Novel recombinant binary vectors harbouring Basta (bar) gene as a plant selectable marker for genetic transformation of plants

Reham M. Nada¹

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Abstract Genetic transformation is one of the most widely used technique in crop improvement. However, most of the binary vectors used in this technique, especially cloning based, contain antibiotic genes as selection marker that raise serious consumer and environmental concerns; moreover, they could be transferred to non-target hosts with deleterious effects. Therefore, the goal of this study was reconstruction of the widely used pBI121 binary vector by substituting the harmful antibiotic selection marker gene with a less-harmful selection marker, Basta (herbicide resistance gene). The generated vectors were designated as pBI121NB and pBI121CB, in which Basta gene was expressed under the control of Nos or CaMV 35S promoter, respectively. The successful integration of the new inserts into both the vectors was confirmed by PCR, restriction digestion and sequencing. Both these vectors were used in transforming *Arabidopsis*, Egyptian wheat and barley varieties using LBA4404 and GV3101 *Agrobacterium* strains. The surfactant Tween-20 resulted in an efficient transformation and the number of *Arabidopsis* transformants was about 6–9 %. Soaked seeds of wheat and barley were transformed with *Agrobacterium* to introduce the bacteria to the growing shoot apices. The percentage of transgenic lines was around 16–17 and 14–15 % for wheat and barley, respectively. The quantitative studies presented in this work showed that both LBA4404 and GV3101 strains were suitable for transforming Egyptian wheat and barley.

Keywords pBI121CB · pBI121NB · *Arabidopsis* · Wheat · Barley · LBA4404 · GV3101

Abbreviations

BA	Benzyl adenine
CaMV 35S	Cauliflower mosaic virus 35S promoter
DMSO	Dimethyl sulfoxide
DMF	<i>N,N</i> -Dimethyl formamide
LB medium	Luria-Bertani medium
Nos	Nopaline synthase

Introduction

Many binary vectors have been constructed and used to transform plants for improvement of various crop traits. Most of these vectors especially cloning based ones contain antibiotic resistance genes as the selection marker. Proteins related to these genes are continuously produced in the transgenic plants which have raised consumer and environmental concerns (Kuiper et al. 2001). Furthermore, these genes could be transferred to non-target organisms like bacteria (Akbarzadeh et al. 2010). Additionally, some of these antibiotics could inhibit the germination of the transgenic as well as the non-transgenic seeds of some plants when used in the selection media (Abogadallah unpublished data). Therefore, attempts have been made to substitute these genes with less harmful ones like the herbicide Glufosinate (Wang et al. 2006).

Non-selective herbicide Glufosinate (trade name Basta) contains an active compound called phosphinothricin that inhibits the action of glutamine synthase (Leason et al. 1982), disturbs the gas exchange and chlorophyll

✉ Reham M. Nada
rnada@du.edu.eg

¹ Department of Botany, Faculty of Science, Damietta University, New Damietta 34517, Egypt

fluorescence emission and consequently disrupts photosynthesis that could lead to death of plant cells (Merkel et al. 2004). The Basta gene-based selection system brought about an easy and reproducible selection method and it has successfully produced numerous herbicide-resistant transformed crops e.g. oilseed rape (De Block et al. 1989; Kopertekh et al. 2009), sugarcane (Joyce et al. 2010), cassava (Koehorst-van Putten et al. 2012) and tomato (Khuong et al. 2013). Unfortunately, most of the vectors that contain Basta as a plant selectable marker are gateway based vectors (e.g. Rohila et al. 2004; https://www.Arabidopsis.org/abrc/catalog/vector_1.html) while most of the cloning based ones contain antibiotic genes instead. Although gateway based vectors are now widely used, cloning based vector system is still valuable and utilisable in many laboratories.

Plant genetic transformation brought about a revolution in enhancing crops by introducing new traits. Traditional plant transformation comprises gene transfer into the target tissues by either *Agrobacterium* or biolistics and regeneration of the transformed tissues by different regeneration methods (Subramanyam et al. 2015).

Wheat (*Triticum aestivum* L.) and barely (*Hordeum vulgare* L.) are two of the most economical crops all over the world. *Agrobacterium*-mediated transformation of these species mostly depends on tissue culture technique that is time and money consuming. Moreover, somatic mutation or somaclonal variation may occur during in vitro culture and some plants obstinate to regeneration (Supartana et al. 2006). Additionally, transformation of wheat is restricted to narrow range of genotypes (Jones 2005). Supartana et al. (2006) have reported new method for transforming *Triticum aestivum* L. (Var Shiranekomugi) by inoculating *Agrobacterium* into apical meristem of soaked

seeds, from which various organs and leaf primordia will be evolved. In the present study, Egyptian wheat and barley varieties were transformed using this method to evaluate its efficiency in transforming different genotypes of wheat and different species where this method has not been reported for barley transformation.

The objectives of the present study were reconstruction of pBI121 binary vectors harbouring Basta as a selection marker under the control of two different promoters and establishing an efficient and easy method for *Agrobacterium*-mediated transformation of Egyptian wheat and barley varieties.

Materials and methods

Kits and bacterial strains

Restriction enzymes, polymerases, T4 DNA ligase and purification kits used in this study were purchased from Thermo Scientific, USA. *Escherichia coli* TOP 10 bacterial strain was purchased from Life Technologies, USA. *Agrobacterium tumefaciens* LBA4404 was purchased from Takara, Japan.

Construction of pBI121NB binary vector

Nos::Basta fragment was amplified from ctapi.289.gw vector (Rohila et al. 2004) using primer set (Table 1) with PmeI restriction site in the forward and XhoI in the reverse primer. The PCR conditions were adjusted to initial denaturation 95 °C for 5 min and 29 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min and final extension at 72 °C for 5 min. A mixture of *Pfu* and hot start *Taq*

Table 1 Primer sets used in amplification Nos::Basta, CaMV 35S and Basta fragments

Nos::Basta F	<u>AGCTTTGTTTAAACGATCATGAGCGGAGAATTAAG</u>
Nos::Basta R	<u>CCGCTCGAGCTAGGGGTCATCAGATCTCG</u>
CaMV 35S F	<u>AGCTTTGTTTAAACAGATTAGCCTTTTCAATTTTCAG</u>
CaMV 35S R	<u>TTGGCGCGCCCGTGTCTCTCCAAATGAAATG</u>
Basta F	<u>TTGGCGCGCCCATGAGCCCAGAACGACGC</u>
Basta R	<u>CCGCTCGAGCTAGGGGTCATCAGATCTCG</u>
Basta F (wheat)	GCACGGTCAACTTCCGTAC
Basta R (wheat)	CTTCAGCAGGTGGGTGTAG
Basta F (barley)	CATGAGCCCAGAACGACGC
Basta R (barley)	CTAGGGGTCATCAGATCTCG
Adaptor	<u>CGTGGGCCCCGTCCAGCACTAGATCAACGAATTCAG</u> CCTTCGCATCCTGCATTTTCGCCTCGTGGATAGATT CTACTGCACTATTATGTACTATGTATCCATGCAT ATGGTCTCCATTCCTTTGAACATGTCCGCCATA TGTTGCTTCCCGCTCGAG

The synthesized adaptor used in re-ligating the reconstructed vectors. Restriction sites are underlined in each primer. Basta (wheat) primers were used in examining the transformants of wheat. Basta (barley) primers were used in examining the transformants of barley

polymerases in ratio 2:1 was used in the amplification. The amplified fragment was excised from agarose gel and purified using PCR gel extraction kit, about 1 g of purified cassette was digested by PmeI and XhoI restriction enzymes. Nos::Basta fragment was double digested by adding 5 l of each restriction enzyme and the reaction was kept overnight at 37 °C.

After digestion, fragment was purified using PCR extraction column. pBI121 (Chen et al. 2003) was double digested by PmeI and ApaI restriction enzymes. All digested fragments were loaded onto 0.8 % agarose gel to check for complete digestion.

After purification, PmeI:pBI121:ApaI and PmeI:-Nos::Basta:XhoI were ligated using ApaI:adaptor:XhoI fragment. To set up the ligation mixture, the three fragments were mixed in 1:3:3 ratios, respectively. The ligation reaction was kept at 4 °C for 48 h or at 22 °C for 4 h. 5 units of T4 DNA ligase was used instead of 2 units (according to the manufacturer's protocol) for complete ligation.

Construction of pBI121CB

Both of CaMV 35S promoter and Basta gene were amplified from pBI121 (Chen et al. 2003) and ctapi.289.gw (Rohila et al. 2004) vectors, respectively using the primer sets described in Table 1. Forward primer of CaMV 35S was designed with PmeI restriction site and AscI on its reverse primer and Basta forward and reverse primers were designed to have AscI and XhoI restriction sites with the forward and reverse primers, respectively. Amplification conditions using PCR were adjusted as previously mentioned. After purification, CaMV 35S and Basta fragments were double digested by PmeI & AscI and AscI & XhoI restriction enzymes, respectively. In each reaction, 5 l of each restriction enzyme was added and the reactions were kept overnight at 37 °C. pBI121NB was double digested by PmeI and XhoI.

As previously motioned and after column purification of all the digested fragments, the ligation reaction was set up by mixed those fragments in 1:3:3 ratio (vector: CaMV 35S promoter: Basta gene) and kept at 4 °C for 48 h or at 22 °C for 4 h.

Transformation of chemically competent *E. Coli* TOP 10 strain

About 10 µl of the ligated mixtures was added to chemically competent *E. coli* TOP 10 cells (50 µl) and the mixture was incubated for 50 s at 42 °C. After 1 h in shaking incubator (37 °C), *E. Coli* was plated on LB

medium (10 g trypton, 5 g yeast extract, 10 g NaCl and 15 g agar per one litre) containing 50 mg/L kanamycin and incubated overnight at 37 °C.

Examining of positive colonies

The transformant colonies were picked and cultured in liquid LB at 37 °C overnight. Plasmids were isolated from 10 ml of bacterial liquid culture using plasmid extraction kit (Thermo Scientific, USA).

The extracted plasmids were used as templates to amplify either Nos::Basta or CaMV 35S::Basta fragments by PCR. The extracted plasmids were digested by different combinations of restriction enzymes to check the orientations of cassettes, 100–150 ng of the extracted plasmid was restriction digested according to the manufacturer's protocol. The orientation of new cassettes was confirmed by sequencing (Macrogen, Korea).

Transformation of electrocompetent *Agrobacterium tumifaciens* strains

Two different strains of *Agrobacterium* (LBA4404 and GV3101) were used.

The recombinant vectors pBI121NB and pBI121CB were transformed in both the strains using BTX electroporator system.

After transformation, bacterial cells were plated on LB media containing 50 mg/L kanamycin and 50 mg/L rifampicin in addition to 50 mg/L streptomycin for LBA4404 and 25 mg/L gentamycin for GV3101.

As previously, mentioned, the plasmids were extracted from 10 ml bacterial liquid culture and then the purified plasmids were examined for the presence of the ligated fragments by PCR and restriction digestion.

Preparation of bacterial cultures for plant transformation

Two methods were used in preparing *Agrobacterium* cultures for plants transformation.

For the first one, 2 days before plant transformation, about 10 µl of stored glycerol stock of *Agrobacteria* harbouring the new vectors was plated on LB media containing the proper antibiotics and incubated at 28 °C, one colony was picked and cultured for 24 h in liquid LB containing the appropriate antibiotics as pre-inoculum or primary inoculum. Secondary inoculation of the culture was used for plant transformation. Cells were collected by centrifugation at 5000 rpm for 5 min and then washed by 0.5× MS pH 5.8 containing different additives according to the type of transformation as follows:

For transient gene-expression analyses, 0.02 % Tween-20 (V/V), 15 mg/L acetosyringone and 1 % sucrose (W/V) were added.

For *Arabidopsis* floral dip, 1 µl/100 ml BA (100 mg was dissolved in 1 ml 1 N KOH as stock solution), 0.02 % Tween-20 (V/V) and 5 % sucrose (W/V) were added.

For wheat and barley co-cultivation, 15 mg/L acetosyringone was added.

After washing, bacterial cells were re-collected by centrifugation at 5000 rpm for 5 min.

The second method: about 100 µl of *Agrobacterium* glycerol stock was plated in LB medium containing the suitable antibiotics and then incubated for 2–3 days at 28 °C. Bacteria were collected by scraping (Logemann et al. 2006) and washed as previously mentioned and then re-suspended in transformation media as following for final O.D.₆₀₀ = 0.6 and incubated at 28 °C with shaking for about 2 h.

Transient gene-expression analyses

With some modifications, transient gene-expression analyses were carried out according to Li et al. (2009). *Arabidopsis* seeds were surface sterilized in 70 % ethyl alcohol (EtOH) for 1 min followed by one wash with autoclaved, distilled water and then with a mixture of 0.1 % mercuric chloride (HgCl₂) and 0.001 % Tween-20 for 10 min. Thereafter, seeds were washed with autoclaved distilled water for 30 min. Sterilized seeds were germinated in 0.5× MS media pH 6.0 containing 1 % sucrose and 0.6 % agar for 4 days.

Similarly, wheat and barley seeds were surface sterilized but kept in 0.1 % mercuric chloride and 0.1 % Tween-20 for 7 min. Seeds were germinated in 0.5× MS media containing 1 % sucrose and 0.6 % agar for 5 days.

About 30 *Arabidopsis* seedlings (4-days old) and about 10 wheat and barley seedlings (5-days old) were soaked in co-cultivation media prepared by suspending the harvested bacterial cells (from one of the previous mentioned methods) into 0.5× MS media (pH 6.0) containing 0.02 % Tween-20, 1 % sucrose and 15 mg/L acetosyringone. Co-cultivation was carried out for 48 h for *Arabidopsis* seedlings and 6 days for wheat and barley seedlings.

After co-cultivation period, seedlings were washed with 100 mg/L cefatoxime for 1 h and then with distilled autoclaved water for 30 min for the subsequent analyses.

Arabidopsis floral dip

Arabidopsis floral dip was done according to Dehestani et al. 2010 with some modifications. Bacterial cells were re-suspended in infiltration media containing 0.5× MS pH 5.8, 0.02 % Tween-20, 5 % sucrose and 1 µg/mL BA.

Healthy inflorescences were dipped in infiltration media for few seconds with gentle agitation; moreover, inflorescences were painted by bacterial suspension to increase the transformation chances. The transformed plants were wrapped with plastic film and incubated for 24 h at 25 °C in dark condition.

Wheat and barley co-cultivation

Bacterial cells were re-suspended in 0.5× MS media pH 5.8 containing 15 mg/L acetosyringone. Seeds of wheat and barley (seed coat of barely was removed) were surface sterilized using two methods. The first: seeds were sterilized with 70 % EtOH for 1 min and washed with autoclaved distilled water and then with a mixture of 0.1 % mercuric chloride and 0.001 % Tween-20 for 7 min and that followed by several washes of distilled, autoclaved water for 30 min. The second method was similar to the first except for the removal of using 70 % EtOH step. Seeds were soaked in sterile wet cotton and kept in dark for 24 h at 23 °C.

Wheat and barley were transformed according to Supartana et al. 2006. Each seed was pierced with small needle to facilitate the introduction of the *Agrobacterium* cells to the shoot apex. Seeds were co-cultivated in 0.5× MS media pH 5.8 containing 5 % sucrose, 1 % glucose, 0.6 % agar and 15 mg/L acetosyringone for 5 days. Germinated seedlings were washed in 1000 mg/L cefatoxime for 1 h and then in autoclaved distilled water for 30 min and then transplanted in pots containing a mixture of compost and clay (1:1). Pots were wrapped with transparent, plastic film until the emergence of new leaf and kept at 24/22 °C and 8/16 h day/night conditions. Plastic films were then removed and plants were grown under the same conditions.

Selection of transgenic plants

For *Arabidopsis*, seeds of T₀ plants were surface sterilized as previously mentioned and germinated on 0.5× MS medium pH 5.8 containing 7.5 mg/L Basta (Sigma Aldrich). After 15 days, seedlings in which the second leaves emerged were transplanted onto another media containing 6 mg/L Basta until the emergence of the fourth leaf. Seedlings were transplanted in pots contained a mixture of compost and perlite (2:1) and then each pot was covered with transparent, plastic film and kept at 25/22 °C and 16/8 h day/night conditions. Plastic film was removed after the full acclimatization was observed (healthy growth).

For wheat and barley seedlings, one leaf from each transformed plant was painted with 7 mg/L Basta.

Gus histochemical assay

Gus analyses were carried out according to (Li et al. 2009) except X-Gluc was dissolved in DMSO instead of DMF.

Confirmation of positive transgenes by RT-PCR

Total RNA was extracted from 50 mg Basta-resistant wheat and barley leaves using TRI-reagent (Sigma, UK). To prevent DNA contamination, the extracted RNA was treated with DNase I according to the manufacturer's protocol. cDNA was synthesized using RevertAid cDNA synthesis kit according to the manufacturer's protocol. Basta gene was amplified using forward and reverse primers (Table 1); two sets of primers [Basta (wheat) and Basta (barley)] were used to amplify different regions inside the Basta gene. PCR conditions were adjusted to initial denaturation for 3 min at 95 °C and 35 cycles of denaturation at 95 °C for 30 s, annealing at 51.5 °C for 30 s and extension at 72 °C for 50 s and final extension for 5 min at 72 °C. The products were resolved onto 1 % agarose gel stained with ethidium bromide and visualized by gel documentation system UVi-Tec. 18S rRNA was used as internal control.

Statistical analyses

Data was statistically analysed by using Sigma-Plot V 11.0. All the experiments were performed in triplicates.

Results and discussion

Substituting the plant selection marker antibiotic gene with the less-harmful Basta in pBI121 binary vector (Chen et al. 2003) could ease the selection of transgenes and take care of concerns regarding the biosafety problems associated with antibiotic selection marker genes. Moreover, it has been recorded that Basta gene remained stable in the clonal progeny plants (Shu et al. 2005). In the present study, Basta gene was cloned into pBI121 binary vector under the control of Nos or CaMV 35S promoter to generate the new binary vectors pBI121NB and pBI121CB, respectively. Nos promoter is a weak promoter but it is commonly used as a promoter for in most of the plant transformation vectors (Szakasits et al. 2007). However, introducing Basta under the control of a highly constitutive and stronger promoter like CaMV 35S (Guilley et al. 1982; Assaad and Signer 1990; Pobjecky et al. 1990) would lead to an efficient transcription and hence effective selection of transgenic plants especially for a large scale experiment. The restriction sites of the original pBI121 were analysed and discussed previously in Chen et al. (2003). Herein the restriction sites were changed due to addition of new sites as described (Fig. 1a, b).

Although pBI121 is a low copy number plasmid, the methods used in the present study resulted in plasmids (from *E. Coli* or *Agrobacteria* cultures) with good purity and high quantity that were adequate for the subsequent analyses (PCR and restriction digestion). The presence of

Fig. 1 Diagrams show the scheme of the new binary vectors and the restriction enzymes that have only one recognition site. **a** pBI121CB and **b** pBI121NB

Fig. 2 Screening for positive clones from using the extracted plasmids from *E. coli* colonies (C) transformed by pBI121CB or pBI121NB using **a** PCR analyses using different primer pairs, **b** restriction enzyme digestion of pBI121CB with PmeI & AscI and PmeI & XhoI and **c** restriction enzyme digestion of pBI121NB with ApaI & NheI, ApaI & XhoI and NheI & XhoI

Basta, Nos::Basta and CaMV 35S::Basta fragments were confirmed by PCR using different sets of primers (Fig. 2a) and then by restriction digestion. pBI121CB plasmids from *E. coli* cultures were double digested by either PmeI & AscI or PmeI & XhoI and the transformant colony 1 (C1) was found to be positive based on the expected size of the inserts (about 700 or 1300 bp, respectively, Fig. 2b). pBI121NB plasmids were double digested with ApaI & NheI, ApaI & XhoI and NheI & XhoI and the resulted fragments confirmed the clones C1 and C2, (Fig. 2c). In the original pBI121 vector, ApaI restriction enzyme has one recognition site (Chen et al. 2003) but in new pBI121CB or pBI121NB, it has two recognition sites.

Plasmids extracted from *Agrobacterium* colonies (LBA4404 or GV3101 strains) were also checked for positive clones using same methods. After confirmation by PCR, pBI121, pBI121CB and pBI121NB plasmids were double digested with PmeI & XhoI and the expected inserts were found for each one (Fig. 3a, b). For pBI121, one linear band only was detected while two insert bands were

Fig. 3 Screening for positive clones from using the extracted plasmids from *Agrobacterium* colonies (C) transformed by pBI121CB or pBI121NB using **a** PCR analyses using different primer pairs, **b** restriction enzyme digestion of pBI121CB and pBI121NB with PmeI & XhoI restriction enzymes. *L* Extracted plasmid from LBA4404 strain, *G* extracted plasmid from GV3101 strain, *CB* bacteria transformed by pBI121CB vector and *NB* bacteria transformed by pBI121NB vector

observed for the other two vectors because XhoI restriction site is not included in pBI121.

In the present study, FAST technique (Li et al. 2009) was used to examine the reproducibility of pBI121CB and pBI121NB in plant transformation. Tween-20 was added as a surfactant instead of Silwet L-77 in the original method. In agreement with Li et al. (2009), Gus staining was detected in all parts of *Arabidopsis* seedlings except roots but it was detected in all parts of wheat and barley seedlings including roots (Fig. 5c–j). Therefore, this transient expression could be dependent on plant species.

Arabidopsis thaliana is a model plant that is widely used in plant research. Since Bechtold et al. (1993) published the original method for *Agrobacterium*-mediated transformation of *Arabidopsis*, a number of modifications have been suggested (e.g. http://genome-www.stanford.edu/cgi-bin/biosci_Arabidopsis). Two main factors that affect the efficiency of *Arabidopsis* transformation via floral dip method are addition of sugars and surfactant (Clough and Bent 1998). Silwet L-77 is a common surfactant used in *Arabidopsis* transformation but it may not be available in many laboratories. Therefore, Dehestani et al. (2010) have examined the effect of adding other surfactant such as Tween-20 and Triton X-100 on the efficiency of *Arabidopsis* transformation via vacuum infiltration. In

Fig. 4 Basta selection of transformants. **a–d** *Arabidopsis* plants germinated in $0.5 \times$ MS media containing 7.5 mg/L Basta. **e–g** Applying 7 mg/L Basta to wheat leaves using paint-brush methods.

Arrows pointed to transformants that were resistant and able to grow in the presence of Basta and still have green leaves

addition, Das and Joshi (2011) compared between adding Silwet L-77 or Tween-20 on transformation efficiency of *Arabidopsis* and they concluded that both surfactants brought about the same results. Here in the present study, the percentage of transformants (about 6–9 %) was similar to that resulted from using Silwet L-77 in *Arabidopsis* transformation (e.g. <http://www.jove.com/video/1952/floral-dip-transformation-Arabidopsis> thaliana-to-examine-ptso2; Das and Joshi 2011). Therefore, this result proved that Tween-20 could successfully be used as an alternative to Silwet L-77 in floral dip transformation of *Arabidopsis*.

According to Basta kill curve (data not shown), 7.5 mg/L Basta was the best concentration for selection of transformants. This concentration may differ from lab to another according to growth conditions (e.g. Tian et al. 2015; Wang et al. 2006). Resistant seedlings that were able to produce the second leaf (Fig. 4a–d) were transplanted to another media containing 6 mg/L Basta to reduce the negative effect of Basta and enhance the healthy growth of transformants.

Gus stain is a reliable indicator for the efficiency of transformation for *Arabidopsis* seedlings (T1) transformed with LBA4404 or GV3101 *Agrobacterium* strains (Fig. 5a, b, respectively).

Wheat and barley are two of the most important crops all over the world. Transformation of these species is usually based on tissue culture technique that is time and

money consuming (Supartana et al. 2006). Establishing new method will increase the opportunity to transform these species. In the present study, soaked seeds of wheat and barley were inoculated with small needle to introduce *Agrobacterium* harbouring pBI121CB or pBI121NB to shoot apices from which many organs and primordia will emerge (Supartana et al. 2006). Co-cultivation was done for 5 days to increase the chance and efficiency of transformation (Fig. 6). After painting the leaf with 7 mg/L Basta, about 16–17 and 14–15 % from wheat and barley T₀ plants, respectively showed resistance to Basta compared to non-transformed or wild type plants whose painted leaves exhibited chlorosis and died after 4 days from painting (Fig. 4e–h). Brush-painting method is a simple and cheap method and it was previously used to detect the hygromycin-resistant plants (Li et al. 2009). RT-PCR with specific primers for Basta gene showed that the above resistant wheat or barley leaves amplified Basta fragment with right size (Fig. 7a, b), whereas this fragment was not detected either in wild type or in non-transformed plants. Different primer sets were used to amplify different regions of Basta gene from wheat or barley leaves to realize the highest specificity. For wheat, one band was detected in transgenic plants but it was not observed in non-transformed or wild type ones (Fig. 7a). For barley plants, the primer set amplified two bands; one of them was only detected in the transgenic plants (the above arrow, Fig. 7b).

Fig. 5 Gus-staining analyses. **a** and **b** Gus-staining analyses for *Arabidopsis* transformants resulted from floral dipping technique; **a** *Arabidopsis* transformed with LBA4404 bacterial strain and **b** *Arabidopsis* transformed with GV3101 bacterial strain. **c–j** Gus-staining analyses for transformants resulted from FAST technique; **c–f** represents transgenic *Arabidopsis* seedlings transformed by **c** GV3101::pBI121CB, **d** LBA4404::pBI121CB, **e** GV3101::pBI121NB and **f** LBA4404::pBI121NB. **g–j** represents wheat and barley seedlings transformed by **g** GV3101::pBI121CB, **h** LBA4404::pBI121CB. **i** and **j** represent magnified parts from wheat and barley

Wheat and barley T1 plants were examined as well and the result was similar to those recorded by Supartana et al. (2006).

Many studies have examined the effect of different disinfectants including ethanol on the germination of

different plant seeds (e.g. Miyoshi and Sato 1997; Piernas and Guiraud 1997; Barampuram et al. 2014). Most researches used 70 % EtOH in surface-sterilizing seeds (e.g. Sretenović-Rajičić et al. 2004; Supartana et al. 2006; Kumari et al. 2016) but others warned the usage of ethanol

as it might inhibit seed germination of many plant species (personal communication). In the present study, using 70 % EtOH in sterilization of wheat and barley seeds did not affect the percentage of germination (Fig. 8a) and that could be attributed to the elimination of the negative effect of EtOH by washing the seeds with sterilized, distilled water.

The match between the host and the bacterial stains could be important for efficient transformation (Clough and

Bent 1998). Here in the present study, both LBA4404 and GV3101 were successfully transform Egyptian wheat and barley varieties.

Logemann et al. (2006) have improved methods to eliminate the risk of contamination, to reduce the amount of chemicals required and to decrease the time consumed during preparing large volumes of bacterial cultures needed for *Arabidopsis* transformation via floral dip method. In the present study, this method was used (see “Materials and methods” section, second method for preparing bacterial culture) for the same reasons but with some modification in which the collected *Agrobacterium* cells were washed twice with 0.5× MS media to remove antibiotic contaminants that may affect plant development. This method was used in not only *Arabidopsis* transformation but also in wheat and barley transformation and the efficiency obtained from this simplified method was similar to that resulted from the usual method.

Nowadays, plant transformation technology plays a vital role in modern agriculture. Therefore, constructing new vectors that provide an efficient system for selecting the transformants and establishing simplified and easy transformation techniques for different plant species would improve the application of this approach. The present study represents the reconstruction of pBI121 binary vector for harbouring Basta as a plant selectable marker. Basta gene was introduced under the control of Nos or CaMV 35S promoter in two new vectors designated as pBI121NB and pBI121CB, respectively. These vectors were used in the transformation of *Arabidopsis*, Egyptian wheat, and barley varieties with some vital modifications that were added to

Fig. 6 Co-cultivation of wheat and barley with *Agrobacterium* cells for 5 days. **a** Soaked seeds of wheat and barley co-cultivated with GV3101::pBI121NB and **b** Soaked seeds of wheat and barley co-cultivated with LBA4404::pBI121CB

Fig. 7 RT-PCR analyses for transformed T_0 lines of wheat and barley using Basta forward and reverse primers. **a** RT-PCR for wheat transformants, faint band represents Basta gene (top arrow) and **b** RT-PCR for barley transformants, faint band represents Basta gene (top arrow). *18S rRNA* was used as internal control for both varieties

Fig. 8 a The percentage of germinated seed of wheat and barley after sterilizing with HgCl_2 only for 7 min or with 70 % EtOH for 1 min followed by HgCl_2 for 7 min. **b** The effect of different *Agrobacterium* strains in transformation efficiency of *Arabidopsis*, wheat and barley

the usual techniques. The success of transformations was confirmed by the data obtained from Gus-staining and RT-PCR analyses.

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