.A. Creelman and J.K. Zhu. 2004. From laboratory to field: Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiol*., 135: 615-621.

Zhang, S., M.J. Cho, T. Koprek R. Yun, P. Bregitzer, P.G. Lemaux. 1999. Genetic transformation of commercial cultivars of oat (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) using shoot meristematic cultures derived from germinated seedlings. *Plant Cell Rep*., 18: 959-966.

(Received for publication 12 October 2015)