VECTOR CONSTRUCTION STRATEGIES FOR TRANSFORMATION OF WHEAT PLANT

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ABSTRACT

Gene cloning and vector construction for plant genetic transformation are routine procedures in plant functional genomic studies. The availability of effective transformation vector is one of the pre-requisites for plant transformation studies. Here, we describe the construction of a series of transformation vectors through different genetic engineering techniques for wheat transformation. For this purpose, NAC-type transcription factor genes *TaNAC69-1*and*TtNAM-B2* were isolated from *T. aestivum* and *T. turgidum*, respectively. Then they were cloned into different types of cloning and expression vectors. Besides traditional restriction enzyme digestion and ligation method, Gateway cloning technology which is a fast and reliable alternative cloning method were used for construction of wheat transformation vectors. The transformation vectors constructed in this study are suitable for use in both particle bombardment (biolistic) and *Agrobacterium* based transformation protocols for wheat plant.

Keywords: Traditional gene cloning, Gatewaygene cloning, Vector construction, Wheat transformation

Abbreviations: NAC (NAM, ATAF1/2, CUC2); NAM (No Apical Meristem); *Pfu(Pyrococcusfuriosus);* NCBI (National Center for Biotechnology Information); BLAST (Basic Local Alignment Search Tool); *GUS* gene (Beta-glucuronidase gene)

INTRODUCTION

One of the most important grain crop cultivated in the world is wheat, providing greatest part of the daily nutritional requirement for human diet. Annual world production of wheat was around 685 million tons in 2009. In order to meet the growing need for wheat, production should be raised to an annual rate of 2%, without any additional land (Patnaik, 2001). Because of being a slow process, conventional plant breeding strategies have failed to fulfill this demand. So, application of recombinant DNA technology and its allied disciplines certainly hold a great promise to increase wheat production (Razzaq *et al.,* 2004).

Gene or DNA cloning is a basic technique in genetic engineering and used for getting a large number of copies of specific DNA fragment, recovering large quantity of protein produced by the concerned gene, production of genomic DNA and cDNA libraries and DNA sequencing studies. In molecular cloning studies, gene of interest or DNA fragment can be copied from both genomic DNA and complementary DNA (cDNA). In recent year, bases of DNA fragments or concerned gene are artificially synthesized using chemical agents. Gene cloning into plasmid vectors involves several steps. Digestion of DNA and plasmid vector with same restriction enzyme is initial step of cloning. Then, ligation of digested products is done using ligation enzymes. Finally, ligation product is transformed into competent *E.coli* cells via different transformation methods and selection and screening of desired recombinant is performed.

Among the gene cloning methods, sticky-ended cloning is the most efficient and commonly used method (Conze *et al.,* 2009). Generally, to produce sticky or complementary ends for sticky-end DNA cloning, insert DNA and vector are separately cut with same restriction endonuclease enzymes. Then, insert DNA is ligated into plasmid vector by DNA ligase. Although this method works efficiently, shortage of suitable restriction sites either in plasmid vector or in insert DNA limits the usage of this method (An *et al.,* 2010). Blunt-end DNA cloning is another cloning technique which introduces the inserting of blunt-ended DNA or 5'-end-phosphorylated PCR product into a linearized and blunt-ended vector (Upcroft *et al.,* 1987). The limitations of blunt-end cloning are none-directional ligation and self-ligation of vector. Therefore, compared to sticky-end cloning, it is less efficient. Because of these disadvantages, blunt-end cloning should not be preferred for cloning of very large DNA fragments or constructing of comprehensive mutant libraries (An *et al.,* 2010). Generally, ligation reactions are affected from different parameters including temperature, components of buffer, ratio of DNA insert

and plasmid vector. To obtain optimal ligation condition for blunt-ended DNA ligation, several studies were done (Liu and Schwartz 1992; Costa and Weiner 1994). However, the efficiency of ligation reaction is still low and a significant improvement should be very necessary. TA cloning is achieved by *Taq* DNA polymerase which has non-template-dependent terminal transferase activity which helps adding of a single deoxyadenosine (A) to the 3 ends of PCR products. As a result, the PCR product can be directly cloned into a linearized T-vector that has single base 3'-T overhangs on each end (Zhou *et al.,* 1995). Despite of widely used, the major drawback of cloning is not directional, because overhanging T and A bases are flanked each sites of linearized vector. So, DNA or PCR product is inserted in linearized T-vector for both orientations. Gateway® Technology, developed by Invitrogen, is a universal cloning method which uses the site-specific recombination properties of bacteriophage lambda (Landy, 1989). This cloning method allows transferring of DNA fragment into different cloning vectors without using restriction endonucleases and ligase. Orientation of DNA insert and open reading frame are maintained because of the site-specific recombination properties of Gateway® Technology. Once DNA insert has entered the Gateway system, it can be transferred other Gateway vectors which provides functional analysis, protein expression, and cloning and subcloning of DNA segments.

Plant growth, development and productivity are adversely affected from environmental stresses, resulting in reduction of average yields for most of economically important crop plants. Any conventional, biotechnological or 'omics' technologies have therefore concentrated on increasing the grain yield, quality characteristics and minimizing crop loss coming from biotic and abiotic stress conditions. *TaNAC69-1* gene provides salt, drought and cold tolerance for plants. On the other hand, *TtNAMB-2* gene greatly influences grain protein content, Fe, and Zn concentrations in wheat plant. Nowadays, wheat transformation still presents more difficulties than transformation of other cereals, such as rice and maize, with lower transformation efficiencies and greater genotype dependence (Shewry and Jones 2005). The most used genetic transformation methods for wheat transformation are particle bombardment and *Agrobacterium* mediated. In this study, different gene cloning strategies were shown to be used for wheat transformation methods. For this purpose, *TaNAC69-1* and *TtNAM-B2* genes were isolated from *T. aestivum* and *T. turgidum*, respectively. Then they were cloned into different types of cloning and expression vectors.

MATERIALS AND METHODS

Isolation of *TaNAC69-1* and *TtNAM-B2* genes: The seeds of wheat cultivars, Kızıltan-91 (*T. turgidum*) and Yüre ir-89 (*T. aestivum*) were provided by Turkish Ministry of Agriculture and Rural Affairs, Central Research Institute for Field Crops, Ankara. Seeds were germinated in plastic pots and irrigated with ½ strength Hoagland`s solution in a growth chamber with 16 h light period at 25 °C for 14 days. Salt stress treatment was performed to increase *TaNAC69-1* and *TtNAMB-2* genes expression levels. So, after the 14th day of growth, salt stress was initiated with ½ strength Hoagland`s solution containing 200 mM of NaCl and kept for 5 days. Total RNA was extracted from leaf tissues according to the Qiagen R Neasy Plant Mini Kit procedure. cDNAs were synthesized from RNA samples according to the manual of Revert Aid™ First Strand cDNA Synthesis Kits from Fermentas. *TaNAC69-1* and *TtNAM-B2* genes were amplified using wheat cDNA samples. *Pfu* DNA Polymerase (Fermentas, Cornaredo, Italy) which is highly thermo stable polymerase was used for amplification of genes for all of the cloning studies. Two types of gene specific primers for each gene (Table 1) were designed based on cloning vectors used in cloning reactions of *TaNAC69-1* and *TtNAMB-2* genes in different expression vectors. Specificity of designed primers was checked in NCBI/Primer-BLAST web tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Cloning of *TaNAC69-1* and *TtNAM-B2* genes into Cloning Vectors: Different cloning strategies were performed to clone *TaNAC69-1* and *TtNAMB-2* genes into expression vectors. The first approach contains using restriction enzyme treatment of both genes and vectors. PCR products of *TaNAC69-1* and *TtNAM-B2* genes were cloned into blunt sides of pJET1.2/blunt cloning vector (Fig. 1). For sticky-end cloning, pJET1.2/blunt vector containing *TaNAC69-1* and *TtNAM-B2* genes and pORE-E3 binary vector were treated with *Not* I and *Cla* I restriction enzymes. So, genes and linear form of pORE-E3 binary vector carried *Not* I and *Cla* I restriction enzyme sites on 5` and 3`ends, respectively. Through the ligation reaction, they were combined each other. For blunt-end cloning, pORE-E3 binary vector carried *TaNAC69-1* and *TtNAM-B2* genes and pAHC25 monocot expression vector were cut with *Sma* I and *Sac* Irestriction enzymes. However only 5` end of genes cut with *S ma* I enzyme produced blunt end. 3` end of both genes and pAHC25 vector were also shifted from sticky end to blunt end. Finally, ligation reaction was performed to ligate genes into pAHC25 vector. The second approach contains TOPO® Cloning and Gateway® Cloning strategies. For TOPO® Cloning, *TaNAC69-1* and *TtNAM-B2* genes were firstly cloned into pENTR™/D-TOPO vector. It enables directional cloning of double-strand DNA using TOPO-charged oligonucleotides by adding 3` overhang to the incoming DNA (Cheng and Shuman, 2000). In this system, forward primer contains CACC which provides directionally cloning of PCR product into

pENTR™/D-TOPO vector which has GTGG overhang. So, PCR product is stabilized in the correct orientation (Fig. 2).Gateway cloning is based on homolog recombination of appropriate Gateway-compatible recombination sites (e.g. attL1 and attL2). It involves a two-step process including cloning of genes into an entry vector and LR reaction between the entry clone and the destination vector. Through the LR reaction between attL and attR sites, *TaNAC69-1* and *TtNAM-B2* genes were transferred into homolog recombination sites of pIPKb002 expression vector.

Detection of Recombinants: To analyze the presence and orientation of the DNA insert into recombinant clones, some methods including colony PCR, restriction analysis and sequencing were performed depending on the purpose. The colony PCR method was firstly applied to detection of recombinants. Individual colony was picked and resuspended in 20 µl of the PCR master mix. PCR was performed as; 95°C, 3 min; 94°C, 30 s, 55°C, 30 s, 72°C 1 min/kb; 25 cycles. Also, restriction analysis was performed using appropriate restriction endonuclease enzymes. Plasmid DNA was isolated from an overnight bacterial culture and cut with restriction endonucleases which found on the map of cloning vector. If the colony carries right orientation of the DNA insert, plasmid was sequenced with forward and reverse sequencing primers.

Molecular Genetic Methods: Rapid DNA Ligation Kit from Fermentas (Cornaredo, Italy) was used for ligation reactions. Concentration of the plasmid and insert DNA was determined by using Alpha Spect µL spectrophotometer. For successful ligation reaction, ratio of plasmid and insert DNA is crucial and should be adjusted to 1:4. So, ligation reaction mixture contains 1:4 ratio of plasmid and inserts DNA, T4 DNA Ligase (5u/µl), 5X Rapid Ligation Buffer and nuclease-free water. The ligation mixture was incubated at 22°C for 1 hour. Ligation products were added into 100 µl of competent *E.coli* cells which thawed on ice. This cell and DNA mixture was incubated on ice for 30 minutes for cold treatment. Then a heat shock at 42°C for 90 seconds was applied to facilitate the entrance of ligation products or plasmids into the cells. The mixture was immediately placed on ice and incubated for 5 minutes. Then liquid SOC medium was added on bacteria cell suspension and grown at 37°C for 60 minutes. Finally, the bacteria was plated on pre-warmed LB medium with appropriate antibiotics and incubated overnight at 37°C.

RESULTS AND DISCUSSION

Cloning of NAC Type Transcription Factors in Cloning Vectors: To indicate different types of gene cloning techniques, two NAC type transcription factor genes *TaNAC69-1* and *TtNAM-B2* were firstly isolated from *T. aestivum* and *T. turgidum*, respectively. The full length of gene amplicons were then cloned into cloning vectors, pJET1.2/blunt and pENTR™/D-TOPO® cloning vectors which were used for conventional cloning and Gateway cloning studies, respectively. In order to show each gene insertions into cloning vectors, colony PCR and restriction digestion of cloning vectors were performed. pJET1.2/blunt cloning vector contains *Bgl*II sites that flank the vector to simplify excision of the cloned PCR product. Restriction digestion with *Bgl*II was performed on the pJET1.2/blunt cloning vector to show integration of *TaNAC69-1* and *TtNAM-B2* genes. Empty vectors and inserts were visualized in Fig. 3a. Insertions in pENTR™/D-TOPO® was also checked by colony PCR using gene specific primers to indicate *TaNAC69-1* and *TtNAM-B2* genes insert. The amplification products were visualized on a 1% agarose gel and indicated in Fig. 3b. In addition to these molecular detection methods, sequence analyses of the promising colonies were also performed using M13 primers. Nucleotide blast from NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for sequence comparison. According to NCBI results, *TaNAC69-1* gene insert fully matched with previously known sequence of *NAC* gene (Genebankaccesion number: AY625682). *TtNAM-B2* gene insert was also identical to *NAM* gene (Uauy *et al.,* 2006) (Genebankaccesion number: DQ869676). Sequence analysis of positive colonies showed that an error free full-length *TaNAC69-1* and *TtNAM-B2* genes were successfully cloned into cloning vectors and used for construction of different plant transformation vectors.

Vector Constructionfor Sticky and Blunt end Cloning: *TaNAC69-1* and *TtNAM-B2* genes from pJET1.2/blunt cloning vector were transferred into binary vector, pORE-E3. After digestion of both plasmids with *Cla* I and *Not* I restriction enzymes, pORE-E3 binary vector became a linear plasmid form and *TaNAC69-1* and *TtNAM-B2* genes had sticky ends that are compatible with pORE-E3 binary vector (Fig. 4). Ligation of binary vector with *TaNAC69-1* and *TtNAM-B2* genes was achieved using T4 DNA ligase. Ligation products belonging to both genes were transferred into competent *E.coli*.Colony PCR was performed to check positive colonies.

    TaNAC69-1 and *TtNAM-B2* genes from pORE-E3 expression vector were transferred into expression vector, pAHC25. pAHC25 (Christensen and Quail 1996) was designed as a monocot expression vector for particle bombardment transformation. It contains both a selectable marker *bar* gene (*hpt*) and a scorable marker *GUS* gene (*uid A*), each under the transcriptional control of a separate Ubi-1 promoter. *GUS* gene was removed from pAHC plasmid using restriction enzymes, *Sma*I and *Sac* I (Fig. 5a). Digested linear plasmid was ligated to *TaNAC69-1* and *TtNAM-B2* genes, separately. Ligation products were transferred into competent *E.coli*. To

confirm position of genes into pAHC25, restriction mapping was used.

For confirmation of *TaNAC69-1* gene position in pAHC25 vector, *Hind*III restriction enzyme was selected because its restriction site is found in both *TaNAC69-1* gene and pAHC25 vector. *TaNAC69-1* gene contains *Hind*III restriction site at a position of 300bp. pAHC25 vector involves two *Hind*III restriction sites (Fig. 5b). So, three DNA fragments were obtained after the *Hind*III enzyme digestion of positive colonies. According to position of *TaNAC69-1* gene in the vector, different fragment sizes were formed after *Hind*III digestion of pAHC25 vector. If *TaNAC69-1* gene was inserted at right position in the vector, 2300 bp, 1430 bp and 5500 bp fragments would be obtained. If the *TaNAC69-1* gene is found at reverse direction in the vector, in this case 3170 bp, 560 bp and 5500 bp DNA fragments would be produced. If the vector is ligated without gene, 5500 bp and 2300 bp DNA pieces were observed in gel (Fig.5b).

pAHC25 vector was also digested with *Xho*I restriction enzyme to confirm position of *TtNAM-B2* gene in the vector. *Xho*I restriction sites are found both *TtNAM-B2* gene and vector. *TtNAM-B2* gene contains *Xho*I restriction site at a position of 700bp. pAHC25 vector involves two *Xho*I restriction sites within the Ubi-promoter. (Fig. 5c). So, three DNA fragments were obtained after the *XhoI* enzyme digestion of positive colonies. After digestion of pAHC25 vector containing *TtNAM-B2* gene with *Xho*I restriction enzyme, 2300 bp, 1000 bp, 600 bp and 5500 bp DNA fragments were observed. It was expected that 1600 bp fragment should be a single band but it probably contains another restriction site not indicated in the map. So, four DNA fragments were visualized after restriction digestion of pAHC25 vector containing *TtNAM-B2* gene at right position. Self ligation of pAHC25 vector was also observed. (Fig. 5c).

Vector Construction for Gateway Cloning: Gateway compatible vectors contain specific recombination sites including attL, attR or attB, attP. Two recombination reactions, namely LR and BP reactions are catalyzed by LR and BP recombinases (clonases), respectively. Two types of selection systems including a positive (antibiotic resistance) and a negative (the cytotoxic *ccdB* gene) systems are mainly used for selection of final construct. Because of lethal gene, negative selection system does not permit the growth of *E.coli* (Magnani *et al.,* 2006). Gateway compatible pIPKb002 vector (Himmelbach *et al.,* 2007) was designed as binary destination vector for *Agrobacterium*-mediated transformation of cereals. *TaNAC69-1*and *TtNAM-B2* genes from pENTR™/D-

TOPO® cloning vector weretransferred into expression vector, pIPKb002 which contains suitable recombination sites (attR1 and attR2) for Gateway cloning (Fig. 6a,b). Through the LR reaction, site-specific recombination occurred and an attB-containing expression clone of *TaNAC69-1*and *TtNAM-B2* genes wereformed. To analyze the presence of *TaNAC69-1*and *TtNAM-B2* genes, colony PCR was performed using both gene specific primers and *hygromycin* gene (hpt) primers (Fig. 6c).

To develop stress resistant transgenic plants, NAC-type transcription factor genes, *TaNAC69-1* and *TtNAMB-2* were firstly isolated from *T. aestivum* and *T. turgidum*, respectively. Then they were cloned into different expression vectors to use for wheat genetic transformation studies.
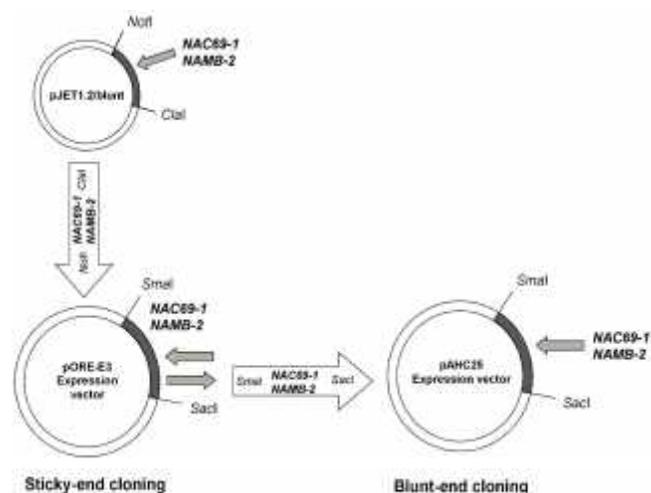

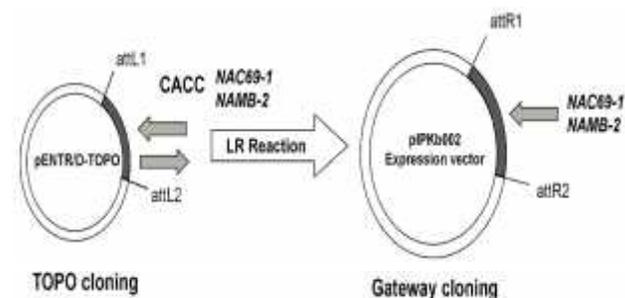**Fig. 1 Presentations of sticky and blunt-end cloning of *TaNAC69-1* and *TtNAM-B2* genes into expression vectors**

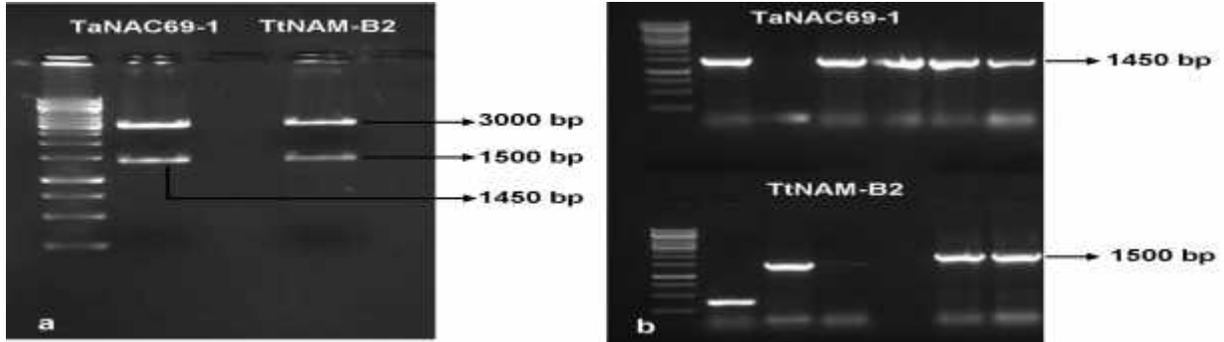**Fig. 2 Presentations of TOPO® Cloning and Gateway® cloning strategies for cloning of *TaNAC69-1* and *TtNAM-B2* genes**

Fig. 3 Agarosegel electrophoresis results of (a) pJET1.2/blunt cloning vector containing *TaNAC69-1* and *TtNAM-B2* genes cut with *Bgl*II(b) colony PCR amplification of *TaNAC69-1* and *TtNAM-B2* genes found in pENTR™/D-TOPO® cloning vector
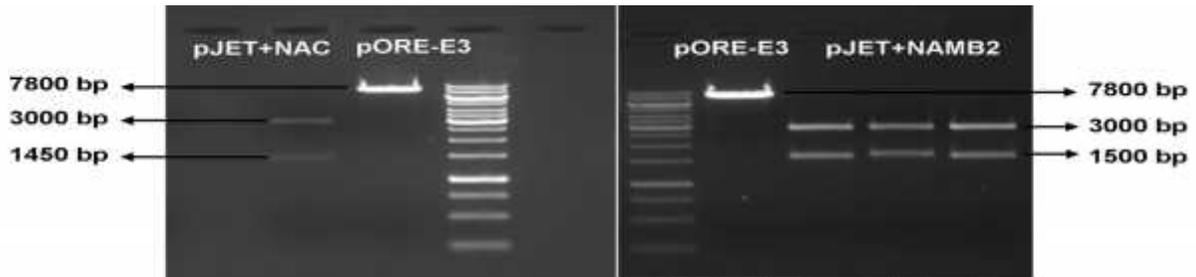

Fig. 4 Agarose gel electrophoresis results of pJET1.2/blunt cloning vector and pORE-E3 binary vector cut with *Cla*I and *Not*I. *TaNAC69-1* and *TtNAM-B2* gene inserts were excised from cloning vector with sticky ends
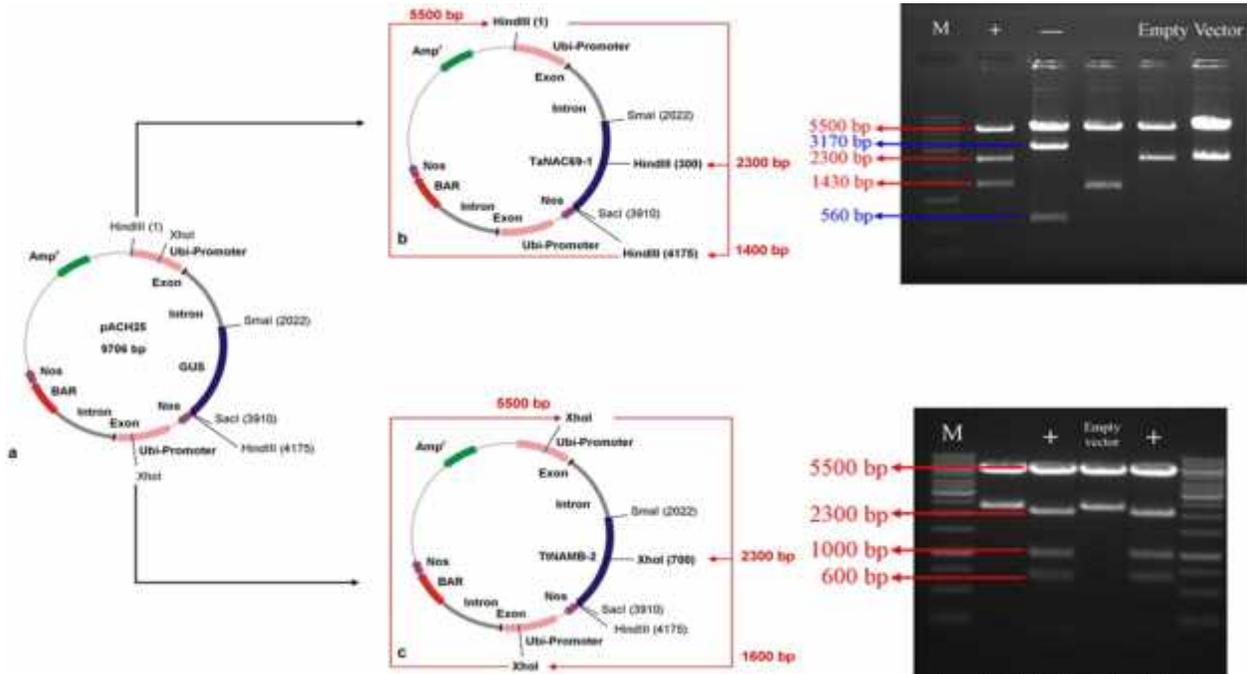

Fig. 5 (a) Map of pAHC25, monocot expression vector. (b)Position of *Hind*III restriction sites in pAHC25 vector containing *TaNAC69-1* gene. Agarose gel electrophoresis results of pAHC25 vector containing *TaNAC69-1* gene digested with *Hind*III. Red arrows indicate DNA fragments that belong to insertion of *TaNAC69-1* gene at right direction. Blue arrows show DNA bands of *TaNAC69-1* gene at reverse direction. (c)Position of *Xho*I restriction sites in pAHC25 vector containing *TtNAM-B2*gene. Agarose gel electrophoresis results of pAHC25 vector containing *TtNAM-B2* gene digested with *Xho*I. Red arrows indicate DNA fragments that belong to insertion of *TtNAM-B2* gene at right direction
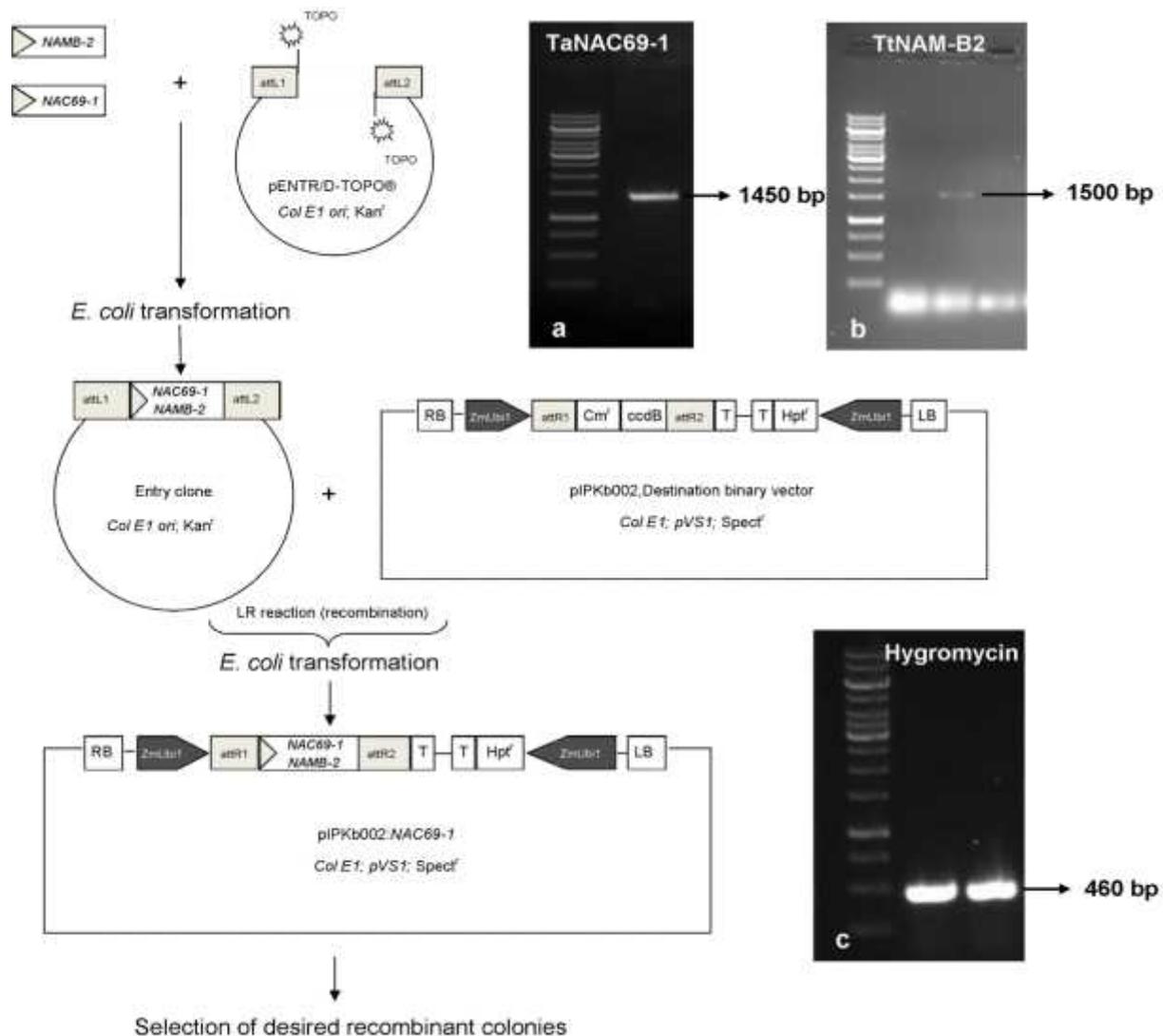
Fig. 6 Agarosegel electrophoresis results of **(a)** pENTR™/D-TOPO® cloning vector containing *TaNAC69-1***(b)***TtNAM-B2* genes **(c)** colony PCR amplification of *hygromycin* gene (hpt) found in **pIPKb002**expression vector

Table 1 Gene specific primers designed for different cloning vectors.

Genes	pJet1.2/blunt	pENTR™/D-TOPO®
NAC69	5`-GCCATCTTCTCCTCCTCCTC-3`	5`-CACCATGCCAATGGGCAGCAGC-3`
	3`-TTCTTATCGGTCGGTCGGTC-5`	3`-TCACATGTGCAGCTGCTGGCTG-5`
NAMB2	5`-ATGGGCAGCTCGGACTCATC-3`	5`CACCATGGGCAGCTCGGACTCATC3`
	3`-TCAGGGATTCCAGTTCACGC-5`	3`-TCAGGGATTCCAGTTCACGC-5`

REFERENCES

An, Y., A. Lv, and W.Wu (2010).A QuikChange-like method to realize efficient blunt-ended DNA directional cloning and site-directed mutagenesis simultaneously. Biochem.Bioph. Res. Co.397: 136-139.

Christensen, A.H., and P. H. Quail (1996).Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants.Transgenic Res.5: 213-218.

Conze, T., A.Shetye, Y.Tanaka, J.Gu, C.Larsson, J.Göransson, G.Tavoosidana, O.Söderberg, M.Nilsson, and U.Landegren(2009). Analysis of genes, transcripts, and proteins via DNA ligation.Annu. Rev. Anal. Chem.Palo Alto Calif.2: 215-239.

Costa, G.L., and M.P.Weiner(1994).Protocols for cloning and analysis of blunt-ended PCR-generated DNA fragments. PCR Meth. Appl. 3: S95-S106.

Himmelbach, A., U.Zierold, G.Hensel, J.Riechen, D.Douchkov, P.Schweizer, and J.Kumlehn (2007).A Set of Modular Binary Vectors for Transformation of Cereals.Plant Physiol. 145: 1192-1200.

Landy, A (1989).Dynamic, structural, and regulatory aspects of lambda site-specific recombination.Annu. Rev.Biochem. 58: 913-949.

Liu, Z.G., and L.M. Schwartz (1992).An efficient method for blunt-end ligation of PCR products.*Biotechniques* 12: 28-30.

Magnani, E., L.Bartling, and S.Hake(2006).From Gateway to MultiSite Gateway in one recombination event.BMC Mol. Biol.7: 46.

Patnaik, D (2001). Wheat biotechnology : A minireview. Electron. J.Biotechn. 4: 1-29.

Razzaq, A., Z.Ma, and H.Wang(2004). Genetic Transformation of wheat (*Triticumaestivum* L): A review. Mol. Plant Breeding 2: 457-466.

Shewry, P.R., and H.D.Jones (2005). Transgenic wheat: where do we stand after the first 12 years? Ann. Appl. Biol. 147: 1-14.

Uauy, C., A.Distelfeld, T.Fahima, A.Blechl, and J.Dubcovsky (2006). A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* 314: 1298-1301.

Upcroft, P., and A.Healey (1987). Rapid and efficient method for cloning of blunt-ended DNA fragments. Gene 51: 69-75.

Zhou, M.Y., S.E.Clark, and C.E.Gomez-Sanchez (1995).Universal cloning method by TA strategy.Biotechniques19: 34-35.